Chittaranjan Kole *Editor*

Genomic Designing for Biotic Stress Resistant Technical Crops

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Editor
Chittaranjan Kole
Raja Ramanna Fellow
Department of Atomic Energy, Government
of India
ICAR-National Institute for Plant
Biotechnology
New Delhi, India

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Dedicated to

Prof. Roger D. Kornberg
Nobel Laureate in Chemistry 2006
Professor of structural biology at Stanford University School of Medicine

With regards and gratitude for his generous appreciations of my scientific contributions and service to the academic community and constant support and encouragement during my professional journey!

Preface

Crop production is drastically affected due to external or environmental stresses. The biotic stresses cause significant yield losses in the range of 31–42% together with 6–20% loss during the post-harvest stage. The abiotic stresses also aggravate the situation with crop damage in the range of 6–20%. Understanding the mechanisms of interaction of plants with the biotic stresses caused by insects, bacteria, fungi, viruses and oomycetes, etc., and abiotic stresses due to heat, cold, drought, flooding, submergence, salinity, acidity, etc., is critical to develop resilient crop varieties. Global warming and climate change are also causing emergence of new diseases and insects together with newer biotypes and physiological races of the causal agents in one hand and aggravating the abiotic stress problems with additional extremes and unpredictability. Development of crop varieties resistant and/or adaptive to these stresses is highly important. The future mission of crop improvement should, therefore, lay emphasis on the development of crop varieties with optimum genome plasticity by possessing resistance or tolerance to multiple biotic and abiotic stresses simultaneously. A moderate estimation of world population by 2050 is about 9.3 billion that would necessitate an increase of crop production by about 70%. On the other hand, the additional losses due to climate change and global warming somewhere in the range of 10–15% should be minimized. Therefore, increase in the crop yield as well as minimization of its loss should be practiced simultaneously focusing both on 'adaptation' andon 'mitigation.'

Traditional plant breeding practiced in the last century contributed a lot to the science of crop genetic improvement. Classical plant breeding methods including selection, hybridization, polyploidy and mutation effectively catered to the basic F^5 need—food, feed, fiber, fuel and furniture. The advent of molecular breeding and genetic engineering in the latter part of that century complimented classical breeding that addressed the increasing needs of the world. The twenty-first century came with a gift to the geneticists and plant breeders with the strategy of genome sequencing in Arabidopsis and rice followed by the tools of genomics-aided breeding. More recently another revolutionary technique, genome or gene editing, became available for genetic correction of crop genomes! The travel from 'plant breeding' based on visual or perceivable selection to 'molecular breeding' assisted by linked markers to

'transgenic breeding' using genetic transformation with alien genes to 'genomics-aided breeding' facilitated by known gene sequences has now arrived at the age of 'genetic rectification' employing genome or gene editing.

Knowledge on the advanced genetic and genomic crop improvement strategies including molecular breeding, transgenics, genomic-assisted breeding and the recently emerged genome editing for developing resistant, tolerant and/or adaptive crop varieties is useful to students, faculties and scientists in the public and private universities and organizations. Whole genome sequencing of most of the major crop plants followed by genotyping-by-sequencing has facilitated identification of exactly the genes conferring resistance, tolerance or adaptability leading to gene discovery, allele mining and shuttle breeding which in turn opened up the scope for 'designing' or 'tailoring' crop genomes with resistance/tolerance to biotic and abiotic stresses.

To my mind, the mission of agriculture in this century is FHNEE security meaning food, health, nutrition, energy and environment security. Hence, genome designing of crops should focus on breeding of varieties with higher yields and improved qualities of the five basic F^5 utilities; nutritional and neutraceutical compounds; and other industrially and aesthetically important products and possibility of multiple utilities. For this purpose of 'precise' breeding, employment of the genetic and genomic techniques individually or in combination as and when required will play a crucial role.

The chapters of the 12 volumes of this twin book series entitled, *Genomic Designing for Biotic Stress Resistant Crops* and *Genomic Designing for Abiotic Stress Resistant Crops* will deliberate on different types of biotic and abiotic stresses and their effects on and interaction with crop plants; will enumerate the available genetic diversity with regard to biotic or abiotic stress resistance among cultivars; illuminate on the potential gene pools for utilization in interspecific gene transfer; will brief on the classical genetics of stress resistance and traditional breeding for transferring them to their cultivated counterparts; will discuss on molecular mapping of genes and QTLs underlying stress resistance and their marker-assisted introgression into elite crop varieties; will enunciate different emerging genomics-aided techniques including genomic selection, allele mining, gene discovery and gene pyramiding for developing smart crop varieties with genetic potential to produce F^5 of higher quantity and quality; and also will elaborate the case studies on genome editing focusing on specific genes. Most of these chapters will discuss on the success stories of genetic engineering in the relevant crops specifically for generating crops with resistance and/or adaptability to diseases, insects and abiotic stresses.

There are obviously a number of reviews and books on the individual aspects of plant molecular breeding, genetic engineering and genomics-aided breeding on crops or on agro-economic traits which includes the 100-plus books edited by me. However, there is no comprehensive reviews or books available that have coverage on crop commodity groups including cereals and millets, oilseeds, pulses, fruits and nuts, vegetables and technical or industrial crops and modern strategies in single volumes with precise focuses on biotic and abiotic stresses. The present volumes will fill this gap with deliberations on about 120 important crops or their groups.

This volume on *Genomic Designing for Biotic Stress Resistant Technical Crops* includes 11 chapters focused on cassava, cocoa tree, Coconut, coffee, cotton, floricultural crops, jute, mulberry, sugarcane, tobacco and yam contributed by 92 scientists from 10 countries including Brazil, China, Costa Rica, D. R. Congo, France, India, Iran, Nigeria, USA, and Venezuela. I remain immensely thankful for their highly useful contributions.

I am indebted to my wife Phullara who as always has assisted me directly in editing these books and indirectly through maintaining an academic ambience to pursue my efforts for science and society pleasantly and peacefully.

New Delhi, India Chittaranjan Kole

Contents

Contributors

Abah S. P. Department of Biotechnology and Product Development, National Root Crops Research Institute, Umuahia, Abia State, Nigeria

Adebola Patrick International Institute of Tropical Agriculture (IITA), Abuja, Nigeria

Agre Paterne A. International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria

Akoroda Malachy Department of Agronomy, University of Ibadan, Ibadan, Nigeria

Alves Rafael Moysés Embrapa Amazônia Oriental, Belém, Pará, Brazil

Anna Durai A. Plant Breeding Section, Division of Crop Improvement, ICAR-Sugarcane Breeding Institute, Coimbatore, India

Aparna V. ICAR-CPCRI, Kasaragod, Kerala, India

Appunu C. Plant Breeding Section, Division of Crop Improvement, ICAR-Sugarcane Breeding Institute, Coimbatore, India

Arias Andres Gatica School of Biology, University of Costa Rica, San José, Costa Rica

Ariyoshi Caroline Biological Science Center, State University of Londrina, Londrina, Paraná, 86057-970 Brazil;
Area of Genetic Improvement and Plant Propagation, Paraná Rural Development Institute, Londrina, Paraná, 86047-902 Brazil

Arunakumar G. S. Central Sericultural Research and Training Institute, Srirampura, Mysuru, Karnataka, India

Asfaw Asrat International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria

Asiedu Robert International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria

Babu Merin ICAR-CPCRI, Regional Station, Kayamkulam, Kerala, India

Baghyalakshmi K. ICAR-Central Institute for Cotton Research, Research Station, Coimbatore, India

Barbosa Ceslaine Cocoa Research Center (CEPEC), CEPLAC, Ilhéus, Bahia, Brazil

Bhargava Bhavya CSIR-Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh, India

Bhattacharjee Ranjana International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria

Caixeta Eveline Teixeira Institute of Applied Biotechnology to Agriculture, Federal University of Viçosa, Viçosa, Minas Gerais, 36570-900 Brazil;
Empresa Brasileira de Pesquisa Agropecuária (Embrapa Café), Brasília, Distrito Federal, 70770-901 Brazil

Carvalho Mariana Cocoa Research Center (CEPEC), CEPLAC, Ilhéus, Bahia, Brazil

Corrêa Ronan Xavier Centro de Biotecnologia e Genética, Universidade Estadual de Santa Cruz, Ilhéus, Bahia, Brazil

Darvishzadeh Reza Department of Plant Breeding and Biotechnology, Faculty of Agriculture, Urmia University, Urmia, Iran

Egesi C. N. School of Integrative Plant Science, College of Agriculture and Life Sciences, Cornell University, Ithaca, USA;
Department of Biotechnology and Product Development, National Root Crops Research Institute, Umuahia, Abia State, Nigeria

Edemodu Alex International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria

Ferreira Cláusio Antônio Centro de Biotecnologia e Genética, Universidade Estadual de Santa Cruz, Ilhéus, Bahia, Brazil

Gangadhara K. ICAR-Central Tobacco Research Institute, Research Station, Kandukur, Andhra Pradesh, India

Gedil Melaku International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria

Geetha N. Entomology Section, Division of Crop Protection, ICAR-Sugarcane Breeding Institute, Coimbatore, India

Gnanesh B. N. Central Sericultural Research and Training Institute, Srirampura, Mysuru, Karnataka, India

Gotyal B. S. ICAR-Central Research Institute for Jute and Allied Fibres, Barrackpore, Kolkata, India

Gramacho Karina Peres Cocoa Research Center (CEPEC), CEPLAC, Ilhéus, Bahia, Brazil

Hegde Vinayaka ICAR-CPCRI, Kasaragod, Kerala, India

Ikeogu U. N. School of Integrative Plant Science, College of Agriculture and Life Sciences, Cornell University, Ithaca, USA

Ivamoto-Suzuki Suzana Tiemi Biological Science Center, State University of Londrina, Londrina, Paraná, 86057-970 Brazil;
Area of Genetic Improvement and Plant Propagation, Paraná Rural Development Institute, Londrina, Paraná, 86047-902 Brazil

Manikanda Boopathi N. Department of Plant Biotechnology, CPMB&B, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Micheli Fabienne Centro de Biotecnologia e Genética, Universidade Estadual de Santa Cruz, Rodovia Ilhéus-Itabuna Km 16, Ilhéus, Bahia, 45662-900 Brazil;
CIRAD, UMR AGAP, 34398 Montpellier, France

Mondo Jean M. International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria;
Institute of Life and Earth Sciences, Pan African University, University of Ibadan, Ibadan, Nigeria;
Université Evangéliqueen Afrique (UEA), Bukavu, Democratic Republic of Congo

Mucherino-Muñoz Jonathan Javier Centro de Biotecnologia e Genética, Universidade Estadual de Santa Cruz, Rodovia Ilhéus-Itabuna Km 16, Ilhéus, Bahia, 45662-900 Brazil;
Department of Forest Management, Facultad de Ciencias Forestales y Ambientales, Universidad de Los Andes, Mérida, 5101 Venezuela

de Oliveira Fernanda Freitas Biological Science Center, State University of Londrina, Londrina, Paraná, 86057-970 Brazil;
Area of Genetic Improvement and Plant Propagation, Paraná Rural Development Institute, Londrina, Paraná, 86047-902 Brazil

da Rocha Matheus Ricardo Department of Agronomy, Center of Agricultural Sciences and Engineering, Federal University of Espírito Santo, Alegre, Espírito Santo, Brazil

dos Santos Menezes Fredson Centro de Biotecnologia e Genética, Universidade Estadual de Santa Cruz, Rodovia Ilhéus-Itabuna Km 16, Ilhéus, Bahia, 45662-900 Brazil

da Silva Angelita Garbossi Biological Science Center, State University of Londrina, Londrina, Paraná, 86057-970 Brazil;
Area of Genetic Improvement and Plant Propagation, Paraná Rural Development Institute, Londrina, Paraná, 86047-902 Brazil

da Silva Chaves Saulo Fabrício Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil

Jibuwa L. C. Department of Biotechnology and Product Development, National Root Crops Research Institute, Umuahia, Abia State, Nigeria

Jordana Natalia Cocoa Research Center (CEPEC), CEPLAC, Ilhéus, Bahia, Brazil

Josephrajkumar A. ICAR-CPCRI, Regional Station, Kayamkulam, Kerala, India

Kolade Olufisayo International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria

Kumar Akhil CSIR-Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh, India

Kumar Lava P. International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria

Lemos Livia Santos Lima Universidade Federal Do Sul da Bahia, Teixeiras de Freitas, Bahia, Brazil

Mandal Kunal ICAR-Central Research Institute for Jute and Allied Fibres, Barrackpore, Kolkata, India

Matsumoto Ryo International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria

Moraes Willian Bucker Department of Agronomy, Center of Agricultural Sciences and Engineering, Federal University of Espírito Santo, Alegre, Espírito Santo, Brazil

Muralikrishna K. S. ICAR-CPCRI, Kasaragod, Kerala, India

Nanda C. ICAR-Central Tobacco Research Institute, Research Station, Hunsur, Karnataka, India

Nithya K. Plant Pathology Section, Division of Crop Protection, ICAR-Sugarcane Breeding Institute, Coimbatore, India

Nkere C. K. Department of Biotechnology and Product Development, National Root Crops Research Institute, Umuahia, Abia State, Nigeria

Nnaji I. C. Department of Biotechnology and Product Development, National Root Crops Research Institute, Umuahia, Abia State, Nigeria

Nwachukwu L. A. Department of Biotechnology and Product Development, National Root Crops Research Institute, Umuahia, Abia State, Nigeria

Nwadili C. Department of Biotechnology and Product Development, National Root Crops Research Institute, Umuahia, Abia State, Nigeria

Okereke N. R. Department of Biotechnology and Product Development, National Root Crops Research Institute, Umuahia, Abia State, Nigeria

Okwuonu I. C. Department of Biotechnology and Product Development, National Root Crops Research Institute, Umuahia, Abia State, Nigeria

Onyeka J. T. Department of Biotechnology and Product Development, National Root Crops Research Institute, Umuahia, Abia State, Nigeria

Parameswari B. ICAR-National Bureau of Plant Genetic Resources Regional Station, Hyderabad, India

Pereira Luiz Filipe Protasio Area of Genetic Improvement and Plant Propagation, Paraná Rural Development Institute, Londrina, Paraná, 86047-902 Brazil;
Empresa Brasileira de Pesquisa Agropecuária (Embrapa Café), Brasília, Distrito Federal, 70770-901 Brazil

Partelli Fábio Luiz Center of North of Espírito Santo, Federal University of Espírito Santo, São Mateus, Espírito Santo, Brazil

Pires José Luis Cocoa Research Center (CEPEC), CEPLAC, Ilhéus, Bahia, Brazil

Prabhakara Rao K. ICAR-Central Tobacco Research Institute, Rajahmundry, Andhra Pradesh, India

Prathibha V. H. ICAR-CPCRI, Kasaragod, Kerala, India

Prathima P. T. Plant Biotechnology Section, Division of Crop Improvement, ICAR-Sugarcane Breeding Institute, Coimbatore, India

Priti CSIR-Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh, India

Rajesh M. K. ICAR-CPCRI, Kasaragod, Kerala, India

Ramasubramanian T. Entomology Section, Division of Crop Protection, ICAR-Sugarcane Breeding Institute, Coimbatore, India

Ramesh S. V. ICAR-CPCRI, Kasaragod, Kerala, India

Ramesha A. Seribiotech Research Laboratory, Bengaluru, Karnataka, India

Ray Soham ICAR-Central Research Institute for Jute and Allied Fibres, Barrackpore, Kolkata, India

Roy Suman ICAR-Central Research Institute for Jute and Allied Fibres, Barrackpore, Kolkata, India

Sangannavar Prashanth A. Central Silk Board, BTM Layout, Madiwala, Bengaluru, Karnataka, India

Sarala K. ICAR-Central Tobacco Research Institute, Rajahmundry, Andhra Pradesh, India

Satya Pratik ICAR-Central Research Institute for Jute and Allied Fibres, Barrackpore, Kolkata, India

Selvi A. Plant Biotechnology Section, Division of Crop Improvement, ICAR-Sugarcane Breeding Institute, Coimbatore, India

Sera Gustavo Hiroshi Area of Genetic Improvement and Plant Propagation, Paraná Rural Development Institute, Londrina, Paraná, Brazil

Shigueoka Luciana Harumi Area of Genetic Improvement and Plant Propagation, Paraná Rural Development Institute, Londrina, Paraná, Brazil

Silva Raner José Santana Centro de Biotecnologia e Genética, Universidade Estadual de Santa Cruz, Ilhéus, Bahia, Brazil

Sreedhar U. ICAR-Central Tobacco Research Institute, Rajahmundry, Andhra Pradesh, India

Verma Jyoti CSIR-Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh, India

Verma Vipasha CSIR-Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh, India

Vijayan K. Central Silk Board, BTM Layout, Madiwala, Bengaluru, Karnataka, India

Villalta-Villalobos Jimmy School of Biology, University of Costa Rica, San José, Costa Rica

Viswanathan R. Division of Crop Protection, ICAR-Sugarcane Breeding Institute, Coimbatore, India

Zhang Jinfa Department of Plant and Environmental Sciences, New Mexico State University, Las Cruces, NM, USA

Zhao W. Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, Jiangsu, China

Abbreviations

2D gel	Two-dimension gel
Aaa	*Acidovorax avenae* subsp. *avenae*
ABA	Abscisic acid
ABS	Access–benefit sharing
ACAC	Agro-Chemical Advisory Committee
ACGC	Arabica Coffee Genome Consortium
ACMV	African cassava mosaic virus
ACT	Artemis comparison tool
AE	Array express
AFLP	Amplified fragment length polymorphism
AGBs	Active Germplasm Banks
AgNPs	Silver nanoparticles
AGO	Argonaute
Ai	Active ingredient
AICEM	All India Coordinated Experiment on Mulberry
AICRP(S)	All India Coordinated Research Project on Sugarcane
AM	Association mapping
AMP1	Antimicrobial protein 1
AMT	*Agrobacterium*-mediated transformation
AS	Alternative splicing
Avr	Avirulence gene
AVT	Advanced varietal trial
AYT	Advanced yield trial
BAC	Bacterial artificial chromosome
BB	Bacterial blight
BC	Backcross
BG	Bollgard
BHB	Bacterial halo blight
bHLH	Basic helix–loop–helix
BILs	Backcross inbred lines
BLS	Brown leaf spot

BSA	Bulk segregant analysis
Bt	*Bacillus thuringiensis*
BTH	Benzothiadiazole
Bti *B.*	*Thuringiensis* serovar *israelensis*
bZIP	Basic Leucine Zipper Domain
CAB	Cocoa from the Brazilian Amazon
CABI	Centre for Agriculture and Bioscience International
CAD	Cassava anthracnose disease
CaMXMT1	Chrysanthemum methylxanthinemethyltransferase 1
CAPS	Cleaved amplified polymorphic sequence
Cas	CRISPR-associated protein
Cas 9	CRISPR-associated protein 9
CaXMT1	Chrysanthemum xanthosinemethyltransferase 1
CBB	Cassava bacteria blight
CBB	Coffee berry borer
CBD	Convention on biological diversity
CBSD	Cassava brown streak disease
CCCVd	Coconut cadang–cadang viroid
CcPDS	Phytoene desaturase
CDB	Coffee berry disease
cDNA	Complementary DNA
CE	Carboxylesterase family
CFDV	Coconut foliar decay virus
CGIAR	Consultative Group for International Agricultural Research
CGM	Cassava green mite
CGRD	Coconut Genetic Resources Database
CICF	Centro de investigação das ferrugens do cafeeiro
CIM	Composite interval mapping
CIPO	Canadian Intellectual Property Office
CIRAD	Centre de Coopération Internationale en Recherche Agronomique pour le Développement
CLM	Coffee leaf miner
CLR	Coffee leaf rust
cM	Centimorgan
CMD	Cassava mosaic disease
CMGV	*Cassava mosaic geminivirus*
CMS	Cytoplasmic male sterility
CMV	*Cucumber mosaic virus*
CNRA	Center National de Recherche Agronomique
CNTs	Carbon nanotubes
CNV	*Cacao necrosis virus*
COGENT	International Coconut Genetic Resources Network
CORESTA	Cooperation Centre for Scientific Research Relative to Tobacco
COS	Conserved ortholog sequences
COS-II	Conserved ortholog set II

COSTREL	Combinatorial super transformation of transplastomic recipient lines
CP	Coat protein
CPs	Cuticular proteins
CpYGFP	Chiridius poppei yellow-green fluorescent protein
CRIN	Cocoa Research Institute of Nigeria
CRIP	Coffee gRNA Identification Program
CRISPR	Clustered regularly interspaced short palindromic repeats
cry1Ab	Crystal protein 1 Ab
CSR&TI	Central Sericultural Research and Training Institute
CSSLs	Chromosome Segment Substitution Lines
CSSV	*Cacao swollen shoot virus*
CTRI	Central Tobacco Research Institute
CVB	*Chrysanthemum virus B*
CVRC	Central Varietal Release Committee
CWR	Crop wild relatives
CYD	Coconut yellow decline
CYMV	Cacao yellow mosaic virus
dai	Days after inoculation
DArT	Diversity array technology
DAS	Days after sowing
DAVID	Database for annotation, visualization and integrated discovery
dCAPS	Derived CAPS
DDBJ	DNA Databank of Japan
DDVP	Dichlorvos
DGA	Defence gene analog
DH	Doubled haploid
DPMA	German Patent and Trademark Office
DRs	Disclosure requirements
DSBs	Double-strand breaks
dsRNA	Double-stranded RNA
DUS	Distinctness, uniformity and stability
EACMV	East African cassava mosaic virus
EBI	European Bioinformatics Institute
EC	Effective concentration
EC	Emulsified concentrate
EFSA	European Food Safety Authority
eIF4E	Eukaryotic translation initiation factor 4E
EIL	Economic injury level
ELISA	Enzyme-linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
EMS	Ethyl methanesulfonate
EPO	European Patent Office
ERF	Ethylene-responsive factor
ERF	ETS2 repressor factor
ERF1	*Ethylene-responsive factor 1*

ERIC-PCR	Enterobacterial repetitive intergenic consensus-PCR
EST	Expressed sequence tag
EST-SSR	EST-derived SSR
ET	Ethylene
ETI	Activating effector-triggered immunity
ETR	Electron transport rate
F1	First filial generation
FaNES1	Frankellianerolidol synthase 1
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
FCF	Fungus culture filtrate
FCTC	Framework Convention on Tobacco Control
FCV	Flue cured virginia
FDV	*Fiji disease virus*
FEC	Friable embryogenic calli
FISH	Fluorescence in situ hybridization
FOV	*Fusarium oxysporum* f. sp. *Vasinfectum*
FPO	Free Patents Online
FW	Fusarium wilt
FYM	Farm yard manure
GA3	Gibberellic acid
GAB	Genomics-assisted breeding
GAP	Good agricultural practice
GBLUP	Genomic best linear unbiased prediction
GBS	Genotyping-by-sequencing
GE	Genome engineering
GEA	Genomic Expression Archive
GEBVs	Genomic-estimated breeding values
GEO	Gene Expression Omnibus
GhAAT	*Gerbera hybrida* aspartate aminotransferase
GhFAH	*Gerbera hybrida* fumarylacetoacetate hydrolase
GhHGD	*Gerbera hybrida* homogentisate 1,2-dioxygenase
GhHPD	*Gerbera hybrida* 4-hydroxyphenylpyruvate dioxygenase
GhTAT	*Gerbera hybrida* tyrosine aminotransferase
GLM	General linear models
GM	Genetically modified
GMD	Golm Metabolome Database
GMO	Genetically modified organism
GO	Gene ontology
GRLs	Guidance residue levels
gRNA	Guide RNA
GRs	Genetic resources
GS	Genomic selection
GSRs	Genome space sequence reads
GSS	Genomic survey sequences

GTE	GATA transcription factor
GUS	*β-glucuronidase*
GWAS	Genome-wide association study/studies
H_2O_2	Hydrogen peroxide
hai	Hours after inoculation
HdT	Timor hybrid
HESP	Haustorial-expressed secreted protein
HG	Homologous groups
hpaGXoo	HarpinXoo
hpRNA	Hairpin RNA
HR	Hypersensitive response
HSF	Heat shock factor
HSP	Heat shock protein
ICAR	Indian Council of Agricultural Research
ICAR-SBI	ICAR—Sugarcane Breeding Institute
ICG	International Coconut Gene Bank
ICGD	International Cocoa Germplasm Database
ICGT	International Cocoa Genbank, Trinidad
ICMV	*Indian cassava mosaic virus*
ICO	International Coffee Organization
ICS	Imperial College Selection
ICTV	International Committee on Taxonomy of Viruses
IDM	Integrated disease management
IGH	Intergeneric hybrids
IGS	Intergenic spacer
IITA	International Institute of Tropical Agriculture
IM	Interval mapping
Indels	Insertion or deletion mutations
INM	Integrated nutrient management
INSDC	The International Nucleotide Sequence Database Collaboration
IPM	Integrated pest management
IPM/IDM	Integrated management of pests and disease
IPR	Intellectual Property Rights
IRAD	Institute of Agricultural Research for Development
IRD	Institut de Recherche pour le Développement
ISAAA	International Service for the Acquisition of Agri-biotech Applications
ISH	Interspecific hybrids
ISSCT	International Society of Sugarcane Technologists
ISSR	Inter-simple sequence repeat
ISTR	Inverse sequence tagged repeat
ITPGRFA	International Treaty for Plant Genetic Resources for Food and Agriculture
ITS	Internal transcribed spacer
JA	Jasmonic acid

JPO	Japan Patent Office
KASP	Kompetitive allele-specific PCR
KEGG	Kyoto Encyclopedia of Genes and Genomes
LD	Linkage disequilibrium
LD50	Lethal dose 50
LG	Linkage group
LlWRKY	Lilium W-box binding transcription factor
LlWRKY10	Lilium W-box binding transcription factor 10
LlWRKY12	Lilium W-box binding transcription factor 12
LlWRKY3	Lilium W-box binding transcription factor 3
LlWRKY4	Lilium W-box binding transcription factor 4
LlWRKY5	Lilium W-box binding transcription factor 5
LMOs	Living modified organisms
LMoV	Lily mottle virus
LOD	Logarithm of odds
LrPR10-2	Lilium regale pathogenesis-related gene—2
LrPR10-4	Lilium regale pathogenesis-related gene—4
LrPR10-5	Lilium regale pathogenesis-related gene—5
LrPR10-6	Lilium regale pathogenesis-related gene—6
LrPR10-7	Lilium regale pathogenesis-related gene—7
LrPR10-9	Lilium regale pathogenesis-related gene—9
LRR	Lucien-rich repeat
LRR-RLK	Llr-like kinase
LSA	Locus-specific amplification
LSD	Leaf scald disease
Lxx	*Leifsoniaxyli* subsp. *xyli*
LY	Lethal yellowing
MAB	Marker-assisted breeding
MABC	Marker-assisted backcrossing
MACC	Marker-assisted complex or convergent crossing
MAGIC	Multiparent advanced generation intercross
MAPK	Mitogen-activated protein kinase
MARB	Marker-assisted resistance breeding
MAS	Marker-assisted selection
mcbt	Modified *cry1Ab* gene of *Bacillus thuringiensis*
MegaN	Meganuclease
MeJA	Methyl jasmonate
miRNA	Micro-RNA
MLO	*Mildew resistance locus O*
MLT	Multi-location trial
MMS	Methyl methane sulfonate
MNPs	Magnetic nanoparticles
MNs	Meganucleases
MoNA	Massbank of North America
MRL	Maximum residue level

MSNs	Mesoporous silica NPs
MTD	Mannitol dehydrogenase
MTI	Mite tolerability index
MYB	Myeloblastosis
MYC	Master regulator of cell
NACN	Acetylcysteine
NaDH	*Nicotiana attenuata* data hub
NattCyc	*N. attenuata*
NBS	National Bureau of Standards
NBS	Nucleotide-binding site
NCBI	National Center for Biotechnology Information
nCBP	Novel cap-binding protein
NDR1	Non-race-specific disease resistance
NFYC	Nuclear transcription factor Y
NGS	Next-generation sequencing
NHEJ	Non-homologous end-joining
NIH	National Human Genome Research Institute
NILs	Near-isogenic lines
NIX	*Nicotiana* multiple (X) genome
NMR	Nuclear magnetic resonance
NPBTs	New plant breeding technologies
NPL	Non-patent literature
NPR	Non-expressor or pathogenicity related
NPs	Nanoparticles
NPV	Nuclear polyhedrosis virus
NSKS	Neem seed kernel suspension
OPR3	*Oxoplytodienotae reductase 1-like*
ORF	Open reading frame
OXO	Oxalate oxidase
PAM	Proto spacer adjacent motif
PAMPs	Pathogen-associated molecular patterns
PB	Pokkahboeng
PBR	Plant Breeders' Rights
PCA	Principal coordinate analysis
PCT	Patent Cooperation Treaty
PCV	*Peanut clump virus*
PDB	Protein Data Bank
PDR	Pathogen-derived resistance
PGDBs	Pathway–genome databases
PI	Proteinase inhibitor
PIB	Population of improved *S. barberi*
PIO	Population of improved *S. officinarum*
PIR	Population of improved *S. robustum*
PIR	Protein Information Resource
PIS	Population of improved *S. spontaneum*

POGs	Peroxidase genes
PPB	Participatory plant breeding
PPVFRA	Protection of Plant Varieties and Farmers' Rights Authority
PR	Pathogenesis related
PR10	*Pathogenesis-related 10*
PRR	Pachymetra root rot
PRRs	Pattern recognition receptors
PRT	Progeny row trial
PTGS	Post-transcriptional gene silencing
PTIPAMP	Triggered immunity
PVP	Plant variety protection
PVY	*Potato virus Y*
PYT	Preliminary/primary yield trial
QTA	Quantitative trait allele
QTL	Quantitative trait locus
QTLs	Quantitative trait loci
R	Resistance
RAD sqe	Restriction site-associated DNA sequencing
RAMP	Random amplified microsatellite polymorphism
RAPD	Random amplified polymorphic DNA
REP-PCR	Repetitive element sequence-based PCR
RFLP	Restricted fragment length polymorphism
RFS	Rainfed selection
RGA	Resistance gene analog
RGAP	Resistance gene analog polymorphism
R-gene	Resistance gene
RhMLO	Rhodopsin mildew resistance locus O
RhMLO1	Rhodopsin mildew resistance locus O 1
RhMLO2	Rhodopsin mildew resistance locus O 2
RhMLO3	Rhodopsin mildew resistance locus O 3
RILs	Recombinant inbred lines
RISC	RNA-induced silencing complex
RKHS	Reproducing Kernel Hilbert space
RKN	Root-knot nematode
RLK	Receptor-like kinase
RLKs-LRRs	Receptor-like kinases LRR
RNAi	RNA interference
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
RP	Recurrent parent
Rpp1	Recognition of *Peronospora Parasitica*
RR	Roundup Ready
RSD	Ratoon stunting disease
RTP1	Rust transferred protein 1
RT-qPCR	Quantitative reverse transcription PCR

RWD	Root (wilt) disease
SA	Salicylic acid
SACMV	South African cassava mosaic virus
SAGE	Serial analysis of gene expression
SAH	Semi-autotrophic hydroponic
SAR	Systemic acquired resistance
SCAR	Sequence-characterized amplified region
SCBV	Sugarcane bacilliform virus
SCGS	Sugarcane grassy shoot disease
SCMMV	Sugarcane mild mosaic virus
SCMV	Sugarcane mosaic virus
SCSMaV	Sugarcane striate mosaic associated virus
SCSMV	Sugarcane steak mosaic virus
SCW	Silicon carbide whiskers
SCWL	Sugarcane white leaf disease
ScYLV	Sugarcane yellow leaf virus
SD	Single dose
SES	Standard evaluation system
SFP	Single feature polymorphism
SGN	Sol Genomics Networks
sgRNA	Single guide RNA
shRNA	Short hairpin RNA
SIB	Swiss Institute of Bioinformatics
siRNA	Small interfering RNA
SLCMV	Sri Lankan cassava mosaic virus
SNP	Single nucleotide polymorphism
Solana Cyc	Solanaceae database
SRA	Sequence Read Archive
SRAP	Sequence-related amplified polymorphism
SrMV	Sorghum mosaic virus
SSA	Sub-Saharan Africa
SSAP	Sequence-specific amplification polymorphism
SSH	Suppression subtractive hybridization
SSLP	Simple sequence length polymorphism
SSNs	Sequence-specific nucleases
SSR	Simple sequence repeat
ssRNA	Single-stranded RNA
STR	Short tandem repeat
STRs	Short repetitions in tandem
STS	Sequence tagged site
SUCEST	Sugarcane expressed sequence tags
SV1	Somaclonal variant-1
TAC	Transformation-competent artificial chromosome
TAL	Transcription activator-like
TALENs	Transcription activator-like effector nucleases

TBIA	Tissue-blot immunoassay
TDFs	Transcript-derived fragments
T-DNA	Transfer DNA
TEV	Tobacco etch virus
TF	Transcription factor
TGATGACG	Binding (TGA) transcription factors
TGI	Tobacco Genome Initiative
TIGR	The Institute of Genome Research
TK	Traditional knowledge
TLCV	Tobacco leaf curl virus
TMV	Tobacco mosaic virus
TobEA	Tobacco Expression Atlas
TOBFAC	Tobacco transcription factors
TPA	Third-party annotation
TPS1	Trehalose-6-phosphate synthase
TRAP	Target region amplification polymorphism
TRIPs	Trade-Related Aspects of Intellectual Property Rights
TSH	Trinidad selected hybrids
TSWV	Tomato spotted wilt virus
TVMV	Tobacco vein mottling virus
UC	Davis University of California, Davis
UNICAMP	Universidade estadual de Campinas
UniParc	UniProt Archive
UPOV	Union for the Protection of New Varieties of Plants
USDA	United States Department of Agriculture
USDA-ARS	USDA—Agricultural Research Service
UV	Ultraviolet
UYT	Uniform yield trial
VIGS	Virus-induced gene silencing
VPg	Viral genome-linked protein
VW	Verticillium wilt
WCSRG	World Collection of Sugarcane and Related Grasses
WFT	Western flower thrips
WGP	Whole genome profile
WHO	World Health Organization
WIPO	World Intellectual Property Organization
WLS	White leaf spot
WNTD	World No Tobacco Day
WRKY	W-box binding transcription factor
WTO	World Trade Organization
WUE	Water use efficiency
Xam	*Xanthomonas axonopodis* pv. *manihotis*
Xcm	*Xanthomonas citri* pv. *malvacearum*
YAC	Yeast artificial chromosome
YAD	Yam anthracnose disease

YLD	Yellow leaf disease
YLS	Yellow leaf syndrome
YMV	Yam mosaic virus
ZFN	Zinc finger nuclease
ZnONP	Zinc oxide nanoparticle

Chapter 1
Genomic Designing for Biotic Stress Resistant Cassava

U. N. Ikeogu, I. C. Okwuonu, N. R. Okereke, L. C. Jibuwa, C. Nwadili, S. P. Abah, L. A. Nwachukwu, I. C. Nnaji, C. K. Nkere, J. T. Onyeka, and C. N. Egesi

Abstract Cassava is essential food security, mostly in Africa, South America, and other major regions of the world where cassava is cultivated. It is very high in caloric value and resilient to climate change, drought, and low fertility. Biotic stress limits cassava cultivation and utilization with an impact that could range from 20 to 90% loss in yield and food quality. Diseases including viral, fungal, bacterial, and nematodes as well as diverse kinds of pests such as cassava whitefly and cassava green mites (CGM) are considered important biotic factors that impact cassava production. Diverse measures and techniques have been implored in cassava towards genomic designing for biotic stress resistance. These techniques range from traditional breeding to genomic selections and other new breeding technologies such as genetic engineering and genome editing. This chapter outlines the most significant biotic stresses in cassava, their prevalence, and impact on yield as well as different technologies being utilized towards the development of biotic stress-resistant cassava.

Keywords Cassava · Biotic stresses · Genomic selection · Genetic engineering · Genome editing

1.1 Biotic Stress in Cassava

Cassava, *Manihot esculenta* Crantz, (Family Euphorbiaceae) is an essential staple crop cultivated across the tropics and subtropics primarily for its starchy roots, which for over a billion serves as a source of calories and for industrial purposes (Lyons et al.

U. N. Ikeogu (✉) · C. N. Egesi
School of Integrative Plant Science, College of Agriculture and Life Sciences, Cornell University, Ithaca, USA
e-mail: uni3@cornell.edu

C. N. Egesi
e-mail: cne22@cornell.edu

I. C. Okwuonu · N. R. Okereke · L. C. Jibuwa · C. Nwadili · S. P. Abah · L. A. Nwachukwu · I. C. Nnaji · C. K. Nkere · J. T. Onyeka · C. N. Egesi
Department of Biotechnology and Product Development, National Root Crops Research Institute, Km 8 Ikot Ekpene Road, Umuahia, Abia State, Nigeria

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2021; Rabbi et al. 2014a). Cassava leaves also have considerable nutritional qualities and serve as food for humans and animals alike (El-Sharkawy 2004; Wasonga et al. 2020). Cassava is an ideal food security crop with the ability to produce optimal yields and can be stored in the ground for long periods allowing harvest flexibility while adapting to the effects of drought and marginal soils (Ceballos et al. 2020). Despite its many strengths, cassava production is hindered by a myriad of abiotic and biotic stresses. Across cassava growing regions, diverse pathogens (including viruses, phytoplasma, bacteria, or fungi) have been implicated in several cassava diseases, and approximately 200 pests (insects and mites) are known to feed off the crop inadvertently causing severe damages and enabling the spread of diseases (Herren and Neuenschwander 1991; Lozano and Booth 1974). Over the years, pests and disease management strategies have seen some successes in mitigating the spread of cassava pests in regions where they are alien, however, mitigation or elimination efforts for diseases caused by viruses or pests native to a region had been challenging (Herren and Neuenschwander 1991; Legg et al. 2015). Current efforts to combat cassava diseases include the development of early phenotyping and detection tools (Okereke et al. 2017; Ramcharan et al. 2017; Sambasivam and Opiyo 2021), cleaning infected planting materials (Maruthi et al. 2019), and genetic improvement for disease resistance through conventional and molecular breeding techniques (Ezenwaka et al. 2018; Rabbi et al. 2014b; Tembo et al. 2017; Wolfe et al. 2015), genetic engineering (Vanderschuren et al. 2012), and genome editing (Gomez et al. 2019; Mehta et al. 2019). This book chapter will focus on the most economically important biotic factors that impede cassava production and we will be reviewing the current biotechnological strategies to develop disease-resistant cassava varieties. In so doing, we identify challenges from these approaches, highlight avenues for further research and conclude with an outlook for pest and disease management in cassava.

1.1.1 Prevalent Cassava Biotic Factors

1.1.1.1 Cassava Diseases

Like most root and tuber crops, cassava is propagated vegetatively to ensure crop uniformity from one planting season to the next. This, unfortunately, contributes to the proliferation and spread of diseases throughout cassava-producing regions. With increasing globalization and urbanization in cassava growing regions, as well as the variability and pervasiveness of climate change, native and emerging cassava disease outbreaks are on the rise in Africa, the Asia–Pacific, and Latin America. Both foreign or alien and native pests and disease pathogens that negatively impact cassava production are not so easy to mitigate (Legg et al. 2015). In sub-Saharan Africa (SSA), viral diseases including cassava mosaic disease (CMD) and cassava brown streak diseases (CBSD) cause devastating losses, affecting the food and income of, especially limited-resource farmers. Many economically important diseases significantly

contribute to yield losses of the host crop, attacking the roots, stems, or leaves. The pathogen, *Xanthomonas axonopodis* pv. *manihotis* which causes cassava bacterial blight (CBB) disease ranks as the 6th most relevant bacterial pathogen in the world (Mansfield et al. 2012). For some of these diseases, their transmission to a cassava host plant is carried out by destructive pests, which feed off the crop, including its succulent green leaves and stems.

1.1.1.2 Cassava Pests

By damaging the leaves, cassava pests affect the photosynthetic capacity of the crop. Several pests damage cassava while feeding, but only a few are considered to be economically important (Table 1.1). There is a significant variation among pests that attack cassava by continent, and foreign pests that are inadvertently introduced in a region where they are not common, cause devastating losses. For example, arthropods such as the cassava mealybug (*Phenacoccusmanihoti* Mat.-Ferr.) and CGM (*Mononychellus tanajoa* Bondar), introduced into Africa and South-East Asia in the 1970s and early 2000s, respectively, potentially cause up to 50% yield losses in local cassava crops (Graziosi et al. 2016). Also, prevailing seasons impact the activities of pests. Arthropod pest complexes for instance, mostly occur in the dry season and not so much in humid regions of heavier rains (Lebot 2008).

Table 1.1 Some economically important biotic stresses across cassava growing regions

Type of pathogen	Disease	Regions		
		Africa	Asia–Pacific	Latin-America
Virus	Cassava mosaic disease	x	x	
	Cassava brown streak disease	x		
	Cassava frogskin disease		x	x
Bacteria	Cassava bacterial blight	x	x	x
Fungi	Cassava brown leaf spot	x	x	x
	Cassava white leaf spot	x	x	x
	Cassava root rot disease	x	x	x
	Cassava anthracnose disease	x	x	x
Pests	Cassava mealybug disease	x	x	x
	Cassava green mite disease	x	x	x

Source Lebot (2008)

1.1.2 Regional Incidence of Cassava Pests and Diseases

There are molecular and archaeological pieces of evidence that support the origin and domestication of cassava from Latin America (Ceballos et al. 2012). In the sixteenth century, cassava was introduced to the Gulf of Guinea by Portuguese slave traders and adopted as a staple in several African countries. Similarly, it is believed that cassava was introduced in southern Asia within the late eighteenth and early nineteenth centuries from Mexico (Liu et al. 2011). Compared to Latin America, Asia and Africa have relatively lower incidences of cassava pests and diseases with studies attributing these low incidences to cassava being an exotic plant in these regions and the presence of cyanogenic glucosides as a deterrent to native pests or disease pathogens (Herren and Neuenschwander 1991). In SSA, the viral diseases, CMD and CBSD are the most economically important, hindering cassava production. Cassava mosaic disease was initially detected in 1894 in Tanzania and has currently become prevalent across multiple cassava-growing regions in Africa. The disease is also a major limiting biotic factor of cassava production in south Asia. Cassava brown streak disease, on the other hand, is most prevalent in East Africa and the Great Lakes region while the bacterial pathogen that causes CBB occurs in all cassava growing regions across the globe. Cassava bacterial blight was previously reported in Brazil in 1920 and has been implicated, in over 40 countries, for devastating losses with a significant economic impact on production and utilization. Apart from the wider continental disparity in incidence, within a country and local agroecological variations in cassava biotic stress prevalence exist (Chikoti et al. 2019; Eni et al. 2021).

1.1.3 Economic Impact of Biotic Stress on Cassava Production and Utilization

Unlike in SSA, where cassava is still primarily a food security crop, the use of Asian grown cassava is incredibly diverse, providing raw materials for biofuels, industrial starch, animal feed, and other cassava-derived products. Over the years CBB has been implicated in several severe epidemics across Africa and south-east Asia (Graziosi et al. 2016). Usually exacerbated by environmental conditions, CBB may cause up to 100% loss of yield or planting materials in regions with very low cassava diversity. Similarly, CBSD and the fungal cassava root rot disease are the major cause of post-harvest losses, rendering cassava roots unusable (Kawuki et al. 2016; Kayondo et al. 2018). This has had huge implications for regions, especially, smallholder farmers that rely strongly on cassava roots for food, income, and industrial raw material. Diseases including brown leaf spot (BLS) and white leaf spot (WLS), as well as pests, have deleterious effects on the quality of cassava leaves which is the most nutritional component of the crop (Wasonga et al. 2020). Broadly, cassava diseases potentially threaten these affordable healthy alternatives, especially in small rural

communities where access to healthcare facilities is limited. Therefore, diseases and pests pose significant threats to the agricultural food sectors of several economies, and embracing control measures, including the development and adoption of cassava varieties resistant to these biotic stresses is imperative to save the livelihood of over 800 million people around the world.

1.2 Biotic Factors Affecting Cassava Production

1.2.1 Diseases

1.2.1.1 Viral Diseases

Cassava Mosaic Disease

Prevalence and Distribution of Cassava Mosaic Disease

The mosaic disease in cassava was first observed more than a century ago in Tanzania and was later in other SSA countries in the twentieth century (Fargette et al. 1990; Fauquet 1990; Thresh and Cooter 2005). The disease is common in all the cassava-growing regions across Africa but has not been verified in Asia, or America (Thresh et al. 1994). It is the most fully documented disease of cassava in Africa in the twentieth century and has been given more attention than any other disease, even before the recent increase in cassava research (Legg and Thresh, 2003). In some African countries, CMD is considered a major disease of cassava whereas, in some other areas, it is regarded as less damaging than CBB and CBSD (Akinbo et al. 2007). The prevalence of CMD exceeds 50% in the leading cassava-producing countries in Africa—Nigeria, the Republic of Congo (DCR), and Ghana (Thresh et al. 1994). Cassava varieties differ largely in their response to CMD with various degrees of symptoms on the crop tissues. Some are from severe stunting, with little or no yield of foliage leaves, stem cuttings, or tuberous roots, whereas other varieties are relatively unaffected and sustain little or no damage (Eni et al. 2021; Fargette et al. 1990; Hillocks et al. 2000).

Causative Agent and Pathogenesis of Cassava Mosaic Disease

Cassava mosaic gemini virus belonging to the genus *Begomovirus* is the actual causative agent of CMDs (Ndunguru et al. 2005). The viral etiology of CMD was first stipulated in 1936 when the disease was suspected to be a viral disease and graft transmissible from cassava to cassava was observed (Swanson and Harrison 1994). In 1975, virus particles were detected following isolation and observation by electron microscopy of geminivirus particles and the successful mechanical transmission of sap from infected cassava to the experimental herbaceous host, *Nicotiana benthamiana,* and back to a susceptible Brazilian cassava cultivar (Bock 1983).

Three CMGs including *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV), and *Indian cassava mosaic virus* (ICMV) have been characterized serologically (Hong et al. 1993; Ndunguru et al. 2005). These three CMGs consist of three different types of iteron sequences, identified and classified into three groups namely ACMV type with isolates of ACMV, EACMZV, and Sri Lanka cassava mosaic virus (SLCMV), EACMV type encompassing all the other EACMV-like viruses and South African mosaic virus (SACMV) and the ICMV type with ICMV isolates alone (Kittelmann and Jeske 2008). Gemini viruses make up a large and diverse group of plant viruses and are characterized by geminate shaped particles of 30 × 20 nm that replicate their circular single-stranded DNA genome (genome size, 2.7–2.8 kb) in the nuclei of infected cells through double-stranded DNA intermediates by rolling circle replication or a recombination-dependent replication mechanism (Jeske et al. 2001). The geminate particles which encapsulate the ACMV genomic components are believed to release their genomic DNA via breaks that occur at the top and shoulders of the virus particles (Kittelmann and Jeske 2008). The *Begomovirus* genome components *DNA-A* and *DNA-B* share a common region of about 200 nucleotides having a high nucleotide sequence identity above 80% (Harrison and Robinson 1999). The common region possesses several regulatory elements as well as two *TATA* motifs and multiple copies of *cis*-elements known as iterons, which are the binding sites for the replication-associated protein (Hanley-Bowdoin et al. 1999).

Mode of Cassava Mosaic Disease Transmission

Cassava mosaic disease is commonly propagated through infected planting material—stem cuttings (Delaquis et al. 2018). Also, the whiteflies species complex plays a natural role in the transmission of CMD in a non-propagative and circulative approach (Islam et al. 2018). The species status of *B. tabaci* (whitefly) has been determined by the phylogenetic relationship of nucleotide sequences of the mitochondrial cytochrome C-oxidase subunit I gene (*cox1*) (de Barro et al. 2011). While whiteflies play a role as disease vectors in short-distance dissemination that is within 20 m (Maruthi et al. 2017), the dissemination of infected cassava planting materials plays a significant role in the long-distance spread of CMD (Delaquis et al. 2018). The use of insecticides that target whitefly density helps to limit short-distance dissemination (Horowitz and Ishaaya 2014). Also, integrated pest management could be adopted to control the population and reduce the build-up of resistance to insecticides by whiteflies.

Symptoms of Cassava Mosaic Disease

Mosaics caused by CMD could be green mosaics or yellow mosaics. Generally, CMD symptoms differ by type, extent, and severity. Plants that are affected by green mosaics have leaves with different sectors of dark and light green tissue and the green mosaics symptoms are clear only when the plants are observed closely. Green mosaics are not usually associated with a distinct decrease in leaf area, leaf number,

plant size, or yield of tuberous roots. On the other hand, plants affected by yellow mosaics are much clearer, as they have leaves with different areas of normal green and yellow tissue (Thresh and Cooter 2005). The chlorotic areas may expand less than other parts of the leaf lamina, which can lead to the folding of the leaflets and rupturing of the tissues, and some CMG strains produce more severe symptoms and greater damage to the growth and yield of cassava than others (Legg et al. 2006). However, there is no evidence for regular disparities between the symptoms caused by the different CMGs so far. Studies have shown that complex infections with more than one different CMGs cause more severe symptoms than a single virus infection (Fondong et al. 2000; Pita et al. 2001). Also, the impact of the environment on CMD symptoms has been documented. Usually, leaves produced during cool weather tend to show more symptoms than those produced during hot seasons (Gibson 1994). Multiple infections with other disease complex or nutrient deficiency, especially, zinc deficiency, make CMD diagnosis difficult since similar damages are observed on the plant tissue (Howeler 2017).

Cassava Brown Streak Disease

Prevalence and Distribution of Cassava Brown Streak Disease

Different strains of CBSD have been identified to be most prevalent in different parts of East Africa. The *cassava brown streak virus* (CBSV) is mainly found in the coastal lowlands of Mozambique and Tanzania, while the *Uganda cassava brown streak virus* (UCBSV) mainly occurs in the highland regions of Uganda and Tanzania (Mbanzibwa et al. 2009). Yield losses associated with CBSD could range from 70 to 100% in the most susceptible cultivars (Hillocks et al. 1999, 2001; Kawuki et al. 2016). Cassava brown streak disease impacts the number, weight, and quality of cassava roots through pitting, constrictions, and root necrosis (Legg and Hillocks 2003). There are several undocumented economic impacts of CBSD and the indirect or unquantifiable consequences on yield related to the deleterious effects on starch quality of non-necrotic portions of affected roots, as well as the additional labor cost incurred in separating necrotic from non-necrotic portions of affected roots have been highlighted (Kawuki et al. 2016).

Causative Agent of Cassava Brown Streak Disease

The etiology of CBSD was uncertain over the past decades since it was thought to be initiated by a virus when the disease was successfully transmitted by grafting (Legg et al. 2015; Tumwegamire et al. 2018). However, the first actual evidence of the causal agent of CBSD was confirmed by mechanical transmission of the virus in a range of herbaceous hosts plants (Kawuki et al. 2016; Legg et al. 2015). These viral particles were subsequently detected by electron microscopy in leaf samples showing symptoms of CBSD (Hillocks et al. 2000). Recent characterization based on a comparison of coat protein and full-length sequences has shown that CBSD is

caused by isolates of at least two phylogenetically distinct species of single-stranded RNA (ssRNA) filamentous virus particles belonging to the *Potyviridae* family and genus *Ipomo* virus (Mbanzibwa et al. 2009; Winter et al. 2010). The first species of *Ipomo* viruses to be discovered was monopartite with a linear, positive-sense, ssRNA genome consisting of about 9000 nucleotides and predicted to produce a polyprotein of 2902 amino acids (Mbanzibwa et al. 2009).

Mode of Transmission of Cassava Brown Streak Disease

In the early 1930s, cassava brown streak viruses were believed to be transmitted by whitefly, *Bemisia tabaci* (Genn.), and was reported in the coastal zone of Tanzania, in East Africa (Storey 1936). Later in the years, the viral etiology was confirmed with a classical demonstration of Koch's postulates (Maruthi et al. 2005; Winter et al. 2010). Unlike CMGs, which are persistently transmitted by *B. tabaci*, CBSVs are transmitted less persistently and are not preserved for more than 24 hours (Dombrovsky et al. 2014). For the later years of the 20th century, the distribution of CBSD was largely restricted to lowland coastal regions of Kenya, Tanzania, and Mozambique, and the surroundings of Lake Malawi in Tanzania and Malawi. The disease is mostly disseminated through cuttings taken inadvertently from infected parent materials (Hillocks et al. 1999; Kawuki et al. 2016; Maruthi et al. 2005).

Symptoms of Cassava Brown Streak Disease

All parts of the cassava plant including leaves, stem, and roots may show CBSD symptoms although, there are differences in the degree of the symptoms based on the factors such as environmental conditions, the growth stage of the crop, relative humidity, the time of infection, and the genetics of the cultivar (Hillocks et al. 1999). There are two types of foliar symptoms from CBSD, type 1 and type 2. Type 1 initially appears as chlorosis along the margins and later tertiary veins, while type 2, is characterized by chlorosis spread in areas between the main veins and later covering much of the leaf lamina (Legg et al. 2014). The root symptoms are considered very lethal and usually develop following foliar symptoms (Hillocks and Jennings 2003). In the most susceptible cultivars, root necrosis may appear within six months of planting cassava cuttings derived from infected plants (Okul Valentor et al. 2018). As the cassava grows into maturity, root symptoms become increasingly destructive and an undocumented secondary impact is the early harvesting of the roots to further spoilage (Hillocks et al. 2001) (Fig. 1.1).

1.2.1.2 Bacterial Diseases

Bacterial and viral diseases constitute the major diseases of economic importance in cassava production and utilization while most others are regarded as minor or of local importance (Hillocks and Wydra 2002). Some of the prevalent bacterial diseases in cassava include CBB caused by *Xanthomonas axonopodix* pv. *manihotis* (Xam),

Fig. 1.1 Healthy (**a**) versus CMD (**b**) and CSBD (**c**) infested cassava tissues (Ano et al. 2021)

prevalent in Africa, South America, and Asia, angular leaf spot (bacterial necrosis) caused by *Xanthomonas campestris* pv *cassava*, reported in East Africa and South Africa, and soft rot of stems and roots caused by *Erwinia carotovora* spps. *carotvora.* Most recently, a new bacterial disease caused by *Enterobacter cloacae* was identified in South America (Santana et al. 2012).

Cassava Bacterial Blight

Prevalence Cassava Bacterial Blight

Cassava bacterial blight is regarded as the major bacterial disease and limits the productivity of cassava in different major cultivation regions including South America, Asia, and Africa, where yield losses could be as high as 80–90% during high epiphytotic periods (Fanou et al. 2018). Disease epidemic and impact on yield vary from year to year which makes its incidence unpredictable and a major risk to subsistent cassava cultivation by smallholder farmers. Cassava bacterial blight is believed to originate from South America due to the high diversity of the pathogen in the region, however, with the dissemination and cultivation of cassava to many

other regions, CBB is currently widespread and prevalent in various regions of the world (López and Bernal 2012).

Symptoms of Cassava Bacterial Blight

The initial symptoms of CBB appear at the start of rains following the end of dry seasons and reach their climax at the height of the rainy season (Hillocks and Wydra 2002). The documented symptoms of CBB include wilting, leaf spotting, die-back, discoloration of vascular tissues in mature stems, and roots of susceptible cultivars with the production of gum exudates on young shoots (Fanou et al. 2018). The CBB cycle is characterized by an epiphytic and parasitic phase. The pathogen survives on seemingly healthy stems during the dry season at the epiphytic phase while at the onset of the raining season it transcends into the parasitic phase when the pathogen multiplies and gains access into the plants through epidermal wounds or natural openings on the leaf (Zárate-Chaves et al. 2021). Initial symptoms appear after about one week as visible translucent water-soaked spots on the abaxial surface of the leaves which eventually become angular dark green spots. These spots later enlarge with adjoining spots joining together to form large brown patches. Lesions formed on the leaves produce creamy white oozes that eventually turn yellow. As the disease progresses, the blight symptom characterized by a superficially burnt appearance usually appears on the affected leaves and leaf tips (Fanou et al. 2018; Hillocks and Wydra 2002; Lozano 1974).

The disease progresses systemically from the leaves into the petiole and woody stem then throughout the entire plant. Multiplication of the pathogen in the vascular system obstructs the movement of water and nutrients thereby inducing leaf wilting which leads to defoliation of the shoot tip. Also, the young growing shoot tip dies producing a characteristic candlestick symptom or tip-dieback. This also affects the newly growing shoots at the lower stem part which also begins to wilt and experience tip dieback. A characteristic brownish discoloration of the vascular systems is easily observed in stems. Primary CBB symptoms in fields newly planted with infected materials are the wilting of the young germinating sprouts with subsequent dieback (Fanou et al. 2018; Lozano 1974).

Etiology of Cassava Bacterial Blight

Cassava bacterial blight is caused by a gram-negative rod Xam which is related to the bacteria from the genre Xanthomonads that cause disease in a host of other plants including rice (He et al. 2010), soya bean (Chatnaparat et al. 2016), citrus (Ference et al. 2018), pepper and tomatoes (Potnis et al. 2011). Though related, each bacterium is proven to be host-specific and Xam is known to be associated only with cassava and other closely related plant species (López and Bernal 2012). *Xanthomonas axonopodis* pv. *manihotis*, produces disease in cassava by delivering type III effector proteins into the plant cell to suppress or modulate host innate immunity and promote pathogenesis. Studies have shown that most Xam strains studied contain from one to five transcription activator-like (TAL) effectors (Castiblanco

et al. 2013; Cohn et al. 2014), which has been associated with virulence for other xanthomonads (Cernadas et al. 2014; Cohn et al. 2014). The TAL effectors when delivered into the host cell are translocated to the plant nucleus where they bind to specific DNA sequences to activate the expression of host genes that facilitates the proliferation and colonization of the host by the bacteria (Cohn et al. 2014; Mak et al. 2012).

There is evidence of classical genetic approaches from mutagenesis and complementation to determine the pathogenicity determinant of the causative agent of CBB. As applicable in other Xanthomonas, different clusters of genes important for pathogenicity in Xanthomonas species have been traced using these methods and other studies and the important pathogenicity factors identified include genes in the Type III secretion system that secretes and translocate effector proteins into the plant cells, cell wall degrading enzymes, exopolysaccharides, and toxins (López and Bernal 2012).

Comparative evaluation of Xam and other Xanthomonas shows a high degree of conservation in gene content and order with no prominent differences found in important pathogenicity factors in the genus. This then infers that the rate of infection lies in differences in specific gene sequences and/or regulatory regions (López and Bernal 2012). Studies of factors that contribute to Xanthomonas disease in their corresponding host plants show that Xanthomonas has developed a range of well-regulated and coordinated traits necessary to adhere to plant tissue, acquire nutrients, suppress plant defense responses, and eventually lead to disease (An et al. 2019).

Response of cassava cultivars to CBB varies and ranges from very susceptible to moderately resistant (López and Bernal 2012). These responses could be expressed as variations in the rate of colonization of the bacterium. Genetic diversity studies of Xam carried out in different regions of the world including African and South America show an increase in Xam diversity over the past three decades which makes the adoption of resistant cassava varieties intricate. Therefore, the continuous evaluation of the bacterial population in fields cultivated with cassava is highly recommended (López and Bernal 2012).

Epidemiology and Control Measures of Cassava Bacterial Blight

Cassava bacteria blight is the most important bacteria affecting cassava production in all the cassava-growing regions globally. Disease occurrence and impact on yield vary from year to year. The first symptom of CBB is usually at the onset of the first rain after the dry season and reaches its maximum when the rains are at their peak (Hillocks and Wydra 2002). Pathogen dispersal within a field is aided by high wind conditions which enable the transfer of Xam from plant to plant in water droplets while dispersal between fields occurs mainly with the exchange of infected propagative material (Lozano 1974). Symptom development on infected plants is favored by rainfall, high temperature, high relative humidity, the incidence of insect vectors, and wounds on the leaves, in addition to high differences between day and night temperatures. The disease is usually managed by employing an integrated control strategy which includes the use of resistant cultivars and improved cultural

methods such as the use of clean planting materials, planting outside of peak epidemic periods, weeding, excluding bush fallow around cassava field, and use of quarantine measures that prevents the introduction of the disease into low disease pressure areas (Banito 2003; Fanou 1999). An excellent method for breaking the life cycle of the pathogen that might persist in the environment in the epiphytic phase is to employ crop rotation between successive cultivation of cassava as well as removing infected leaves by burning and burying infected debris (Banito 2003). Although the use of resistant cultivars has proven to be the most effective control measure for CBB, there is a tendency for reduced durability of resistance as the diversity of the pathogen population increases (Restrepo et al. 2000). It is therefore pertinent to ensure that implementing varietal resistance is coupled with assessing the diversity of pathogen populations in the field and deploying cultivars resistant to the local pathogen populations.

Impact of Cassava Bacterial Blight on Yield

Up to 80–90% yield losses could be attributed to CBB during high epiphytotic periods affecting both fresh root yield and planting materials (Santana et al. 2012). The devastating symptoms of CBB contributing to cassava loss include wilting and leaf defoliation which can be high under favorable climatic conditions. Various climatic, inoculum pressure and cultivar responses affect the rate of susceptibility to the disease. Also, CBB can contribute to a low accumulation of starch in cassava roots (Fanou et al. 2018).

Angular Leaf Spot (Bacterial Necrosis) of Cassava

Angular leaf spot is caused by *Xanthomonas campestris* pv. cassava reported only from East Africa and Southern Africa. The disease is characterized by the secretion of bright yellow exudates from infected leaf tissue during periods of high humidity. Oftentimes, severe necrosis leads to the defoliation of plants. The disease is localized in the cortex of the stems and spreads to the vascular tissues. Predisposing factors to the disease include cultivation on very poor soils and plant injuries resulting from severe rainstorms (Hillocks and Wydra 2002).

Soft Rot of Cassava Stems and Roots

Cassava soft rot of stems and roots is caused by *Erwinia carotovora* sp. *carotovora.* The disease is characterized by internal rotting of stems and branches, dark lesions, external cankers, root necrosis, wilting of young shoots, and tip dieback. Control of diseases could be achieved by the use of uninfected, healthy cuttings of varieties resistant to fruit fly and the use of insecticides (Hillocks and Wydra 2002).

1.2.1.3 Fungi Diseases of Cassava

Fungi threaten cassava production in different parts of the world, destroying the stems or killing the roots of the plant (Bellotti et al. 2012a). The degree of damage, however, is mostly affected by different cassava clones, strains of the pathogen, and the environment (Hillocks and Wydra 2002). Most of the soil-borne fungi spread within the root system and will attack healthy cassava if their specially adapted hyphae come in contact with the young roots, leaf petioles, or stems (Hillocks and Wydra 2002). Some of them can attack older cassava plants by penetrating through old wounds and lesions in stems and through cracks and lesions in the leaves. Some fungal pathogens attack all forms of cassava, causing anthracnose, leaf spots, leaf blight, stem rot, root rot, and sometimes complete crop loss (Hillocks and Wydra 2002). Fungal attack on susceptible varieties causes premature aging of plants, yellowing, or even death of leaves, petioles, or stems. Under favorable conditions, some fungal populations can build up very rapidly resulting in expanse infections on cassava.

The control measures of cassava fungal diseases include the use of well-established scientific methods and good cultural practices (Legg et al. 2006). Cassava varieties with improved fungal resistance can be used for disease control as they reduce the rate of spore penetration on the leaves, allow extended cassava growth under field conditions, and generally help cassava plants resist infection within the field even under high fungal populations (Ahmad et al. 2021; Hussain 2015). Fungi diseases are still major agricultural problems in cassava production and require a biotechnological approach for sustainable management (Aerni 2006). A scientific approach based on the understanding of pathogen biology, epidemiology, and ecology of plant disease has been promoted and should apply to fungi disease control in cassava (Rimbaud et al. 2015). The fungal diseases that attack cassava with their biology, epidemiology, and ecology are discussed below.

Cassava Anthracnose Disease

Cassava anthracnose disease (CAD) caused by *Colletotrichum gloeosporoides* f.sp. *manihotis*, Henn. (Penz) Sacc. is a common and widespread disease that attacks cassava in nearly all cassava growing areas (Akinbo et al. 2007; Owolade et al. 2005). It is one of the major economic diseases of cassava in the tropics. The fungal pathogens can attack cassava stems, leaves, and root tubers, causing disease symptoms ranging from brown to necrotic spots on the leaves, stem and root lesions, and girdling of the plant (Theberge 1985). Cassava anthracnose is regarded as a serious and important disease of cassava with a record of epidemic levels in high rainfall regions.

Symptoms of Cassava Anthracnose Disease

Cassava anthracnose disease (CAD) is characterized by the presence of sunken leaf spots on the leaf lamina measuring 1–10 mm in diameter and up to 30 mm in diameter

in advanced cases. When the disease is fully developed, the center of the spot is white and studded with pinkish fruit bodies of the fungus (Fokunang et al. 1997, 1999b). Infection on the petiole is initiated by hyphae growing from the infected lamina through conducting tissues. In highly susceptible cassava varieties, the leaves blight, wilt, and sometimes defoliation may occur. The pathogen weakens the sprouting of planted cassava cutting where the infection sets in early, causes young stems to wilt and induces cankers on mature stems (IITA 1990). Fresh leaves produced at the beginning of the rainy season are usually the most susceptible and the disease tends to disappear when the dry season begins. At a relative humidity below 70%, the fungus will stop invading plant tissues (IITA 1990). The symptoms of CAD include the development of cankers on stems, branches, and fruits, leaf spots and tip dieback, green portions with shallow oval depressions that are usually pale brown, but with a point of normal green tissue in the center (Fokunang et al. 1999a). In the mature portions of the stems, anthracnose lesions are round, swollen, and in bands, forming deep cankers on the epidermis and cortex, and sometimes deforming the stem as it destroys the eye buds on cassava stems thereby reducing the sprouting potential of cassava stems.

Etiology Cassava Anthracnose Disease

The organism causing this disease has been variously called *Glomerellamanihotis, Colletotrichum manihotis*, *Gloeosporiummanihotis*, and *Glomerellacingulate* (Weir et al. 2012). These are likely traceable to one species, generally characterized by a relatively short straight cylindrical to broadly ellipsoidal conidia, with obtuse ends and truncated attachment points. Under natural conditions, *C. gloeosporioides* f.sp. *manihotis*, penetrates the host through mechanical wounds or sucking insect vectors (*Pseudotheraptusdevastans*, Dist Het. Coriedae) punctures (Weir et al. 2012). The CAD pathogen penetration after insect puncture of young cassava stems, the cracks extend through the cork layer or the epidermis up to the underside of the sclerenchyma, then immediately the lesion extends tangentially under lignified fibers (Owolade et al. 2005; Theberge 1985). The host reacts to the invasion through the generative zone, the layer of cork more precariously formed at the level of the initial necrotic lesion than in the primary healthy stem (Sinkovics 2011). New races of the pathogen can arise by mutation in somatic cells, recombination of nuclear genes during sexual reproduction, by reassortment or exchange of genetic material in somatic cells, and by mutation of extrachromosomal or cytoplasmic genes (Esser 2016). *Colletotrichum loeosporioides* f.sp. *manihotis*, is a specialized fungus on the cassava host, and sporulation and germtube development of the physiologic races of the fungus is highly correlated with pathogenicity and virulence on the cassava host plant (Fokunang et al. 2001).

Epidemiology of Cassava Anthracnose Disease

Cassava anthracnose disease is one of the important cassava diseases and prevalent in most cassava growing regions of Africa, South America, and Asia (Fokunang et al.

2000, 2002). The pathogen survives environmental conditions in its perfect state and *Gloeosporiumcingulata* appears to increase during the dry season. The prevalence, occurrence, and dissemination of CAD pathogens are huge and occur across many ecozones in Africa with incidences ranging from 68 to 100% (Fanou et al. 2018; Fokunang et al. 1999a). Cassava anthracnose disease survival and transmission occur through breeder seeds and post-harvest debris in the field (Fokunang et al. 1997). Also, water splash, air current, insect, or other forms of contact can disperse the disease. The rainfall regime plays an important role in the development and dispersal of CAD pathogens. The rain splash dislodges spores from the acervuli and spreads them along the young cassava stems.

Impact of Cassava Anthracnose Disease on Cassava Yield

Precise yield loss due to CAD has not been quantified specifically due to the occurrence of multiple disease complexes in the field, however, losses could be devasting in certain areas (Makambila 1994). There are also many unreported cases of infection in farmers' fields and experimental plots severely invaded by CAD in rainfall cassava growing zones. Cassava anthracnose disease severity could lead to a significant loss in planting materials and low biomass production. Severely infected cassava stem in some cases results in a 40–60% loss in germination rate. Total crop failure has been reported in cases where infected stem cuttings have been used for planting (Fokunang et al. 2001, 2002).

Control Strategies of Cassava Anthracnose Disease

Cultural Control
The cultural control measures implemented to reduce or eradicate the spread of CAD range from crop rotation, fallow, and manipulation of planting times (Latah 2016).

Chemical Control Measures
The use of chemicals offers control that may not be sustainable due to bioaccumulation and pathogen mutation.

Resistant Varieties
The use of resistant varieties remains a sustainable and most efficient means of controlling CAD.

Quarantine/Sanitation Measures
Major means of dissemination of cassava diseases and pests have been from the exchange of disease-infested cassava stem cuttings for propagation. The origin of CAD has not been reported but its ubiquitous nature in the cassava growing regions is an indication of exchange through the distribution of infected materials. A recent report shows that anthracnose is seed-borne and can be transmitted by infected cassava seeds (Fokunang et al. 1997). Several sanitation measures, in addition to those

legally established by quarantine regulations, could reduce the risk of disseminating the disease (Lozano and Booth 1974).

White Leaf Spot (*Phaeoramularia manihotis*)

White leaf spots caused by *phaeoramularia manihotis* are small in spot size diameter and with a difference in whiteness to the white color spot induced by *Cercospora henningsii.* They vary from circular to angular spots with a diameter between 1 and 7 mm. They are normally clear white, but in some cases, yellowish tending towards brown.

Symptoms of White Leaf Spot

White leaf spot lesions are sunken on both sides of the leaf lamina when compared to half of the thickness of a normal healthy leaf blade. The white spots on the lower leaf surface can be distinguished, although they are sometimes diffusely colored which makes them appear as brown-violet irregular lines that are usually surrounded by brown to yellowish halos. The centers of the spots have light grayish characteristics during the pathogen's fruiting.

Etiology Cassava White Leaf Spot

During the process of infection, the pathogen (*P. manihotis)* forms thin stomata in lesions on leaves. The stromata produce conidiophores in loose fascicles that emerge through the stomata (Fokunang et al. 1997). The fungus penetrates the host through stomatal cavities and then invades the host's tissues through the intercellular spaces. When leaf spots reach 5–7 mm in diameter, a stroma is formed which produces conidiophores. The secondary cycles of the disease are repeated throughout the rainy season as conidia are dispersed by wind or rain splash.

Epidemiology of Cassava White Leaf Spot

The white leaf spot fungus survives the dry season in old, infected tissues and resumes activity at the beginning of the rainy season following the host's new growth.

Impact of White Leaf Spot on Cassava Yield

Yield loss due to WLS is not quite damaging in cassava production but can seriously affect cassava leaves, which can negatively impact the food and income of the population that uses cassava leaves as vegetables.

Management and Control of Cassava White Leaf Spot

The best control for this disease is by using resistant varieties. Significant differences in varietal resistance have been reported. To reduce the severity of infection, recommended cultural practices include reducing excess humidity during planting.

Cassava Brown Leaf Spot

The brown leaf spot is caused by *Passalora henningsii* (previous names include: *Cercosporidium henningsii, Cercospora henningsii,* and *Mycospaerella henningsii*, named after the sexual state). The disease has a broad geographical distribution and can be found in Asia, North America, Africa, and Latin America. The disease naturally attacks *M. esculenta*, *M. glaziovii,* and *M. piauhynsis* (Legg and Alvarez 2017). In the tropics, *C. henningsii* is an important pathogen, causing severe defoliation (Legg and Alvarez 2017; Teri et al. 1981).

Symptoms and Life Cycle of Cassava Brown Leaf Spot

The BLS disease usually occurs between 5 and 6 months after planting on the older and lower leaves of the plant. Leaf spots caused by BLS are circular and up to 15 mm in diameter, becoming angular and limited by veins. The spots are brown on upper surfaces with dark borders, sometimes surrounded by indistinct yellow margins. On the underside, the spots are gray with less distinct borders. Minor veins crossing the spots appear as black necrotic lines. The centers of the spots dry crack and may fall out. As the spots enlarge, the leaves will turn yellow and fall off.

Warm and humid weather increases the severity of cassava BLS. Spores of the fungus produced on the lower surface are spread by wind and water splash. Long-distance spread occurs when spores are carried on planting materials. In general, older leaves are more susceptible to the disease than young leaves. The fungus can continue to live on old fallen leaves until favorable conditions return (Legg and Alvarez 2017).

Impact of Cassava Brown Leaf Spot on Yield

Cassava yield losses up to 30% in Africa, 23% in South America, and 17% in India have been reported due to BLS infection. It generally affects older leaves and is usually late in the growth of the plants.

Management and Control of Cassava Brown Leaf Spot

Cultural Method of Controlling Cassava Brown Leaf Spot
The disease is of minor importance, and good cultural practices such as wide spacing, crop rotations, and early planting should reduce any potential impact. Spacing

between plants helps to lower relative humidity which increases the spread of the disease (Ayesu-Offei and Antwi-Boasiako 1996).

The Use of Resistant Varieties in Controlling Cassava Brown Leaf Spot
There are no known varieties that are resistant to the BLS disease in the Pacific islands, although in other regions there are reports of potential resistant clones although constant evaluation and selection for resistance are recommended (Ayesu-Offei and Antwi-Boasiako 1996; Teri et al. 1981).

Chemical Control of Brown Leaf Spot
If warranted, use copper fungicides or mancozeb for chemical control.

Super-Elongation Disease

The causal agent of cassava super-elongation disease is the fungus *Sphaceloma manihoticola.* The pathogen produces distortion or curling in young leaves, and cankers on the underside of leaves, petioles, and stems. These cankers are lens-shaped and show different sizes. The affected leaves show white irregular spots. The over-amplified elongation of the stem internodes is a characteristic symptom of this disease. The affected stem is thin, weak and the diseased plants are much taller and spindly than the healthy ones. The disease causes progressive dieback of the plant and partial or total necrosis of the leaf blades, resulting in severe defoliation.

Symptoms Cassava Super-Elongation Disease

Super-elongation disease symptoms are more common in the wet season than at other times and that is when the infection spreads rapidly because the spores are distributed easily by wind and rain. High humidity is required for spore germination. Infected plants are taller than surrounding healthy ones. Young shoots, leaves, and petioles become distorted, and bear eye-shaped cankers appear along the midribs and veins. White, irregular spots may occur on the leaf lamina. There may also be dieback and defoliation.

Management of Cassava Super-Elongation Disease

Planting material should be selected from disease-free plants and treating cuttings with captafol solution can control the spread of the disease. The use of cassava cultivars that are resistant to the disease is the most sustainable management measure. In areas where the pathogen is endemic, planting should be carried out during periods with the least precipitation (Table 1.2).

Table 1.2 Major cassava fungal diseases and mean severity in Nigeria

Cassava fungal disease	Responsible pathogen	Distribution	Mean severity
Cassava foliar fungal diseases			
Super-elongation	*Sphaceloma manihoticola*	41.82% in rainforest areas	1.51
Cassava anthracnose disease (CAD)	*Colletotrichum gloeosporioedes*	98.80% of the fields surveyed	2.84
Cassava brown leaf spot (CBLS)	*Cercosporidium henningsii*	93.98% of the fields surveyed	2.4
Cassava white leaf spot	*Phaeomularia manihotis*	84.22% of the fields in Southeast Nigeria	1.62
Cassava root rot fungal diseases			
Phytophthora	*Phytophthora* spp		
Fusarium spp.	*Fusarium solani* *Fusarium oxysporum*	9.1–11.7% in rain forest zones	1.25
Rhizoctonia solani	*Rhizoctonia solani*	1.9% in rain forest zones	1.7
Botryodiplodia theobromae	*Botryodiplodia theobromae*	66.7% in all fields surveyed	2.22

Source Nwokacha and Nwadili (2011), Onyeka et al. (2004)

Cassava Root and Stem Rot

The next most important group of diseases in both Africa and South America are the root rots. The major pathogens of economic significance for root diseases include *Sclerotium rolfsii, Botryodiplodia theobromae, Fomes lignosus, Rosellinia necatrix, Rhizoctonia solani, Phytophthora* spp., and *Fusarium* spp. (Hillocks and Wydra 2002). Cassava root rots are caused by a complex of soilborne pathogens which induce damages that eventually reduce the yield (Ibrahim and Shehu 2014). Cassava yield losses of up to 80% due to rot diseases have been reported (Cock 1987; Theberge 1985). In some areas, total crop losses have been attributed to rot diseases. Among the organisms commonly reported are *Phytophthora drechsleri*, *Rosellinia necatrix*, *Armillaria mellea*, *Rigidoporus lignosus*, *Botryodiplodia theobromae, Sclerotium rolfsii*, and *Colletotrichum gloeosporioides* f.sp. manihotis (Fokunang et al. 2002). The prevalence of cassava of root and stem rot diseases could be higher in the forest than in the wet savanna zones, especially in Africa.

Cassava Soft Root Rot Disease

Several *Phytophthora* species have been associated with soft rots of cassava roots and these often occur with a few other soil-borne fungi, particularly *Pythium* spp. and *Fusarium* spp. *Phytophthora drechsleri* Tucker has been reported from Latin America and *Phytophthora erythropseptica* Pethy from Africa. Soft rot, in general,

is a worldwide problem and cool wet conditions and root damage predispose the tuberous roots to infection. In areas close to drainage ditches or poorly drained soils, losses can be up to 80% (Hillocks and Wydra 2002; Theberge 1985).

Symptoms of Cassava Soft Root Rot Disease
In soft root rot disease, young roots initially show water-soaked patches that eventually turn brown and death of the feeder roots. As the rot progresses, the starch-bearing tissues disintegrate and the affected roots develop a pungent odor. Root dysfunction causes dieback of the terminal shoots and leads to sudden wilting in advanced stages of root decay.

Impact of Cassava Soft Root Rot Disease on Yield
Soft rot infection can cause greater than 80% total root yield loss and loss of planting material (Cock 1987). This happens mainly in susceptible cultivars.

Management of Cassava Soft Root Rot Disease
The use of cultural practices such as good drainage, selection of loose-textured soils, crop rotation, early harvest, and avoiding soils prone to flooding are important in controlling soft root rot in cassava. Treatments with fungicides may help establish the crop, preventing root rots from attacking during the crop's first months. Fungicides based on plant extracts, oils, and cytokinins help control soil fungi while offering a nonpolluting organic alternative. The use of resistant varieties is also important in controlling the disease.

Cassava Dry Root Rot

Cassava dry root rot is usually caused by *Rosellinia necatrix* (Hartig) Berl. or *Armillariella mellea* (Vahl.) Pat. (*Armillaria mellea* (Vahl) Fr.), or by both fungi together (Msikita et al. 2007; Onyeka et al. 2021). These pathogens have been recorded on cassava in different parts of the world, especially in places where the crop is growing in moist soils high in organic matter. The pathogens have a wide host range among woody perennials. The dry root rot fungi normally attack cassava planted after forest clearance and can destroy the roots of affected plants. It can be managed by planting annual crops after forest clearance before planting cassava (Makambila 1994).

Symptoms of Cassava Dry Root Rot
Both fungi produce rhizomorphs which appear as thickened mycelial strands on the outside of the roots. They are white at first and later begin to turn black. Infected roots are discolored and exude a watery liquid when squeezed. Rhizomorphs penetrate the infected tissues. The above-ground symptoms include plant wilting though the plants do not shed their leaves but eventually desiccating to assume a scorched appearance (Theberge 1985).

Impact of Cassava Dry Root Rot on Yield
Cassava yield reduction due to dry rot infection is relatively reduced as it rarely occurs except in newly opened forests and pathogen endemic areas.

Management/Control of Cassava Dry Root Rot

Although the disease has not been reported in young plants, the recommendation control measure is to avoid selecting planting materials from infected crops. Also, establishing cassava fields in newly opened forests should be avoided or proceeded with caution. The use of resistant varieties as planting materials is a good alternative. Crop rotation with grasses should be carried out whenever the incidence of plant death or root rot reaches 3% in the field. Infected cassava residues and/or litter from perennial trees (e.g., trunks and decaying branches) should be eliminated as they can serve as alternate hosts. Loose-textured soils with improved soil drainage should be used for planting cassava.

1.2.2 Pests

1.2.2.1 Cassava Green Mite

Life Cycle of Cassava Green Mite

Cassava green mite is a very serious dry season pest in Africa. Reproduction in CGM is arrhenotokous (Roy et al. 2003); it is a form of parthenogenesis in which unfertilized eggs develop into males. There are four active stages: a six-legged larva, two nymphal stages (proto- and deutonymph), and the adult stage. The growth rate and development of CGM depend on temperature, rainfall, humidity, host plant, and sex (Yaninek and Hanna 2003). In a study in Nigeria, at a temperature of 27 °C, relative humidity of 70% and a photoperiod of 12 h light and 12 h darkness, the development of the egg, larva, protonymph, and deutonymph on leaves of cassava (TMS 30,572) have been recorded at 5.4, 3.0, 1.1 and 2.8 days, respectively (Yaninek et al. 1989a). At the same temperature of 27 °C, the adult female mite lives for 11.6 days including a day for preoviposition and 9.8 days of oviposition, and lays an average of 62.8 eggs over 9.8 days with a maximum reproduction rate of 43.2 progeny. Egg to adult developmental periods has been estimated to be 21.3, 15.5, 12.3, 7.7, and 6.9 days at 20 °C, 24 °C, 27 °C, 31 °C, and 34 °C, respectively. The average life span of these females is 24.4 days. There are two population peaks per year of CGM especially in the tropics. The first peak occurs at the end of the wet season around November to December while the second peak happens at the start of the rainy season between March and April. Cassava green mite is considered a dry-season pest and thrives in the lowlands where high temperatures prevail. Low temperatures and constant rainfall increase the mortality rate of the mites and population density.

Distribution and Prevalence of Cassava Green Mite

Mites have been reported to feed on cassava in the Americas, Africa, and Asia (Bellotti et al. 2012a; Bellotti 2009). The most important are *Mononychellus tanajoa*,

M. caribbeanae, *M. mcgregori*, *Tetranychus cinnabarinus, T. urticae*, *T. truncates*, *T. kanzawai, T. neocalidonicus, Oligonychus biharensis* and *O. peruvianus*. Cassava is the major host for the *Mononychellus* species, while the *Tetranychus* species tend to have a wide host range. The *M. tanajoa* is native to the Neotropics and is the most important mite species, limiting cassava production, causing huge yield losses in both the Americas and Africa.

The first record of a mite attack on cassava was in 1971 in Uganda, East Africa. The mite was identified as *Mononychellus tanajoa* (Bondar), an exotic species of Neotropical origin (Lyon 1973; Yaninek and Herren 1988). This mite was introduced into Uganda on cassava cuttings imported from Colombia, South America and by 1974, the mite had spread to all countries bordering Uganda (Lyon 1973; Yaninek and Herren 1988). It continued to spread from Tanzania into Zambia, Malawi, and Mozambique (Bellotti et al. 1987; Yaninek and Herren 1988). By 1977, it had infested most of the cassava in East Africa (Yaninek et al. 1993). Spreading west from Uganda, *M. tanajoa* made a sudden leap across much of central Africa to Congo and was first found in West Africa in Nigeria in 1979 where it moved rapidly across the broadly similar vegetation from Nigeria to Benin, Togo, Ghana, Ivory Coast at an average speed of spread of 600 km/year (Yaninek et al. 1989b). By 1985, the speed of spread has decreased to 250 km/year as the mite moved through the rain forest in the Ivory Coast, Liberia, Sierra Leone, and Guinea Conakry (Yaninek et al. 1989b). The mite continues to spread throughout the African cassava belt and is threatening a crop that is often the last major food source available for harvest during drought conditions (Herren and Neuenschwander 1991). Cassava green mite could be spread by infected planting material however, the most important method by which CGM is dispersed is by the wind. In the morning, adult mites lower themselves from the leaves on silken threads so that even low wind currents can carry them over long distances. This may account for the rapid spread of the mite (300 km/ year) (Mutisya et al. 2016). The *M. tanajoa* can easily survive on leaves and stems removed from the field. Cassava leaves gathered and sold locally as a vegetable usually remain infested with mites for up to five days before the leaves become shriveled.

Mode of Infestation, Symptoms, and Economic Impact Cassava Green Mite

Cassava green mite is greenish-yellow in color and can hardly be seen with naked eyes. It is a dry-season pest that has piercing and sucking feeding habits (Jiwuba et al. 2020). It feeds by inserting their chelicerae (stylets) into the abaxial surface of cassava leaves and extracting the fluid content of palisade and spongy mesophyll cells (Yaninek and Hanna 2003). This causes chlorosis which increases from a few whitish to yellowish appearance to complete loss of green pigment (Bellotti et al. 2012b). The damage first appears on the surface of developing and newly formed leaves. Symptoms vary from a few chlorotic spots to complete chlorosis, stunted and deformed leaves that it is often mistaken for CMD symptoms.

Heavy infestations of CGM can cause defoliation starting from the apical tip of the plant and lateral buds down to the shoots, resulting in severe candlesticks and

possible dieback. Cassava green mite diminishes the plant's photosynthetic capacity and growth rate, by reducing the leaf area of the plant (Tomkiewicz et al. 1993). Damage by the mite affects the quantity and quality of planting material, increases weed infestation and root rot disease in cassava (Yaninek et al. 1989a).

Cassava green mite incidence is high in the dry season and leads to a 20–80% tuber yield loss, depending on the severity of the attack. Cassava yield and economic losses due to CGM severity threaten rural household incomes and global food security. The results of the multivariate regression analysis studied by Jiwuba et al. (2020) revealed the negative significance of CGM severity on FRY which caused a loss of 20% average yield. From that study, negative correlations between CGM and yield traits (biomass, fresh root yield, and root dry matter content) were also observed. Increasing populations of CGM during the dry season can significantly reduce the dry matter content on the leaves, stems, and roots up to 10–30% during the dry season and 25–45% during the wet season in the roots (Yaninek 1994). Environmental stress such as drought can contribute to a significant reduction of up to 73% root yield loss and a 67% reduction in stem yield in susceptible cultivars (Byrne et al. 1983). Also, a 10% drop in the photosynthetic rate of cassava leading to a 20% decline in dry matter production has been reported (Cock 1982). Schulthess et al. (1987), found that the dry matter lost during the dry season depended on the amount of stress caused by drought, other pests, or pathogens. From that study, the presence of CGM on drought-stressed plants significantly increased dry matter losses in cassava. Losses in unimproved varieties were generally much higher. A strong negative correlation was observed between plant height and CGM severity (Egesi et al. 2007). Ezenwaka et al. (2018), results showed that leaf pubescence, leaf retention, stay green, shoot tip size, and shoot tip compactness are significantly and negatively correlated with CGMs.

Cassava plants recover from drought stress during the subsequent rainy season, but not from mite damage (Yaninek and Hanna 2003). New plant growth is triggered by rainfall and mites are washed off the leaves during rainy seasons. Mites can survive on leaves, stems, and cuttings removed from the field for a period of up to 60 days (Yaninek and Hanna 2003).

Measures of Controlling Cassava Green Mite

Chemical Control of Cassava Green Mite

Cassava is a long-cycle crop and is mostly grown by small-scale farmers with few resources. Chemical control, although technically possible, is not economically feasible for low-income farmers (Bellotti et al. 2012b). Even low doses of pesticides have adverse effects on natural enemies and reduce the yield by up to 33%. Chemical treatments usually cause secondary pest outbreaks and pose a threat of pest resurgence due to rapidly induced pesticide resistance in the long term (Maclntyre and Graham 1976; Nyiira 1982).

Cultural/Agronomic Practices for Cassava Green Mite Control

Early cassava research concentrated on modifying cultural/agronomic practices to reduce losses due to mites. Most of the recommendations are still useful, but their impact is limited owing to technical, social, and economic factors (Bellotti et al. 1999; Byrne et al. 1983; Nyiira 1982) Cassava plants that are 2–9 months are the most vulnerable to CGM infestation (Bellotti et al. 2012). Adjusting the planting times, the way the cuttings were planted, intercropping, de-topping, and removal of infested leaves have been the principal forms of protecting cassava plants from CGM attack. Ezulike et al. (1993), reported that the cuttings planted in a slanting position had mites on the leaves soon after sprouting, but those planted horizontally did not. Cassava intercropped with pigeon pea suffered less damage from CGM and gave higher yield than those grown on a pure stand (Ezulike and Egwuatu 1990). Also, de-topping of the infested shoot tip has been recommended (Lyon 2009), but that aggravates the problem since the resulting lateral shoot growth produces even more new leaves.

Host-Plant Resistance in Cassava Green Mite Management

Cassava clones with pubescent leaves, large compact shoot apices, and enhanced leaf retention and stay green ability suppress the initial buildup of the CGM population and offer higher levels of resistance to CGM than cultivars that lack these characteristics (Jiwuba et al. 2020). Ultimately, leaf pubescence has been shown to favor the colonization of the predatory mite (*T. aripo*) and enhance the ability of the predator to find the prey (CGM) due to the production of herbivore-induced plant volatiles (Onzo et al. 2012).

Biological Control of Cassava Green Mite

The biological control method involves the use of CGM natural enemies to control the population of CGM. Natural enemies of CGM are found in the families of Chrysopidae, Cecidomyiidae, Syrphidae, Anthocoridae, Lygaeidae, Staphylinidae, Coccinellidae, and Phytoseiidae (Byrne et al. 1983; Murphy 1984; Yaseen and Bennett 1977). However, Phytoseiidae is the most common predator of mite in the Neotropical region (Bellotti et al. 2012; Byrne et al. 1983; Yaseen and Bennett 1977). The first introduction of a natural enemy of CGM into Africa was by scientists from the Commonwealth Institute of Biological Control. Later in 1980, IITA began the Africa-wide Biological Control Project (ABCP) to control exotic cassava pests using enemies introduced from the Neotropical region. More than ten species of phytoseiids were shipped from Colombia and Brazil to Africa. The Colombian species were *Galendromus annectens* (De Leon), *Euseius concordis* (Chant) and *Amblyseius limonicus* Garman and McGregor, *Euseius ho*, *Typhlodromalus tenuiscutus, Neoseiulus californicus*, and *Galendromus annectens* while the Brazilian were *Neoseiulus idaeus*, *Typhlodromalus aripo* and *Typhlodromalus manihoti*. None of the Colombian species survived in Africa but the three Brazilian species did. In 1993, *T. aripo* was reported

to be the most successful species released in Africa. A post-release survey showed that the natural enemies are associated temporally and spatially with CGM (Yaninek et al. 1993). The disappearance of *T. aripo* increased the severity of CGM on cassava plants. Cassava green mite is still a serious arthropod pest causing considerable damage to cassava in Nigeria, the largest producer of cassava, so there is a need to look for a genetic source of resistance to CGM.

Genetic Control of Cassava Green Mite

There has been limited work on the genetic control of CGM to uncover genomic regions associated with natural resistance to *M. tanajoa.* Some of the successes recorded include the identification of two SSR markers, NS 1099 and NS 346 which showed high association with CGM resistance (Choperena et al. 2012). Also, two quantitative trait loci (QTL) namely, qCGMc5Ar and qCGMc10Ar were identified on chromosomes 5 and 10 using a biparental population (Nzuki et al. 2017). Also, the genetic mechanisms underlying the resistance to CGM and its phenotypically associated traits (leaf pubescence, leaf retention, stay green, shoot tip size, and compactness) have been studied in diversity panels using genome-wide association mapping (Ezenwaka et al. 2018). The study identified the most significant SNP marker S8_5962253 on chromosome 8. Seventeen candidate genes including, Manes.08G058500, Manes.08G048200, Manes.08G048800, Manes.08G034200, Manes.08G046400, Manes.08G041900, Manes.08G026500, Manes.08G053900, Manes.08G060500, Manes.08G058000, Manes.08G045400, Manes.08G035100, Manes.08G043900, Manes.08G024700, Manes.08G046700, Manes.08G044000, and Manes.08G026900 were found to have a strong association with the genes conferring resistance to insects/pests in plants. Recently, using 109 F_1 progeny derived from a cross between CGM resistant parent, TMEB778, and a very susceptible parent, TMEB419, nine novel candidate genes on chromosome 12 were reported to be linked to CGM resistance (Ezenwaka et al. 2020).

Conventional phenotype-based recurrent selection to breed CGM-resistant varieties is lengthy and resource-intensive due to several biological aspects associated with cassava including low seed set, slow multiplication rate of planting materials, and 12-month growing cycle (Ceballos et al. 2012). Moreover, phenotypic selection for CGM resistance requires dry environmental conditions that favor *M. tanajoa* infestation for the identification of resistant varieties. When and where such conditions occur irregularly or non-uniformly, screening for the trait can be difficult. These challenges can be overcome using genomic-assisted and marker-assisted breeding tools that can facilitate indirect selection (Wolfe et al. 2016) (Fig. 1.2).

1.2.2.2 Cassava Mealybug

This is a distinct dry season pest in Africa that builds up during drought and high temperatures. It is indigenous to South America but was accidentally introduced into Africa in the early 1970s through vegetative planting material. First reported in the

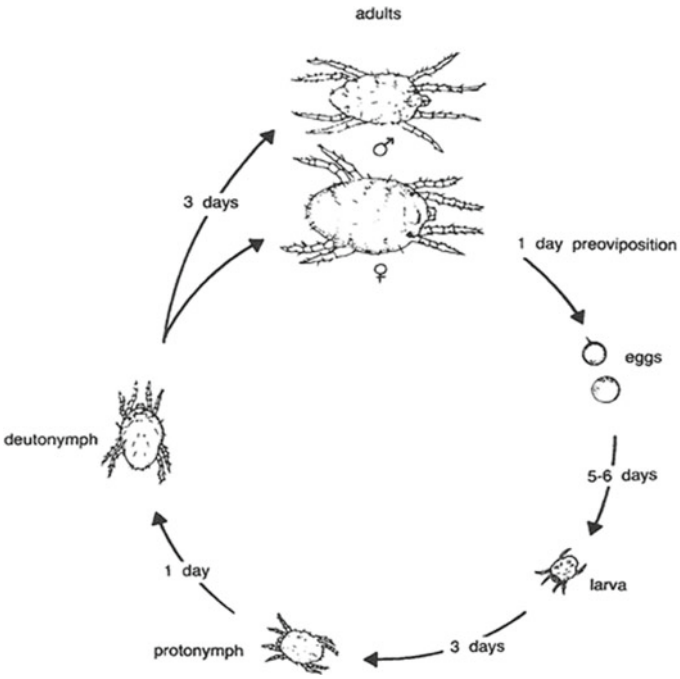

Fig. 1.2 Life cycle of CGM at a temperature of 27 °C with a relative humidity of 70%. Eggs to adult—12.5 days, adult life span—24 days, and fecundity—60 days. *Source* Yaninek et al. (1989a)

Congo Republic in 1973, it has spread to almost all cassava-growing areas in Africa from West through to East Africa and down to the eastern edge of South Africa (Herren and Neuenschwander 1991). The mealybug sucks sap from the phloem. Initially, it attacks the terminal ends of cassava shoots and later spreads to the petiole and expanded leaves. Shoot stunting and shortening of the internodes are believed to be caused by a toxigenic substance present in the insect's saliva. In cases of severe infestation, green shoots die but die-back may not occur. Tuber loss resulting from mealybug infestation has been estimated to range from 70 to 80%.

1.2.2.3 Cassava Whitefly

Worldwide, one of the biggest problems in the production of cassava is the presence of whiteflies (Hemiptera: Aleyrodidae), these whiteflies are complex species that cause direct damage by feeding on the phloem and indirectly by transmitting more than 100 different virus species (Navas-Castillo et al. 2011). A total of 1556 species of whitefly has been described (Forero 2008), but only 15 have been identified to be associated with cassava cultivation (Vásquez-Ordóñez et al. 2015). The genera, Bemisia and Trialeurodes, are only vectors of disease-causing viruses (Navas-Castillo et al. 2011).

In East Africa, the whitefly, *Bemisia tabaci* is among the most challenging to control because it is recognized as a pest species complex which means that multiple biologically distinct species exist within the species complex but cannot be readily differentiated due to the lack of distinct morphological attributes (Boykin and de Barro 2014). The *B. tabaci* cryptic species complex is agriculturally important because members within this complex are important vectors for the transmission of several plant viruses including begomoviruses, which are the most devastating group of viruses in the tropics (Maruthi et al. 2007). In Uganda, several *B. tabaci* putative species have been observed on cassava, including sub-Saharan Africa 1 (SSA1), SSA2, and the Indian Ocean (Boykin et al. 2018). In this group of species, SSA1 transmits viruses that cause the two most devastating cassava diseases namely, CMD and CBSD. These two diseases greatly reduce yields and compromise the quality of cassava tubers (Pennisi 2010). Many CMD-infected cassava varieties produce few or no tubers depending on the severity of the disease and the age of the plant at the time of infection. Phloem-feeding by *B. tabaci*, indirect damage caused by sooty mold, and transmission of plant viruses can cumulatively reduce yields by up to 80% (Kalyebi et al. 2018).

According to records, there has been an increase in the frequency of outbreaks of indigenous SSA members of the *B. tabaci* complex in the cassava growing regions of East Africa over the last 20 years, without much understanding of the ecological factors driving population peaks in *B. tabaci* (Legg et al. 2014; MacFadyen et al. 2018). However, there is no general agreement on managing African cassava *B. tabaci*. Instead, the breeding of new cassava varieties that are resistant or tolerant to CMD and CBSD continues to be the main approach for the management of cassava yield losses. In Uganda, about five improved cultivars are currently in use, including the numerous landraces that vary in yield and general tolerance to pests and diseases (Tumwegamire et al. 2018). Although the improved cassava varieties are generally preferred by farmers because of higher yields, early maturity, and greater resistance to diseases, most smallholder farmers engage in a mixture of varieties either as a mixed planting in one field or planted adjacently to other fields (Akoroda et al. 1987).

During colonization, certain factors may influence *B. tabaci* population growth or lead to adult preferences when colonizing a new plant. Although *B. tabaci* adults are known for long-distance migration, most times, not all movements to new plants are in response to reduced availability of feeding and oviposition sites (Isaacs and Byrne 1998; Mansveld et al. 1982). The *B. tabaci* may have a preference to host plants on which to oviposit via small-scale movements between plants within a field (Costa et al. 1991). Knowing the preferences of *B. tabaci* to oviposit on different cassava varieties and plants of different ages may lead to new management strategies that disrupt the colonization process. These strategies which could reduce *B. tabaci* population growth and ultimately the transmission of diseases may include changing planting time, alternating the variety planted, and increasing planting space (Fig. 1.3).

Fig. 1.3 Whitefly-infested cassava. *Source* Photo: L. A. Nwachukwu

1.3 Approaches for Developing Biotic Stress Resistant Cassava Varieties

1.3.1 Conventional Breeding Approach for Developing Biotic Stress Resistant Cassava Varieties

The use of the conventional breeding approach although it takes a longer time has been practically useful in developing outstanding cassava varieties that are resistant to major pests and diseases (Malik et al. 2020). The conventional breeding approach involves the production of full- or half-sib seeds in crossing blocks. Cassava genotypes are highly heterozygous as a result gives rise to polymorphic progenies that are genetically diverse during hybridization, which simply means that cassava at the F_1 stage is genetically distinct with a high level of segregation taking place. This invariably implies that many crosses are needed to generate seedlings with desirable interest. Cassava as an annual crop required three or more years to achieve a breeding cycle to produce enough stem cuttings for multi-location testing.

Conventional cassava breeding method shows only minor variations across many breeding programs with an emphasis on breeding objectives for pests and diseases resistance among other important agronomic and quality traits for both eastern and western Africa (Fukuda et al. 2002; Jennings and Iglesias 2002; Kawano and Cock 2005).

Breeding objectives depend on the ultimate use of the crop. In cassava, however, increased yield, drought resistance, multiple pests and disease resistance, desirable agronomic traits such as appropriate plant architecture, early bulking of storage roots, with high dry-matter content, low cyanide content, resistance to drought, and other biotic stress and consumer preference traits, e.g., easy peeling and early

vigor in plant growth (for high foliage yield for leaf vegetable) have been the main breeding objectives. Recently breeding for improved micronutrient content has been emphasized (Gregorio 2002).

The breeding values of the parents are evaluated through progeny testing in seedling nurseries. Based on evaluations, selected parental clones or half-sib progenies are hybridized for further improvement in a recurrent selection scheme. Backcrossing has also been a useful procedure for the transfer of resistance into elite populations by providing resistant lines quickly to prevent the severe infestation of relevant pests.

In cassava, resistance to pests and diseases such as super-elongation disease, or reaction to whiteflies or thrips, are considered to have high heritability. For example, the resistance to the trips *Frankliniella williamsi* depends on the pubescence on the leaves in the apical shoot which is stable and readily identifiable. Resistance to the whitefly *Aleurotrachelus socialis* is linked to antibiosis (Bellotti 2009).

Conventionally, breeding stages can be classified into the development phase (hybridization and seedling nursery stage) and evaluation phase (preliminary yield trial—PYT, advance yield trial—AYT, and uniform yield trial—UYT) (Fig. 1.4). To breed for biotic stress resistance cassava conventionally, genetically diverse populations from different backgrounds of agro-ecologies through recurrent selection and backcrossing methods are developed using multiple crossing schemes. Individuals that are resistant to biotic stress are selected as parents and segregating families are generated by multiple crosses among these resistant clones, complementing various agronomic and consumer-quality traits. These parental lines are evaluated

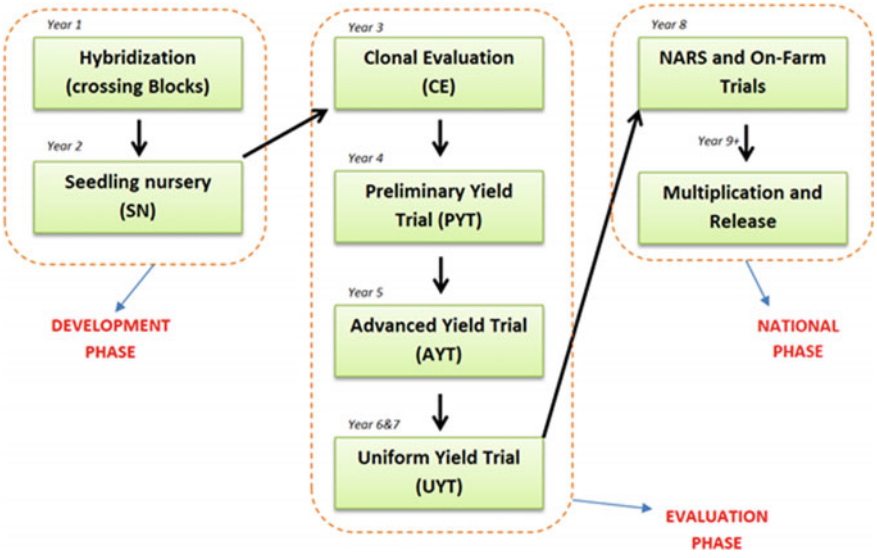

Fig. 1.4 Conventional cassava breeding pipeline

and selected using the progeny testing and breeding values of the seedlings. Resistance can be introduced into the elite population by further making crosses among the selected parental clones in both recurrent and backcrossing breeding methods to prevent infestation and infection of pest and disease respectively.

Thousands of botanical seeds produced from the crosses within one year will be grown in the F1 seedling nursery in year 2. When the seedling nursery field is matured within one year, selection will be carried out based on traits that have high heritability for vigor, architecture, quality, potential yield, pests, and diseases; After which, hundreds of selected individual plants that are pest and disease resistant in the seedling nursery are cloned for single row trials of 5–8 plants per genotype in year 3. At the subsequent stages (PYT, AYT, and UYT stages) of evaluations and selections, the number of genotypes to be evaluated reduced widely from hundreds to tens of genotypes respectively while the number of plants per clone per plot in the different stages of the evaluation process increases progressively (Ceballos et al. 2012). Multilocation evaluation of the selected genotypes is required at the AYT and UYT stages to evaluate resistance and performance across different agro-ecological zones.

High and stable cassava varieties with biotic stress resistance have the opportunity of expanding and exploiting genetic variability that would generate clones with increased value for the different industrial processes where cassava can be a strategic raw material. When cassava is used as a food security crop, additional requirements need to be addressed for a variety to be adopted (Teeken et al. 2018).

1.3.1.1 Breakthroughs of Conventional Breeding for Biotic Stress in Cassava

Conventional breeding has been useful in breeding a range of selected cassava genotypes that combine high stable yields, agronomic and consumer quality with acceptable levels of resistance to CMD, CBB, CGM, and CBSD (Ezenwaka et al. 2020; Kawuki et al. 2016; Okogbenin et al. 2012, 2007). The introduction of Latin American germplasm into the breeding programs in Africa has significantly broadened the genetic base of cassava in Africa. Genotypes with resistance to several pests and diseases have been selected and recombined by genetic crosses to form the basis of selection and breeding for different agro-ecosystem (Ceballos et al. 2016; CIAT 2009; Kawano and Cock 2005). Many breeding approaches including farmer participatory schemes have been employed to evaluate recombinant progenies and select several varieties positive for several traits and widely adapted to a range of environments (Ceballos et al. 2012a).

The presence of the major biotic constraint like CMD, which was not found in Latin America, limits the immediate use of the germplasm from that region and requires introgression of CMD resistance into the Latin American germplasm before being used for breeding. Resistance to CMD has been developed using backcrosses and wide evaluation to account for genotype-by-environment variation. The development of improved germplasm promotes the extension of cassava cultivation beyond

its traditional area in the humid and sub-humid tropics into the semi-arid zones (Kawano 2003).

1.3.1.2 Limitations of Conventional Breeding in Biotic Stress Resistant Variety Development

Most of the important traits in cassava are polygenic (Amma and Sheela 1995). Variation in polygenic traits is attributed to QTL. Quantitative traits in plants are studied using a variety of genetic models and designs including the analysis of mating designs in segregating populations to estimate effective factors using biometrical techniques. Biometrical methods can also be used to estimate useful factors for quantitative traits (Lynch and Walsh 1998). As a result of limitations found in using conventional breeding techniques, advances in molecular breeding techniques are required for breeding for the resistance of cassava varieties to major biotic stress.

1.3.2 Molecular Techniques for Biotic Stress Improvement in Cassava

Although the traditional breeding approach in cassava has been used for a long time, it still has notable drawbacks that require improvement. The traditional approach is mostly based on a single individual phenotypic selection where scanty information including family structure is available and utilized at the early stages of selection. Also, many genotypes are eliminated at this early stage of selection where trials are not replicated. For traits that are highly affected by the constantly changing environmental variables, selections without extensive multi-location and multi-year observation could be unreliable. The evaluation of many genotypes has cost implications in crop improvement. Conventional methods depend on the vast evaluation of genotypes over many years and multiple locations and require a lot of resources to sustain such efforts. Therefore, coupled with the low and somewhat variable flowering and seed-set pattern in cassava, improvement strategies based on the traditional breeding approach could be inefficient, expensive, and difficult (Ceballos et al. 2004, 2012a; Kawano et al. 1998). Therefore, the adaptation of new breeding technologies is necessary to make genetic improvement in cassava more efficient and effective.

The continuous dwindling of sequencing cost brought about by the recent innovations in genomics, molecular biology, and statistical genetics has led to the affordability and availability of molecular markers in many breeding programs, which in turn encourages the adoption of advanced marker-based breeding techniques.

Wide-distributed molecular markers have been important in dissecting the association between the markers and variation in various phenotypes of interest using distinct parents in QTL mapping or a wider diverse population in genome-wide association studies (GWAS). In marker-assisted selection (MAS), molecular markers in

or near genes that affect the phenotype of interest can be used in screening genotypes, to track if the specific gene or chromosome segment(s) known to affect the phenotype is present in a given selection of candidates, individuals, or populations. Various population designs have been adopted for QTL mapping and may include F_2, F_2-derived, backcross inbred lines (BILs), doubled haploids (DHs), recombinant inbred lines (RILs), near-isogenic lines (NILs), chromosomal segment substitution lines (CSSLs), multi-parent advanced generation intercross (MAGIC), nested association mapping (NAM), etc. (Collard and Mackill 2008; Singh and Singh 2015). Evidence of QTL mapping in cassava using mostly F_1, F_2, and backcross populations have been reported for agronomic, productivity, quality, and post-harvest traits (Akinbo et al. 2012; Fernando Cortés et al. 2002; Okogbenin et al. 2008; Okogbenin and Fregene 2003).

Specifically, many QTLs have been identified for biotic stress improvement in cassava including CMD (Akano et al. 2002; Luisa Garcia-Oliveira et al. 2020; Masumba et al. 2017; Nzuki et al. 2017; Okogbenin et al. 2007), CBB (Jorge et al. 2000; Soto Sedano et al. 2017a, b; Wydra et al. 2004), CAD (Boonchanawiwat et al. 2016), CBSD (Luisa Garcia-Oliveira et al. 2020; Masumba et al. 2017; Nzuki et al. 2017), and CGM (Ezenwaka et al. 2020; Luisa Garcia-Oliveira et al. 2020; Nzuki et al. 2017). The potentials for practical application of the dissected QTLs for selection in breeding programs have also been demonstrated, although more evidence, especially for polygenic traits are needed (Bi et al. 2010; Lokko et al. 2006; Okogbenin et al. 2007, 2012; Olasanmi et al. 2021).

Recently, following the increase in funding for cassava research and the availability of improved genomic and other resources, the adoption of GWAS for QTL identification has been on the increase due to the inherent limitations of QTL mapping using narrow populations. Quantitative trait loci mapping detects only a small number of QTL with major effects compared to the total genetic variation underlying most of the polygenic quantitative traits; and it leverages only on the allelic diversity present in a specific family which might not be relevant in other mapping families (Dekkers 2004; Korte and Farlow 2013). Genome-wide association studies, on the other hand, evaluate the association between each genotyped marker and a phenotype of interest that has been scored across a large number of individuals. Genome-side association studies are relevant in detecting causative/predictive factors for a given trait or to determining aspects of the genetic architecture of the trait (Korte and Farlow 2013; Spindel et al. 2015). The adoption of GWAS in the detection of the genetic basis for biotic stress resistance in cassava has been reported (Ezenwaka et al. 2018; Kayondo et al. 2018; Rabbi et al. 2014a; Somo et al. 2020; Wolfe et al. 2016, 2017).

Unlike GWAS, genomic selection (GS) combines marker data with phenotypic and pedigree data (when available) in an attempt to predict the merit or the genomic estimated breeding values (GEBVs) based on the marker genotypes of selection candidates or populations from a prior developed model on observed records (Goddard and Hayes 2007; Lin et al. 2014; Meuwissen et al. 2001). Genomic selection promises to promote rapid selection of superior clones or populations and fast-tracks breeding cycles of plants and animals. Since GS reduces breeding cycles, it saves

phenotyping and the overall cost of breeding. The option of combining genome-wide markers, phenotypic, pedigree, environmental, and omics information such as transcriptomics and metabolomics in GS creates an endless possibility of improving prediction accuracies in many settings (Pott et al. 2021; Xu et al. 2017).

Besides, the ability to leverage on correlations between traits, the possibility of multiple traits prediction and selection have been demonstrated with improved prediction accuracies over the single trait GS (Calus and Veerkamp 2011; Ikeogu et al. 2019; Jia and Jannink 2012; Okeke et al. 2017). The use of genome-wide markers ensures that all QTL in linkage equilibrium with a minimum of a single marker and hopes to capture most of the genetic variation of mostly quantitative traits. The breeding lines with high GEBVs could serve as a potential material in breeding programs. The implementation of GS in cassava breeding serves as a model for the improvement of root and tuber as well as orphan crops. It provided empirical evidence on the potential of GS in crop improvement. The practical application of GS for biotic traits improvement in cassava is well underway (Kayondo et al. 2018; Ozimati et al. 2018; Wolfe et al. 2016, 2017). It is being adopted as the modern breeding tool in many cassava breeding programs.

1.3.3 The Adoption of Genetic Engineering in Genomic Designing for Biotic Stress Resistant Cassava

Studies have shown that the introduction of genetically engineered crops has had significant impacts on disease management (Bart and Taylor 2017). Over the years, there has been a concerted effort to engineer resistance against several economically important cassava pests and diseases, due to bottlenecks encountered using conventional or molecular breeding approaches. These bottlenecks mostly stem from cassava being propagated asexually resulting in severe inbreeding depression (Ceballos et al. 2015; de Oliveira et al. 2018). Increasing investments in cassava genomics research and reducing costs of next-generation sequencing have led to the development of genomic resources that provide insight into the cassava genome, accelerating progress in cassava research and genetic improvement (Malik et al. 2020; Mbanjo et al. 2021). Engineering for genetic improvement and most especially disease resistance in cassava has mostly relied on stable *Agrobacterium-mediated* transformation of friable embryogenic calli (FEC) or pathogen-derived resistance. This transgenic technology was adapted from the mechanism by which *A. tumefacians* causes a plant tumor called "crown gall" (Howell et al. 2018). It was discovered in the '70s that a large portion of the *A. tumefaciens* tumor-inducing plasmid, called transfer DNA (T-DNA), could be transferred to plant cells causing a permanent genetic change. The T-DNA could be replaced with a gene of interest and transferred to plant cells inducing specific permanent changes. Compared to other methods of transformation like the microprojectile bombardment method, *Agrobacterium-mediated* transformation is highly efficient, with reduced risks of carrying chimeras, highly reproducible,

requiring simple equipment, and expresses stable transgenes (Bull 2015). Under contained conditions like a growth chamber or greenhouse, both methods have been used to inoculate cassava plantlets with CMD in a bid to screen for resistance among plantlets (Fofana et al. 2004; Vanderschuren et al. 2009). These methods of evaluation for resistance are usually lengthy (12–22 weeks). In 2017, (Beyene et al. 2018) reported on the use of a virus-induced gene silencing method that rapidly (2–4 weeks) screens for resistance to CMD.

In 2005, Zhang et al. (2005) demonstrated that high levels of resistance to CMD were a result of a natural defense mechanism of plants called post-transcriptional gene silencing or RNA silencing. Over the years, different laboratories have optimized interference RNA produced from this mechanism for CMD and CBSD resistant genes in the model cassava genotype TMS60444. This has produced varying levels of resistance against several *Begamoviruses* and both *Ipomoviruses* (Ntui et al. 2015; Rey and Vanderschuren 2017; Vanderschuren et al. 2012). Subsequent control field trials have been carried out to assess the efficacy of the transgenic plants in high disease locations in East and West Africa (Lin et al. 2019). In the model cassava cultivar, 60,444, there have been attempts to engineer resistance against CBB by identifying microRNAs that respond to the pathogen, over-expression, and silencing of the resistant gene *RXam1* and the over-expression of R gene *Bs2*, with varying levels of success (Quintero et al. 2013; Zárate-Chaves et al. 2021). To sustain the efficient generation of transgenic cassava transformation protocols must enable the production of stable FEC that can be sub-cultured to establish embryogenic suspensions (Zhang et al. 2017). Studies have shown that FEC induction in cassava is genotype-specific. For example, Ugandan scientists were able to produce high-quality FEC from local varieties Ebwanatereka and Aladu but not Bukalasa (Apio et al. 2015). It is therefore necessary that FEC production protocols are developed and optimized for each cassava genotype. Recent reports have identified a phenomenon where transgenic cassava carrying the CMD-type 2 resistant gene loses their disease resistance. Due to this drawback, gene editing technologies are currently being used to circumvent this sudden susceptibility.

1.3.4 Genome Editing in Genomic Designing for Biotic Stress Resistant Cassava

Pathogens in a plant-pathogen interaction have adopted different forms of circumventing a host plant immune response. One of such forms involves the pathogen secreting proteins or effectors that interact with a singular host susceptibility gene or a gene family (Bastet et al. 2017). Mutations of these susceptibility genes have been used as sources of resistance for many years (Dangl et al. 2013). Genome editing allows a rapid and more precise approach to modifying specific sequence sites conferring resistance or other genetic gains to the host organism. Because of its simple design and ease of use, clustered regularly interspaced

short palindromic repeats/Cas systems (CRISPR/Cas systems) are fast becoming the genome-editing tool of choice. It is a mechanism adapted from the defense pathway of bacteria and archaea. This technology uses *Cas* 9 (endonuclease of *Streptococcus pyogenes*) guided by a synthetic single-guide RNA to create double-stranded breaks at targeted sites of invading phages or conjugative plasmids. These breaks undergo repairs frequently through an imprecise non-homologous end-joining repair machinery that generates insertions or deletions (INDEL) altering the target gene's function. This system has been demonstrated to confer resistance to several destructive geminiviruses, *Xanthomonas,* and potyviruses in model plants like *Nicotiana benthamiana, Arabidopsis thaliana,* and tomato (Chandrasekaran et al. 2016; Nekrasov et al. 2017; Pyott et al. 2016). Gene editing technologies can accelerate this process of conferring resistance, by allowing the transfer of strategies from model plants to non-model crops like cassava. The frequently used CRISPR-Cas9 system is still in its infancy in cassava genetic improvement with varying levels of success (Bull et al. 2018; Odipio et al. 2017). The genetic editing strategy has been implemented against three major cassava diseases in SSA; CBSD, CBB, and CMD. Against CBSD, previous studies on how potyviruses evade a plant host immune system indicated that the pathogens generate viral genome-linked protein (VPg) that must interact with members of host eukaryotic translation initiation factor 4E (eIF4E). Of the five members of cassava's eIF4E, yeast two-hybrid and co-immunoprecipitation experiments showed that the viral VPg interacts mostly with novel cap-binding protein (nCBP). Editing the nCBP produced mutants in the model cassava 60,444 and on graft inoculating the plant with the pathogen revealed a delayed response with reduced symptom severity (Gomez et al. 2019). Against CBB, the effector protein *Xam-TAL20*, secreted by the pathogen, interacts with the promoter of cassava *MeSWEET10a*, altering its expression (Cohn et al. 2014). When it does not interact with this susceptibility factor, symptoms are markedly reduced (Lin et al. 2019). By exploiting the repair machinery at the cassava *MeSWEET10a* a tool was recently developed to visualize the initial steps of CBB infection, for the first time in vivo (Veley et al. 2021) Unlike the other pathogens, CMD does not have a known susceptibility factor to be altered, but the pathogen itself can be a target for genome editing (Gomez et al. 2019). Although genome editing of the CMD pathogen produced mutant forms it failed to confer resistance to the disease in cassava plantlets and subsequently produced novel viral mutants that cannot be altered by the CRISPR/Cas system. Unintended consequences like this are one of the reasons why stringent biosafety regulations are required for handling transgenic materials.

1.4 Future Perspectives in the Genomic Designing for Biotic Stress Resistant Cassava

Biotic stress affects the yield of cassava and negatively impacts the economic well-being as well as the nutrition of millions of people around the globe that depend on

the crop for their livelihood and source of food. Fortunately, besides the integrated approaches adopted in controlling biotic stress, there are genetic resources in many breeding programs that are available in combating and breeding for resistance to known and immerging pathogens of biotic stress in cassava. Although traditional plant breeding methods for improving biotic stress resistance in cassava have been successful, the method is generally laborious, inefficient, and time-consuming. The adoption of emerging technologies is therefore relevant to meet the rising demand for cassava as food, feed, and a source of industrial raw material for so many processes. Similar to integrated pests and disease management schemes, the strategic design of integrated techniques will be important in developing new products that will meet the need of farmers. The sustainability of the cheap sequencing method will continue to play important role in supporting the ongoing adoption of new breeding tools for the development of varieties that combine biotic stress resistance with high yields and good quality traits in cassava. Integrating high-throughput phenotyping or phenomics with other techniques including transcriptomics, metabolomics, bioinformatics, and genome editing will continue to revolutionize cassava breeding leading to improved selection accuracy, higher genetic gain, and reduced breeding cycle in the crop. Good data storage and management system has been pivotal and will continue to play a key role in modern cassava breeding for biotic stress improvement.

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Chapter 2
Genomic Designing for Biotic Stress Resistant Cocoa Tree

Fredson dos Santos Menezes, Jonathan Javier Mucherino-Muñoz, Cláusio Antônio Ferreira, Saulo Fabrício da Silva Chaves, Ceslaine Barbosa, Livia Santos Lima Lemos, Natalia Jordana, Mariana Carvalho, José Luis Pires, Raner José Santana Silva, Karina Peres Gramacho, Rafael Moysés Alves, Ronan Xavier Corrêa, and Fabienne Micheli

Abstract Cocoa tree (*Theobroma cacao* L.) is cultivated mainly in tropical regions and produces beans that are used for chocolate manufacture. Worldwide, cocoa bean production is threatened by biotic stresses, mainly fungus, oomycetes, virus and other pests. The understanding of the determinism of the plant-pathogen interactions as well as the different and integrated ways to manage the cocoa diseases at field level began the focus of several research groups. Here, we did an overview of the several

F. dos Santos Menezes · J. J. Mucherino-Muñoz · C. A. Ferreira · R. J. S. Silva · R. X. Corrêa · F. Micheli (✉)
Centro de Biotecnologia e Genética, Universidade Estadual de Santa Cruz, Rodovia Ilhéus-Itabuna Km 16, Ilhéus, Bahia 45662-900, Brazil
e-mail: fabienne.micheli@cirad.fr

R. X. Corrêa
e-mail: ronanxc@uesc.br

J. J. Mucherino-Muñoz
Department of Forest Management, Facultad de Ciencias Forestales y Ambientales, Universidad de Los Andes, Mérida 5101, Venezuela

S. F. da Silva Chaves
Universidade Federal de Viçosa, Av. Peter Henry Rolfs, s/n, Campus Universitário, Viçosa, Minas Gerais 36570-900, Brazil

C. Barbosa · N. Jordana · M. Carvalho · J. L. Pires · K. P. Gramacho
Cocoa Research Center (CEPEC), CEPLAC, Km 22 Rod. Ilhéus-Itabuna, Ilhéus, Bahia 45600-970, Brazil

L. S. L. Lemos
Universidade Federal Do Sul da Bahia, Campus Paulo Freire, Praça Joana Angélica, 250, Bairro São José, Teixeiras de Freitas, Bahia 45988-058, Brazil
e-mail: livia.lemos@cpf.ufsb.edu.br

R. M. Alves
Embrapa Amazônia Oriental, Trav. Dr. Enéas Pinheiro, s/n, Bairro Marco, Caixa Postal 48, Belém, Pará 66095-903, Brazil
e-mail: rafael@cpatu.embrapa.br

F. Micheli
CIRAD, UMR AGAP, 34398 Montpellier, France

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cocoa diseases, of the traditional breeding methods as well as the molecular assisted ones recently developed, of the molecular and omics resources currently available, and of the new biotechnology approaches—including genome edition and nanotechnologies—that are used at basic and applied research levels. We also described the main germplasm and collections worldwide as well as the use of the cocoa diversity as main source of disease resistance.

Keywords Biotechnology · Cocoa genes and genomes · Diversity · Plant diseases · Traditional and assisted cocoa breeding

2.1 Introduction

Cocoa tree (*Theobroma cacao* L.), the main source of raw material for the manufacture of chocolate, is a plant originated from the Upper Amazon Basin and currently cultivated mainly in tropical regions of the world, where it finds favourable conditions for its development (Gardea et al. 2017). It is a culture of high economic importance, especially for the African and American continents, which are responsible for about 75% and 17% of world production, respectively (https://www.icco.org/). At a social level, this agricultural activity generates jobs, directly and indirectly, throughout its entire production chain, thus ensuring a greater source of income, especially for smallholder farming (https://www.worldcocoafoundation.org/). According to the world region, cocoa tree also has relevant environmental importance, as observed in Latin America where it is cultivated under the Atlantic Forest covering in a system called *Cabruca*, which contributes to the maintenance of biodiversity and carbon sequestration (Franzen and Borgerhoff Mulder 2007; https://www.tnc.org.br/o-que-fazemos/nossas-iniciativas/cacau-floresta/).

Even its socioeconomical and environmental importance, the cocoa production has been highly threatened by biotic stresses, especially by phytopathogenic attacks such as *Phytophthora* ssp. (Kellam and Zentmyer 1986), *Moniliophthora perniciosa* (Aime and Phillips-Mora 2005), *M. roreri* (Phillips-Mora and Wilkinson 2007), *Ceratocystis cacaofunesta* (Engelbrecht et al. 2007a) and *Cacao swollen shoot virus* (CSSV) (Abrokwah et al. 2016), which, finding susceptible hosts and favourable environments, are able to establish compatible interactions, to trigger the characteristic symptoms of the corresponding diseases, and finally to drastically affect the productivity levels of this crop.

Aiming to contain or mitigate the damage caused by these pathogens, research has been intensively invested in supporting *T. cacao* breeding programs around the world (Bekele and Phillips-Mora 2019), with the main intentions of providing new knowledge to help in adoption of control measures based on genetic resistance. For this, a multidisciplinary knowledge is needed, ranging from classical techniques to modern and sophisticated molecular approaches, which were enhanced thanks to the sequencing of the genome of this species (Argout et al. 2011, 2017; Motamayor et al. 2013). The application of such methodologies, individually or together, mainly

provides knowledge of the genetic diversity and characterization of this species. This serves as a basis to assist in the selection of potential genotypes for resistance through classical breeding and genome-wide association study (GWAS), that can be recommended as crop varieties or as parents for further establishment of promising hybrids (Osorio-Guarín et al. 2020; McElroy et al. 2018; Marita et al. 2001; Lanaud et al. 2004; Romero Navarro et al. 2017), as well as, through genetic engineering-based applications, to select genes potentially involved in triggering defence responses, which can be later used to increase the degree of resistance in a given genotype that already has other favourable characteristics for cultivation (Scotton et al. 2017; Maximova et al. 2006; Fister et al. 2018; Helliwell et al. 2016; Shi et al. 2010, 2013).

As mentioned above, to reach this final result, it is necessary to form a solid and diversified base of knowledge about the genetics and molecular biology of *T. cacao*, and this foundation was divided into topics that will be explained throughout this chapter.

2.2 Description on Different Biotic Stresses

The cocoa diseases with the greatest threat to world production are caused by the basidiomycete fungi of the genus *Moniliophthora.* These are *Moniliophthora roreri* (frosty pod rot) and *Moniliophthora perniciosa* (witches' broom). The third most important disease is black pod of cacao caused by *Phytophthora* ssp., which is practically ubiquitous to all cacao producing countries. Virus disease such as the *Cacao swollen shoot virus* (CSSV) disease, has reduced cacao production and is of particular concern in Africa. In Indonesia, *Ceratobasidium theobromae* is of economic importance. The vascular streak dieback or the *Sahlbergella singularis* (mirids) are also responsible for cocoa production damages, but with less intensity and/or in a geographically localized way. These diseases will be highlighted in this chapter (Table 2.1).

2.2.1 Frosty Pod Rot of Cocoa

The frosty pod rot of cocoa caused by *Moniliophthora roreri* is restricted to the western continent, and it is present in 11 countries of Tropical America. Brazil was free of the disease, but the report of the disease in July 2021 in a small city of Acre, western of north Brazil, is a call for the Brazilian cacao chain. The first official report of *Moniliophthora roreri* was in 1917 in Ecuador, but its centre of origin and distribution is Colombia (Phillips-Mora and Wilkinson 2007). Losses by the disease vary from 25 to 100% depending on climate conditions and plant genetic background, culminating in the field abandonment, having already caused serious socioeconomic problems (Bailey et al. 2018). In natural conditions, only the pods of the genera *Theobroma* and *Herrania* are affected by the disease.

Table 2.1 Information on the distribution of the most prevalent cacao diseases

Disease	Pathogen	Geographical spread
Virus diseases	*Cacao necrosis virus (CNV)*; *Genus Nepovirus*	Ghana, Nigeria
	Cacao swollen shoot virus; *Genus Badnavirus* (CSSV)	Benin, Côte d'Ivoire, Ghana, Liberia, Nigeria, Sierra Leone, Togo Reports also in Sri Lanka
	Cacao yellow mosaic virus; *Genus Badnavirus* (CYMV)	Sierra Leone
	Trinidad cocoa virus; Genus Badnavirus	Trinidad; isolated occurrences
Witches' broom disease	*Moniliophthora perniciosa*	Brazil (Bahia, Espirito Santo, Amazonian regions), Bolivia, Colombia, Dominican Republic, Ecuador, French Guiana, Grenada, Guyana, Panama, Peru, St. Lucia, St. Vincent, Suriname, Trinidad and Tobago, Venezuela
Moniliophthora pod rot (frosty pod rot or moniliasis disease)	*Moniliophthora roreri*	Belize, Bolivia, Colombia, Costa Rica, Ecuador, El Salvador, Guatemala, Honduras, Jamaica, Mexico, Nicaragua, Panama, Peru, and western Venezuela
Black pod rot	*Phytophthora* spp.	Most cocoa-producing countries worldwide
	P. citrophthora	Brazil, Cameroon, Costa Rica, Côte d'Ivoire, Dominican Republic, El Salvador, French Guiana, Guatemala, India, Indonesia, Jamaica, Mexico, Panama, Peru, Trinidad, Venezuela
	P. heveae	Brazil, Cameroon, Cuba, India, Malaysia, Mexico, Philippines
	P. megasperma	Brazil, Cuba, India, Malaysia, Venezuela, Philippines
	P. nicotianae var. *parasitica*	Brazil, Cuba, India, Malaysia, Philippines
	P. megakarya	Bioko (Fernando Po), Cameroon, Côte d'Ivoire, Gabon, Ghana, Nigeria, São Tomé and Principe, Togo

(continued)

Table 2.1 (continued)

Disease	Pathogen	Geographical spread
Ceratocystis wilt	*C. cacaofunesta*	Brazil, Cameroon, Colombia, Costa Rica, Ecuador, French Guiana, Trinidad, Venezuela
Vascular streak die-back	*Ceratobasidium theobromae*	Most cacao-growing areas in South and South East Asia: China (Hainan Island), India, Indonesia, West Malaysia and Sabah, Myanmar, PNG, (islands of New Guinea, New Britain, New Ireland), southern Philippines, Thailand, and Vietnam
Mealybug	Several species	All cacao-growing regions

M. roreri has a hemibiotrophic lifestyle with well-defined prolonged biotrophic phase. The spores, the only infective propagule, can infect fruits at any stage of development, but fruits are more susceptible during the first stages (45–60 days old); the older the pods lower the susceptibility. Thus, the pods are the primary sources of dissemination. Spore germination requires high humidity and an average temperature of 22 °C. Infection occurs mainly through the cuticle or stomata, first colonizing the apoplasm of the cortical parenchyma cells. After a prolonged incubation period that can last 45–90 days after the infection (Griffith et al. 2003), brown lesions are developed. The biotroph/necrotroph shift is coordinated with the shift from green to necrotic pods (Bailey et al. 2018). A snow-white pseudostroma and powdery spores of *M. roreri* develop rapidly on the pod surface following necrosis (about 3–10 days later). From then on, the pod becomes sporulating and necrotic (Phillips-Mora and Wilkinson 2007). The potential of inocula is very high, and one pod can produce between 44 million and 7 billion spores/cm^2 (Ram et al. 2004). The average temperature between 22 and 30 °C, air moisture higher than 80%, and available water, favour the germination and penetration of the spores (Evans 1981; Orea et al. 2017). Spores are long-lived, abundant during the dry season, and survive on crop residues. Thus, the dry season is a critical factor in determining *M. roreri* survival between harvests. It is a guarantee of inoculum sources available at the beginning of the wet season (Evans 1981). The spores are dispersed by wind and rain. But, human-mediated dispersal is the main form of long-distance dissemination.

M. roreri propagates mainly clonally (Bailey et al. 2018; Díaz-Valderrama and Aime 2016) and remains in a primarily haploid stage throughout its life cycle. Through the amplified fragment length polymorphism (AFLP) and inter-simple sequence repeat (ISSR) methodology, the genetic groups of *M. roreri* were determined and four main genetic groups were identified: Co-Central, Co-West, Bolivar, and Gileri. Corroborating to the *M. roreri* Colombian origin idea, four of the genetic groups were found in Colombia, with Co-East and Co-Central being apparently

endemic in this country. The Gileri group was the only group not found in Colombia and probably is exclusively found in Ecuador (Phillips-Mora 2003).

Control of frosty pod rot is done mainly by genetic control associated with good agronomic practices. Cultural control consists of the removal of potentially diseased pods right at the onset of necrotic lesion. In addition, cultural treatments such as mowing, threshing, pruning, fertilization of cacao trees, drainage, and treatment with anti-sporulates are efficient (Ram et al. 2004). Chemical control with protective fungicides, such as copper fungicides and oxychloride-based fungicides (Suarez Contreras and Rangel Riaño 2013), and systemic fungicides such as trizols and strobirulines is recommended to avoid build-up of epidemics. Biological control is carried out using antagonistic microorganisms such as bacteria of the genus *Pseudomonas* and *Bacillus* and fungi of the genus *Trichoderma* and *Clonostachys* (Krauss et al. 2003). The use of resistant varieties is also a promising alternative, but clones descending from Scavina (SCA), a reference for resistance to witches' broom disease, do not show resistance to frosty pod rot attack. However, some clones from Peru, Costa Rica, Colombia, and Ecuador show levels of resistance to the disease (Phillips-Mora et al. 2005).

2.2.2 Witches' Broom Disease

Witches' broom disease is the second most important threat of a cocoa plantation in South America. It's causal agent *Moniliophthora perniciosa* (Stahel) Aime Phillips-Mora (Aime and Phillips-Mora 2005), belongs to the phylum Basidiomycota, class Agaricomycetes, order Agaricales, family Marasmiaceae. Although the witches' broom disease was first identified in the Brazilian Amazon Forest in 1785, the disease was only officially mentioned in 1904, reporting the occurrence of the disease in Suriname in 1895 (Went 1904). In the following years, the disease spread rapidly and is currently distributed in the main cocoa-producing countries in tropical America such as Brazil, Bolivia, Ecuador, Colombia, Peru, Panama, Guyana, Grenada, Caribbean Islands, Trinidad and Tobago (Mondego et al. 2008; http://www.cabi.org/isc/datasheet/16054).

Moniliophthora perniciosa has co-evolved with cacao in the western Amazon basin in an area that includes eastern Ecuador (the Orient region), north eastern Peru and south central Colombia (Pound 1938; Clement et al. 2010). Thus, the *Malvaceae, Theobroma,* and *Herrania* species are its primary hosts; the economical ones being cocoa (*Theobroma cacao* L.) and cupuaçu (*T. grandiflorum).* However, after the witches' broom disease introduction in Bahia, in 1989, several alternative hosts belonging to *Solanaceae, Malpighiaceae*, *Bignoneaceae*, *Bixaceae* have been reported as *M. perniciosa* hosts (Griffith and Hedger 1994a, b). However, this is still debatable if these species are indeed *M. perniciosa* hosts as there is a lack of pathogenicity tests to prove these as host's range of *M. perniciosa.* To our knowledge, Brazil harbors the greatest diversity of hosts ever recorded, with 29 wild and cultivated species (Lisboa et al. 2020; Patrocínio et al. 2017). If these species are proven as

primary hosts of *M. perniciosa,* the natural distribution of this fungus will be probably one of the largest ever described for an obligate plant pathogen. A phylogenetic analysis and pathogenicity studies support the hypothesis of specificity of the fungus to the host of origin.

Studies on fungal populations showed that *M. perniciosa* has high genetic variability, with populations varying according to country of origin, and host-specificity. Thus, there are differences in pathogenicity and genetic diversity among Peruvian, Ecuadorian, and Brazilian isolates. Further studies in Brazil showed that *M. perniciosa* strains harvested from cocoa resistant selected varieties recommended for planting are genetically different from the strains collected from unselected local genotypes (Pires 2003). Also, temporal studies have shown a significant increase in disease severity in progenies of the primary source of resistance (Scavina) and varieties recommended to farmers and derived from that source (Pires 2003; Gramacho 2003).Samples of *M. perniciosa* isolated from resistant and susceptible cacao genotypes, differed genetically, thus indicating an evolutionary process towards the Scavinas resistant sources.

The *M. perniciosa* life cycle is characterized by the basidiocarp formation, in which basidiospore, the only infective propagule is produced. Basidiospores are very sensitive and short-lived. Germination occurs under high humidity and average temperatures of 22 °C. Spores are disseminated to the infection courts; any meristematic plant organs such as fruits, flower, and apical shoots, adhering to and penetrating the natural opening, through stomata, the base of trichomes, or directly through the cuticle (through enzymatic digestion) (Purdy and Schmidt 1996; Sena et al. 2014). At this phase, colonization is mainly in the apoplastic tissues, and 45–60 days later *M. perniciosa* shifts to intracellular colonization. In this stage, the mycelia are characterized by thin, dikaryotic hyphae with clamp connection and have a saprophytic life-style (Sena et al. 2014).

The symptoms of witches' broom disease depend on the type and stage of the tissue that is infected by *M. perniciosa.* Infected branches increased in thickness, and compared with uninfected branches, infected branches appear distinctly swollen. Large numbers of smaller branches or witches' brooms are often formed eventually on the infected branches. Symptoms appear 30–60 days after the infection. Afterward, they begin dry and about 90 days later until they reach complete dryness. Infected flower buds can produce several flowers and fruits stuck by the peduncle, forming the floral cushion brooms that eventually dry and produce fruits. Young fruits there may be from abortive parthenocarpic fruits (in the shape of strawberries and carrots) to black, hard, and irregular lesions (Silva et al. 2002). These symptoms can be so severe that they can lead to a loss of 90% of cocoa production in a region.

2.2.3 Black Pod Rot

The black pod is one of the main diseases of the cacao tree, being responsible for losses of 20–30% of the annual cocoa production, which can lead to the loss of 10%

of the plants (Bailey and Meinhardt 2016; de Oliveira and Luz 2005). It is caused by many species of *Phytophthora*—Kingdom Straminipyla, Phylum Oomycota, Class Oomycetes, Order Peronosporales, Family Phytiaceae—considered a pseudo-fungus with varied geographical distribution (Acebo-Guerrero et al. 2011; Ho 2018).

There are about 300 species of *Phytophthora* reported in the world, with only 7 causing black rot in cacao trees (Bailey and Meinhardt 2016; Ho 2018) and only four species have a commercial impact—*Phytophthora palmivora, Phytophthora megakarya, Phytophthora citrophthora*, and *Phytophthora tropicalis (P. capsici)* (Ho 2018; Bailey and Meinhardt 2016; End et al. 2017). All these species affect the plants with the same types of symptoms, differing in aggressiveness and geographic distribution. *Phytophthora megakarya* and *Phytophthora palmivora* are considered the most important for cacao cultivation in Central and West Africa and most studies are related to them (Guest 2007; Adomako 2007), however *P. megakarya* (considered the most aggressive) has been reported only in Africa and it is still in an invasive phase. Since *P. megakarya* is more aggressive and causes higher yield losses than *P. palmivora*, special care should be given when moving plant/soil materials and cocoa beans to production areas and countries that are not yet affected by *P. megakarya* (End et al. 2017). On the other hand, *P. palmivora* is the widest world distribution species in Africa, Asia, and America (Guest 2007).

High incidence levels of black pod disease have been reported in Brazil, being induced by *Phytophthora capsici*, *Phytophthora citrophthora*, *Phytophthora heveae*, and *Phytophthora palmivora* (Luz et al. 2001). Recently, the first description of the species *P. theobromicola* was reported, which seems to be prevalent in plantations in the state of Bahia, Brazil, inducing more extensive lesions in various genetic materials (Decloquement et al. 2021).

The pathogen's life cycle is divided into a sexual and asexual phase, the first being more commonly found in nature and the second marked by the formation of chlamydospores (vegetative structures) that germinate giving rise to sporangia that in turn release zoospores that can be dispersed over long distances by water and indirectly infect other plants, as opposed to the direct infection that occurs through the mycelium (Ho 2018; Luz et al. 2001; Oliveira et al. 2014; Kudjordjie 2015).

The most evident symptom of the disease in cacao is the formation of black pods. Initially, small dark spots appear on the pods approximately 30 h after infection. These symptoms appear in pods of any age, but in more developed pods, the beans can be totally or partially used, while in younger pods, spots, wrinkling, and darkening are common and may be confused with physiological withered. Although the pod is the most affected site, the disease can also affect leaves and roots that can serve as a source of inoculum, resulting in dark sunken lesions in the stem that often develops as a result of mycelium spread from pods into flower cushions and further along the stem or directly through wounds (End et al. 2017). In addition, extensive necrosis of leaves and shoots of seedlings, flower cushions, and root infections can occur, and these lesions can even cause the death of the plant (de Oliveira and Luz 2005; Luz et al. 2001; Silva Neto et al. 2001). The trunk can also be a target of infection due to the dispersion of pathogen structures, which can even cause the death of the plant (de Oliveira and Luz 2005; Luz et al. 2001; Silva Neto et al. 2001).

In this scenario, to prevent new pathogens from spreading or prevent them from multiplying further if they have already gained entry and have been established in new restricted areas are used legal enforcement of the quarantine measures. The plant parts that are likely to carry pathogen in trade and transport are: pods, roots, budwood, trunk/branches, leaves, and soil or growth media accompanying plants, while seeds originating from healthy pods are unlikely to carry the disease (End et al. 2017).

2.2.4 Ceratocystis Wilt of Cacao

The Ceratocystis wilt of the cocoa tree, also known as machete disease, is characterized by drying out and completely killing the cocoa tree. This disease is caused by Ascomycota *Ceratocystis cacaofunesta* Engelbrecht & T.C. Harr (Engelbrecht and Harrington 2005), a species within the Latin American clade of the *Ceratocystis fimbriata* species complex. *Ceratocystis cacaofunesta* belongs to the class Sordariomycetes, order Microascales, and family Ceratocystidaceae. The first report of *Ceratocystis* infecting cacao trees was in 1918 in Ecuador, where the disease was confined until the 1950s. In that same decade, the disease was reported in other countries in South and Central America (Silva et al. 2004; Rorer 1918).

When Ceratocystis wilt of cacao emerged in Ecuador there was not much damage to cacao production, as the country's cacao trees were resistant to this disease. However, in 1957 a more virulent form of the fungus was identified and since then it has affected many cacao trees in South American countries such as Brazil, Colombia, Guyana, Peru, Venezuela and, Trinidad and Tobago; in Central America such as Guatemala, Costa Rica, and Mexico and the Caribbean island of Haiti (Cabrera et al. 2016). Ceratocystis wilt is considered one of the most important emerging diseases of cacao because the agricultural practices of crop management (such as pruning and harvesting the fruits) favor the penetration of the fungus into the host tissue when the tools used are contaminated with the fungus (Engelbrecht et al. 2007a).

Species of the genus Ceratocystis have relatively low genetic diversity. This fact may be related to the type of reproduction of the fungus (homothalic), the limited flight range of the dispersing beetles and the introduction of the disease via human action, generally having few genetic variants in long-term dispersion (Engelbrecht et al. 2007b) report that populations in Colombia, Costa Rica and Bahia have limited genetic diversity, characteristic of introduced populations that have suffered recent genetic bottlenecks. While the populations of Rondônia (Brazil) and western Ecuador have similar diversity to populations of other species of the genus, indicating that they are natural populations.

The main route of colonization of *C. cacaofunesta* is through natural wounds on the surface of the host, which can be caused by beetles (carrying the conidia adhered to their paws) or contaminated tools. The conidia of this pathogen infect the plant's xylem parenchyma cells and move in a radial direction, invading the xylem vessels (Araujo et al. 2014). Conidia of *C. cacaofunesta* are very small in the early stages of colonization; this characteristic may be related to the rapid distribution of this

fungus in the plant and allows the fungus to more easily penetrate the host cell wall (Santos et al. 2013). The plant defence system, in turn, recognizes the pathogen, and a containment barrier against the fungus is formed, causing vascular occlusion. This barrier can favour both host resistance or pathogenesis, depending on the rate of occlusion formation. That is, if this obstruction prevents the spread of the pathogen, it will generate resistance to the host, but if this obstruction makes it difficult to transport water in many vessels, it may cause the plant to collapse (Talboys et al. 1972).

Ceratocystis cacaofunesta is a necrotrophic fungus, as such, it causes necrosis of the vascular parenchyma cells, which contributes to triggering the main symptoms of the disease, which is the wilt and dryness of the cocoa tree. Even after death, the leaves can still stick to the tree for weeks. In addition to these, there may be symptoms on the stem of the plant, presenting as brownish-brown lesions with lighter regions, seen in advanced stages of the disease. And in the roots, similar symptoms can manifest, which indicates that there is the transmission of the pathogen through the soil and contact between roots (Cabrera et al. 2016; Santos et al. 2013; Silva et al. 2004).

2.2.5 Cocoa Swollen Shoot Virus

Virus diseases are the most critical disease limiting cacao production in West Africa (Table 2.1), a region responsible for ~70% of the world's cocoa production. The *Cocoa swollen shoot virus* (CSSV) is one of the most destructive phytopathogens for the cocoa crop, especially in West Africa, where it causes significant economic damage (Padi et al. 2013). Marelli et al. (2019) accounted for an annual loss of 96,000 metric tons in production due to the virus. CSSV is considered endemic to West Africa and its importance in Malaysia and Sri Lanka was highlighted (Steven 1936; Geering and Hull 2012). Other viral diseases have been reported in Trinidad, but they were not associated with swellings. An attenuated and localized form of CSSV is reported in Indonesia, Sabah, and Sri Lanka (Muller 2016).

CSSV was reported in West Africa, Ghana, in 1936, but it was probably present since 1922 (Muller 2016). CSSV dissemination followed the cacao cultivation in the Eastern Region, and although few scattered outbreaks were observed in the Western region, in the 20th decade, this region became the most severely affected area in Ghana. After that, the disease has spread throughout the entire widely cacao-growing areas in West Africa, and nowadays, the disease is considered endemic to the Eastern Hemisphere.

Cacao swollen shoot virus disease is caused by a complex of badnaviral species (family, Caulimoviridae) referred to as pararetroviruses. Several different strains of the virus exist and can cause defoliation, dieback of the plant, and severe yield losses. Following the International Committee on Taxonomy of Viruses (ICTV), CSSV taxonomy is based on the nucleotide diversity in the RT/RNaseH region (Kouakou et al. 2012; Oro et al. 2012; Chingandu et al. 2017), and five different species, A, B–C,

D, E, and G have been described to cause CSSV disease. The virus is naturally transmitted to cocoa by at least 16 species of mealybugs (Hemiptera: Pseudococcidae) (Ameyaw et al. 2014; Hughes and Ollennu 1994), the vector of most badnaviruses. Particles are bacilliform and measure 121–130 × 28 nm. The virus does not multiply in the vector and is not transmitted to its progeny. CSSV can infect cacao at any stage of plant growth, and so far, there is no evidence of seed or pollen transmission. CSSV natural hosts are species from the Malvaceae families: *Adansonia digitata, Bombax* spp., *Ceiba pentandra, Cola chlamydantha, Cola gigantea, Theobroma cacao* and other tree species of the Malvaceae.

The mealybugs are the vectors responsible for spreading the disease over a short distance (radially) by crawling the canopy from tree to tree. New outbreaks are associated with jump spread over greater distances by wind-borne viruliferous mealybugs or by the very active small first instar nymphs (Strickland 1950; Thresh et al. 1988). Once a plant is infected, it cannot be cured. Like most plant viral diseases, the disease can be contained or prevented if healthy plants are isolated within barriers of CSSV-immune crops. The disease is not seed-borne, but it may be introduced in clones imported as plants or budwood.

Additionally, experimental pathogenicity tests with indigenous plants of West Africa also revealed the families Bombaceae, Sterculiaceae, and Malvaceae as potential alternative hosts of CSSV (Posnette et al. 1950). Several plant species have been reported as CSSV hosts, among those, 30 plant families are used as shade for cacao and other crops (Abrokwah et al. 2016; Friscina et al. 2017). Their geographical origin influenced phylogenetic relationships between Ghanaian and Togolese sequences rather than whether they originate from mild or severe isolates. CSSV populations have now been analysed molecularly in the major West African countries (Ivory Coast, Ghana, Nigeria, and Togo) (Oro et al. 2012; Kouakou et al. 2012), and six structural groups were proposed according to the diversity in the first part of open reading frames (*ORF3*) with high genetic variability within them.

It is admitted that no virus disease has been found in cocoa in South America. The high variability within CSSV populations and the combined knowledge of CSSV disease-badnavirus on molecular and pathogenicity studies and the historical data of the disease emergence have led researchers to hypothesize that CSSV in cocoa emerged from host jumps indigenous plants. Likely its introduction in Africa is due to several host shifts from indigenous hosts. Selective pressures in alternate host plants may induce a differential evolution of the virus compared to its evolution in cacao. This diversity is reflected in the differential preference of each species, as well as in symbiotic interactions with other organisms, making it difficult to control the insect and, therefore, the virus (Muller 2016; Ofori et al. 2015; Padi et al. 2013; Roivainen 1976).

Control measurements include preventive measures through the use of resistant material eradication of the infected tree and trees around it. Upper Amazon hybrids with good agronomic characteristics have been demonstrated more resistant to infection than other genotypes being grown (Amelonado type) (Amon-Armah et al. 2021).

2.2.6 Other Diseases and Pests

2.2.6.1 Vascular Streak Dieback

The vascular streak dieback, caused by *Ceratobasidium* (Oncobasidium) *theobromae*, is a disease of paramount importance to cacao orchards in Southeast Asia and Melanesia regions where the pathogen is endemic (Bekele and Phillips-Mora 2019; McMahon and Purwantara 2016). As the common name suggests, the symptoms of *C. theobromae* in cocoa progressively evolve, culminating in the plant's death. It can infect both adult and seedling individuals (Samuels et al. 2012). This basidiomycete causes considerable losses in the countries where it occurs. Marelli et al. (2019) estimated an annual loss due to disease of 61 thousand metric tons in almond production by 2016. Vascular streak dieback has been reported in a lot of cacao-growing areas as South and Southeast Asia, Melanesia, Kerala (India), Myanmar, Thailand, Hainan Island (China), Vietnam, Malaysia and Indonesia (Ploetz 2007).

The symptoms of vascular streak dieback initially begin with a chlorosis in the leaf, flushes behind the shoot apex and scattered islets of green tissue. The symptomatics leaves become fully chlorotic in a few days and the symptoms progressively develop in all leaves. Internally, the steams present infected xylem with dark streaks within the vascular tissue. During the wet weather, when an infected leaf falls, the hyphae can emerge from the leaf scar and develop into a tullasneloid basidiocarp. After that, it results in the formation of a white, flat, velvety covering of the leaf scar and bark. When it occurs on a dry day, the scar heals over and the fungus fails doesn't develop. Basidiospores keep viable for a few hours and require free water for the germ tube growth (Prior 1979).

The *C. theobromae* basidiospores are dispersed by wind and the effective spore dispersal is probably limited to a few hours in the early morning with high humidity and wet leaves (Keane 1981). In this way, just a few infections occur beyond 100 m from the diseased cacao.

The management of vascular streak dieback includes the use of resistant varieties. Crosses using Trinitarios clones have shown some resistance to the disease. Despite biocontrol of *C. theobromae*, treatment with *Trichoderma harzianum* T-22 suppressed vascular streak dieback development (Vanhove et al. 2016). Also, fungicides including flutriafol, hexaconazole, propiconazole, tebuconazole and triadimenol had been used to control vascular streak dieback but none of them showed to be commercially viable in cacao plantations (Holderness 1990).

2.2.6.2 Pests

Some pests of greater economic importance can be cited, such as *Distantiella theobroma*, *Monalonion bondaris*, *Selenothrips rubrocinctus*, *Conotrachelus humeropictus,* as well as some species of the genus *Helopeltis*. However, the bug *Sahlbergella singularis*, the cocoa borer, *Conopomorpha cramerella*, as well as the species of the

family Pseudococcidae, vectors of the CSSV, have been reported as causing the greatest losses in production (Bekele and Phillips-Mora 2019; Muller 2016).

2.3 Genetic Resources of Resistance Genes

Resistance genes to the main cocoa diseases have been sought almost exclusively in the species itself (primary gene pool), in wild or cultivated materials. The first technical reports of works aimed at this search are from the beginning of the 1930s, in Latin America and the Caribbean; and it is from collections conducted in Peru (Pound 1938) that the first genotypes that proved to be highly resistant to witches' broom disease were obtained (Bartley 1994). Since then, several collection expeditions were conducted in areas of natural dispersal of the species in Brazil, French Guyana, Ecuador and Peru (http://www.icgd.rdg.ac.uk), with a strong focus on resistance, especially to witches' broom disease. And, seeking resistance, countless collections and selection procedures in populations of traditional varieties and improved seminal varieties, and selection procedures in breeding cycles, have been conducted in many of the producing areas around the world—the International Cocoa Germplasm Database presents, in detail, the genotypes identified as resistant or promising for the main cocoa diseases and some pests. Currently, 49 national and international ex situ collections of germplasm participate in the preservation of the genetic resources of resistance genes of cocoa (http://www.icgd.rdg.ac.uk).

2.4 Glimpses on Classical Genetics and Traditional Breeding

2.4.1 Breeding Objectives

The breeding of cocoa, carried out by classical methods or by modern biotechnological techniques, plays a fundamental role in promoting the development of this species. The continuous technological advances obtained to promote the orchards' sustainability, earning a differentiated financial return, favours economic viability; and guarantees food security for both the producer and the consumer. Furthermore, it provides ecologically friendly farming, either by reducing the use of pesticides or reducing the use of chemical fertilizers (Dennis et al. 2008). Therefore, there are several goals to be achieved, which are dynamic, with synergistic or antagonistic interactions between them. They concern the productive, vegetative and health aspects of cocoa, and breeding will be challenged to develop genotypes that bring together favourable genes in these three aspects. Thus, the producer will be able to count on cultivars that will provide security, stability, productivity and longevity for field cultivation.

2.4.1.1 Productive Aspects

It aims to improve productivity and the qualitative characteristics of almonds, cacao's main product. Genotypes with a high number of fruits per plant, a high amount of almonds per fruit, and a high weight of almonds are sought (Lopes et al. 2011). These characters are related and can be expressed in a series of indexes such as fruit index, seed index, fruit value, among others (Bekele and Phillips-Mora 2019; Dias 2001), or even indexes that group other characters, such as production and resistance (Jaimez et al. 2020). The almond weights component, which makes up the seed index, tends to have less environmental influence, and consequently, greater heritability. However, it suffers interference from other factors, such as the length of the production cycle, the quantity and location of the almonds in the fruit (Doaré et al. 2020). Market demands exert pressure on breeding programs to also pay attention to the qualitative characteristics of almonds, which influence the quality of chocolate. Among them are the content and consistency of the butter, from the almond, as well as the nutritional and organoleptic characteristics themselves (Adeigbe et al. 2021; Araújo et al. 2009; Pinheiro et al. 2012).

2.4.1.2 Vegetative Aspects

It considers the plant's vigour, as well as the characteristics that facilitate the management, such as size, uniformity, precocity and genotypes' adaptability to different growing conditions, to which the cocoa tree can be subjected (Bekele and Phillips-Mora 2019; Mustiga et al. 2018). Particularly, for this last character, economic factors should be considered, such as the increase in planting density aiming at higher productivity, and/or plant in full sun (Olufemi et al. 2020); ecological, such as cultivation in agroforestry systems with different compositions and, consequently, levels of competition (Schneider et al. 2016); and climatic, due to the increasingly frequent changes as a result of global warming (Farrell et al. 2018).

2.4.1.3 Health Aspects

These refer to biotic stresses and will have a special focus in this chapter. Cocoa tree cultivation brings with it phytosanitary problems, among pests and diseases that, depending on the level of infestation, can drastically reduce the viability of the orchards. Therefore, breeding programs primarily aim at the development of genotypes with long-lasting resistance to these pathogens, preferably of the horizontal type, promoting adaptability and geographic and temporal stability against a range of phytopathogens simultaneously (Dias 2001; Bekele and Phillips-Mora 2019).

2.4.2 Classical Mapping Efforts

Markers are important tools for optimizing classical breeding. They provide fundamental information for the adequacy of the approach, as well as the selection methods used. In addition, they are essential in the characterization of genetic materials, as well as in the detection of genetic variability, raw material for breeding (Kordrostami and Rahimi 2015). In cocoa, several markers have already been developed and used as auxiliary to classical breeding. In chronological order of use, morphoagronomic, biochemical and DNA markers can be mentioned. The latter will be covered in the following sections of this chapter.

2.4.2.1 Morphoagronomic Markers

They constitute the basic tool of the cocoa breeder, being historically the most used in the crop breeding. In Active Germplasm Banks—AGB's, for example, the use of morphoagronomic markers is fundamental for the characterization and phenotypic distinction of accessions. These markers provide relevant information about the morphological characteristics of the vegetative and reproductive structures of each accession, to differentiate between them. They also refer to agronomic characteristics, such as production components and resistance to pests and diseases, allowing the efficient pre-selection of accesses to form working collections (Bekele et al. 2006; Dias 2001). Through simple morphological markers, it is possible, for example, to distinguish between the main cocoa tree ecotypes: Forastero, Criollo and Trinitário (Bekele et al. 2006, 2020; Bidot Martínez et al. 2017). As they are phenotypic, and, in most cases, related to polygenic characteristics, morphoagronomic markers suffer an expressive environmental influence, which interferes with heritability. However, its use is not unnecessary, having value as a complementary tool to genetic markers, increasing the reliability of information (Bidot Martínez et al. 2017; Gopaulchan et al. 2019). Robust information on morphological markers applied to germplasms can be found in the International Cocoa Germplasm Database (http://www.icgd.reading.ac.uk/icgd/; ICGD).

2.4.2.2 Biochemical Markers

Biochemical markers are less used in cocoa than morphological and DNA markers. Even so, this methodology has already been used for the assessment of genetic diversity and accession conservation strategy at the International Cocoa Genbank, Trinidad (ICGT), proving to be as effective as random amplified polymorphic DNA (RAPD)-type DNA markers, for example (Sounigo et al. 2005; Warren et al. 1995). Furthermore, these markers were used to study the pattern of self-compatibility and incompatibility in the species (Warren et al. 1995), as well as for making a gene linkage map (Lanaud 1986; Lanaud et al. 1995), fundamental for subsequent molecular studies.

2.4.3 Classical Breeding Achievements

Cocoa farming would not be at the technological level that it is today, were it not for the advances provided by research on genetic improvement. Only from the turn of the 20th to the twenty-first century, biotechnology became a reality in the species' improvement. Therefore, most of the results that were and are still being obtained, especially the development of cultivars, are the result of the classical approach, showing its importance (Bekele and Phillips-Mora 2019; Dias 2001). Below, the main advances provided by classical breeding are highlighted, with emphasis on resistance to biotic stresses.

2.4.3.1 Resistance to *Moniliophthora perniciosa*

The concern with *M. perniciosa* dates back to the initial stages of cocoa breeding, in the first half of the twentieth century, when pioneering prospecting was carried out in search of sources of resistance to the fungus (Pound 1943). The supposedly resistant SCA 6 and SCA 12 clones were material collected in these first expeditions (Dias 2001). These clones became, for a long period, the main source of resistance to the fungus, with clone SCA 6 still being classified in this way (Rodrigues et al. 2020). However, this resistance was overcome with the diversification of the pathogen in some countries such as Brazil, Peru and Ecuador (de Albuquerque et al. 2010; Gramacho et al. 2012; Lopes et al. 2011). This revealed the need to diversify the sources of resistance to the fungus, as a strategy to maintain the durability of resistance in the field.

Several studies were carried out to find clones and hybrid combinations with high resistance indexes, without, however, disregard the durability and geographic range. Currently, genetic materials that have resistance alleles to the pathogen, in addition to Scavina clones, are ICS (Imperial College Selection) 1, 60, 98, 45, 85 and 10; CAB (Cocoa from the Brazilian Amazon) 0371, 0388, 0392, 0410, 0169, 0352, 0214, 0208 and 0270; IMC (Iquitos Mixed Calabacillo) 67 and 47, PA (Parinari) 121, EET 272, POUND 18 and CC 41, as well as their hybrid combinations, such as the TSH (Trinidad Selected Hybrids) and TSA series (de Albuquerque et al. 2010; Bekele and Phillips-Mora 2019; Dias 2001; Benjamin et al. 2016).

2.4.3.2 Resistance to *Moniliophthora roreri*

Genetic resistance to the pathogen is the main strategy for controlling the disease. In line with the polygenic nature of the trait in question, the species' breeding programs seek to pyramid resistance genes, that is, to concentrate genes from different sources in a few genotypes (Phillips-Mora et al. 2018). In this sense, there is a need to constantly search for genetic materials that contain different sources of resistance to moniliasis, as well as hybrid combinations that manifest such resistance, preferably

horizontally. Thus, a range of genotypes have already been identified and are currently being used in breeding programs as clones or parents for controlled crosses, such as, for example, resistant clones UF 273 and 712, ICS 95 and 10, and PA 169, which, crossed with each other and with other clones with varied resistance indices, constituted the hybrids of the CATIE series, such as, for example, CATIE-R1, CATIE-R4 and CATIE-R6, promising materials for both resistance and almond production (Bekele and Phillips-Mora 2019; Jaimes et al. 2011; Phillips-Mora et al. 2009, 2018). Osorio-Guarín et al. (2020) report that several genetic materials with evidence of resistance to moniliasis and witches' broom have been identified, such as FCM 19, SUI 72, SCC 85, EBC 09, among others. The authors also indicate that there is a low correlation between the symptoms of the two diseases, explained by the competition for the same site of infection. The search for materials that can add resistance alleles for both pathogens of the *Moniliophthora* genus is currently the motto of the main Latin American breeding programs (Bekele and Phillips-Mora 2019; Lopes et al. 2011; Osorio-Guarín et al. 2020).

2.4.3.3 Resistance to *Phytophthora* spp.

It is noticed that the breeding for resistance to *Phytophthora* has a complicating factor, concerning the aforementioned pathogens: the great variety of etiological agents, which demands a proportional diversity of resistance sources capable of withstanding the pressure of most species, that is, efficient use of the available cocoa germplasm (Nyadanu et al. 2012). As with moniliasis, the nature of resistance to pod rot is quantitative and additive (Fister et al. 2019; Nyadanu et al. 2012), with relatively high heritability (Bekele and Phillips-Mora 2019; Nyassé et al. 2007) which allows the pyramiding of resistance genes from different origins. Several tolerant genotypes to different species have been developed in the main breeding programs in the world, such as, for example, POUND 7, SPA 9, ICS 1, IMC 47, SCA 6, IFC 5, PA 150, UF 12, as well as their hybrids, from crosses with each other and with genotypes with similar or slightly lower resistance (Bekele and Phillips-Mora 2019; Dias 2001; Fister et al. 2019; Nyadanu et al. 2012; Nyassé et al. 2007; Thevenin et al. 2012).

2.4.3.4 Resistance to CSSV

Besides, the recommended control actions, such as the elimination of diseased plants and the use of chemical pesticides, have not been efficient in reducing the viral spread. For this reason, the most sustainable and least costly option for disease control is the breeding route, with the development of genotypes that are not attractive to scale insects, as well as resistant to viral symptoms (Trebissou et al. 2020). As a result of these researches, several genotypes with evidence of genetic resistance to the virus were identified. It was observed that Amelonado-type genetic-based materials, which were widely used in the African continent, are highly susceptible to the disease, unlike genotypes originating from the Lower and Upper Amazon (Padi et al. 2013). With

these, there was even a successful attempt to obtain tolerant clones via mutagenesis, using gamma rays (Adu-Ampomah et al. 1996). The additive nature of resistance to CSSV was found, indicating the possibility of accumulation of resistance alleles via hybridization (Lockwood 1981). Furthermore, the influence of shading management to mitigate the effects of CSSV was defined, with the possibility of integrating the shade tolerance character with virus resistance in breeding programs (Andres et al. 2017, 2018). Thus, plantations in full sun tend to be more favourable to the rapid evolution of this virus (Andres et al. 2018).

To date, genotypes that are completely resistant to CSSV have not been developed, and there were low genetic gains for the trait (Padi et al. 2013). However, some materials with evidence of genetic resistance have already been identified, which are being used in breeding programs, such as Upper Amazon clones IMC 67 and 47, NA 33; the Lower Amazon clones Catongo, RB 49 and C-Sul 7, some Guyanese materials such as GU 239/H, 225/V and 290/H, some hybrid combinations between the genotypes of these origins, such as TC65 (PA 7 × IMC 35), in addition to clones from mutation-induced mvP30 and mvT85 (Bekele and Phillips-Mora 2019; Trebissou et al. 2020; Padi et al. 2013; Muller 2016; Ofori et al. 2015).

2.4.3.5 Resistance to *Ceratocystis cacaofunesta*

Ceratocystis cacaofunesta is difficult to control due to the rapid progression of the visible symptoms of the disease until the plant's death (Silva et al. 2007). Added to this fact is the way the fungus penetrates the plant, through the action of some Coleoptera of the Scolytidae family, as well as injuries caused by the management of cacao (pruning, crowning, etc.) (Santos et al. 2012b). After the plant is infected, tissues dry out and plant death, but leaves and fruits remain attached to the plant for a long time. For these reasons, development of resistant varieties via genetic improvement of the species is the most viable option to mitigate the damage caused by this pathogen (Yamada et al. 2015).

Analysing the pattern of plant mortality in the field in di-allele analyses, Gardella et al. (1982) found the influence of several genes in the manifestation of resistance to the fungus, which was later confirmed by Sanches et al. (2008) and Santos et al. (2012b). This polygeny attaches great importance to the additivity of the genes involved, attesting to the need to direct crosses between genotypes with different sources and resistance, for the pyramiding of favourable genes. Dominance also plays an important role, making it possible to exploit heterosis from crosses through cloning.

The search for sources of genetic resistance generated significant results, having identified genotypes that were later used in crosses. These include BMI 67, ICS 6, EET 400, PA 7, POUND 18, EET 272, TSH 1188, VB1151, CEPEC 2008, among others (Bekele and Phillips-Mora 2019; Santos et al. 2012b; Sanches et al. 2008; Silva et al. 2013; Yamada et al. 2015).

2.4.3.6 Resistance to *Ceratobasidium theobromae*

Cultural and chemical control methods, even when integrated, are not efficient in mitigating the harmful effects of the pathogen on crop production. However, genetic breeding, associated with such methods, constitutes an effective tool to control the fungus (Asman et al. 2021; McMahon and Purwantara 2016). As observed for resistance to the aforementioned pathogens, the additivity of the genetic resistance trait to *C. theobromae* was also observed, evidencing the importance of selecting parents with favorable genes for the production of hybrids and clones. For this character, intra-allelic dominance relationships are less important (Tan and Tan 1988). Despite being quantitative, studies show that few genes govern the trait (McMahon and Purwantara 2016).

Some clones with evidence of genetic resistance to *C. theobromae* that are currently being used in the main breeding programs are of the Trinitarian and Upper Amazon type, as well as their hybrids. As an example, we have KA2-106, KA2-101, PBC 123, BR 25, PA 191, TSH 858, SCA 9, ICS 95, UF 667, among others (Bekele and Phillips-Mora 2019; McMahon et al. 2015; McMahon and Purwantara 2016; Guest and Keane 2018).

2.4.3.7 Resistance to Major Pests

Although a range of pests coexists with the cocoa crop, reducing production, most have local economic importance, unlike the aforementioned diseases, which have a global scope and, therefore, greater appeal. For this reason, and due to the difficulty in identifying efficient sources of resistance, the search for genotypes that manifest a genetic resistance mechanism is still little explored by the classical breeding of cocoa (Bekele and Phillips-Mora 2019).

For *S. singularis*, an important pest for the African continent, Upper Amazon and Guyanese genotypes have more concrete signs of resistance, with a complex defence structure that combines antibiosis, antixenosis and tolerance itself. Some clones can be highlighted, such as ICS 1, UF 676, PA 102, EET59, in addition to the hybrids T65/7 × T57/22, T65/7 × T9/15, among others (Anikwe et al. 2009; N'Guessan et al. 2008).

Together with the pathogens *C. theobromae* and *P. palmivora*, the *Conopomorpha cramerella* borer is one of the main responsible for promoting significant losses in cocoa crops on the Asian continent (McMahon et al. 2009; Niogret et al. 2020). Estimates made by Marelli et al. (2019) attributed to the action of the pest an annual loss of 81,000 metric tons in production. For this reason, the most advanced pest-related studies aimed at achieving genetic resistance have been dedicated to *C. cramerella.* Characteristics related to insect tolerance were identified, consisting mainly of physical barriers in the genotypes, such as the thickness of almonds' sclerotic layer (Soesilo et al. 2015). There is a differential manifestation of these characteristics between genotypes, denoting genetic control and, therefore, amenable to selection

(Soesilo et al. 2015; Teh et al. 2006). In this context, some clones have greater potential for resistance to the pest, such as ARDACIAR 10, Na 33, Paba/V/81L/1, Aryadi 2, SCA12 KKM22, BR25, among others. The last three, despite having low infestation rates, do not have thick fruits and/or almonds, suggesting an alternative resistance mechanism, such as, for example, antixenosis (Soesilo et al. 2015; McMahon et al. 2009).

The resistant clones mentioned in each topic are just examples of the wide range of genotypes developed and used by classical cocoa breeding programs around the world. In the consulted literature, other genotypes available in these programs can be found.

2.4.4 Limitations of Traditional Breeding and Rationale for Molecular Breeding

As a perennial species, the cocoa tree has particularities that make it difficult to generate short-term results and, therefore, demand that selection is as accurate and efficient as possible. Among these specificities are the long reproductive period, the modification of characters over the years of cultivation, the need for large areas for genetic evaluation of a substantial number of genotypes, among others. It is noteworthy that, during the development of a cultivar, each breeding cycle takes around 10–12 years (Bekele and Phillips-Mora 2019; Dias 2001; Resende 2002).

On the other hand, the current market dynamic requires more speed and precision in generating results. Therefore, with this growing demand for research, which has intensified in recent years, the cocoa genetic breeding, using only a classical approach, will not be enough to deliver the assets required by the production chain promptly, given the particularities of the species mentioned in the previous paragraph. In this sense, biotechnological tools such as molecular markers, genomic maps, high-throughput phenotyping, among others, are strategic to increase the efficiency of the species improvement (Wickramasuriya and Dunwell 2018).

The use of molecular markers, for example, makes it possible to shorten the breeding cycle, by prematurely identifying the presence of favourable alleles in an individual. In terms of biotic stresses, the identification of marks attributed to resistance genes is essential to save months or even years of field trials, with inoculations and/or phenotyping (Osorio-Guarín et al. 2020). Once the genotypes have been sequenced, it is possible to predict the performance of their progenies or clones, enabling the targeting of crosses and thus contributing to the pyramiding of resistance genes to a particular pathogen, or tolerance to some category of abiotic stress (McElroy et al. 2018).

With the advancement of computer resources, the use of large databases is already a reality in most economically relevant crop breeding programs. For cocoa, it should be no different. Therefore, combining classical breeding with sophisticated biotechnology and statistical genetic tools can—and must—significantly increase selection

gains, and make cocoa breeding more efficient and responsive to the new challenges and opportunities that currently present themselves.

2.5 Brief on Diversity Analysis of Cocoa Germplasm

2.5.1 Phenotype-Based Diversity Analysis

Diversity analysis is supported by two actions: (i) the genetic improvement of culture aiming to overcome the environmental adversities that the culture faces throughout its domestication and use, such as biotic stress factors; and (ii) the genetic study that supports the main practices involving plant genetic resources (Henry 2005; Acquaah 2020). The estimation of a given crop that has phenotypic diversity can be understood as the differences in the expression of phenotypic characteristics between individuals or populations (Fuccillo et al. 2007). *Theobroma cacao* has a long history of domestication (Motamayor et al. 2002, 2003), which started in Mesoamerica despite the primary centre of origin and diversity being regions of the upper Amazon. Archeological findings indicate that the use of cocoa as food, in rituals and in medicine by Mesoamerican populations dates back to around 5300 years ago, indicating the pre-Columbian use of this genetic resource (Zarrillo et al. 2018).

In the last 250–300 years, cocoa cultivation spread to several tropical regions, and cultivars or varieties were used to establish productive systems in several regions of the Americas and Africa. This distribution combined with local and regional breeding has led to a wide diversity in qualitative and quantitative terms in the phenotypic descriptors of the plant and productivity mainly with the production of new cultivars and hybrids (Mustiga et al. 2018). However, the crossing between plants does not increase the genetic diversity of a crop, but evolutionary events such as the artificial selection of plants that are increasingly promising and more resistant to biotic and abiotic factors are important factors for the increase in genetic diversity (Henry 2005; Coates et al. 2018).

In cocoa, the different characteristics associated with the fruits have been the main differentiating descriptors of variety and cultivars, because it is the most desired plant organ to improve itself (Acquaah 2020; Bartley 2005). This greater emphasis on the cocoa fruit as a differentiating factor, led to estimate and divide the crop into distinct genetic groups and has also been used as a way to measure genetic diversity.

The characteristics of the fruits linked to geographic origin have also been two important classification factors in the subdivision of the crop into genetic groups, such as the varieties of the Criollo and Forastero groups. Varieties of the Criollo group are native and their origin is well known. The Forastero group, on the other hand, encompasses the varieties cultivated in various regions, and their origin is widely discussed. Both groups have a range of varieties and cultivars and even hybrids that are part of a third Trinitarian genetic group, expanding the classification system of cocoa (Bartley 2005). However, the revision of the classification of the species has been carried

out, proposing a total of ten different genetic groups, also taking the geographic distribution as a basis (Motamayor et al. 2008).

The genus *Theobroma* comprise about 22 species of origin Neotropics, with low species number diversity compared to other genera of angiosperm (Cuatrecasas 1964). However, the genus *Theobroma* is phylogenetically close to the genus *Herrania* (Schultes 1958). The intra- and intergeneric variations are based on morphological descriptors. The genus *Theobroma* has the species *T. cacao* as one of the most diversified in morphoagronomic descriptor variability, as a result of domestication and breeding. The Amazon region is an important centre of species diversity and contains genetic groups and germplasm with endemic characteristics to this region and also species at risk of extinction (Hammer and Khoshbakht 2005; González-Orozco et al. 2020).

The characteristics of fruits and seeds are the most important from an economic point of view, although wild species usually present smaller quantitative data for these descriptors, which are used in yield estimates. The species *T. grandiflorum* revealed higher fruit weight compared to *T. cacao* (Santos et al. 2012a). However, the descriptors for fruit and seeds are extremely variable in *T. cacao*, depending on the variety, clone or cultivar (Bartley 2005). The knowledge between the phenotypic and genetic relationships between wild species of the genus *Theobroma* and their wild relatives is important not only to understand the phylogeny and evolution of the group, but also to support genetic improvement with the use of biotechnological tools aimed at gene introgression and agronomically important characteristics (Henry 2005; González-Orozco et al. 2020).

The cocoa phenotypic study in locally or regionally used varieties is important, as the environment and the agronomic management associated with the crop considerably influence the descriptors related to fruits and seeds (Bartley 2005). In the coastal region of the state of Espírito Santo in Brazil, the evaluation of phenotypic and chemical descriptors associated with the fruit in seven promising cultivars for cultivation and regional production, distributed the germplasm into three statistically distinct groups, revealing that the clones CCN10 and CCN51 are the most suitable for the production of almonds aimed at the chocolate industry (Alexandre et al. 2015). The phenotypic and chemical characterization of cocoa was also carried out in regions with little availability of water (Brazilian semi-arid), in cultivars that are already widely used in the region; this demonstrate the use of the Brazilian genetic resource in several regions of the country. CCN51 and CEPEC2005 clones were the most promising for almond fermentation, while other clones (PS1319 and CEPEC2004) are also indicated for the production of other food items, such as jellies, nibs and sweets with low fat content (Reges et al. 2021). In Indonesia, several clones and hybrids resulting from the crossing between clones were evaluated for genetic diversity using qualitative and quantitative phenotypic descriptors, indicating that the generated cluster was more influenced by qualitative descriptors, generating two main groups and three subgroups in the larger group “A”, with only two clones in group “B” (AD-04 and M-01) (Lembang et al. 2019). However, it has already been observed that the length and width of the fruits have a greater impact on the multivariate distribution of cultivars (Alexandre et al. 2015). In recent years, the

characterization of cocoa has considered parameters directly related to its commercial use, with organoleptic characteristics among the products generated by different cultivars or clones. The sensory profile is important to direct the agricultural product to its final destination and consequently to the consumer market. In Peru, characteristics aimed at the production of fine-flavour cocoa have been evaluated in hundreds of plants, indicating the variability of 64 unique characteristics in combinations, expanding the possibility of using the cocoa genetic resource for the production of various items of great commercial value (Eskes et al. 2018).

In the Southern region of Bahia in Brazil, the characterization of local cocoa varieties has revealed a broad genetic base, due to the long history of introduction and breeding, fostered by local research and production institutions. In this region the local varieties Comum, Pará and Maranhão have been widely exploited in the formation of production systems, representing a good portion of the genetic resource available in the region, along with several clones, such as CCN51, TSH1188, SCA 6 and many others, whose characteristics are not only linked to the commercial value of the fruits and seeds, but also the resistance to biotic factors. In this sense, the Bahia's genetic resource has been considered a rich system in terms of genetics and phenotypic expression, revealing germplasm for the most diverse actions of improvement and selection of plants with agronomically desirable characteristics (Santos 2019) (Fig. 2.1).

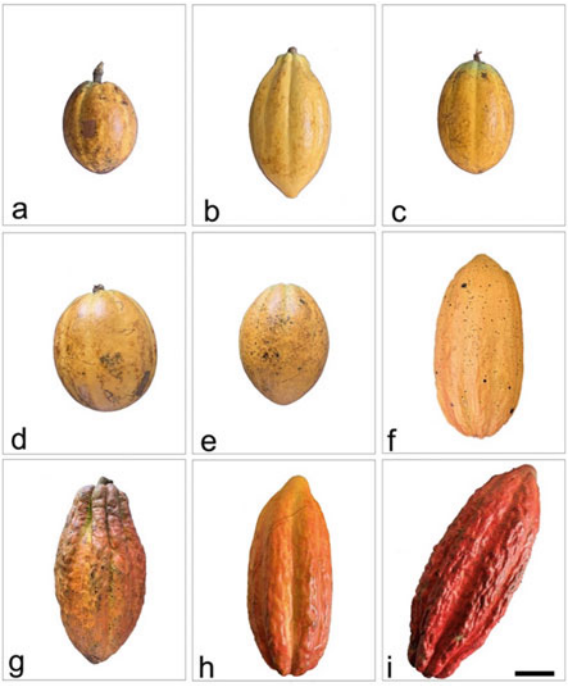

Fig. 2.1 Some shapes and sizes of ripe cocoa fruits found in plantations in southern Bahia. Local ancient varieties (**a**—Pará, **b**—Maranhão, **c**—Comum). Local mutant varieties (**d**—Redondo; **e**—Catongo). Commercial cultivars available from seedling producers (**f**—PS1319, **g**—TSH1188, **h**—SJ02, **i**—CCN51) (bar = 5 cm)

2.5.2 Genotype-Based Diversity Analysis

Until the 1980s, in general, the evaluation of genetic variation was performed based on phenotypic descriptors (Engels 1986; Nevo 1988). Additionally, the evaluation by enzymatic profile was also widely performed as a way to assess the genotypic variation using isoenzymes (Elliot and Kennedy 1988). From the 1990s onwards, molecular markers were developed, expanding the analytical perspectives of the cocoa genome. The different types of markers proposed and applied in the study of cocoa vary depending on the genetic and evolutionary principle of the analysed sequences and also the technical variation (Henry 2013).

In cocoa, molecular markers have been used to evaluate population and geographic distribution in relation to classification parameters (Motamayor et al. 2008), detecting and relating genomic and phenotypic characteristics (Barreto et al. 2018), assisting breeding programs and assessing reproductive characteristics such as self-incompatibility (Royaert et al. 2011).

Polymorphism among populations from different genetic groups was an important target for initial investigations with molecular markers-linked to the study of genetic diversity - genome size estimates, and polymorphic and specific sequence hybridizations (Figueira et al. 1992; N'Goran et al. 1994). Molecular markers based on sequence hybridization, such as restriction fragment length polymorphisms (RFLPs)—used for the evaluation of polymorphisms together with random markers based on polymerase chain reaction (PCR) random amplification of polymorphic DNAs (RAPDs), enabling the evaluation of diversity genetics, but with little certainty in the relationship between the germplasm and the proposed genetic groups (N'Goran et al. 1994). RFLP probes with low-copy sequences enabled the detection of variability between different genetic groups, with indications of introgression by hybridization between these genetic groups of different origin in germplasm. However, there is a certain genomic purity with low heterozygosity and other genetic and diversity specifications of each group already described, such as Criollo, Forastero and Trinitário (Lerceteau et al. 1997).

In the last three decades, around 50 different diversity studies based on molecular markers have resulted in highlighting the genetic diversity of cocoa in the different regions where it is cultivated (Fig. 2.2). The genome sequencing, and the use of nuclear and chloroplastic microsatellite sequences (SSR—simple sequence repeat) have been an effective strategy for the analysis of genetic polymorphism in cocoa (Lanaud et al. 2004, 2009; Motamayor et al. 2008; Santos et al. 2015; Lachenaud and Zhang 2008). SSR markers in cocoa have been efficient in the methodological standardization for the evaluation of the genetic diversity (Saunders et al. 2004).

The diversity of cocoa has brought about great discussions about the number and classification of genetic groups, a factor that often reflects on the phenotypic variability. The differentiation of Amazonian cocoa populations revealed, based on geographic distribution and molecular markers, the formation of ten distinct genetic groups: Amelonado, Contamana, Criollo, Curaray, Guyana, Iquitos,

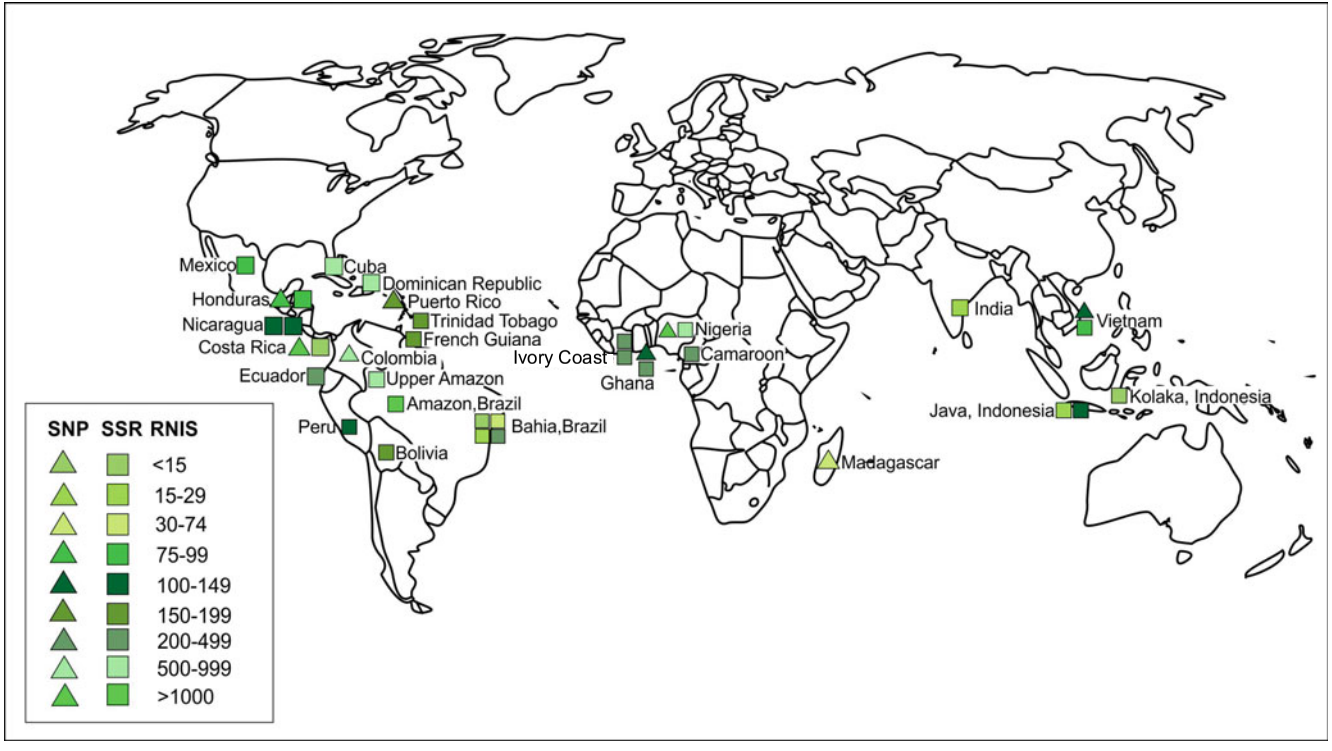

Fig. 2.2 Studies on the genetic diversity of cacao around the world, based on SNP and SSR markers. RNIS, range of the number of individuals per each study in different locations

Marañon, Nacional, Nanay and Purús expanding the possibilities of use of the cocoa genetic resource in breeding and conservation programs (Motamayor et al. 2008).

In Brazil, both the diversity and the genetic structure of plants cultivated in Bahia, revealed that the genetic resource of this location has particular characteristics, presenting a specific identity in terms of variability, differing from plant populations from other regions. In this case, cocoa produced in the Bahia State showed great genetic divergence between plants from other cultivation locations (Santos et al. 2015). Another approach to study the diversity of local cocoa in Bahia was to assess the diversity of materials that showed resistance to witches' broom after its occurrence in this cocoa-producing region. Then, RAPD markers were used, enabling access to genetic diversity and subsequent selection of potentially resistant plants (Faleiro et al. 2004a; Leal et al. 2008). Later, SSR markers were used for the same purpose of finding genetic novelties as alternative sources of plants resistant to witches' broom (Yamada et al. 2009; Faleiro et al. 2004b; Lima et al. 2013).

A population of Ecuador was evaluated for polymorphic SSR loci; it has been shown the trend of genetic erosion caused by the breeding and introgression of germplasm, reducing the number and diversity of native allele or wild plants (Loor et al. 2009). In Peru, high diversity has been revealed, depending on the region, and also due to the spatial structure of the collected accessions. However, the introduction of germplasm from other regions has altered the region's native germplasm, forming other populations in natural and semi-natural farming systems (Zhang et al. 2009b). In West Africa, genetic analysis of cocoa trees from different collection sites revealed great allelic richness with only 12 loci evaluated, indicating a greater diversity in germplasm from the upper Amazon compared to the genetic resource introduced from other regions (Aikpokpodion et al. 2009).

Expressed sequence tag (EST)-SSR markers have been used for functional genomic analysis, serving as a basis for the study of genome-phenotype relationship (Argout et al. 2011). Additionally, SSR markers have been related to genetic regions responsible for disease resistance in cocoa, one of the biggest problems associated with the crop in terms of reduced productivity, crop dropout by producers and plant death (Pugh et al. 2004; Brown et al. 2007; Lanaud et al. 2009; Akaza et al. 2016).

In the 2010 decade, the genome sequencing of cocoa was reported, expanding the perspectives in the evolutionary and genetic study of the species (Argout et al. 2011, 2017). The information generated by the cocoa genome sequencing allowed the identification and development of single nucleotide polymorphisms (SNPs). SNPs markers were also obtained to analyse the origin of traditional varieties cultivated in Madagascar, indicating that the genetic resource of this region has descent from the Criollo, Amelonado and Trinitário groups (Li et al. 2021).

2.6 Brief Account of Molecular Mapping of Resistance Genes and QTLs

2.6.1 Genetic Maps of Cocoa, Marker Evolution and Segregating Populations

The genetic mapping of cocoa based on molecular markers allowed the identification of genes and QTLs for resistance to different diseases that affect this crop, as well as other characteristics, mainly those that affect production, consequently with an economic effect. The genetic mapping of the first cocoa populations segregating for witches' broom and black pod resistance predominantly used the RFLP, AFLP and RAPD markers. They were carried out with F1 population and backcross population involving Catongo and Pound 12, identifying QTLs for resistance to black pod disease (Crouzillat et al. 2000), as well as with the population derived from the artificial self-pollination of the hybrid TSH 516, identifying QTLs with large effect for resistance to witches' broom (Queiroz et al. 2003). This map containing QTLs for resistance to witches' broom was expanded from 193 to 342 markers and each individual was multiplied by grafting for repetition of phenotypic evaluations, enabling greater accuracy in the phenotyping step (Faleiro et al. 2006). In this case, the focus was to identify genomic regions related to the resistance of cocoa to witches' broom, in order to assist in genetic improvement steps. Subsequently, different populations and marker types were used to identify QTLs and candidate genes for resistance to these two diseases (Tables 2.2 and 2.3).

The first genetic map from segregating populations for frosty pod rot resistance involved the F1 population derived of Pound 7 × UF 273, using SSR markers or single strand conformation polymorphism (SSCP) of resistance gene homolog (RGH) and WRKY markers (Brown et al. 2007). The two genetic maps of cocoa population segregating for Ceratocystis wilt resistance used SSR and SNP markers. These maps involved F2 population derived from SCA6 × ICS1 (Santos et al. 2012b), and population F1 population derived from TSH1188 × CCN51 (Fernandes et al. 2018).

Different strategies were used for the development and mapping of SNP markers in cocoa. For example, almost a hundred conserved ortholog set II (COS-II) have been identified and mapped in cocoa, with SNPs being identified for 83 genes, among which 19 cosegregated with QTLs (Kuhn et al. 2012). The strategies used in this study consisted of (1) evaluating polymorphisms in the mapping population, (2) identifying SNPs in a diversity panel through DNA amplification from 15 different cocoa accessions based on conserved genes, (3) analysing the transcriptome from leaf RNA from these 15 cocoa accessions. The 15 different genotypes used by Kuhn et al. (2012) to identify SNPs in conserved sequences were previously allocated to 10 different genetic groups based on SSR markers (Motamayor et al. 2008).

Another SNP discovery and mapping initiative was carried out based on a germplasm bank of 249 accessions and two linkage genetic mapping populations.

Table 2.2 Generation, size and genealogy of different populations of cocoa genetic mapping

Generation	Genealogy[a]	Size	QTL or sequence[b]	References
F1 BC1	Catongo × Pound 12 → F1 (Catongo × Pound 12) × Catongo → BC1	55 131	BP	Crouzillat et al. (2000)
F2	SCA6 × ICS1 → TSH516 ⊗ → F2	82	WB	Queiroz et al. (2003)
F2	SCA6 × ICS1 → TSH516 ⊗ → F2	82	WB	Faleiro et al. (2006)
F2	SCA6 × ICS1 → TSH516 ⊗ → F2	146	COS-II	Kuhn et al. (2012)
F1	UPA402 × UF676 → F1	125	Candidate gene	Fouet et al. (2011)
F2	SCA6 × ICS1 → TSH516 ⊗ → F2	143	CW	Santos et al. (2012b)
F1	TSH1188 × CCN51 → F1	459	WB	Royaert et al. (2016)
F1	TSH1188 × CCN51 → F1	265	BP	Barreto et al. (2018)
F1	TSH1188 × CCN51 → F1	266	CW	Fernandes et al. (2018)
F1	Pound 7 × UF 273 → F1	256	BP, FP	Brown et al. (2007)
F1	Pound 7 × UF 273 → F1	179	BP, FP	Gutiérrez et al. (2021)
F1	EET 95 × Silecia 1 → F1	733	WB, FP	Livingstone et al. (2017)
F1	SCA 12 × unknown genotype → F1	251	WB, FP	Livingstone et al. (2017)
Trihybrid	(SCA6 × H) × C1 → F1 (P7 × ICS100) × C1 → F1 (P7 × ICS95) × C1 → F1	179 173 183	BP	Akaza et al. (2016)
F1	DR1 × Catongo → F1 S52 × Catongo → F1 IMC78 × Catongo → F1	96 94 125	BP	Clement et al. (2003)
Trihybrid	(Na34 × IMC60) × IFC2 → F1	59	BP	Flament et al. (2001)
F1	T60/887 × IFC5 → F1	56	BP	Flament et al. (2001)
Trihybrid	(SCA6 × H) × C1 → F1	151	BP	Risterucci et al. (2003)

[a]For more details on the genetics of each genotype denoted here by the acronyms, see the original articles. ⊗ , selfcross. BC, backcross. F1, first generation. F2, second generation

[b]QTL for different diseases (BP, black pod; WB, witches' broom; FP, frost pod; CW, Ceratocystis wilt) and sequence or candidate genes (COS-II, conserved ortholog set of resistance genes; candidate genes; EST sequence)

Table 2.3 QTLs associated with resistance to pathogens in cocoa

Disease	Species	Population	Marker	Phenotyping	Software linkage/QTL	Statistical methodology	QTL number	LOD	LG	%VE (R^2)	References
BP	*Phytophthora palmivora*	Catongo × Pound 12(F1), F1 × Catongo (BC)	RFLP, RAPD, AFLP	Artificial inoculation in fruits	MAPMAKER/Q-gene	One-way ANOVA, simple interval mapping	6	>2	1, 2, 4, 5, 9(× 2)	7.4 to 47.9	Crouzillat et al. (2000)
BP	*Phytophthora palmivora*	T60/887 × IFC2, T60/887 × IFC5	AFLP, RFLP	PRR, Artificial inoculation in fruits and leaf-disc	JOINMAP/MAPQTL	Nonparametric marker by marker	7	2.2 to 4.2	2(×2), 3(×2), 6(×2), 10	9 to 17	Flament et al. (2001)
BP	*Phytophthora palmivora (×2), P. megakarya(× 2), P. capcisi(× 2)*	SCA6_H × IFC1	AFLP, SSR	Artificial leaf-disc inoculation	JOINMAP/MAPQTL	Composite interval mapping	13	2.93 to 4.06	1(×3), 3, 5(× 5), 6(×3), 7	7.5 to 12.4	Risterucci et al. (2003)
BP	*Phytophthora palmivora*	IMC78 × Catongo, DR1 × Catongo, S52 × Catongo	AFLP, RFLP, SSR	PRR	MAPMAKER-EXP/QTLCartographer	Composite interval mapping	2	2.5, 7.4	4(×2)	10.1, 22.6	Clement et al. (2003)

(continued)

Table 2.3 (continued)

Disease	Species	Population	Marker	Phenotyping	Software linkage/QTL	Statistical methodology	QTL number	LOD	LG	%VE (R^2)	References
BP	*Phytophthora palmivora*	Pound7 × UF271 (F1)	SSR	Artificial inoculation in fruits	JOINMAP/MAPQTL	Restricted multiple QTL mapping	3	6.1 to 14.6	4, 8, 10	7.3 to 23	Brown et al. (2007)
BP	*Phytophthora palmivora*	(SCA6 × H) × C1, (P7 × ICS100) × C1 and (P7 × ICS95) × C1	SSR	PRR, artificial inoculation in leaf-disc	JOINMAP/MAPQTL	Single marker locus analysis, simple interval mapping	11	2 to 4.35	1(×2), 2, 3, 4(×2), 6(×3), 8, 10	13.2 to 27.6	Akaza et al. (2016)
BP	*Phytophthora palmivora, P. citrophthora, P. capsici*	TSH1188 × CCN51 (F1)	SRR	Artificial inoculation in leaf-disc	OneMap/OneMap	Composite interval mapping	6	3.224 to 3.873	1, 2, 3, 4, 6(× 2)	1.776 to 3.299	Barreto et al. (2018)
BP	*Phytophthora palmivora*	Pound7 × UF273 (F1)	SNP	Artificial inoculation in fruits	JOINMAP/GenStat	Marker regression, simple interval mapping, composite interval mapping, multiple QTL mapping	4	3.82 to 10.88	2, 4, 8, 10	8.23 to 23.07	Gutiérrez et al. (2021)

(continued)

Table 2.3 (continued)

Disease	Species	Population	Marker	Phenotyping	Software linkage/QTL	Statistical methodology	QTL number	LOD	LG	%VE (R^2)	References
WB	*Moniliophthora perniciosa*	SCA6 × ICS1 (F2)	RAPD, AFLP	Average number of vegetative brooms per canopy area	MAPMAKER-EXP/Q-gene	Single factor, composite interval mapping	1	>4	11	34.8	Queiroz et al. (2003)
WB	*Moniliophthora perniciosa*	SCA6 × ICS1 (F2)	SSR	Number of brooms produced by infection	JOINMAP/MAPQTL	Simple interval mapping, multiple QTL mapping, restricted multiple QTL mapping	3	3.38 to 10.55	1, 9(× 2)	6.7 to 51.1	Brown et al. (2005)
WB	*Moniliophthora perniciosa*	TSH1188 × CCN51 (F1)	SSR	Number of brooms produced by infection (natural and artificial)	-/SAS	Simple regression	3	–	3, 9(× 2)	74.6 to 82.7	Santos et al. (2007)
WB	*Moniliophthora perniciosa*	TSH1188 × CCN51	SNP	Vegetative brooms, cushion brooms	JOINMAP/GenStat	Restricted maximum likelihood	7	2.318 to 11.24	3, 4, 6(×2), 7, 9(× 2)	0.6 to 13.5	Royaert et al. (2016)

(continued)

Table 2.3 (continued)

Disease	Species	Population	Marker	Phenotyping	Software linkage/QTL	Statistical methodology	QTL number	LOD	LG	%VE (R^2)	References
FP	*Moniliophthora roreri*	Pound7 × UF273 (F1)	SSR	Artificial internal and external inoculation of pods	JOINMAP/MAPQTL	Restricted multiple QTL mapping	5	3.9 to 7.2	2(×2), 7, 8(×2)	4.5 to 9.8	Brown et al. (2007)
FP	*Moniliophthora roreri*	Pound7 × UF273 (F1)	SNP	Artificial inoculation of pods	JOINMAP/GenStat	Marker regression, simple interval mapping, composite interval mapping, multiple QTL mapping	11	3.87 to 9.19	2, 4(×2), 7(×2), 8(×2), 9(×2), 10(×2)	4.96 to 11.19	Gutiérrez et al. (2021)
CW	*Ceratocystis cacaofunesta*	SCA6 × ICS1 (F2)	SSR, EST-SRR	Stem inoculation	JOINMAP/MAPQTL	Simple interval mapping, composite interval mapping, multiple QTL mapping	2	2.57 to 3.27	3, 9	7.7 to 9.6	Santos et al. (2012b)
CW	*Ceratocystis cacaofunesta*	TSH1188 × CCN51 (F1)	SNP	Stem inoculation	JOINMAP/MAPQTL	Interval mapping, multiple QTL mapping	2	4 to 48.85	4, 6	3.8 to 62.6	Fernandes et al. (2018)

BP black pod; *CW* Ceratocystis wilt; *FP* frosty pod; *LG* linkage group; *WB* witches' broom

Among these different cocoa genotypes, 15 were used to identify EST-SNPs, among which the four genotypes used by Allegre et al. (2012) for the linkage genetic mapping of the discovered EST-SNPs. Two-population maps and a saturated consensus genetic map were developed for cocoa; they included SNPs and multiple markers, some of them located in coding sequences of candidate genes (Fouet et al. 2011; Royaert et al. 2011; Fernandes et al. 2018).

The three genetic maps developed from the TSH1188 × CCN51 population represent an effort to identify genomic regions involved in the resistance response to witches' broom, Ceratocystis wilt and black pod, which are the three main diseases that affect cocoa in several regions where it is cultivated. SNP and SSR markers were co-localized with genome sequences to correlate gene function to observed traits such as plant resistance (Royaert et al. 2016; Fernandes et al. 2018; Barreto et al. 2018).

DNA ships containing species-representative SNPs were used for the genetic mapping of cocoa. A chip with 6 k cocoa SNPs was used to map and identify QTLs for resistance to witches' broom and Ceratocystis wilt (Royaert et al. 2016; Fernandes et al. 2018). Additionally, the 15 k strategy consisted of identifying thousands of SNPs, many of them within genes annotated in the cocoa genome, and transforming them into an Illumina Infinity II array. These SNPs were identified within a diversity panel of 11 different cocoa accessions. The mapping of these SNPs was performed in two F1 populations of full-sib: one was derived from the cross between EET 95 × Silecia 1; the other was from SCA12 × unknown genotype (Livingstone et al. 2017). In this last work, genomic information and plant phenotyping were intensively used for resistance to frosty pod rot, the incidence of witches' broom disease and other characteristics.

Cocoa genetic mapping populations include different generations, genealogies and sizes (Table 2.2). These segregating populations range from 82 to 733 individuals, with a predominance of medium-sized populations (150–250 individuals), which are suitable for genetic mapping. They were generated from controlled crosses between wild varieties (SCA 6), mutants (Catongo), simple hybrids (self-pollination of TSH516) and complex hybrids (TSH1188, CCN51, etc.). Thus, these populations are full-sib progenies.

2.6.2 QTL Regions Disease Resistance in Cocoa

Efforts to identify QTL regions associated with biotic stress in cocoa have focused on the four main pathogens that affect culture: black pod, witches' broom, frosty pod and Ceratocystis wilt.

2.6.2.1 Black Pod Resistance

Since the early 2000s, most of the efforts have focused on the elucidation of the QTL regions involved in resistance to the different species of the genus *Phytophthora* that cause black pod, due to the devastating consequences of the pathogens in culture; these efforts have used various molecular markers such as AFLP, RAPD, RFLP, EST-SSR, SSR and more recently SNP. A QTL region located in the linkage group (LG) 4, initially identified by Crouzillat et al. (2000), represents one of the most promising regions in the interaction with the different species of *Phytophthora*. This region has been validated in different genotypes and in different countries (Table 2.3). Other regions located in the LGs 1, 2, 4, 8 and 10 have been recurrent in various studies. The polygenic character of black pod resistance was initially proposed by Spence & Bartley (Spence and Bartley 1966), which has been validated by the identification of various QTLs, from QTLs of major effect to minor effect by different studies (Table 2.3). It is important to highlight that the values of % phenotypic variation explained (%VE) presented by the various authors have been influenced by the different phenotyping methodologies and statistical methodologies implemented in each study, ranging from point-to-point analysis to multipoint analyses potentiated with machine learning algorithms like HMM.

2.6.2.2 Witches' Broom Resistance

The main phenotyping methodology used in the studies that have addressed to the witches' broom resistance is the quantification of infected vegetative brooms in the field and the studies have focused on two crosses, SCA6 × ICS1 and TSH1188 × CCN51. The markers used were restricted to RAPD, AFLP, SSR and more recently SNP. A first study identified a QTL in LG 11 with a %VE = 34.8 (Queiroz et al. 2003). Although it has been refined in terms of phenotyping and of use of a greater number of markers (REF), this results most likely was due to the use of a linkage map wih insufficient coverage for the date. Subsequently, Brown et al. (2005) identified a QTL with a major effect with %VE = 51.1 in LG 9, among others with a minor effect (Table 2.3). In 2007, Santos et al. also identified QTLs in the same LG and LG 3 with %VE between 74.6 to 82.7, and this study used a simple regression statistical approach without the use of a linkage map. More recently, Royaert et al. (2016) using SNP also identified two QTLs in LG 9, but with substantially lower %VE and also identified several QTLs of minor effect varying between 0.6 and 13.5 of %VE, due to a different genetic background used to generate the segregating population (Table 2.3).

2.6.2.3 Frosty Pod Resistance

Efforts to elucidate QTL regions associated with resistance to frosty pod caused by the *M. roreri* fungus, are less compared to black pod and witches' broom. This may

be due to the less cosmopolitan distribution of the disease, present only in northern South America and have reports in Central America to southern Mexico. To date, only two studies have been carried out on detection of QTLs associated with resistance to frosty pod (FP) (Table 2.3), both using the same genotypes, Pound7 × UF273 (F1). Initially Brown et al. (2008) identified five QTLs in LG 2, 7, and 8 with a %VE varying between 4.5 and 9.8 using SSR markers. Later, Gutiérrez et al. (2021) using SNP markers identified 11 QTLs confirming those previously identified by Brown et al. (2008), and identified 6 more regions with a %VE varying between 4.96 and 11.19.

2.6.2.4 Ceratocystis Wilt Resistance

The vascular disease Ceratocystis wilt, caused by the fungus *Ceratocystis cacaofunesta*, is of utmost importance since it causes the death of infected plants and it has also been proven that the vast majority of witches' broom resistant genotypes show susceptibility to Ceratocystis wilt. The first study carried out by Santos et al. (2012b) used a linkage maps previously developed using SSR and EST-SSR markers, and identified two QTLs in LG 3 and 9, with a %VE of 7.7 to 9.6 on the cross SCA6 × ICS1 (F2). The first and only effort in the identification of QTLs associated with Ceratocystis wilt resistance based on a linkage map constructed with the use of SNP markers using the TSH1188 × CCN51 (F1) cross, was carried out by Fernandes et al. (2018), identifying two QTLs in LG 4 and 6, with a %VE between 3.8 and 62.6, the last in LG 6, being the first QTL of major effect reported as associated with resistance to Ceratocystis wilt in cocoa.

2.6.2.5 Brief Summary of QTL Regions Associated with Resistance to Biotic Stresses in Cocoa

The first decade of QTL mapping of disease resistance in cacao was examined in detail using meta-analysis (Lanaud et al. 2009). Nowadays, the number of QTLs and types of diseases have been increased (Table 2.3). A summary of QTL regions is shown from the main QTLs associated with resistance to biotic stress of the four diseases addressed in QTLs studies (Fig. 2.3) (Akaza et al. 2016; Gutiérrez et al. 2021; Brown et al. 2005, 2007; Royaert et al. 2016; Santos et al. 2012a, b; Fernandes et al. 2018). In this summary, only the QTLs identified by interval mapping methodologies in studies with $n \geq 150$ individuals, and showing the highest %VE (R^2) by disease are shown.

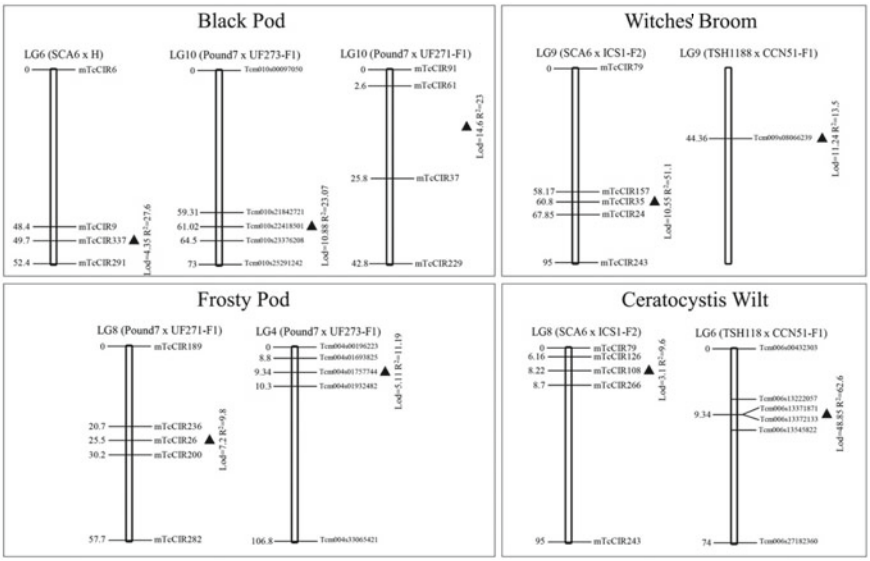

Fig. 2.3 Main linkage groups, crosses and QTLs associated with resistance to biotic stresses in cocoa. In each linkage group the relative position (cM) is shown on the left and the markers on the right. ▲: QTL Region, LG: Linkage group, Lod: LOD peak value of the logarithm of the likelihood ratio observed, R^2: proportion of the phenotypic variation explained by the QTL. This map was built with data from previous studies (Akaza et al. 2016; Gutiérrez et al. 2021; Brown et al. 2005, 2007; Royaert et al. 2016; Santos et al. 2012b; Fernandes et al. 2018)

2.7 Cocoa Germplasm Characterization

The characterization of a plant germplasm gives crucial information to understand the genetic bases of the given species, allowing the comprehension of the identity of the individuals that make it up, the genealogical and geographic relationships, the genetic variability present in populations, and the geographic origin of the accessions present in the germplasm bank, which together serve as a basis for both the conservation of genetic resources and for the improvement of certain characteristics of agronomic interest (Lindo et al. 2018; Zhang et al. 2009a). In the case of *T. cacao*, morphological and/or molecular characterizations have already been performed in germplasm banks and/or farms present in several countries, such as Costa Rica (Zhang et al. 2009b), Peru (Zhang et al. 2009a), Colombia (Osorio-Guarín et al. 2017), Jamaica (Lindo et al. 2018), China (Wang et al. 2020), Trinidad (Bekele et al. 2020), Brazil (Santos et al. 2015), Cameroon (Efombagn et al. 2009), Dominican Republic (Boza et al. 2013), Honduras and Nicaragua (Ji et al. 2013), among others.

In Costa Rica, 688 accessions of *T. cacao* from the International Cocoa Collection of CATIE (IC3) maintained in the country were used, which were genotyped using 15 SSR molecular markers. The level redundancy analysis revealed that, in a random sample, 113 accessions were representing 90% of the allelic diversity

present in the germplasm collection. Two hundred and thirty-one alleles distributed in 548 genotypes were identified. These 548 genotypes, based on geographic origin, were classified into 12 groups. The groups from Brazil and Ecuador accounted for about 56% of the total number of exclusive alleles, noting that the collection was composed of only 18.4% from these two groups. Through genetic structure analyses, it was observed that within-country/within-region was responsible for 84.6% of the total molecular variation, while between-the-countries/between-regions represented 15.4%. The results found in this work mainly indicated that the IC3 contains a high level of redundancy (Zhang et al. 2009b).

In Peru, the evaluation was made using a total of 612 cocoa accessions from the Pound collection, the first to be established in South America and which has important sources of genetic variation that can be exploited in breeding programs, especially for resistance to diseases. These accessions were genotyped using 15 microsatellite markers. As main results, 180 cases of mislabelling and a total of 116 duplicates were detected, and 316 accessions were then selected for the diversity analyses. The half-sib and full-sib families were rebuilt for the five access groups in the collection, with 78 half-sib and 48 full-sib families being rebuilt. Through analysis of probability simulations, eight parents were identified as probably responsible for 117 pairs of mother–child relationships present in the collection. The principal coordinate analysis (PCA) together with the Bayesian method of clustering indicated a marked structure of genetic diversity stratified by the fluvial systems of the Peruvian Amazon (Zhang et al. 2009a).

In Colombia, a set of 565 accessions was used for evaluation, of which 450 came from the Colombian Corporation for Agricultural Research (Corpoica) germplasm bank and 115 from breeding collections. For genotyping, these 565 accessions plus 252 accessions from reference populations were characterized using 87 SNP markers. For phenotyping, 141 accessions were analysed using 18 morphological characters and 94 accessions were analysed with four biochemical characters, both from the UPOV descriptor list. PCA analysis of the morphological characters showed that 60.6% of the total variation was represented in seven descriptors, while for the biochemical characters 100% of the variation was explained in the four characters evaluated, noting that for both sets of characters the genotypes analysed were grouped into four clusters. Genotyping analysis showed that this collection from Corpoica has a high genetic diversity (Osorio-Guarín et al. 2017).

In Jamaica, 160 accessions from the germplasm banks of the Orange River Agricultural Research Station in the parish of St. Mary and Montpelier Agricultural Research Station in St. James, plus 150 reference accessions from farms have been characterized, both sets of accessions being genotyped using 94 SNP markers. The results indicated that most of the genotypes present in Jamaica collection are hybrids originating mainly from the genetic groups Parinari, Iquitos Mixed Calabacillo, Scavina, Amelonado and Criollo, with the greatest contributions being from the Parinari and Amelonado groups. Through the construction of the Neighbour-joining dendrogram, the formation of two large clusters was observed, in cluster 1 the accessions from the Nacional and Scavina groups were inserted and in cluster 2 the accessions from the Amelonado, Criollo, Trinitario, Nanay, Parinari and Iquitos Mixed

groups Calabacillo. Through the analysis of molecular variance, it was also found that the highest level of differentiation occurred among individuals within the population (97%) (Lindo et al. 2018).

In China, where a collection of 170 accessions of *T. cacao* is maintained in Yunnan, a sample of 88 accessions was selected for characterization and was genotyped with 91 SNP markers. It is also worth mentioning the use of over 140 reference accessions. Through PCA, it was observed that most accessions belonging to the Yunnan collection were dispersed in the Amelonado group and other reference groups. Through the UPGMA dendrogram, the formation of large clusters was observed, cluster 1 with all reference populations, except Amelonado, and cluster 2 with all accessions of the Yunnan collection together with the reference population Amelonado. These results showed that the introductions of cocoa accessions in the Yunnan collection consisted mainly of hybrids derived from the Amelonado group, thus demonstrating a low genetic diversity and suggesting the need for new introductions of genotypes from the other groups, to obtain a greater representation of the genetic diversity of this species (Wang et al. 2020).

In Cameroon, 300 accessions from farms of the South and West regions, and 77 accessions from the Institute of Agricultural Research for Development (IRAD) germplasm bank were selected for characterization, and both sets were evaluated using eight quantitative and nine qualitative descriptors. Morphological variations were observed between accessions from farms and those from the germplasm bank. When evaluating the quantitative characters related to the pods, it was possible to find differences between the sets of accessions from farms in the South and West regions. By PCA, it was found that 86% of the total phenotypic variance was contained in four principal components. Comparing morphological and molecular results, it was observed that the differences found between farms in the South and West region were mainly influenced by non-genetic factors. While accessions from the germplasm bank and those from farms were considered genetically distant, which suggested a low introduction of genotypes from breeding programs in farm crops (Efombagn et al. 2009).

In the Dominican Republic, 803 accessions from the germplasm bank and 55 accessions cultivated in local farms were selected for characterization by genotyping with 14 SSR markers. It was detected in the set of accessions from the germplasm bank the presence of 15 synonymous groups containing 48 accessions, while for the set coming from local farms, the occurrence of 13 synonymous groups containing 30 accessions was identified. Analysing the genetic diversity, the presence of 117 and 113 alleles for the germplasm and farm accessions, respectively was observed. When evaluating the population structure, a predominance of accessions in the Amelonado group was detected, which represented 43.9% for germplasm accessions and 72.1% for the farm accessions (Boza et al. 2013).

In Trinidad, 1900 accessions from the International Cocoa Genbank Trinidad (ICGT) germplasm, of which 260 belong to the Trinitario group were characterized using 25 descriptors from the International Board for Plant Genetic Resources. Upon observation of the Pod Index, which is used as an indicator of potential yield, a variation between 13.9 and 92.8 was found, with values below 21.0 being considered

favourable. It was also observed that 23% of the accessions considered superior belonged to the Trinitario group. Through PCA, it was identified that 69% of the total variation was contained in nine principal components. Observing the characters 'number of seeds' and 'cotyledon mass' for the 260 accessions of the Trinitario group, six were considered as elite for potential yield (Bekele et al. 2020).

In Honduras and Nicaragua, 84 accessions collected from traditional cocoa farms and 31 reference clones from the IC3 were selected and genotyped with 70 SNP markers. It was found, in the group of 84 accessions collected on farms, the presence of six synonymous groups, in which 16 accessions were included, representing 11.9% of the varieties included in this group. Through multivariate analysis, the formation of five clusters was identified, which represented the genetic groups Antigo Criollo, Amelonado, Trinitario from Nicaragua, Trinitario from Honduras and Forastero from Superior Amazon. It was found that the classification of accessions by farmers had high coincidence with the groups formed through genotyping by SNPs, highlighting that from the 28 accessions named as Criollo, 22 were in fact inserted in the Antigo Criollo group; two out of three accessions named as the Trinitario of Nicaragua were grouped into the Trinitario reference group; and 12 of the 13 accessions named as Trinitario de Honduras actually belonged to the Trinitario de Honduras group (Ji et al. 2013).

In Brazil, 279 accessions of *T. cacao* were selected for characterization, from which 179 were from the IFBAIANO and CEPLAC institutions, located in Bahia State in the cities of Canavieiras, Camacan, Gandu and Uruçuca, and another 103 genotypes from the CEPEC/CEPLAC institution, in the cities of Ilhéus and Igrapiúna in Bahia State. Genotyping was carried out with 30 SSR markers. Genetic structure analysis allowed the formation of two groups, one named as Bahia cocoa and the other as Non Bahia cocoa. In the Bahia cocoa group, two subgroups were observed, one where the genotypes cultivated on farms were inserted and the other composed of SIAL/SIC clones. Based on genetic analysis, the 30 SSR markers identified 209 alleles. Analyzing the groups separately, the genetic diversity present in the Bahia cocoa group was considered low, when compared to that presented in the Non Bahia cocoa group (Santos et al. 2015).

By analyzing the work already carried out, it is observed that the characterization of germplasm banks provides results that mainly allow to understand the genetic diversity of the species present in a given collection, influencing both genetic conservation measures and breeding programs. These data can support the selection of superior accessions that can be indicated for planting, as well as genotypes that can serve as parents in crosses in order to insert or increase traits of agronomic importance (fruit quality, yield, flavor and disease resistance) or to identify genes that can be used in genetic engineering work, mainly for disease resistance.

2.8 Map-Based Cloning of Resistance Genes

2.8.1 BAC Libraries

The construction of genomic DNA libraries based on bacterial artificial chromosomes (BAC) can be used for genomic mapping, positional cloning, complex genome sequencing and gene annotation (Holmes et al. 2015; Luo and Wing 2003). For *T. cacao*, a BAC library was built and validated in 2004, aiming to develop molecular resources to enable the study of the structure and evolution of the genome of this species (Clement et al. 2004). For the construction of the *T. cacao* BAC library, DNA extraction was performed from collected leaves of the SCA6 genotype from the germplasm collection of the Center National de Recherche Agronomique (CNRA) of Ivory Coast. As a result, this library has 36,864 clones with an average size of 120 kb each, with about 80% of the clones having a size greater than 100 kb and 13% greater than 150 kb, thus representing a coverage around 10 times greater in relation to the size of the haploid genome. Additionally, from the establishment of this library, the authors characterized and refined regions associated with disease responses. Relationships between physical and genetic distances were performed, and a screening was carried out in the BAC library using nine resistance gene analog (RGA) and defence gene analog (DGA) probes previously obtained (Lanaud et al. 2004). Two resistance clusters were identified: a region of 3.4 cM located on chromosome 4 and another of 1.1 cM on chromosome 7, which, respectively, were used to construct two contigs, one of 1004 kb and another of 404 kb, thus providing the first clues about the relationships between physical and genetic distances in the genome of *T. cacao* (Clement et al. 2004).

2.8.2 Cytogenetic Studies

Cytogenetic studies provided a great contribution to the understanding of the genomic structure and the evolutionary biology of cultivated plants, mainly because they provide important integrative tools allowing genetic and genomic analyses of plant chromosomes and genomes (Figueroa and Bass 2010). Such studies may facilitate the understanding of behaviour, as well as genetic regulations and mechanisms that, at the gene level, control chromosomal dynamics, and may also contribute to the identification and transfer of resistance genes from exotic to native species (Younis et al. 2015).

For *T. cacao*, some cytogenetic studies have already been carried out, proving that this species has a symmetrical karyotype consisting of $2n = 20$ chromosomes (da Silva et al. 2017; Dantas and Guerra 2010; Figueiredo et al. 2013), with size haploid around 0.45 pg (da Silva et al. 2017) and length of each chromosome ranging from 1.19 to 2.00 μm, with arm ratios from 1.12 to 1.32 μm (Dantas and Guerra 2010). Specifically, the varieties Cacau Comum (native to the Lower Amazon Region)

and Cacau Rui (originated in the Cocoa Region of Bahia State in Brazil) contain exclusively metacentric chromosomes, while the varieties Cacau Jaca (originated in the Cocoa Region of Bahia State in, Brazil) and Cacau Papala (originated in Peru) presented one pair of submetacentric chromosomes each, pairs 5 and 7, respectively (Figueiredo et al. 2013). Analysing commercial cocoa species, by conventional staining with DAPI, an interphasic reticulate type of nucleus was observed, with 19–20 chromocenters with regular shape and size. In prophase, through conventional staining and C-Banding, it was observed that the chromosomes presented high condensation in the proximal region and decondensation in the terminal region (Dantas and Guerra 2010). Using the CMA+/DAPI− double staining technique, it was observed the presence of this band in the terminal region of the long arm in only one pair of chromosomes, the CMA+ band being often heteromorphic in size and distended in one or both homologues (da Silva et al. 2017; Dantas and Guerra 2010). Using the fluorescence in situ hybridization (FISH) technique, analyses of rDNA sites were performed, observing the occurrence of a 5S rDNA site in the proximal region of one of the three largest chromosomes and a 45S rDNA site located together with the CMA+ band. By using C-banding followed by staining with DAPI or Giemsa, it was observed in the interphase nuclei the presence of 20 well-defined chromocenters, all presenting centromeric or proximal heterochromatic bands of similar sizes (Dantas and Guerra 2010).

From cytogenetic studies, 20 EST-SSR type markers were also developed, and informative and satisfactory results on allelic variations between wild and cultivated species were obtained. Thus, 60% of these markers were polymorphic and 40% monomorphic in *T. cacao* (da Silva et al. 2017), strengthening the assertion that these studies can bring essential contributions both to structural and functional genomics as well as to the evolutionary biology of species (Figueroa and Bass 2010).

2.9 Genomics-Aided Breeding for Resistance Traits

2.9.1 Large Scale Transcriptomic Resources

A transcriptome is a snapshot of the gene expression profile of a cell and/or tissue at a given time and/or physiological situation, which can be analysed through the establishment of transcript libraries and subsequent application of specific tools (Ward et al. 2012). In *T. cacao*, functional genomics analyses have mainly sought to understand the roles of certain genes, from redundant and shared functions to unique functions (Gesteira et al. 2007), which has led to the construction of EST libraries from cocoa submitted to various factors (Argout et al. 2008; Gesteira et al. 2007; Jones et al. 2002) or disease resistance inducers (Verica et al. 2004) and more recently to high throughput analysis such as RNAseq (Teixeira et al. 2014; Fister et al. 2015).

The first effort to establish large scale transcriptomics data from *T. cacao* was made in 2002 using complementary DNA (cDNAs) from seeds and leaves of five

cocoa varieties (Amelonado, P7B, R10, Spec54, UF221 and Sic 5) (Jones et al. 2002). Two libraries from Amelonado seeds and leaves, respectively, were obtained, and an unigene of 1380 sequences was identified and annotated. In the seeds, transcripts associated mainly with storage and defence were found (e.g. vicilin, 21-kDa seed protein), while leaves mainly contained sequences related to photosynthetic process (Jones et al. 2002). In 2004, suppression subtraction hybridization (SSH) libraries resulting from *T. cacao* leaves (Forastero and Comum varieties) treated with the defence response inducers benzothiadiazole (BTH), Nep1 and methyl jasmonate/ethylene were built. A dataset of 2114 ESTs, which resulted in the assembly of 1256 unigenes was obtained. From them, 865 had corresponding genes already annotated in the database, and 330 corresponded to genes induced during the defence response (Verica et al. 2004). In 2007, an EST library was developed specifically for the *T. cacao-Moniliophthora perniciosa* interaction, using Catongo (susceptible) and TSH1188 (resistant) cocoa varieties as plant material. In this study, the authors established two EST libraries, one originating from the interaction of *M. perniciosa* with resistant genotype (RT) and another with susceptible genotype (SP), generating a dataset of 1719 unigenes and 1207 for RT and SP, respectively. From them 1371 and 859 unigenes were specific for RT and SP, respectively, and 16 functional classes were established (Gesteira et al. 2007). In 2008, 56 libraries were built, of which 25 corresponded to cocoa tissues subjected to different biotic stresses, highlighting pods infected by the *P. palmivora*, *P. megakarya*, *M. perniciosa* and *M. roreri*; leaves infected by *P. palmivora* and *P. megakarya*; stems inoculated with *M. perniciosa* and *C. fimbriata*; and stems attacked by *Sahlbergella singularis*. From the 56 libraries, 146,650 ESTs were generated, corresponding to 48,594 unigenes, 12,692 contigs and 35,902 singletons. The authors identified 1001 genes associated with stress responses, mainly resistance proteins or proteins involved in plant defence mechanisms, such as chitinases, 1-3 beta glucanases and pathogenesis-related proteins (PRs) (Argout et al. 2008). In 2014, a transcriptomic analysis of the *T. cacao-M. perniciosa* interaction was obtained by RNA-seq (Teixeira et al. 2014). The cocoa cultivar Comum was inoculated with the BP10 *M. perniciosa* isolate and material from infected seedlings was harvested 30 days after inoculation. The authors identified 562 million and 436 million of reads for infected seedlings and healthy seedlings (control), respectively. The alignment of reads against the Cacao Genome Database (Motamayor et al. 2013) (see also Sect. 2.9.2) and the differential expression analysis between infected and control samples allowed the identification of 1967 differentially expressed genes, 1269 upregulated and 698 downregulated (Teixeira et al. 2014). In 2015, through microarray analysis, genes expressed in the SCA6 and ICS1 cocoa genotypes inoculated with *P. tropicalis* and treated with salicylic acid (SA) were identified. As main results, the authors found that the treatment with SA reduced the size of the lesions and limited the growth of the pathogen in the two cocoa genotypes, especially in SCA6. Among other results, it was observed that the ICS1 genotype treated with SA showed a greater number of upregulated PR genes compared to the SCA6 genotype, also treated with SA (Fister et al. 2015) (Table 2.4).

Table 2.4 Main large-scale omics data from cocoa related to biotic stress

Resource type	Technical data	Cocoa genotype	Pathogen or inducer	Data amount	Database or accession ID	Publication year	References
Transcriptomics	ESTs, microarrays	Amelonado, P7B, R10, Spec54, UF221, Sic5	Non-inoculated leaves and seeds	1380 unigenes	np	2002	Jones et al. (2002)
	SSH, microarrays	Forastero, Comum	Benzothiadiazole, Nep1, methyl jasmonate/ethylene	1256 unigenes	CF972636–CF974749	2004	Verica et al. (2004)
	ESTs	TSH1188, Catongo	*M. perniciosa*	2926 unigenes	ES439783-ES440989 ES440990-ES442709	2007	Gesteira et al. (2007)
	ESTs, SSH	ICS1, SCA6	*P. palmivora*, *P. megakarya*, *M. perniciosa, M. roreri*, *C. fimbriata*, *Sahlbergellasingularis*	48,594 unigenes	https://esttik.cirad.fr/	2008	Argout et al. (2008)
	RNA sequencing (RNA-seq)	Comum	*M. perniciosa*	562,491,362 paired reads (infected)	SRA066232	2014	Teixeira et al. (2014)
	Microarrays	SCA6, ICS1	*P. tropicalis*, salicylic acid	1974 differentially expressed genes (salicylic acid)	GPL18260	2015	Fister et al. (2015)
Genomics	Genome-wide shotgun strategy, Roche/454, Illumina and Sanger sequencing	Belizean Criollo	na	76% genome, 82% genes anchored (version 1)	https://cocoa-genome-hub.southgreen.fr/	2011	Argout et al. (2011)

(continued)

Table 2.4 (continued)

Resource type	Technical data	Cocoa genotype	Pathogen or inducer	Data amount	Database or accession ID	Publication year	References
	Illumina large insert size mate paired libraries, 52× long reads, Genotyping-by-sequencing	Belizean Criollo	na	75.5% genome, 99% genes anchored (version 2)	https://cocoa-genome-hub.southgreen.fr/	2017	Argout et al. (2017)
	Genome-wide shotgun strategy, Roche/454 sequencing, fosmid and BAC libraries	Matina 1,6	na	29,408 loci, 98.9% of the scaffolds anchored	https://www.cacaogenomedb.org/ PRJNA51633, ALXC00000000 Phytozome 12	2013	Motamayor et al. (2013), Goodstein et al. (2012)
Proteomics	2D gel, mass spectrometry	TSH1188, Catongo	*M. perniciosa*	554 proteins	np	2020	dos Santos et al. (2020)

np non-provided; *na* non-applicable

The overall data obtained through transcriptomic approaches allowed the identification of genes specifically involved in resistant *vs* susceptible cocoa genotypes (from different resistant sources, e.g. SCA6, TSH), in different metabolic pathways responsible for important agronomic traits (e.g. resistance, defence) or in specific plant organs (e.g. pod, seeds, leaves) (Table 2.4). Such data provided solid bases for an increase of the global knowledge of the plant-pathogen interaction, but also as source of information useful for molecular strategies (e.g. marker development) associated to cocoa breeding programs.

2.9.2 Genome Sequencing

The genome is the set of all the genetic information of an organism or a cell, which can be determined in an increasingly broad, fast and efficient way thanks to the development of new sequencing platforms, known as next generation sequencing (NGS), enabling the whole genome sequencing (WGS) (Giani et al. 2020). The genomic sequencing of species of agronomic interest has allowed breeders more consistent approaches, especially when they are dealing with multigene inheritance traits, which consequently suffer a greater environmental influence and are more difficult to be evaluated and selected based only on classical breeding techniques (Ray and Satya 2014).

For the *T. cacao* species, the first genome sequencing was accomplished in 2011, from the Belizean Criollo (B97-61/B2) genotype, which is appreciated because its almonds result in the production of fine chocolate. For this sequencing, the authors used the genome-wide shotgun strategy incorporating Roche/454, Illumina and Sanger technologies, generating a total of 26 Gb of data. The raw data were used for assembly using the Newbler tool, generating 25,912 contigs and 4792 scaffolds, which represented a total assembly length of 326.9 Mb, covering approximately 76% the genome size of this species. Through the use of 1259 molecular markers, 94% located in the assembly, it was possible to anchor 67% of the sequenced genome of *T. cacao* along the 10 linkage groups. By linking genetic distance to physical distance, relationships of 1 cM per 444 kb and 1 cM per 146 kb were observed for centromeric and chromosomal regions, respectively. It was identified, through a search for homology and functional annotation, that the genome of *T. cacao* has 28,798 genes that encode proteins, each gene having an average size of 3346 bp and 5.03 exons (Argout et al. 2011). In 2017, a second version of the Belizean Criollo genome was established (Argout et al. 2017) using some data from the first version (Argout et al. 2011) and adding new results generated by sequencing Illumina HiSeq 2000 and 52× long reads sequencing. In order to fill gaps existing in the first annotation system, these new data were integrated, and a consensus annotation was established, containing a total of 29,071 contigs encoding for proteins. In summary, this new version of the *T. cacao* genome added data compared to the previous version, improving assembly, correcting some inconsistencies, reducing the

number of scaffolds and unanchored regions, and updating structural and functional information (Argout et al. 2017).

Independently, another *T. cacao* genome was sequenced: the cultivar Matina 1-6 was used, standing out for belonging to the most cultivated cocoa group (Motamayor et al. 2013). For sequencing, the Sanger and Roche 454 pyro-sequencing methods were used, with subsequent assembly performed via Arachne. Altogether, around 32.4 Gb of sequences were generated, as well as 20,103 contigs, which formed 711 scaffolds, representing a total length of 346.0 Mbp. It was observed that 98.9% of these data were mapped on the 10 chromosomes, where 29,408 loci were evidenced. Aiming to establish a comparison between this genome of Matina 1-6 with that of Criollo, the authors performed a synteny analysis, resulting in the identification of 271 orthologous regions between the two genomes. It was found that most genes responsible for LLR-RLK protein synthesis, flavonoid and lipid biosynthesis, and terpenoid synthesis are inserted outside orthologous regions, which led the authors to suggest that genes located outside these regions or in orthologous regions between non-orthologous chromosomes may be responsible for the existing differences related to disease resistance and fruit quality between the two genotypes (Motamayor et al. 2013).

From the sequencing, annotation and availability in databases of the Belizean Criollo and Matina 1-6 genomes, genomic and post-genomic approaches were facilitated, mainly serving as a basis for projects involving comparative and/or evolutionary studies in *T. cacao* and enhancing genetic analysis to identify genes involved in important characteristics of the cocoa tree, which has been directly contributing to the breeding programs of this species.

2.9.3 Proteomics Resources

Currently, only few studies related to proteomics of cacao-pathogen interaction have been developed. However, in 2020, comparative proteomics analysis of the *T. cacao-M. perniciosa* interaction was performed. TSH1188 (resistant) and Catongo (susceptible) cocoa genotypes were inoculated with *M. perniciosa*, samples were harvested through the time course disease, and their proteins analysed by mass spectrometry. The authors obtained a total of 554 protein spots with a greater amount of proteins observed in TSH1188, mainly in inoculated samples. In the Catongo genotype, uninoculated samples (control) presented more proteins than the inoculated ones, indicating a possible gene repression after inoculation with the pathogen. The identified proteins were distributed into eight functional groups, and it was noted that the TSH1188 genotype presented a greater amount of defence and stress proteins than the Catongo one, both inoculated with *M. perniciosa.* Subsequently, these proteins were identified: PRs, chitinases, proteins related to oxidative stress regulation and trypsin inhibitors. For proteins differentially expressed in the two genotypes, interactomics analysis revealed the occurrence of complex networks of protein–protein interactions specific of each genotype (dos Santos et al. 2020).

2.9.4 Bases for Marker Assisted Selection

With the support of structural and functional *T. cacao* omics data, the cocoa breeding has contributed to identify potential sources of resistance to diseases (Wickramasuriya and Dunwell 2018). By accessing the information present in the genome and how it is structured, it is possible to locate genomic regions that are possibly responsible for triggering resistance responses in the plant. These regions have already been described in *T. cacao* for interaction with several pathogens, especially *T. cacao-M. perniciosa* (McElroy et al. 2018; Osorio-Guarín et al. 2020; Schnell et al. 2007; Motilal et al. 2016), *T. cacao-M. roreri* (Romero Navarro et al. 2017; Gutiérrez et al. 2021; McElroy et al. 2018; Osorio-Guarín et al. 2020; Schnell et al. 2007), *T. cacao-Phytophthora* spp. (Gutiérrez et al. 2021; Motilal et al. 2016; Schnell et al. 2007) and *T. cacao-C. cacaofunesta* (Fernandes et al. 2018; Santos et al. 2012b). These and other works cited throughout this chapter have provided important support to know how certain characters are genetically determined and at the same time allowing their selection assisted by markers, thus contributing to the development of new cocoa cultivars with certain characteristics of agronomic interest in a shorter time span (see also Sect. 2.6 above).

2.10 Brief on Genetic Engineering for Resistance Traits and Recent Concepts and Strategies Developed

2.10.1 Review on Achievements of Transgenics

In addition to support by works involving molecular marker assisted selection, genomic data provide a crucial basis for selection of potential genes to be used in genetic engineering applications, mainly through recombinant DNA technology. For the application of this technique, *Agrobacterium tumefaciens* has become the most popular tool for plant transformation, by delivering genes of interest in specific plants including cocoa (Hwang et al. 2017; Maximova et al. 2003). In *T. cacao*, some works involving the use of this technique have already been carried out, highlighting functional studies of *T. cacao* genes such as *chitinase 1* (Maximova et al. 2006), *NPR1* (Shi et al. 2010), *TcNPR3* (Shi et al. 2013), *PI3P* (Helliwell et al. 2016) and *TcBI-1* (Scotton et al. 2017).

The *chitinase 1* gene was overexpressed in the PSU-SCA6 cocoa genotype though *Agrobacterium* mediated-plant transformation (Maximova et al. 2006). Cocoa transgenic plants, compared do the wild-type (non-transgenic plants), showed an increase of the chitinase activity in their leaves as well as a reduction of necrotic lesions when the plants were challenged with the pathogen *Colletotrichum gloeosporioides.* Globally, the results showed that the plants overexpressing the *chitinase 1* gene presented a higher resistance response against the pathogen *C. gloeosporioides* than non-transformed plants (Maximova et al. 2006).

The *TcNPR1* cDNA was isolated from of the SCA6 cocoa genotype and used to complement, by genetic transformation, Arabidopsis plants presenting mutation for *NPR1* (npr1-2 mutant) as well as a high susceptibility to the pathogen *Pseudomonas syringae* pv. tomato (Shi et al. 2010). Complementation showed that the *TcNPR1* gene is a functional ortholog of *A. thaliana* NPR1, and that the overexpression of *TcNPR1* in npr1-2 mutant plants conferred an upregulation to the *PR1* gene after treatment with salicylic acid, and increased the resistance to the pathogen *P. syringae* pv. tomato (Shi et al. 2010). Similar methodology was used to elucidate the function of the cocoa *NPR3* gene, considered as a possible repressor of defence responses mediated by *NPR1*. The *TcNPR3* cDNA was isolated from SCA6 cocoa genotype, and then transferred to *A. thaliana* plants mutated for *npr3*, the Arabidopsis endogenous version of this gene. Functional complementation confirmed that *TcNPR3* is a negative regulator of defence responses in floral tissues, and a functional ortholog of the *A. thaliana NPR3* gene (Shi et al. 2013). *Theobroma cacao* leaves were also submitted to transient transformation with *TcNPR3*: the knockout of this gene led to an increase of the resistance responses against the fungus *Phytophthora capsici*, resulting in smaller lesion sizes and reduction of the pathogen replication (Shi et al. 2013).

Functional study of the *PI3P* gene was made by transient and stable SCA6 cocoa genotype transformation (Helliwell et al. 2016). Cocoa leaves transiently overexpressing the *PI3P* gene showed an increase of resistance against the pathogen *P. palmivora*, while the transient overexpression of *PI3P* directed to the apoplast showed an increase to the resistance against the pathogen *P. tropicalis*. Stable transformation of cocoa plants with *PI3P* showed an increased resistance against the pathogen *C. theobromicola*, while addressing the *PI3P* sequence to the apoplast, stable transformation showed an increased resistance against the *P. palmivora* and *P tropicalis* pathogens (Helliwell et al. 2016).

The negative programmed cell death regulator Bax-1isolated from CAB214 cocoa genotype was used to transformed the Micro-Tom tomato cultivar (Scotton et al. 2017), a plant model for cocoa-*M. perniciosa* interaction (Marelli et al. 2009). As main results, the Micro-Tom TcBI-1-transformed plants inoculated with the necrotrophic pathogens *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, *Botrytis cinerea*, and *M. perniciosa* showed a significant reduction of the severity of the corresponding symptoms. Additionally, the authors suggested that the overexpression of *TcBI-1* affects the penetration of germinating *M. perniciosa* spores into susceptible tissues of *T. cacao*, and may be able to restore part of the non-host resistance against the S-biotype of *M. perniciosa* (Scotton et al. 2017).

These studies showed that genetic transformation of cocoa (or of related plant model) allowed to functionally confirm the role of some cocoa candidate genes in plant resistance to different pathogens. These results also opened up potential opportunities for the development of further work both in conventional genetic breeding and in biotechnological applications, aiming to increase the resistance responses of *T. cacao* (Maximova et al. 2006; Shi et al. 2010, 2013; Helliwell et al. 2016).

2.10.2 Genome Editing

A great advance with the *T. cacao* genome sequencing (see Sect. 2.9.2) is the possibility to efficiently and precisely edit sequences of interest related to important traits such as pathogen response or resistance. Among the gene editing techniques, the one that has been gaining recently more prominence is the clustered regularly interspaced short palindromic repeats-associated protein 9 (CRISPR/Cas9), due to its simplicity, high efficiency, easy use in the production of transgenics (Mali et al. 2013). Additionally, CRISPR/Cas9 has a high versatility and can be used for knockout, insertion, deletion and replacement of genes, as well as in the activation of gene expression, providing approaches that directly or indirectly help researchers in the processes of plant domestication and improvement of traits of agronomic interest (Ahmad et al. 2020). In agriculture, CRISPR/Cas9 stands out above all for the development of works aimed to the resistance of plants to pathogens, with three factors supporting this assertion: (i) there is a high availability of information related to specific pathosystems allowing the identification of potential genes involved in triggering defence responses to be edited; (ii) disease resistance is often achieved after editing a gene; and (iii) targeted mutagenesis is easily applied to disease resistance, as the inactivation of susceptibility genes can lead to the manifestation of a resistant phenotype (Borrelli et al. 2018).

In *T. cacao*, the first work involving the use of the CRISPR/Cas9 technique took place in 2018 (Fister et al. 2018). In this work, the use of CRISPR/Cas9 transient-mediated transformation to introduce the CRISPR/Cas9 components into cocoa leaves and cotyledon cells would limit the expression of *TcNPR3*, a defence response suppressor gene (see also Sect. 2.10.1). It was hypothesized that the knockout of this gene would result in an increase in resistance responses in tissue treated with CRISPR/Cas9. As main results, the CRISPR/Cas9 edition system allowed the deletion of 27% of *TcNPR3* copies in treated tissues (leaves). Moreover, 72 h after inoculation with the pathogen *P. tropicalis*, the tissues treated with the *TcNPR3*-CRISPR/Cas9 construct exhibited a reduction of the lesion size when compared to the control. The transformed leaves inoculated with the pathogen also showed an increase of the expression level of the *PR-2, PR-3, PR-4, PR-5* and *TcNPR1* genes in relation to control. Then, stable transgenics for *TcNPR3* in mutant embryos were obtained, being noted that the expression of single guide RNA (sgRNAs) and Cas9 in developing embryos can increase the proportion of mutated *TcNPR3* in the tissue under study, which would lead to a possible homozygosis for this mutated embryo or plant. The overall results confirmed the role of *TcNPR3* as a repressor of resistance responses in *T. cacao*, and additionally the application of this technique in a pioneering way for this species represents a great advance in functional genomics and allows greater precision in genetic engineering applications in the genetic breeding of cacao (Fister et al. 2018).

2.10.3 Nanotechnology

The advent of nanotechnology has brought new perspectives to face the constant challenges encountered when working with phytopathology, and may in the future be used to assist in the management, diagnosis and genetic transformation of plant diseases (Elmer and White 2018). In *T. cacao*, some studies aiming the development of nanoparticles have already been developed. In 2019, work was carried out to synthesize silver nanoparticles (AgNPs) from three different parts of the cocoa fruit: seed, husk and pulp. As part of this study, the antimicrobial potential of nanoparticles against *Bacillus subtilis* and *Escherichia coli* was tested; the authors found that the nanoparticles affected bacterial growth, probably causing protein leakage and cell death. Additionally, it was observed that AgNPs from the pulp showed better antimicrobial activity than the AgNPs from seed and husk. The authors argued that the these results would open new perspectives, especially for antimicrobial applications in environmental sciences, health and related fields (Thatikayala et al. 2019). In 2021, another work was developed, this one using cocoa husks for the synthesis of zinc oxide nanoparticles (ZnONPs), verifying its antimicrobial potential against foodborne pathogens. As main results, the authors found that ZnONPs showed antimicrobial activity against *E. coli* and *Staphylococcus aureus* when used at concentrations of 6.25 and 12.5 μg /mL, respectively. Additionally, it was noted that ZnONPs had a more potent antimicrobial activity than chloramphenicol. The authors suggested that the results open possibilities to explore underutilized plant materials for the synthesis of ZnONPs in an ecological and economical way, as well as for future applications of ZnONPs in food, cosmetic, textile and therapeutic medicine packaging (Sarillana et al. 2021).

2.11 Brief Account on Tole of Bioinformatics as a Tool

2.11.1 Gene and Genome Databases

Currently available *T. cacao* genomes are from 2 distinct genotypes, the Criollo genotype which can be found in Cocoa Genome Hub (Argout et al. 2017) and National Center for Biotechnology Information (NCBI), Matina genotype has its genome available in Cocoa Genome Database (www.cacaogenomedb.org/) and Phytozome 12 (Goodstein et al. 2012). The genomes of the two genotypes (Criollo and Matina) have an excellent assembly quality and are good reference genomes (see also Sect. 2.9.2 and Table 2.4). Unfortunately, there is no unified database in which to integrate genotype genomes with tools to compare them.

2.11.2 Comparative Genome Databases

Some comparative genomic analyses of *T. cacao* can be done in databases such as NCBI and Phytozome for example. The quality of the *T. cacao* genome is also important for comparative studies of genetic families in other species or plant families such as Brassicaceae (Hofberger et al. 2015), which ends up indirectly providing new information on comparative genomics of *T. cacao.*

2.11.3 Gene Expression Databases

Although transcriptomics studies from *T. cacao* exist, most of the data and results are not available or accessible in public databases. The two main *Theobroma cacao* ESTs data that can be accessed are the ESTtik database (https://esttik.cirad.fr/) (Argout et al. 2008) and the CocoaESTdb bank (http://cocoaestdb.cpcribioinformatics.in/) (Naganeeswaran et al. 2015). A unique ESTs database from ESTtik and dbEST was obtained and is available at http://cocoaestdb.cpcribioinformatics.in/ (Naganeeswaran et al. 2015). Transcriptomics studies of the *T. cacao* and *M. perniciosa* interaction (Teixeira et al. 2014) and more recently gene expression studies related to evolutionary adaptations (Hämälä et al. 2019) were also obtained (see also Sect. 2.9.1 and Table 2.4).

2.11.4 Protein Databases

Although there are no specific proteomic databases for *T. cacao*, there are proteomic studies in somatic embryogenesis (Noah et al. 2013; Alexandra Pila Quinga et al. 2018; Niemenak et al. 2015), seed proteomic studies (Kumari et al. 2018; Scollo et al. 2018), abiotic stress (Monteiro Reis et al. 2020), interaction with pathogens (dos Santos et al. 2020) and fruit husk proteomics (Awang et al. 2010). These studies, among others, reinforce the importance of the proteomics study of *T. cacao* as well as provide information for a better omic knowledge of the species.

2.11.5 Integration of Different Data

In silico tools and *T. cacao* genome analysis provided a great source of knowledge to integrate and correlate data as well as to carry outstudies of genome-wide gene families such as transcription factors (Silva Monteiro de Almeida et al. 2017) or pathogenesis related proteins (Fister et al. 2016). Some studies also focused on understanding plant resistance mechanisms through characterization of a genome-wide

pattern recognition receptors and *R* genes, which make up the system innate immune *T. cacao* (Santana Silva and Micheli 2020; Li et al. 2016). As for the evolutionary aspect, studies of cacao ancestral gene recombination contributed to a better understanding of the relationship between cocoa species (Utro et al. 2012). In general, the literature on *T. cacao* is rich in information, data and omics studies related to evolutionary, agronomic and responses to biotic and abiotic stresses. The biggest challenge would be the integration of omics data from the literature in one or more *T. cacao* databases. Some efforts were made to integrate and modelled data through interactomics or systems biology allowing the drawing of general and integrative schemes related to specific physiological situation (e.g. interaction cocoa-*M. perniciosa*) (da Hora Junior et al. 2012; dos Santos et al. 2020).

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Chapter 3
Genomic Designing for Biotic Stress Resistance in Coconut

S. V. Ramesh, A. Josephrajkumar, Merin Babu, V. H. Prathibha, V. Aparna, K. S. Muralikrishna, Vinayaka Hegde, and M. K. Rajesh

Abstract Coconut (*Cocos nucifera* L.) is an economically important plantation crop grown widely in tropical and sub-tropical regions and coastal ecosystems worldwide. The impact of global warming on agriculture, in general, and perennials such as plantation crops, in particular, warrants the application of novel genomics-based approaches to safeguard the crops against abiotic and biotic stressors. Unlike the seasonal or annual crops, the damage of pests and diseases in coconut plantations is a serious threat to the coconut-based economy owing to the perennial nature of the crop. Against this backdrop, adopting genomic approaches for designing biotic stress tolerant coconut genotypes is inevitable. Coconut molecular breeding has witnessed the application of DNA markers in genetic diversity analysis and mapping of quantitative trait loci (QTLs). Further advancements in genome sequencing and transcriptome profiling have opened enormous avenues for utilising coconut-derived 'big data' in developing biotic stress-tolerant cultivars. This chapter discusses the important diseases and pests of coconut, genetic resources of coconut, approaches in

S. V. Ramesh · V. H. Prathibha · V. Aparna · K. S. Muralikrishna · V. Hegde · M. K. Rajesh (✉)
ICAR-CPCRI, Kasaragod, Kerala, India
e-mail: rajesh.mk@icar.gov.in

S. V. Ramesh
e-mail: ramesh.sv@icar.gov.in

V. H. Prathibha
e-mail: prathibha.vh@icar.gov.in

V. Aparna
e-mail: aparna.veluru@icar.gov.in

K. S. Muralikrishna
e-mail: muralikrishna.ks@icar.gov.in

V. Hegde
e-mail: vinayaka.hegde@icar.gov.in

A. Josephrajkumar · M. Babu
ICAR-CPCRI, Regional Station, Kayamkulam, Kerala, India
e-mail: joseph.rajkumar@icar.gov.in

M. Babu
e-mail: merin.babu@icar.gov.in

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conventional breeding to develop resistant genotypes, molecular mapping of resistance genes, QTLs and marker-assisted breeding, association mapping, glimpses of genome assemblies, and RNA-Seq approaches to develop disease and pest resistant genotypes.

Keywords Biotic stressors · *R*-genes · Molecular tree breeding · Phytoplasmal diseases · Coconut genetic sources

3.1 Introduction

Coconut (*Cocos nucifera* L.) is an economically important plantation crop grown widely in the humid tropical regions of the world. Coconut is cultivated around the globe, in not less than 90 countries, chiefly belonging to island and coastal ecosystems. Coconut production has been estimated to be 68,833 million nuts from an area of little over 12 million ha (ICC 2019). The coconut palm is fittingly called 'tree of life' (*Kalpavriksha*) because of its multitude of utilities ranging from nutrient-dense food and oil, therapeutically important virgin coconut oil (VCO), energy drink, inflorescence sap-based mineral-rich sugar, to other ancillary uses of the fiber, shell, timber for industrial purposes etc. The recent advocacy for nutrient diversity and nutritional security of the masses has opened up multiple avenues for the coconut as an invaluable health food with immense therapeutic potential.

Botanically, the coconut palm belongs to the family *Arecaceae*, sub-family *Coccoidea* and is a monotypic genus. Genetically, the crop is a diploid harboring 32 chromosomes ($2n = 2x = 32$). The genetic resources of coconut have been a critical component all these years, widely exploited in the process of conventional breeding to develop varieties with enhanced yield, productivity potential along with other agronomic features of abiotic and biotic stress tolerance (Niral et al. 2019). In general, breeding approaches of mass selection, hybridization, and elite palm selection to impart novel traits have been accomplished. The inherent traits of the palm, such as its perennial nature, heterozygosity and greatly extended juvenile phase and a requirement for suitable mass propagation techniques, have seriously hindered the varietal development programmes, especially to counter the biotic stresses and related exigencies (Arunachalam and Rajesh 2008, 2017).

Diseases and pests of coconut are major production constraints in addition to the climate-change-induced vagaries. Therefore, breeding for disease and pest resistance in coconut has been a main focus area of research in breeding programs across the globe. Among the diseases, phytoplasma causing lethal yellowing and root (wilt) diseases and fungal diseases such as bud rot, basal stem rot, stem bleeding, etc., are major stressors with serious implications on coconut productivity. Coconut breeding approaches worldwide have taken up the development of phytoplasma resistant varieties as a major thrust area followed by introgression of resistance against fungal diseases (Thomas et al. 2018). Further, the palm is infested by several pests of economic importance, namely rhinoceros beetle, red palm weevil, eriophyid

mite, black-headed caterpillar and emerging or invasive pests like rugose spiralling whitefly. Efforts have been made to develop resistant varieties or identify resistant genetic sources of coconut to withstand pest pressure (Josephrajkumar et al. 2018a, b, c, d; Nampoothiri and Parthasarathy 2018).

Applications of DNA-based molecular markers have made rapid strides in the annual crops, and varieties have been developed to shield them against pests and diseases. The advent of these markers has supported genetic diversity analysis and mapping QTLs of economic and agronomic importance in coconut (reviewed by Rajesh et al. 2018, 2021a). Further, technological advancements in genome sequencing have greatly aided in generating three good quality genome assemblies of coconut depicting genetically diverse genotypes (Xiao et al. 2017; Lantican et al. 2019; Rajesh et al. 2020). In this context, this chapter provides glimpses of major diseases and pests of coconut, breeding efforts in developing varieties to withstand disease and pest attacks and application of genomics-assisted breeding in designing biotic stress tolerance in coconut.

3.2 Diseases of Coconut

A coconut-based cropping system warrants maximization of the returns by incorporating multiple crops and other components in a diversified manner. The concept of integrated disease management (IDM) requires adopting economically viable, ecologically safe and agronomically feasible approaches to ward off the diseases. Extensive research approaches are being evaluated in the research farms and on-field trials to ensure the safety and sustainability of integrated approaches to manage coconut diseases. The various economically important diseases of coconut are as follows:

3.2.1 Leaf Rot

Leaf rot, caused by pathogenic fungi, generally occurs along with root (wilt) diseased palms (Srinivasan 1991). The annual economic loss was estimated to be around Rs. 5.6 million (Menon and Nair 1948) due to the loss of 461 million nuts (Joseph and Rawther 1991). Extensive rotting of leaf tissue is preceded by the formation of water-soaked lesions that enlarge and coalesce. The rotten distal regions of the leaflets fuse to give a 'fish bone-like appearance', and these regions drop off after drying. This disease causes a severe reduction in the photosynthetic efficiency of the palms, thereby reducing the yield and attracting insect pests. It is caused by the fungi *Colletotrichum gloeosporioides* (Penzig) Penzig and Sacc., *Fusarium* spp. and *Exserohilum rostratum* (Drechsler) Leonard and Suggs (Srinivasan and Gunasekaran 1996). Disease management warrants that spindle leaf alone requires protection due to its increased susceptibility. Further, application of fungicide hexaconazole 5 EC on

leaf rot affected palms and applying neem cake-sand mixture in leaf axils to control rhinoceros beetle are found to be effective in managing the disease. Moreover, various economic and environmentally feasible methodologies and integrated approaches have been adopted to manage the disease and the insect pests (Koshy 2000a; Koshy et al. 2002). Though palms in the early stages of infection recover, disease in the advanced stage may take over three years to recover following the recommended IDM practices completely.

3.2.2 Bud Rot

Bud rot of coconut is a disease of sporadic nature; however, epidemic scale outbreaks have also been recorded. Though the disease occurs in palms of all the growth stages, young palms are more susceptible and, therefore, severely affected. The disease was first reported in Grand Cayman in 1834. The disease has since been reported in many countries, including Cuba, India, The Philippines, etc. (Menon and Pandalai 1958; Child 1974; Renard and Darwis 1993). In India, bud rot occurs in coastal regions (Menon and Pandalai 1958; Radha and Joseph 1974; Sharadraj and Chandramohanan 2013; Chandran et al. 2017). In Côte d'Ivoire, infections have killed even up to 50% of the palms depending on the prevailing climatic conditions and the nature of the planting materials (Quillec and Renard 1984). Characteristic symptoms of the disease include spindle withering into pale color, or brown color, bending over of the spindle, rotting of internal tissues which emit a foul odor, slowly affecting the inner leaves and leaving only the matured leaves of the crown. Consequently, the palm succumbs (Menon and Pandalai 1958; Lingaraj 1972). Bud rot is caused by fungal pathogens, *Phytophthora palmivora* Butl., *P. faberi* Maubl. and *P. heveae* (Butler 1906; Shaw and Sundararaman 1914; Joseph and Radha 1975). The primary dry rot caused by *P. palmivora* is colonized by secondary invaders viz., *Fusarium* sp., *Xanthomonas, Pseudomonas* and *Erwinia,* causing wet rotting (Radha and Joseph 1974). Besides, *P. katsurae* was also identified as causal agent of bud rot of coconut (Thomas et al. 1947; Uchida et al. 1992; Chowdappa et al. 2003). Further, a significant inter and intra-specific variability among the *Phytophthora* spp. infecting coconut in India was recorded (Sharadraj and Chandramohanan 2016).

The disease is controlled by adopting IDM practices which involve improving the drainage, effective weed control, increasing the plant spacing, immediate removal of disease affected palms, removal of infected spindle leaf during the early stage, treatment of wound with chlorothalonil 75% WP. Prophylactic measures such as application of Bordeaux mixture around the base of the spindle and placing perforated sachets containing chlorothalonil 75WP (2 sachets palm^{-1}; 3 gm chlorothalonil sachet^{-1}) are also recommended. Also, antagonistic fungi such as *Trichoderma* sp., *Myrothecium roridum* and *M. verrucaria* have been effectively utilized to arrest the growth of *P. palmivora* and *P. katsurae.* Placement of *Trichoderma* coir pith cake in the inner whorls of coconut leaves is also an effective strategy (Chandramohanan et al. 2013). Besides nutrient management, appropriate irrigation scheduling and

replanting of affected palms are necessary to ward off the disease. Growing of tolerant genotypes (Rennel Island Tall and local Indonesian talls, hybrids of Malaysian Yellow Dwarf × Rennel Island Tall or Local Tall, Red Dwarf × Tall coconut hybrids) is an alternate strategy (Mangindaan and Novarianto 1999).

3.2.3 Stem Bleeding

Stem bleeding of coconut is a disease of tropics reported in Sri Lanka (Petch 1906), India (Sundararaman 1922) and other countries like The Philippines, Malaysia, Trinidad, Papua New Guinea, Fiji, Ghana and Indonesia (Menon and Pandalai 1958; Renard et al. 1984). The disease has been reported recently in Brazil and China (Warwick and Passos 2009; Yu et al. 2012). Even though yield loss during the early stages of the disease is very minimal, enormous yield loss and even death of palms occur during later stages (Nambiar and Sastry 1988; Chandran et al. 2017). The characteristic symptoms of the disease are the appearance of dark colored patches at the base of the tree trunk, which progresses upwards, leading to longitudinal cracks on the bark exuding dark liquid. Eventually, these exudates dry up to form black encrustations having brownish-orange margins and tissue underneath and beyond the external encrustations starts to decay. The disease severity in young palms is high, causing considerable yield reduction and death of palms (Ohler 1984; Nambiar and Sastry 1988). Crown symptoms, characterised by premature yellowing of the outer whorl of leaves, are also common during summer and eventually dry up. Nut fall was found to be pronounced in palms that are exposed to drought-like situations. Reduction in crown size is also observed as the main trunk tapers towards the apex. The disease is caused by the fungus *Thielaviopsis paradoxa* (de Seynes) von Hohnel. It's perithecial stage [*Ceratocystis paradoxa* (Dade) Moreau] was also found associated with the disease (Menon and Pandalai 1958; Ohler 1984; Nambiar et al. 1986a, b). At times, the infection is further aggravated due to the infestation with *Diocalandra* weevil causing deterioration of the health of palms. The disease could be effectively controlled following IDM practices, viz., application of paste of talc-based formulation of *T. harzianum* CPTD 28 on bleeding patches, soil application of *Trichoderma harzianum* CPTD 28 (100 g) mixed with neem cake (5 kg), FYM (50 kg), and NPK fertilizer (500; 320; 1200 gm $palm^{-1}$ $year^{-1}$). Summer irrigation, and avoiding any injuries to palm trunk, are important measures to control the disease. Genotypes such as Banawali Green Round Tall, Banawali Brown Round Tall and Malayan Orange Dwarf were found to be less susceptible to the disease. In contrast, Malayan Green Dwarf, Chowghat Orange Dwarf and Philippines Ordinary Tall were found to be more susceptible.

3.2.4 *Ganoderma* Wilt/Basal Stem Rot

Ganoderma wilt or basal stem rot or *Thanjavur* wilt is one of the most destructive diseases in coconut, first reported in the Indian state of Tamil Nadu in 1952 (Vijayan and Natarajan 1972). The fungal pathogen *Ganoderma lucidum* was associated with the disease in other parts of the country (Venkatarayan 1936; Nambiar and Rethinam 1986; Wilson et al. 1987). *G. boninense* Pat. was known to be linked with the disease in Sri Lanka (Peries 1974). In general, palms of 10–30 years old are more susceptible to the disease (43%) than younger ones (Vijayan and Natarajan 1972). The disease progresses with typical symptoms spread over five distinct stages: leaflet wilting, leaflet yellowing, decay and death of fine roots. In the next stage, bleeding starts from the base of the stem, with the lesions spreading upwards, concomitant with extensive root decay and declined or nil bunch production, button shedding and development of barren nuts. Progression of stem bleeding further causes the drooping and drying of leaves of outer whorls, and ultimately all the leaves dry up and stem shrivels. Further, the infection process is aggravated due to the infestation by scolytid beetle, *Xyleborus perforans* and the weevil, *Diocalandra stigmaticollis,* ultimately causing the death of the palm (Anonymous 1976; Rethinam 1984; Bhaskaran 1986). Occurrence of the disease could be detected before the expression of typical symptoms utilizing several methodologies such as colorimetric detection (Natarajan et al. 1986), analysis of physiological parameters like transpiration rate and stomatal conductance or resistance (Vijayaraghavan et al. 1987), and PCR-based detection (Karthikeyan et al. 2006; Rajendran et al. 2014) of *Ganoderma lucidum.*

To control the disease, integrated approaches are followed, including cultural practices that avoid pathogen establishment. Some of the common practices adopted are avoiding hardpan formation in the sub-soil region, avoiding water stagnation during monsoon, and applying summer irrigation (Rao and Rao 1966; Anonymous 1976; Ramasami et al. 1977; Satyanarayana et al. 1985). Growing indicator plants such as *Leucaena leucocephala* and *Gliricidia maculata* is an effective prevention strategy as these species are affected well before the palms show infection (Anonymous 1989). Further, the EDTA test also aids in the early detection of the disease (Natarajan et al. 1986; Vijayaraghavan et al. 1987). The IDM practices standardized by Coconut Research Station, Veppankulam, Tamil Nadu and Agricultural Research Station, Ambajipet, Andhra Pradesh (Anonymous 1990) suggest multiple approaches such as clean crop management practices, regular basin irrigation during summer months, avoiding flooding, application of organic manures, neem cake (5 kg palm^{-1} year^{-1}) fortified with *Trichoderma harzianum* (CPTD 28) talc formulation (50 g palm^{-1} year^{-1}), growing banana as intercrop and soil drenching of Bordeaux mixture thrice annually (1%, 40 L). Root feeding with 1% Hexaconazole 5EC and soil application of *Trichoderma harzianum* (CPTD 28) enriched neem cake @ 5 kg palm^{-1} at quarterly intervals, or soil application of *T. harzianum* (CPTD 28)

enriched neem cake @ 5 kg palm^{-1} at three months interval followed by maintenance of moisture and mulching around palm basin were found very effective in the management of disease (Prathibha et al. 2020).

3.2.5 *Immature Nut Fall*

Immature nut fall is one of the common diseases observed in coconut plantations. The disease has been ascribed to various factors such as poor mother palm selection, extreme soil reactions (high acidity or high alkalinity), poor water management leading to drought or waterlogging conditions, imbalance in plant nutrition and poor pollination (Smith 1969; Ohler 1984; Prasada Rao 1988). Also, infestation due to eriophyid mite (*Aceria guerreronis* Keifer) is one of the prime reasons for nut fall besides encouraging secondary infections due to the fungus causing rot (ChandraMohanan and Baby 2004). Environmental factors like relative humidity and minimum temperature are also attributed to the disease (Venugopal and ChandraMohanan 2010). Nut fall or fruit rot is caused by *P. palmivora* (Butl.) and *Lasiodiploida theobromae* Pat. *P. meadii* Mc Rae has also been found to cause the immature nut fall for the first time in the Kodagu district of Karnataka State, India (Chowdappa et al. 2002). The disease can be managed by removing all the infected nuts from the palm and spraying with Bordeaux mixture 1% to the bunches two sprays at 30 days interval depending on the severity of the disease.

3.2.6 *Grey Leaf Spot*

Grey leaf spot is prevalent in all coconut growing regions, affecting young (nursery) seedlings and adult palms. In the latter, the yield reduction due to disease has been reported to be around 10–24% (Karthikeyan 1996). Further, delayed flowering is also observed when the disease infects the palms (Abad 1975). Minute yellow spots with gray margins appear on the outer whorl of old leaves, which later coalesce to provide a burnt appearance. Under severe conditions, drying and shrivelling of leaves occur, causing a 'blight' appearance. The pathogenic fungi *Pestalotiopsis palmarum* (Cooke) Steyaert has been identified as the causal agent of blight disease. Later, the application of molecular techniques enabled Maharachchikumbura et al. (2012) to describe several species of this pathogen. Incidence of the disease suggests the poor nutritional status of the palms either due to deficiency of potash or excess nitrogen. As a control measure, integrated nutrient management (INM), application of Bordeaux mixture or any copper fungicides or carbamates is suggested. Further, KCl application also significantly reduces disease incidence.

3.2.7 Lasiodiplodia *Leaf Blight of Coconut*

Lasiodiplodia leaf blight of coconut has been reported from almost all the major coconut growing countries such as Trinidad, Brazil, Malaysia, Sri Lanka and India (Ram 1993; Bhaskaran et al. 2007; Monteiro et al. 2013). The disease incidence severely weakens and causes the death of the palms growing in soils lacking drainage or under water stress and imbalance of nutrition. The fungus also infects seed coconuts (Raju 1984). Leaves and nuts are affected where the former appears as charred or burnt due to drying followed by apical necrosis of lower leaves, giving an inverted "v" shape and reminiscing the effects of water-deficit stress. The fungus causes systemic invasion and induces internal necrosis (Souza-Filho et al. 1979). A small black sunken region appears near the perianth of immature nuts. The nuts attacked by eriophyid mite are infected by the pathogen and cause rotting and shedding of immature nuts (Venugopal and Chadramohanan 2006). The fungus *Lasiodiplodia theobromoae* (Pat.) Griffon and Maubl causes the disease. Though 20 species have been identified based on conidial and paraphysis morphology, it was observed that the causal fungus is a complex of cryptic species (Alves et al. 2008). The disease could be effectively controlled by following phytosanitary measures such as (i) removal and burning of severely affected leaves to avoid further spread of inoculum; (ii) application of *Pseudomonas fluorescens* (200 g) along with FYM (50 kg) + neem cake (@5 kg palm^{-1} year^{-1}); (iii) spraying of Hexaconazole 5EC or copper oxychloride (0.25%) two times at 45 days interval during summer months; (iv) root feeding with Hexaconazole 5EC @ 2 ml in 100 ml of water at 3 month intervals.

3.2.8 *Phytoplasmal Diseases of Coconut*

Phytoplasma refers to small cell wall-less bacteria but enveloped by a single membrane and are known to cause various diseases in palms that are known by their characteristic symptoms. The advent of molecular detection of plant pathogens has greatly aided in the taxonomic characterization of many phytoplasmas associated with diseases of coconut.

3.2.8.1 Root (Wilt) Disease

Root (wilt) disease is an economically important, non-lethal yet debilitating disease of coconut. The economic losses due to husk damage and the decline in copra yield have been estimated to be around Rs. 3000 million. The disease was first reported in 1882 in the Kottayam district of the Indian State of Kerala. Later, several researchers have documented root (wilt) disease in Kerala (Butler 1908; Pillai 1911; Menon and Pandalai 1958; Koshy 1999). The spread of the disease received wide attention as it

infects an area of 0.41 million ha in a contiguous manner in Kerala and in certain regions of Tamil Nadu, Goa and Karnataka (Solomon et al. 1999a, b; Koshy 2000a, b; Koshy et al. 2002). Further, the disease intensity survey had revealed that severity ranges from 2.1% (in Thiruvananthapuram district) to 48.0% (Alappuzha district) (Solomon et al. 1999a, b). Though earlier reports by Mathew et al. (1993) recorded a decline in yield of 45–60%, in West Coast Tall variety and D × T hybrids, respectively yield reduction of nuts to the extent of 80% is not uncommon when the disease is in advanced stage (Radha et al. 1962; Ramadasan et al. 1971). The characteristic foliar symptoms of the disease include flaccidity (in 67–97% palms), yellow discoloration (38–67% palms) and marginal necrosis (28–48% palms) of the leaflets (Varghese 1934; Menon and Nair 1952; Menon and Pandalai 1958). Further, the expression of disease symptoms varies with the age, variety, nutritional status of the palm, and crop management practices. In addition, inflorescence necrosis characterized by the lack of female flowers and sterile pollen and immature nut shedding are some of the symptoms (Varghese 1934; Varkey and Davis 1960). As the name suggests, root rotting or decay is another important symptom observed in more than 50% of the main roots (Butler 1908). Root decay may vary from 12 to 95% depending on the disease intensity (Michael 1964). Besides reducing the number of roots, degeneration of root anatomy, physiological aberrations and impaired water uptake are observed (Davis 1964; Michael 1964; Ramadasan 1964; Indira and Ramadasan 1968; Govindankutty and Vellaichami 1983). Multiple investigations based on electron microscopy, vector transmission studies have established phytoplasma as the causal agent of the disease (Solomon and Govindankutty 1991a, b). *Stephanitis typica* and *Proutista moesta* were reported as the insect vectors of the disease (Mathen et al. 1990; Anonymous 1997). Also, tetracycline treated trees exhibited remission of symptoms corroborating the phytoplasmal etiology of the disease. Manimekalai et al. (2010) reported that 16Sr XI group phytoplasma is associated with diseased palms.

Management of root (wilt) disease is cumbersome due to factors such as the perennial nature of the palms, pathogen persistence, and easy transmission due to vectors. Being a debilitating disease, crop management practices have attained immense importance; hence diverse strategies have been formulated for heavily and mildly infected areas (Anonymous 1986; Muralidharan et al. 1991). In the heavily infected areas, management of leaf rot, application of appropriate fertilizer dose, inclusion of organic manures, summer irrigation, intercropping with cassava, elephant foot yam and greater yam (Menon and Nayar 1978) and mixed farming approaches are suggested. In the mildly affected regions, removal of all the diseased palms by following systematic surveillance, adoption of appropriate disease detection tests including DAC ELISA (Sasikala et al. 2001, 2004) before the appearance of visual symptoms are recommended. It is followed by replanting with disease-free seedlings.

3.2.8.2 Lethal Yellowing

Lethal yellowing (LY) is an important disease that threatens the cultivation of coconut worldwide. The disease was initially documented in Grand Cayman Island in 1834

and Jamaica in 1884. However, at present, LY severely constrains the production potential of palms in the Southern United States, Central America and Caribbean region and east Africa (Harrison et al. 2014). The disease was known differently in diverse geographic regions: Cape St. Paul Wilt in Ghana, Kribi disease in Cameroon, Kaincope disease in Togo, Awka disease in Nigeria, lethal decline in east African countries (Brown et al. 2007). It affects coconut palms of all ages, and palms succumb within six months of the onset of symptoms (McCoy et al. 1983; Been 1995).

The characteristic symptoms of the disease include premature nut fall. The second stage is characterized by inflorescence necrosis followed by yellowing of fronds in the third stage. In this process, the death of the bud happens, and the emerging spear leaf will collapse. In the last or fourth stage of the disease, complete defoliation of the palm causes its decapitation (Brown et al. 2008; Harrison et al. 2014). Initially, electron microscopy and PCR-based detection identified phytoplasma as a causative agent (Heinze et al. 1972; Plavsic-Blanjac et al. 1972). Advancements in the PCR-based assays and serological analysis have helped to characterise the coconut LY group of phytoplasmas as belonging to the members of group 16SrIV having four subclades (16SrIV-A, 16SrIV-B, 16SrIV-C and 16SrIVD). Since phytoplasma is phloem limited, the cixiid, *Haplaxius* (*Myndus) crudus* was known to act as a vector that spreads disease in Florida (Howard et al. 1983). However, in Jamaica, the role of cixiid *Haplaxius* (*Myndus) crudus*, in the disease transmission could not be confirmed (Schuiling et al. 1976; Eden-Green 1978; Eden-Green and Schuiling 1978; Dabek 1974). Even though PCR detection of LY DNA in embryos was proven, seed transmission of this pathogen has not been proven unequivocally (Cordova et al. 2003). As a disease management strategy, clean cultivation practices, crop sanitation and prevention of the spread of insect vectors, and removal of weed hosts are critical (Lee et al. 2000). The application of oxy-tetracycline-HCL also suppresses LY symptoms (McCoy et al. 1976). Cultivating resistant cultivars MayPan hybrid (Malayan Dwarf × Panama Tall) in Jamaica has been an effective strategy; however, resistance breakdown reported in these genotypes is a concern (Wallace 2002).

3.2.8.3 Coconut Yellow Decline (CYD)

CYD is a debilitating disease first reported in Malaysia by Sharples in 1928, which considerably reduces the productivity of coconut in Malaysia. Nejat et al. (2009a) reported classic phytoplasmal symptoms such as yellowing and drying of fronds in Malayan dwarf ecotypes found in Selangor State in Malaysia. Therefore, the disease was called 'coconut yellow decline' (CYD). Leaves of the outer whorls show yellowing which gradually becomes light brown. Later the younger leaves become symptomatic, and the spear leaf also shows chlorotic symptoms. Further, premature nut fall and necrosis of inflorescence is observed. As the disease progresses, fronds collapse and rotting of the growing tip of palms occur, ultimately causing the death of palms (Nejat et al. 2009a). CYD in Malaysia was found to be caused by Bermuda grass white leaf group (16SrXIV, '*Candidatus* Phytoplasma cynodontis' group) (in Malyan Red Dwarf and Malayan Tall palms), and *Candidatus* Phytoplasma

malaysianum (16Sr XXXII-B) (in Malayan Yellow Dwarf) (Nejat et al. 2009a, b, 2013). A real-time PCR assay was developed for quantitative and rapid detection of the 16Sr XXXII-B (Nejat et al. 2010).

3.2.8.4 Tatipaka Disease

Tatipaka disease is a non-fatal but debilitating disease of coconut palms endemic in the east and west Godavari, Srikakulam, Nellore, Krishna and Guntur districts of Southern India (Rethinam et al. 1989; Rajamannar et al. 1993). Relatively young palms (below 20 years) are not generally affected, and also the spread of disease is sporadic (Solomon and Geetha 2004). The typical symptoms include a considerable reduction in the number and size of leaves, presence of chlorotic water-soaked spots on the leaves, abnormal bending of fronds and a marked reduction in crown size. The fasciated appearance of leaves is also a characteristic symptom. The bunches comprise both the normal and abnormal nuts, and the atrophied nuts are generally barren and at times ooze gummy exudates (Ramapandu and Rajamannar 1981). Sap transmission studies and electrophoretic analysis of DNA ruled out the possibility of virus or viroid infection (Rajamannar et al. 1984; Randles and Hatta 1980). Electron microscopy coupled with Dienes stain and fluorescence microscopy analysis of roots, meristem, petioles of developing leaves and rachilla of the tender inflorescence of diseased palms revealed the presence of phytoplasma in the sieve tubes (Rajamannar et al. 1993). There are no prophylactic or curative measures for the disease; hence diseased palms are to be removed immediately to arrest the spread of the disease.

3.2.8.5 Weligama Wilt Disease

Weligama wilt disease was first recorded in the Weligama region in the Matara district of Sri Lanka (Wijesekara et al. 2008; Perera et al. 2010). However, currently, the disease is prevalent in other districts of Sri Lanka (Perera et al. 2012). Flattening and bending of leaflets leading to flaccid appearance are the earliest symptoms. Palm crowns appear dark green, especially in the younger leaves, and it becomes prominent when the leaves are completely opened. Further, intense yellowing of lower whorls of leaves is also a characteristic feature of this disease. Drying up of fronds followed by leaf falling or ragged appearance of the crown occurs during later stages. Due to the reduced number of fronds, the palm crown becomes smaller, and trunk tapering happens. With the progression of the disease, female flower production declines and the productivity of the palm is severely affected (Wijesekara et al. 2008; Perera et al. 2010, 2012). Phytoplasma belonging to 16SrXI *Candidatus* Phytoplasma *oryzae* group has been reported as the causal agent of the Weligama wilt in Sri Lanka (Perera et al. 2012). Molecular detection of the infected palms is possible, and hence the removal of the diseased palms is advocated as a containment strategy.

3.2.8.6 Lethal Wilt Disease

Lethal wilt disease (LWD) of coconut, reported recently from East Coast of Tamil Nadu State of India, is another concern to the coconut farmers. The primary symptom of the disease is abnormal shedding of nuts, which is followed by inflorescence necrosis and yellowing of outer whorls of leaves. The foliar yellowing progresses to inner whorls and subsequently chlorotic leaves turn brown and necrotic. As the disease advances, necrosis and rotting of spear leaves and bud region occur. Affected palms die within 3–5 months leaving a bare trunk. The phytoplasma associated with LWD has been identified as '*Ca.* P. asteris'-related strain belonging to subgroup 16SrI-B (Babu et al. 2021). Since the disease is confined to a limited area, periodic surveillance and eradication of diseased palms form the primary management strategy.

3.2.9 Diseases Caused by Viruses and Viroids

3.2.9.1 Coconut Foliar Decay or Vanuatu Wilt

Coconut foliar decay (alternatively foliar decay *Mindus taffini* or New Hebrides coconut disease) is a disease known to occur in the introduced palms of the Malayan Yellow Dwarf in Vanuatu. The name *Mindus taffini* is derived from the plant hopper that transmits the disease.The local cultivars Vanuatu tall and Vanuatu Dwarf, though carriers, remain asymptomatic (Randles et al. 1992; Hanold and Randles 2003). Yellowing of leaves of positions 7–11 from spear leaf is the initial symptom, and as the yellowing spreads, the fronds break near the base and hang down. These symptoms happen in younger leaves too when they reach the position anywhere from 7 to 11. With the progression of the disease, the trunks tapers towards the top, and palms die in 1 or 2 years. The disease is caused by coconut foliar decay virus (CFDV) belonging to the family *Nanoviridae* (Randles et al. 1986) and transmitted by a planthopper *Myndus taffini* Bonfils (Cixiidae). Interestingly the virus and vector remain confined to the Vanuatu archipelago. Growing of tolerant cultivars (Vanuatu tall or the hybrid, Vanuatu Tall × Vanuatu Red Dwarf) is suggested as a disease management strategy. Further, FAO/IBPGR *Technical Guidelines for the Safe Movement of Coconut Germplasm* warrants the movement of coconut embryos in a sterile medium. The parts of the mother plant must be screened for the presence of virus or viroid before the transport of material.

3.2.9.2 Viroid Diseases

Coconut cadang-cadang and Tinangaja are two viroid diseases documented in the coconuts grown in The Philippines and Guam, respectively. Coconut Cadang-cadang is a lethal disease-causing severe economic losses reported from southern Luzon

in The Philippines (Randles et al. 1987). In general, palms that have attained the flowering stage are affected, and infection of young palms is a rarity. During the early stage of the disease (2–4 years), the young nuts become more rounded, and equatorial scarifications are observed. Inflorescence shows a stunted appearance, and chlorotic spots characterize leaves. In the mid-stage of the disease (about two years), production of spathe, inflorescence and nut production decline but leaf spots become more prominent. In the late stage (5 years), leaf spots coalesce to result in chlorosis, the number and size of fronds decline, crown size is markedly reduced, and palm dies. The etiological agent of the disease has been identified as coconut cadang-cadang viroid or CCCVd (Hanold and Randles 1991). Even though no insect vector has been identified with the spread of the disease, viroid transmission in a small percent of progeny palms was observed when pollen from infected palms was used. The mode of natural transmission in the field is not known. CCCVd was also successfully transmitted to palms through contaminated harvesting scythes. Currently, there are no control measures to eradicate the disease; however, growing resistant palms, strict quarantine, and clean cultivation habits are suggested. The major diseases infecting coconut are described in Table 3.1.

3.3 Pests of Coconut

Coconut is infested by a wide array of pests, including 830 insect and mite species and 78 nematode species causing a serious decline in productivity (CPCRI 1979). Further, insect, mite and vertebrate pests in coconut result in a crop loss to the tune of 30% in the palm (Gitau et al. 2009). Damage due to the pest complex in Kerala State, India, has been estimated to be 618.50 million nuts annum^{-1}, suggesting the severity and extent of infestation (Abraham 1994). The pests of coconut could be classified as borers, defoliators, sap feeders, subterranean pests and emerging pests. The major pests infesting coconut are described in Table 3.2.

Besides the above-stated pests, other pests of importance are *Darna* (*Macroplectra*) *nararia* Moore (Limacodidae: Lepidoptera), *Parasa lepida* (Cramer); spiralling whitefly *Aleurodicus dispersus* Russell (Aleyrodidae: Hemiptera); and scale insects. Also, burrowing nematode (*Radopholus similis*), lesion nematode (*Pratylenchus coffeae*) and red ring nematode (*Rhadinaphelenchus cocophilus)* causing red ring disease and root-knot nematode (*Meloidogyne* spp.) are found to be pests of importance in the coconut ecosystem (Koshy 1986a, b).

Table 3.1 Major diseases infecting coconut and their causative pathogens

Sl. No.	Diseases	Causative agent(s)	Occurrence	References
I. Major fungal diseases				
1	Bud rot disease	*Phytophthora palmivora, P. heveae, P. katsurae P. nicotianae, Fusarium moniliforme, F. solani, Graphium* sp.	India, Côte d'Ivoire, Indonesia, Jamaica, Puerto Rico, Africa, Peninsular Malaysia and The Philippines	Butler (1906), Menon and Pandalai (1958), Quillec et al. (1984), Uchida et al. (1992)
2	Basal stem rot	*Ganoderma lucidum, G. applanatum, G. zonatum, G. boninense*	India, Florida, South America, Java, tropical Africa, Australia, Japan, Indonesia, Malaysia, The Philippines, Samoa, Sri Lanka and Tasmania	Peries (1974a), Satyanarayana et al. (1985)
3	Stem bleeding	*Thielaviopsis paradoxa*/*Chalara paradoxa*	Sri lanka, India, Indonesia, Malaysia, The Philippines, Fiji, Ghana, Trinidad	Petch (1906), Sundararaman (1922)
4	Leaf rot	*Exserohilum rostratum*/*Colletotrichum gloeosporioides*/*Fusarium solani* and *Fusarium moniliforme*	India	Varghese (1934), Menon and Pandalai (1958), Radha and Lal (1968), Srinivasan and Gunasekaran (1999)
5	Grey leaf blight	*Pestalotiopsis palmarum*	Guyana, India, Malaysia, New Hebrides, Sri Lanka, Trinidad, Nigeria	Copeland (1931), Cook (1971), Holliday (1980)
6	Leaf blight	*Lasiodiplodia theobromae*	India	Johnson et al. 2014
II. Phytoplasmal diseases				
1	Lethal yellowing	16Sr IV group Phytoplasma	Western Jamaica, Cuba, southern USA (Florida and Texas), southern Mexico	Nutman and Roberts (1955), Ashburner et al. (1996), Harrison et al. (1994, 2002), Myrie et al. (2007)

(continued)

Table 3.1 (continued)

Sl. No.	Diseases	Causative agent(s)	Occurrence	References
2	Root (wilt) disease	16Sr XI group Phytoplasma	India	Solomon et al. (1983), Manimekalai et al. (2010)
3	Coconut lethal disease	16Sr IV group Phytoplasma	Tanzania, Kenya, Mozambique	Schuiling et al. (1992), Mpunami et al. (1999)
4	Tatipaka disease	Phytoplasma	India	Rethinam et al. (1989)
5	Weligama wilt	16Sr XI group Phytoplasma	Sri Lanka	Perera et al. (2010)
6	Coconut yellow decline	16Sr XIV and 16Sr XXXII-B group Phytoplasma	Malaysia	Nejat et al. (2009a, b)
III. Virus diseases				
1	Coconut foliar decay or Vanuatu wilt	*Coconut Foliar decay virus* (CFDV)	Vanuatu	Calvez et al. (1980), Randles et al. (1986)
IV. Viroid diseases				
1	Coconut Cadang-cadang disease	*Coconut cadang-cadang viroid* (CCCVd)	Philippines	Randles (1975)
2	Coconut Tinangaja disease	*Coconut tinangaja viroid* (CtiVd)	Guam on Marianas Island	Boccardo (1985)
V. Protozoan diseases				
1	Fatal wilt or Heart Rot or Hartrot	*Phytomonas stahellii*	Central America (Costa Rica, Honduras and Nicaragua), South America (Brazil, Colombia, Ecuador, Guyana, Peru, Surinam and Venezuela) and the West Indies (Grenada, Trinidad and Tobago)	Waters (1978), Dollet (1984)

Table 3.2 Major pests infesting coconut, the damage symptoms and their control measures

Sl. No.	Pests (common name)	Biological name	Crop loss, symptoms and damage	Control measures	References
I. Borers					
1	Asiatic rhinoceros beetle	*Oryctes rhinoceros* Linn. (Coleoptera: Scarabaeidae)	• Central crown damage levels of 40–60%, 60–80% and 80–100% cause 12, 17 and 23% nut losses, respectively • Adults bore unopened spear leaves, spathes and tender nuts. When injured spindles open up, the green leaves present a geometric 'V'-shaped cut pattern • In juvenile palms, stunted growth and delayed flowering • In freshly transplanted coconut seedlings, infestation causes the wilting and improper establishment • Beetle attack provides egg-laying sites for red palm weevil and entry of fungal pathogens	• Regularly monitor and identify any damage on the spear leaf of the juvenile palm or at the collar region of seedlings • Hook out the beetle if any chewed up fibres are noticed from the leaf axils • In juvenile palms, place naphthalene balls (4 g/axil) and fill with sand or place chlorantraniliprole (0. 4% GR) or fipronil (0.3% GR) (3 g in perforated sachet/ axil) on top most 3–4 leaf axils • In adult palms, fill the top most 3–4 leaf axils with 250 g neem cake/maroti cake/Pongamia cake mixed with an equal volume of sand • Incorporate *Clerodendrum infortunatum*, a weed, into the manure pits to induce larval-pupal abnormalities in feeding grubs • Apply entomopathogenic green muscardine fungus (*Metarhizium majus*) into the breeding pits @ 5×10^{11} spores/m^3 • Release 10–12 virosed (*Oryctes rhinoceros* nudivirus) beetles for auto-transmission of nudivirus	Ghosh (1911), Pillai (1919), Nirula (1955b), Howard et al. (2001); Rajan et al. (2009), Josephrajkumar et al. (2015), Mohan et al. (2018), Josephrajkumar et al. (2016, 2019c)

(continued)

Table 3.2 (continued)

Sl. No.	Pests (common name)	Biological name	Crop loss, symptoms and damage	Control measures	References
2	Red palm weevil (RPW)	*Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae)	• RPW attack juvenile palms of age less than 20 years • All life-stages are confined within the infested palms with an action threshold determined as 1% • Internal feeder and hence difficulties in early diagnosis • Yellowing and wilting of the inner and middle whorl of leaves. Holes and tunnels trunk, ooze out a brown viscous fluid, emanating fermented odour	• Early diagnosis through acoustics-based RPW detector • Periodic monitoring and regular crown cleaning • Destroy crown toppled palms in a garden to avoid the lateral spread of the pest • Avoid causing any physical injury to the palms and cut the petioles at least 1 m away from the trunk • Spot application of imidacloprid 17.8 SL @1 mL/L of water or spinosad 45% SC @5 mL/L through the bore holes • Stimulo-deterrence for the disorientation of weevils through crop pluralism approach • Follow all regular management practices against rhinoceros beetle and leaf rot disease	Ghosh (1911), Menon and Pandalai (1960), Abraham and Kurian (1975), Abraham et al. (1998), Josephrajkumar et al. (2014a), Rajan et al. (2009), Josephrajkumar et al. (2017, 2018c, 2019c, 2021)
II. Defoliators					
1	Coconut black-headed caterpillar	*Opisina arenosella* Walker (Lepidoptera: Oecophoridae)	• Caterpillars produce silken webs reinforced with excreta and scrapes of leaf bits • Severe pest damage cause complete drying of the middle to the inner whorl of fronds • Annual loss due to infestation has been estimated as INR 7280 ha^{-1}	• In case of very severe infestation, remove and destroy fully dried 2–3 outer whorl of leaves • Augmentative release of stage-specific parasitoids viz., • larval parasitoids *Goniozus nephantidis* (Bethylidae) @ 20 parasitoids/palm, *Bracon brevicornis* (Braconidae) @ 30 parasitoids/palm • pre-pupal parasitoid, *Elasmus nephantidis* (Elasmidae) @ 49 /100 pre-pupae • pupal parasitoid *Brachymeria nosatoi* (Chalcididae) @32/100 pupae at the appropriate time • Ensure balanced nutrition and timely irrigation for recovering palm health	Rajagopal and Arulraj (2003), Mohan and Sujatha (2006) Mohan et al. (2010) Rajan et al. (2009), Josephrajkumar et al. (2018a, 2019c)

(continued)

Table 3.2 (continued)

Sl. No.	Pests (common name)	Biological name	Crop loss, symptoms and damage	Control measures	References
III. Sap Feeders					
1	Coconut eriophyid mite	*Aceria guerreronis* Keifer (Acarina: Eriophyidae)	• Infestation occurs in the developing young buttons after pollination and is seen in the floral bracts (tepals) and the soft meristematic portions beneath the perianth • Dispersal of the pest through wind, honeybees and other insects • Yellow halo around the perianth in the initial stage turns into brown and show necrotic patches, and finally, the damage is manifested as warts and longitudinal fissures on the nut	• Crown cleaning should be taken up routinely • Spray 2% neem oil-garlic emulsion or 0.004% azadirachtin (10,000 ppm ai) @ (4 mL/L) on bunches after pollination or root feeding with neem formulations containing azadirachtin (50,000 ppm ai) at 7.5 mL or azadirachtin 10,000 ppm at 10 mL with an equal volume of water three times during March–April, October–November and December–January • Spray 20% palm oil-sulphur (0.5%) emulsion on bunches after pollination • Application of talc-based preparation of *Hirsutella thompsonii* @ 20 g/l/palm containing 1.6×10^8 cfu per gram with a frequency of three sprayings per year during March–April, October–November and December–January • Adopt integrated nutritional management practices such as applying NPK fertilizer as recommended, recycling biomass or raising green manure crops in coconut basins and in situ incorporation during flowering, timely summer irrigation, and adopting appropriate moisture conservation measures	Moore and Howard (1996), Sathiamma et al. (1998), Haq (2001), Nair (2002), Rajan et al. (2009), Josephrajkumar et al. (2016, 2019c)
IV. Subterranean pests					
1	Coconut white grub	*Leucopholis coneophora* Burm. (Coleoptera: Scarabaeidae)	• Seedling damage occurs due to the feeding of roots, and death happens • In adult palms, root damage causes impaired water and nutrient conduction, yellowing of fronds, and yield loss	• Mechanical collection and destruction of adult beetles emerging during June • Repeated ploughing to expose the grubs to predators/digging and removal of grubs during October–December • Targeting early-instar grubs during July–August using Imidacloprid 17.8 SL @ 0.7 ml/l of water, 3–4 L of spray solution/m^2 area or Bifenthrin 10 EC @ 3 mL/L, 3–4 L of spray solution/m^2 area or Chlorpyriphos 20 EC @ 3 mL/L @ 3–4 L spray solution/m^2 area • Soil application of aqua formulation of entomopathogenic nematode, *Steinernema carpocapsae* @ 1.5 billion IJ/ha during September–October	Nirula et al. (1952), Rajan et al. (2009), Prathibha et al. (2013, 2018), Rajkumar et al. (2019), Josephrajkumar et al. (2019c)

(continued)

Table 3.2 (continued)

Sl. No.	Pests (common name)	Biological name	Crop loss, symptoms and damage	Control measures	References
V. Emerging pests					
1	Rugose spiralling whitefly	*Aleurodicus rugioperculatus* (Aleyrodidae: Hemiptera)	• It is an invasive pest of coconut • Desapping by whitefly could induce stress on the palms due to removal of water and nutrients • The economic loss is restricted mainly to the loss of photosynthetic efficiency due to the formation of sooty mold fungus (*Leptoxyphium* sp.)	• Pesticide holiday approach (i.e., no insecticides are recommended) to conserve habitat niche of natural enemies (*Encarsia guadeloupae, Apertochrysa* sp., lady beetles) and sooty mould scavenger beetle (*Leiochrinus nilgirianus*) by conservation biological control • Installation of yellow sticky traps on the palm trunk and interspaces for trapping adult whiteflies • In severe cases, water or neem oil (0.5%) spray is recommended on the lower surface of palm leaflets • Encourage good palm health by soil-test-based application of nutrients, organic recycling of residual biomass, irrigation, crop pluralism by growing various inter/mixed crops and ecological intensification to infuse biodiversity • Avoid the movement of infested seedlings (coconut, banana, ornamental palms) from endemic regions	Mohan et al. (2017), Josephrajkumar et al. (2018a, b, 2019b, c, 2020a, b)
2	Coreid bug	*Paradasynus rostratus* Dist. (Coreidae: Hemiptera)	• Loss is due to the shedding of developing buttons and immature nuts ranging from 18.2 to 66.4% • Deep furrows, crinkles and gummosis on the nut surface are typical symptoms	• Crown cleaning to destroy eggs and immature stages of the pest • Spray azadirachtin 300 ppm (Nimbecidene) @ 0.0004% (13 mL/L) two times during May–June and September–October • Spray thiamethoxam 25% WG (0.2 g/L) preferably in September–October on bunches after pollination during afternoon hours to safeguard pollinators • Encourage the predatory weaver ant, *Oecophylla smaragdina,* in the palm system	Kurian et al. (1972), Rajan et al. (2009), Josephrajkumar et al. (2019c)
3	Nesting whiteflies	*Paraleyrodes bondari* Peracchi and *Paraleyrodes minei* Iaccarino (Aleyrodidae: Hemiptera)	• Exotic whitefly smaller than RSW and desapping from under the surface of palm leaflets • Exudates honey dew and sooty mould developed on the upper surface of leaflets interfering with photosynthetic efficiency	• In juvenile palms, spraying water with jet speed could dislodge the whitefly and reduce the feeding and breeding potential of the pest • Ensure good nutrition and adequate watering to improve the health of juvenile and adult palms • Effective nitidulid predators belonging to *Cybocephalus* sp. and lady beetles were observed on the palm system and pesticide holiday is advised for conservation biological control	Mohan et al. (2019), Josephrajkumar et al. (2019a, 2020a)

3.4 Genetic Resources of Resistance/Tolerance Genes

The first systematic account of the classification of coconut genetic resources was performed by Narayana and John (1949). Later multiple variants for a specific phenotypic feature of the coconut accessions were recognized. For instance, habit is characterized with dwarf; intermediate forms; and talls, the stem has branching; polyembryony; and suckering variants, vegetative parts of the palm exhibit variants ranging from albinism; chimaera; rosette-type seedlings; the fusion of leaflets ('plicata'); and forking of leaves portions. In inflorescence too, twin spadix; multiple spathes; incomplete spike suppression; secondary; splitting of spikes; spikes unbranched ('spicata'); secondary spikelets; viviparous germination; bulbils; midgets; persistent stem inflorescences are the variants observed. Fruit variants are numerous such as jelly-like; fragrant, and sweet forms, to name a few. Against this backdrop, International Coconut Genetic Resources Network (COGENT) was set up in 1991 to conserve and utilize the coconut genetic resources to achieve sustainable productivity goals. It had led to the establishment of five multi-site International Coconut Gene banks (ICGs), viz., (1) Indonesia—Southeast and East Asia; (2) India—South Asia and the Middle East; (3) Papua New Guinea—South Pacific; (4) Côte d'Ivoire—Africa and the Indian Ocean; (5) Brazil—Latin America and the Caribbean which constitute a network of ex-situ conservation and collection of coconut accessions. The International Coconut Genetic Resources Database (CGRD) under the aegis of COGENT reveals that even though over 1416 coconut accessions were being conserved, the country-specific crop improvement programs utilize less than 5% of the germplasm holdings (Batugal 2005). Nonetheless, the development of catalogues of coconut genetic resources providing descriptors have greatly enhanced the utility of these genotypes in national breeding objectives (Hamelin et al. 2005). At present, 24 gene banks spread worldwide have 1837 accessions for use in varietal development programs considering the local and national requirements (Nampoothiri and Parthasarathy 2018). Accordingly, South American and African countries aim for evolving disease tolerant genotypes to counter lethal yellowing disease (LYD), whereas Vanuatu focuses on coconut varieties that could withstand coconut foliar decay. In India, the objective is to develop root (wilt) disease tolerant lines and accessions in the management of invasive spiralling whitefly, among others.

Efforts in the characterization of coconut genetic resources have discovered trait-specific germplasm, and several of them have been registered with the ICAR-National Bureau of Plant Genetic Resources (ICAR-NBPGR) in India. A robust evaluation and breeding by selection methodology at Indian Council of Agricultural Research-Central Plantation Crops Research Institute, Kasaragod, Kerala, along with the many coordinating Research Centres under the All India Coordinated Research Project on Palms (AICRP on Palms) and State Agricultural Universities (SAUs) resulted in the release of over 30 improved coconut cultivars (Niral et al. 2009). In addition, the multi-pronged approach of screening of accessions for tolerance to biotic/abiotic stressors, laying special emphasis on the root (wilt) disease resistance, drought tolerance, and climate resilience, have yielded an appreciable collection of germplasm

(Rajagopal et al. 1990; Nair et al. 2004; Kasturi Bai et al. 2006; Hebbar et al. 2013, 2018).

3.5 Classical Genetics and Traditional Breeding

Traditionally the main focus of coconut breeding has been the development of high yielding speciality varieties that could withstand the biotic and abiotic stresses (Jerard et al. 2016). Accordingly, the variability in yield and economic parameters were given importance. The traits such as fruit weight, percentage of the husk and the nut yield were considered of paramount importance to develop high yielding varieties (Niral et al. 2009). A defined breeding objective to evolve insect-resistant varieties in coconut does not exist in this context. Nevertheless, conventional breeding approaches have developed coconut varieties and hybrids resistant to some notable biotic stressors (Table 3.3). Preliminary screening of genotypes against leaf-eating caterpillar (*Nephantis serinopa* Meyr.) (Kapadia 1981) and rhinoceros beetle (*Oryctes rhinoceros* Linn.) (Sumangala Nambiar 1991) revealed considerable variability among the coconut genotypes. Screening of coconut accessions for Eriophyid mite (*Aceria guerroronis* Keifer) resistance suggests that multiple nut characteristic features including its color and shape, tightness of tepals around the nut, space between the rim of the fruit and tepal aestivation confer resistance to mite attack (Moore and Alexander 1990; Arunachalam et al. 2013). Hence, genotypes such as Kulasekharam Green Dwarf (KGD) and Chowghat Orange Dwarf (COD) and selection of KGD called Kalpa Haritha and few other accessions viz., Navasi Tall, Gangapani Tall, Jamaica Tall and East Coast Tall displayed lesser mite incidence (Niral et al. 2014; ICAR-CPCRI 2019).

Similarly, whenever COD was a pollen parent, rhinoceros beetle infestation was severe, and this phenomenon was equally observed whenever a dwarf accession constitutes a parent in a hybrid combination. Hence, West Coast Tall (WCT), East Coast Tall (ECT) and hybrid combinations of Laccadive Ordinary Tall × Cochin China Tall and Gangabondam Green Dwarf × East Coast Tall were found to have the least incidence of beetle infestation (Muthiah and Bhaskaran 2000). In a survey for red palm weevil infestation and search for resistant genotypes, CGD, COD, and Benaulium Tall have ovipositional preference implying their susceptible nature to the pest (Faleiro and Rangnekar 2001). Screening for coconut scale insect resistance divulged that the prevalence of leaf glandular trichomes conferred resistance to the insect attack. Hence, Coco Nino Dwarf (194.4 trichomes cm^{-2}) was resistant compared to the susceptible Laguna Tall (81.6 trichomes cm^{-2}) (Galvez et al. 2018).

In the field of disease resistance, root (wilt) disease (RWD) is a serious concern for coconut cultivation, and field screening suggests that the cultivar CGD exhibit > 90% tolerance in field conditions. Various cross combinations and extensive screening for resistance have resulted in the development of a variety Kalparaksha- a selection of a cultivar Malayan Green Dwarf (Nair et al. 2009). Further, CGD was found to be

Table 3.3 Improved coconut varieties and hybrids developed, and other genetic sources of resistance identified to tackle various biotic stresses

Sl. No.	Variety or hybrid or resistance source	Organization(s) involved/Reference	Major characteristic features
1	Kalpa Haritha	ICAR-CPCRI	Less eriophyid mite damage Dual-purpose variety suitable for tender nut and copra
2	Kalparaksha	ICAR-CPCRI	Yields high [in terms of nut/copra yield] in the root (wilt) disease (RWD) prevalent tracts Semi-tall statured variety suitable for tender nut purpose characterized with green fruits
3	Kalpasree	ICAR-CPCRI	Yields high in the root (wilt) disease (RWD) prevalent areas Dwarf variety with green fruits and yields good quality oil
4	Kalpa Sankara	ICAR-CPCRI	A hybrid derived from CGD X WCT cross Exhibits tolerance to root (wilt) disease (RWD), yields high
5	Malayan Dwarf × Panama Tall (Maypan hybrids)	Harries and Romney (1974)	Resistant to lethal yellowing disease (LYD)
6	Sri Lankan Dwarf, Indian Dwarf sand King Coconut	Been (1981)	Promising sources of resistance to Lethal yellowing disease
7	Donají hybrid (Malayo Enano Amarillo cv. Acapulco × Alto Pacífico cv. Escondido)	Experimental field of Oaxaca Coast of INIFAP (Serrano et al. 2011)	Resistant to LYD
8	PB 121 hybrid and progenies	Bourdeix et al. (1992)	Excellent level of tolerance to nut-fall caused by *Phytophthora katsurae*
9	Nias Yellow Dwarf × Palu Tall and Bali Tall	Brahmana et al. (1993)	Resistant to bud rot (*Phytophthora palmivora*) due to high polyphenol content
10	ECT × BSR-resistant ECT	Karthikeyan et al. (2005)	Higher survival percentage, higher nut yield against basal stem rot less disease incidence

(continued)

Table 3.3 (continued)

Sl. No.	Variety or hybrid or resistance source	Organization(s) involved/Reference	Major characteristic features
11	Kenthali Orange Dwarf and Chowghat Orange Dwarf	Ramaraju et al. (2000), Nair (2000)	Lower incidence of eriophyid mite infestation
12	Hybrids Java Giant Tall × East Coast Tall, Ayiramkachi Tall × West Coast Tall, Cochin China Tall × Philippines Ordinary Tall and West Coast Tall × Chowghat Orange Dwarf	Muthiah and Rajarathinam (2002)	Moderately tolerant to eriophyid mite
13	Varieties namely BSI, Chowghat Orange Dwarf, Philippines Ordinary Tall and Spicata Tall, and hybrids, viz. Philippines Ordinary Tall × San Blas Tall and Cochin China Tall × Philippines Ordinary Tall	Muthiah and Natarajan (2004)	Moderately resistant to eriophyid mite
14	Sri Lankan Yellow Dwarf and Gonthembili Tall	Perera (2006)	Tolerant cultivars to eriophyid mite
15	Java Giant Tall and Ceylon Green Tall	Raju et al. (2006)	Moderately resistant to eriophyid mite
16	Gangabondam Green Dwarf	Girisha and Nandihalli (2009)	Less mite incidence attributable to the tight attachment of perianth to nut surface
17	Laccadive Ordinary Tall and East Coast Tall × Godavari Ganga	Sujatha et al. (2010)	Lowest mite damage index
18	Jamaican Tall, BSI, Philippines Lono Tall, Guam Tall and Orange Dwarf	Badge et al. (2016)	Minimum infestation of eriophyid mite
19	Laccadive Ordinary Tall × Cochin China Tall and Gangabondam Green Dwarf × East Coast Tall	Muthiah and Bhaskaran (2000)	Minimum damage to rhinoceros beetle (*Oryctes rhinoceros*)
20	MAWA (Malayan Yellow Dwarf × West African Tall)	Capuno and de Pedro (1982)	Resistant source of Red Spider Mite (*Oligonychus velascoi*)

a promising donor of the RWD resistance gene, which led to the development of a variety 'Kalpasree' and a (D × T) hybrid, 'Kalpa Sankara' (Nair et al. 2006).

Multiple screening for lethal yellow disease (LYD) resistance in Ghana (Vanuatu Tall and the Sri Lanka Green Dwarf), Jamaica (MYD) and Tanzania (dwarfs such as Cameroon Red Dwarf, Equatorial Guinean Dwarf and Brazilian Green Dwarf),

Nigeria (dwarfs MGD, MYD and MOD) identified that the respective genotypes were relatively free from the disease. Hence, it was suggested to deploy a range of partially resistant genotypes, and the accessions of SE Asia and Mexico were found to be promising (Villarreal et al. 2002; Serrano et al. 2011; Odewale et al. 2013). In general, Malayan dwarfs or hybrids involving Malayan dwarfs and talls inherit resistance to LYD. Hence, Maypan hybrid (90%), Malayan Dwarf (96%), Panama Tall (Malayan Dwarf × Fiji Dwarf) and other dwarf cultivars of India and Sri Lanka showed field resistance (Been 1981).

Screening of hybrids for resistance against stem bleeding (caused by *Ceratocystis paradoxa*) in India identified a genotype (from the cross Cochin China Tall × Gangabondam Green Dwarf) as least infected (Radhakrishnan and Balakrishnan 1991). Though detached leaf petiole inoculation of the fungi to screen 26 coconut genotypes did not reveal any resistance to the disease, the lesion size was least in Banawali Green Round (Ramanujam et al. 1998). Among the three juvenile hybrids of Brazil screened for leaf spot (caused by *Bipolaris incurvata*) resistance, PB 121 was promising (Gomez-Navarro et al. 2009). In India, MYD and CGD displayed relatively low disease incidence (Govindan et al. 1991); Tiptur Tall was resistant (Ghose et al. 2006) (Table 3.3).

3.6 Association Mapping Studies

Association mapping is a promising way of resolving the genetic basis of complex traits and identifying trait-associated markers based on naturally existing collections with uncertain pedigree relationships. A concept of natural population-based genetic mapping is well suited for perennial crops like coconut due to its high resolution, allelic richness. Moreover, it does not necessitate a developed mapping population for tracing the QTLs. Thus, linkage disequilibrium or genome-wide association studies (GWAS) utilize the principle of linkage disequilibrium in a set of crop accessions to identify QTLs. Studies on association mapping of traits in coconut germplasm are very limited. To date, a few preliminary association studies have been reported in coconut. Geethanjali et al. (2018) and Zhou et al. (2020) employed GWAS strategy to study the population architecture and the fatty acid content traits, respectively. Analysis of genetic diversity of 79 coconut accessions revealed the presence of 2–7 alleles and two major clusters differentiating talls of Indo-Atlantic and South Asia from the accessions of Indo-Pacific and SE Asia region. Also, association analysis in a subset of 44 genotypes in the same study detected a single SSR locus, CnCir73, putatively associated with fruit yield component traits (Geethanjali et al. 2017). Zhou et al. (2020) performed linkage analysis in 80 accessions for fatty acid content resulting in the grouping of germplasm into sub groups comprising higher-fatty acid and lower-fatty acid groups. Further, SSR markers linked to fatty acid content in chromosome 11 and donor genotype (Aromatica Green Dwarf) carrying an allele CnFAtB3-359 with major positive effects were identified for use in coconut oil breeding.

The adoption of high-throughput sequencing technologies coupled with the developments in bioinformatics and statistical methodologies has greatly accelerated the genetic mapping of economically important crops (Elshire et al. 2011). The whole-genome sequence resources (Xiao et al. 2017; Lantican et al. 2018; Rajesh et al. 2020) have been effectively utilized by Yang et al. (2021) to develop a high-density linkage map of coconut by adopting a genotyping-by-sequencing (GBS) approach. This study had arranged the coconut genome sequence onto 16 pseudomolecules and placed over two-thirds of the coconut genome onto these 16 linkage groups. This chromosome-scale genome assembly of coconut would certainly facilitate the implementation of robust molecular breeding programmes (Yang et al. 2021). Also, recently, GBS technique was employed to study the genetic diversity of 16 coconut accessions originating from diverse regions of the globe and to discover novel SNPs (Rajesh et al. 2021b). GBS strategy yielded a total of 10,835 high-quality SNPs and around 80% of them exhibited polymorphism information content (PIC) values in the range of 0.3–0.4. Further phylogenetic and population structure analysis based on Bayesian model suggest that coconut genotypes clustered depending upon their morphoforms (talls *vs.* dwarfs) although clustering based on geographical origin was also observed. The pattern of Linkage disequilibrium (LD) in coconut reveals that it is reported to decay at a relatively short genetic distance of 9 Kb. This study has paved the way for application of forward genetic approaches such as GWAS and development of GS models in coconut (Rajesh et al. 2021b).

3.7 Molecular Mapping of Resistance Genes and QTLs and Marker-Assisted Breeding

A genetic linkage map is a linear map that shows the relative positions of genes along with a chromosome or linkage group. Genetic distances among them are established by linkage analysis, which determines the frequency at which two gene loci become separated through chromosomal recombination. Availability of a good quality genetic linkage plays a vital role in genetic analysis of a trait, helps in acceleration of breeding programmes, facilitates the identification of novel loci governing important traits. Hence, linkage mapping is considered an integral component of any marker-assisted breeding (MAB) programs. Though the physical maps could provide the order and distances of molecular markers, genetic maps are required to validate them. They would greatly assist in improving the de novo genome assemblies. Characterization and mapping of loci corresponding to quantitative traits refer to QTL mapping would help analyse the segregation pattern of QTLs and assist the genomics-based breeding in coconut. In coconut, the mapping strategies, (a) linkage mapping and (b) association mapping or linkage disequilibrium, are followed to identify QTLs, but the latter is minimally explored.

The first genetic map of coconut was made in the year 1991, with a population developed from cross EAT × LAG ('African Tall' × 'Laguna Tall') using inverse

sequence-tagged repeat (ISTR) markers (Rohde et al. 1999). This work was further extended with a population generated using a cross Malayan Yellow Dwarf (MYD) × Laguna Tall (LAGT) to identify QTLs associated with early germination traits. This was the first opportunity developed for marker-assisted selection in coconut. After that, Ritter et al. (2000) identified QTLs for leaf production, girth using 52 F_1 progenies generated from the cross Laguna Tall (LAGT) and Malayan dwarf (MYD), the markers used for the study were RAPD, ISTR, AFLP. Another genome map has been constructed with half-sib families of CRD × RIT ('Cameroon Red Dwarf' × 'Rennell Island Tall') using 227 markers (AFLP and SSRs), detected QTLs for the yield-related traits like the number of bunches and number of nuts (Lebrun et al. 2001). With the addition of new markers to the same mapping population, i.e. CRD × RIT, 52 putative QTLs were identified for the 11 traits, 34 of them were probably correspond to the single pleiotropic genes, and the others had relatively large effects on the individual traits (Badouin et al. 2006); QTLs linked to fruit components such as weight, endosperm humidity and fruit production were identified at different locations of a genome. Studies were also taken up in coconut to identify QTLs governing major cuticular wax components of coconut, which are involved in the plant's defence against abiotic and biotic stresses. Around 46 QTLs related to biosynthetic pathways of five different wax components were identified by Riedel et al. (2009).

Application of molecular markers in coconut improvement has spanned wide areas including in analyzing the genetic differences and genetic diversity analysis among the genotypes (Lebrun et al. 1998; Ashburner and Been 1997; Perera et al. 1998; Rohde et al. 1992; Manimekalai and Nagarajan 2006; Rivera et al. 1999; Rajesh et al. 2014, 2015; Jerard et al. 2017; Preethi et al. 2020), habit detection (Rajesh et al. 2013, 2014), mite resistance (Shalini et al. 2007), LYD resistance (Konan et al. 2007) among others. As stated above, only a few studies have been conducted in coconut to identify genes or loci associated with biotic stress resistance. DNA-based molecular markers have enormous advantages over conventional phenotypic markers for applications in plant breeding, especially in perennials such as coconut. Identification of AFLP markers linked to root (wilt) disease has greatly aided the process of resistance breeding in coconut (Rajesh et al. 2002). Deciphering the population structure of apparently disease-free and susceptible palms from the disease endemic districts of Southern Kerala towards RWD utilizing microsatellite markers revealed two distinct populations of resistant WCT along with several sub-populations (Deva Kumar et al. 2011). It was suggested to use these populations for genomics-assisted disease resistance breeding. Building on the findings of Rajesh et al. (2015), which unravelled the transcriptomic response of CGD leaf samples against RWD, Rachana et al. (2016) amplified, sequenced and characterized putative resistant gene analogues (RGA) from the same cultivar. Further, the coconut RGAs exhibited a high degree of sequence similarity to monocot NBS-LRRs and were expressed highly in RWD resistant genotypes (Rachana et al. 2016). Whole-genome sequence of root (wilt) disease-resistant cultivar CGD and comparative transcriptomic approach identified a total of 112 NBS-LRR encoding loci of six different classes (Rajesh et al. 2020).

To identify genetic loci associated with lethal yellowing (LY) disease resistance, Cardena et al. (2003) analyzed three different coconut populations with contrasting characteristics [susceptible West African Tall (WAT), the resistant Malayan Yellow Dwarf (MYD), and a resistant population of Atlantic Tall (AT) plants]. Using bulk segregant analysis, RAPD markers were selected if their frequencies were high in MYD and AT and low in WAT. A total of 82 RAPDs could differentiate the DNA pools derived from the MYD and WAT. The 12 RAPDs selected during the analysis of MYD and WAT are invaluable markers for differentiating the genetic makeup of the coconut materials. Konan et al. (2007) utilized 12 microsatellite markers to analyze LYD resistance revealing a total of 58 alleles. This study also identified the 10 specific alleles (CnCir series of SSR loci) associated with LYD resistance by screening tolerant Vanuatu Tall (VTT), Sri Lankan green Dwarf (SGD), and susceptible West African Tall (WAT). Further, the F_{st} index suggests that around 60% of the total allelic variability could explain the differences among the three genotypes studied. And these marker types could be useful for identifying the resistance material for taking up breeding programmes. Later search for resistance-conferring genes in coconut by Puch-Hau et al. (2015) using degenerate primers resulted in amplifying nucleotide-binding site (NBS)-type DNA sequences from coconut genotypes that were either resistant or susceptible to LYD. Interestingly, all the resistant gene analogues derived from coconut clustered with a non-TIR-NBS-LRR subclass of NBS-LRR genes. Further, gene expression analysis suggests that these RGAs exhibited variability in their expression for external salicylic acid. This study has set the stage for the exploration of RGAs in coconut. Putative receptor-like kinase (RLK) genes from coconut genotypes under the threat of Cape St Paul wilt disease was characterized by Swarbrick et al. (2013). Further sequence analysis of intron sequences of these putative RLKs identified three potential single nucleotide polymorphisms (SNPs) that could significantly differentiate susceptible and resistant genotypes.

Efforts were also made to identify molecular markers linked to coconut eriophyid mite (*Aceria guerreronis* 'Keifer') resistance. In the process of identification, coconut genotypes and mite-resistant and -susceptible accessions were collected. Thirty-two simple sequence repeat (SSR) and seven RAPD primers were used to identify the association between resistant trait-associated loci. Based on single marker analysis, nine SSR and four RAPD markers associated with mite resistance were identified. Combinations of 5 markers (SSR and RAPD) associated with eriophyid mite resistance have been discerned based on combined step-wise multiple regression of both SSR and RAPD data (Shalini et al. 2007).

3.8 Genomics-Aided Breeding for Resistance Traits

3.8.1 Whole-Genome Sequence Assemblies

The de novo nuclear genome assembly of coconut (cv. Hainan Tall) unravelled that the genome harbours 28,039 protein-coding genes. In contrast, related palm genera such as *Elaeis guineensis* and *Phoenix dactylifera* have 34,802 and 28,889–41,660 protein-coding genes, respectively (Xiao et al. 2017). Molecular evolutionary analysis based on Bayesian genetics suggests that coconut had diverged from oil palm around 46 million years ago. Comparative genomics further divulge that gene families encoding plasma membrane transporters (especially those involved in K^+ and Ca^{2+}, and Na^+/H^+ antiporters) are prevalent in coconut genome suggesting its adaptability to saline environments (Xiao et al. 2017). It was followed by sequencing of dwarf cultivar 'Catigan Green Dwarf' (CATD) by Lantican et al. (2019) that aided in characterization of novel 7139 microsatellite markers, 58,503 SNP variants and 13 gene-linked SSRs following a comparative analysis of dwarf and tall coconut genomes. Also, SSRs linked to drought tolerance and other biotic stress tolerance identified were promising resources for molecular breeding in coconut. These efforts were further complemented by characterizing the nuclear and organellar genomes of indigenous coconut cultivar, Chowghat green dwarf (CGD), showing resistance against root (wilt) disease (RWD) (Rajesh et al. 2020). Among these three genome assemblies, the efforts of Rajesh et al. (2020) would greatly help in identifying genetic factors responsible for resistance to root (wilt) disease and to introgress those genetic elements in susceptible cultivars by resorting to genomics-assisted breeding.

3.8.2 Transcriptomic Approaches

Transcriptome analysis, using RNA-sequencing (RNA-Seq), has been performed in coconut to decipher the molecular response of diseases such as coconut yellow decline (Nejat et al. 2015) and root (wilt) disease (RWD) (Rajesh et al. 2015, 2018). However, prior to this, researchers have utilized a comparative genomics approach to analyze the expression dynamics of *R* genes of coconut using related palms, mainly date palm. Resistant gene analogs (RGA) derived from coconut were utilized to comprehend the expression dynamics of coconut *R*-genes expression in response to root (wilt) disease. Conserved domains of nucleotide-binding site-leucine-rich repeat (NBS-LRR) class genes of oil palm and date palm were used to design primers and study coconut RGAs (Rajesh et al. 2015). Three putative RGAs were isolated in coconut. Their relatively high expression status in the leaves of RWD resistant cultivar suggests their potential utility in genomics assisted resistance breeding in coconut (Rachana et al. 2016).

RNA sequencing of apparently healthy and diseased coconut cultivar Chowghat Green Dwarf (CGD) revealed the underlying host molecular response to the disease

progression (Rajesh et al. 2018). Differential transcript expression analysis of healthy and diseased RNAs reveals that many transcripts (~2700) are differently regulated in the wake of the disease. Interestingly, a genetic regulatory network analysis based on the transcriptome data shows that RNA encoding calmodulin-like 41, WRKY DNA-binding proteins are upregulated. This transcriptome analysis also put forth a molecular model of coconut's response to RWD involving host protein kinases, calcium-binding proteins and a signaling cascade involving salicylic acid in concurrence with the dynamic expression of TFs such as WRKY and NAC-domain proteins. (Rajesh et al. 2018).

Similarly, comparative transcriptome analysis of healthy and diseased coconut yellow decline (CYD) diseased infected leaves have identified that genes involved in defence response and signal transduction pathways are highly upregulated (Nejat et al. 2015). In the phytoplasma infected tissues, genes coding for pathogenesis-related proteins (PRs) were highly expressed. This study proved that the active defence response of the host is stimulated during the phytoplasma invasion (Nejat et al. 2015).

3.9 Conclusion and Future Perspectives

In summary, it is evident that the application of genomics science in improving coconut has been lagging compared to many other crops. Also, a very handful of successful applications of MAS has been witnessed in coconut, especially in developing insect or disease-resistant cultivars. Further identification and validation of major QTLs linked to traits of agronomic importance are strategically required to unleash the potential of genomics-assisted breeding in coconut. Considering its perennial nature, applying concepts such as 'speed breeding' to reduce the timeline for developing novel varieties warrants progress and the use of robust in vitro propagation techniques. To increase the genetic gain, adopting techniques such as genomic selection (GS) models is imperative as it helps in the shortening of breeding cycles and improves the efficiency of selection procedures. Deployment of genomics assisted breeding has to be integrated with the current conventional breeding strategies for developing biotic stress tolerance in coconut. Applications of transgenics or genetic engineering technologies have long been overlooked in horticultural, perennial crops, unlike the annuals wherein successful deployment of GM crops has helped manage deadly pathogens and pests in the field conditions. Nonetheless, it is anticipated that break-through in coconut in vitro clonal propagation techniques along with developments in the field of gene prospecting would spur the development of GM coconut aimed at pests and disease tolerance. Availability of good quality genome assemblies and application of functional genomics has a great potential to decipher gene-function relationship in coconut. Further, genome-editing approaches utilizing CRISPR/Cas9 tools are imminent in coconut to develop biotic stress-tolerant genotypes by way of suppressing host susceptibility factors. Though applying this technology is challenging in perennials such as coconut, it is highly desirable in the

context of 'fast-forward breeding' approaches envisioned. In addition, resequencing of a large number of coconut accessions possessing specific biotic stress tolerance traits (disease or pests) would provide a genetic blueprint for accelerating the genetic gain in the field of genomics assisted resistance breeding.

In addition, the role of bioinformatics in solving the bottlenecks in breeding for biotic stress tolerance in coconut is required now more than ever because large-scale data pertaining to the genome, transcriptome sequences are available in the public databases. Mining of these databases to develop robust genic markers, EST-based full-length gene sequences, reconstruction of transcriptome profiles, identification of novel functional genetic elements and other gene regulatory elements are fundamental to reap the benefits of big data-enabled molecular breeding in coconut.

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Chapter 4
Current Challenges and Genomic Advances Toward the Development of Coffee Genotypes Resistant to Biotic Stress

Caroline Ariyoshi, Fernanda Freitas de Oliveira, Luciana Harumi Shigueoka, Angelita Garbossi da Silva, Andres Gatica Arias, Jimmy Villalta-Villalobos, Suzana Tiemi Ivamoto-Suzuki, Gustavo Hiroshi Sera, Eveline Teixeira Caixeta, Matheus Ricardo da Rocha, Willian Bucker Moraes, Fábio Luiz Partelli, and Luiz Filipe Protasio Pereira

Abstract Coffee (*Coffea* spp.) is an important agricultural world commodity. Biotic stresses caused by pests or phytopathogens can affect not only the coffee production, but also the grain quality. They interfere with physiological processes affecting plant growth and development, and damaging different plant organs and tissues, such as leaves, roots, and fruits. The use of chemicals to control diseases and pests directly

C. Ariyoshi · F. F. de Oliveira · A. G. da Silva · S. T. Ivamoto-Suzuki
Biological Science Center, State University of Londrina, Londrina, Paraná 86057-970, Brazil
e-mail: caroline.ariyoshi@uel.br

F. F. de Oliveira
e-mail: ffoliveira2@uem.br

A. G. da Silva
e-mail: angelita@idr.pr.gov.br

S. T. Ivamoto-Suzuki
e-mail: suzanatiemi@uel.br

C. Ariyoshi · F. F. de Oliveira · L. H. Shigueoka · A. G. da Silva · S. T. Ivamoto-Suzuki · G. H. Sera · L. F. P. Pereira (✉)
Area of Genetic Improvement and Plant Propagation, Paraná Rural Development Institute, Londrina, Paraná 86047-902, Brazil
e-mail: filipe.pereira@embrapa.br

G. H. Sera
e-mail: gustavo.sera@idr.pr.gov.br

A. G. Arias · J. Villalta-Villalobos
School of Biology, University of Costa Rica, San Pedro de Montes de Oca, San José 11501-2060, Costa Rica
e-mail: andres.gatica@ucr.ac.cr

E. T. Caixeta
Institute of Applied Biotechnology to Agriculture, Federal University of Viçosa, Viçosa, Minas Gerais 36570-900, Brazil

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affects coffee cultivation sustainability and are often inefficient. This scenario could worsen with the rapid insurgence of new and/or more aggressive pathogens and pests as a result of the world climate change. Thus, coffee breeding with a focus on the development of resistant cultivars is the best strategy to control these biotic stresses. In this context, biotechnological tools can help the coffee breeding in a persuasive way. Advances in genomic editing techniques, such as CRISPR, are capable of introducing punctual modifications in the plant genome. As an alternative to the use of chemicals, the sequence-specific gene silencing via RNA interference (RNAi) holds a great promise for effective management of agricultural pests. The emergence of high-throughput sequencing technology has allowed unprecedented advances in genomic and transcriptomic data. The genomic and transcriptomic coffee data can now be used to identify a large number of genes and molecular markers that determine coffee resistance to pathogens and pests. The identification of these genes helps to elucidate plant pathogen interactions, as well as can be targets for genome editing. The implementation of molecular markers, through assisted selection, can help accelerate breeding programs and pyrimidize resistance genes. In addition, the genomic and transcriptomic data of pathogens and pests are useful to identified targets for RNAi approaches. In this review, we address the research in modern genetics and molecular biology related to the main biotic stresses of coffee plants and its implications for coffee breeding.

Keywords *Coffea canephora* · *Coffea arabica* · Pathogens · Transcriptome · Molecular marker · Transgenic · Genome editing · RNA interference

4.1 Introduction

Coffee (*Coffea* spp.) is an important tropical agricultural commodity for the economy of several tropical countries, with a total revenue in 2014 of US$173 million in more than 80 countries (ICO 2019). Coffee also has an important socioeconomic role because it is related to the livelihood of more than 25 million farmers (Dumont 2019), in addition to the participation of more than 125 million workers in its laborious

e-mail: eveline.caixeta@embrapa.br

E. T. Caixeta · L. F. P. Pereira
Empresa Brasileira de Pesquisa Agropecuária (Embrapa Café), Brasília, Distrito Federal 70770-901, Brazil

M. R. da Rocha · W. B. Moraes
Department of Agronomy, Center of Agricultural Sciences and Engineering, Federal University of Espírito Santo, Alegre, Espírito Santo 29500-000, Brazil
e-mail: willian.moraes@ufes.br

F. L. Partelli (✉)
Center of North of Espírito Santo, Federal University of Espírito Santo, São Mateus, Espírito Santo 29933-515, Brazil
e-mail: partelli@yahoo.com.br

production chain (Dumont et al. 2019). Brazil, Vietnam, and Colombia are the world's largest producers and exporters, responsible for more than 60% of both.

The genus *Coffea* belongs to the Rubiaceae family, with more than 140 described species (Davis et al. 2011; Guyot et al. 2020); however, only *Coffea arabica* L. (arabica) and *Coffea canephora* Pierre ex A. Froehner (robusta) are considered commercially important because they represent approximately 60% and 40% of the global market, respectively (USDA 2020). In addition to these species, two other species are cultivated on a small scale to meet the local market demand: *Coffea liberica* and *Coffea racemosa* (Krishnan et al. 2015). Coffee is one of the most consumed beverages in the world, and its consumption has increased by 160% in the last 30 years. Therefore, it is necessary to develop new strategies and technologies to promote productivity increases of sustainable coffee plantations (FAO 2015; ICO 2019).

It is estimated that the reduction in agricultural productivity associated with damage caused by pests and phytopathogens (fungi, bacteria, viruses, and nematodes) is 20–30% in the main crops (Savary et al. 2019). Furthermore, global climatic changes can also increase disease or insect pest outbreaks, as well as expand these problems to areas where they were not previously prevalent (Dubberstein et al. 2018; Ziska et al. 2018). In coffee trees, infestation by pests and the presence of diseases are among the main factors that affect productivity and grain quality (Ventura et al. 2017). Biotic stress impairs physiological processes (photosynthesis, absorption, and translocation of water and nutrients), plant development, and damages different plant organs and tissues, such as leaves, roots, and fruits (Esgario et al. 2020).

Several pathogens that affect coffee production have been identified in different regions worldwide (Maghuly et al. 2020). The most important are coffee leaf rust (CLR), coffee berry disease (CBD), bacterial halo blight (BHB), coffee leaf miner (CLM), coffee berry borer (CBB), and nematodes. Farmer's control of these biotic stresses relies mainly on chemicals that can increase production costs, adversely affect human health, and cause environmental problems (Zambolim 2016; Talhinhas et al. 2017). Furthermore, the use of chemicals in some cases may have low effectiveness, as evidenced mainly in the management of nematodes and CBB (Avelino et al. 2018). In this context, the development and use of coffee cultivars resistant to pests and diseases is the best alternative.

Coffee breeding programs frequently focus on the development and selection of resistant genotypes as protagonists of an integrated management program for the main biotic stresses (Silva et al. 2018). However, coffee breeding programs are laborious, with long-term results (25–30 years). To meet the growing producer, consumer, and market demand, a complex, continuous, and dynamic breeding process is required, resulting in costly long projects for the development of superior cultivars. Therefore, the implementation of statistical and biotechnology tools can help to reduce the time and effort required to produce new coffee cultivars and consequently assist in the urgent need for chemical reduction (Hindorf and Omondi 2011; Kumar et al. 2016; Oliveira et al. 2021).

From this perspective, researchers have been seeking molecular markers linked to genes or lociassociated with coffee resistance (Pestana et al. 2015; Ariyoshi et al.

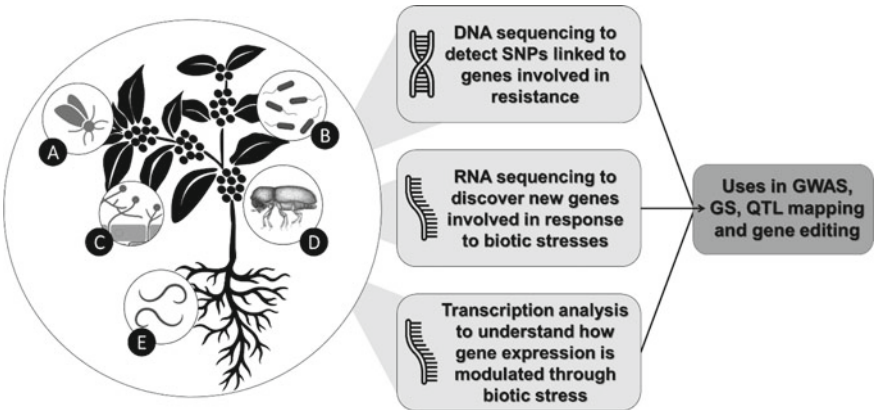

Fig. 4.1 Main biotic stresses that affect coffee trees and major research approaches for genetic improvement. (A) Coffee leaf miner (CLM); (B) bacterial halo blight (BHB); (C) coffee leaf rust (CLR) and coffee berry disease (CBD); (D) coffee berry borer (CBB); and (E) nematodes. GWAS: genome wide association studies; GS: genome selection; QTL: quantitative trait locus

2019; Gimase et al. 2020), elucidating the genes involved in plant defense mechanisms (Florez et al. 2017; Castro-Moretti et al. 2020), and determining the genes related to pathogen and pest effectiveness (Porto et al. 2019). These markers and genes can be incorporated in breeding programs that help breeders select resistant coffee plants, or these traits can be incorporated by genetic engineering and/or genome editing in elite coffee cultivars (Fig. 4.1).

In this review, we have focused on research that has used modern genetics and molecular biology related to the main biotic stresses of coffee plants and their implications for coffee breeding. We have discussed the problems encountered and the prospects for using genomic technologies, with the goal of developing new strategies to combat the main pests and pathogens in coffee farming.

4.2 Genomic Analyses for Major Biotic Stresses in Coffee

The implementation of resistant cultivars is considered the most effective and sustainable methodology for disease and pest management (Naidoo et al. 2019). Thus, genomic resource availability is a key factor in achieving this goal. Genomic DNA data allow researchers to access important information to design coffee breeding program strategies, such as the use of molecular markers for plant selection (Sant'ana et al. 2018; Gimase et al. 2020).

For successful breeding programs, screening for disease-resistant genotypes must be reliable and efficient. Molecular markers linked to resistance loci in coffee genotypes are advantageous for morphological evaluations (Alkimim et al. 2017). Molecular DNA markers are not affected by environmental effects, allow the selection of

resistant individuals in the absence of the pathogen, and can be applied at any stage of plant development. Additionally, molecular markers can effectively aid in the identification of genotypes with multiple resistance factors when there is a dominant or epistatic effect. This is useful for assisting the pyramidization of resistance genes, which is a promising strategy in breeding programs (Alkimim et al. 2017).

In the last few decades, remarkable advances in DNA sequencing technologies have emerged with the advent of next-generation sequencing (NGS) (de Filippis 2017). The cost of sequencing 1 million base pairs (1 Mb) of DNA was reduced from US$1000 in 2004 to US$0.008 in 2020 (NIH 2020). In addition to the evolution of sequencing technology, the equipment to store this volume of data and bioinformatics tools to conduct analyses have also evolved during the same period (de Filippis 2017). Currently, there are data available in several public databases for coffee genomes, pathogens, and pests (Table 4.1).

Table 4.1 High-throughput genome sequencing studies with coffee species and pathogens-pest related

Specie	Study	Bioproject (NCBI)	Reference—Institute
C. arabica Caturra	Genome	PRJNA506972	John Hopkins
C. arabica Red Bourbon	Genome	PRJNA554647	Scalabrin et al. (2020)
C. arabica Geisha	Genome	NA*	UC Davis—Phytozome
C. arabica Et39	Genome	PRJNA698600	ACGC (2014)
C. eugenioides CCC68	Genome	PRJNA508372	John Hopkins
C. eugenioides	Genome	PRJNA698600	ACGC (2014)
C. canephora DH 200-94	Genome	PRJEB4211	Denoeud et al. (2014)
C. canephora BUD15	Genome	PRJNA698600	ACGC (2014)
C. Arabica	Diversity	PRJEB9368	Lashermes et al. (2016)
C. Arabica	Diversity	PRJEB26929	Gimase et al. (2020)
C. Arabica	Diversity	PRJNA401643	Texas A&M
Coffea spp.	Diversity	PRJNA698600	ACGC (2014)
Coffea spp.	Diversity	PRJNA612193	Bawin et al. (2020)
Coffea spp.	Diversity	PRJNA401643	Texas A&M
Coffea spp.	Diversity	PRJNA352624	CRIN
Coffea spp.	Diversity	PRJNA242989	IRD
Coffea spp.	Diversity	PRJNA505204	Huang et al. (2020)
C. canephora	Metagenome	PRJNA526486	UNICAMP
Hypothenemus hampei	Genome	PRJNA279497	Vega et al. (2015)
Hypothenemus hampei	Genome	PRJNA626647	Navarro-Escalante et al. (2021)
Hemileia vastatrix	Genome	PRJNA419278	Porto et al. (2019)
Colletotrichum kahawae	Diversity	PRJEB26929	CICF

* NA: Not available

Genome sequencing provides not only a handful of genes, transposons, and noncoding RNA information (Lemos et al. 2020) but is also the starting point for modern breeding approaches, such as genomewide association studies (GWAS) and genome selection (GS) (Gimase et al. 2020). GWAS have emerged together with NGS technology as powerful tools for identifying molecular markers associated with agronomic traits of interest. GWAS can overcome the limitations of traditional genetic linkage mapping, including maps with little refinement and limited diversity to parents (Bartoli and Roux 2017). In addition to identifying molecular markers to be implemented in molecular marker-assisted selection (MAS), GWAS also allow the identification of gene linkages to the associated markers, enabling the elucidation of the molecular mechanisms of plant defense against pathogens (Chagné et al. 2019; Zhang et al. 2020).

GWAS can explore the genetic diversity found in wild crop relatives. This is extremely important in *C. arabica.* The recent origin of a single hybridization event and its predominantly autogamous reproduction, associated with the limited dispersion of plants worldwide, created a huge bottleneck in cultivar development (Setotaw et al. 2013; Merot-L'anthoense et al. 2019; Scalabrin et al. 2020). The *C. arabica* collections of wild crop relatives, such as from the survey organized by the Food and Agriculture Organization (FAO) in 1964–1965 in Ethiopia (FAO 1968), are essential for the exploration of their genetic variability and for application of modern breeding approaches. Based on the wild germplasm of *C. arabica*, several characterization and evaluation studies have been conducted to identify sources of resistance, such as against the nematodes *Meloidogyne paranaensis*, *M. incognita*, and *M. exigua* (Anzueto et al. 2001; Fatobene et al. 2017), BHB (Mohan et al. 1978), and CBD (van der Vossen and Walyaro 1980). All these agronomic traits have the potential to be used in GWAS to identify markers and genes related to plant defense.

4.3 Transcriptome Studies for Major Biotic Stresses in Coffee

Transcriptomic resource availability and genomic resources are key factors in the design of coffee breeding strategies (Noriega et al. 2019). The study of the transcriptome profile of genes during the plant-pathogen interaction is one of the main molecular approaches to elucidating the biological processes during the infection process.

Once pathogens are able to overcome mechanical defense barriers, the plant has receptors capable of recognizing the pathogen and activating signaling pathways that drive the expression of defense response genes (Andersen et al. 2018). At the first level of recognition, non-mutable molecules of the pathogen, known as pathogen-associated molecular patterns (PAMPs), can be detected by host proteins of the

pattern recognition receptors (PRRs). This first level of pathogen recognition activates PAMP-triggered immunity (PTI), which can be suppressed by pathogen effectors. The second level of recognition involves resistance proteins (R) capable of detecting effectors and activating effector-triggered immunity (ETI). Generally, ETI is accompanied by cell death, known as the hypersensitive response (HR) (Zipfel and Rathjen 2008). Through the loss or diversification of effectors, pathogens can successfully suppress ETI and colonize the host. The coevolution of pathogens and plants, and notably their repertoire of effectors and R proteins, led to the so-called zigzag model (Jones and Dangl 2006). Among the PRRs and R proteins, the classes with the greatest representativeness are the receptor-like kinases leucine-rich repeats (RLKs-LRRs), and the nucleotide binding sites leucine-rich repeats (NBSs-LRRs), respectively (Coll et al. 2011). Although ETI has greater magnitude and duration, defense signaling through PTI and ETI can activate similar downstream molecular events, such as mitogen-activated protein kinase (MAPK) activation, oxidative bursts, ion influxes, increased biosynthesis of plant defense hormones, and transcriptional regulation of defense genes (Tao et al. 2003; Navarro et al. 2004).

Transcriptome data can provide specific information about the class of plant genes (pathogen recognition, signaling, or defense response) and the time course that is being activated or suppressed, an important issue in plant pathogen interactions. Moreover, transcriptome studies have identified pathogen genes, such as effectors, that are active in the infection process. Therefore, expression profiling of hosts and pathogens can provide a new understanding of their interactions and allow the identification of virulence genes in the pathogen and defense pathways in host cells (Boyd et al. 2013; Florez et al. 2017). Despite the research on transcriptome of plant-pathogen interaction in coffee plants that had been developed (Table 4.2), the reduction in the cost of sequencing technologies will allow for a greater number of studies in this area, with a major advance in understanding the mechanisms of resistance to the main pests and pathogens of coffee trees.

Table 4.2 Transcriptomic sequencing data of *Coffea* spp.—pathogens interaction

Specie	Pathogen	Technique	Bioproject (NCBI)	Reference
Ck	CBD	Illumina (HiSeq2000)	PRJNA271934	NP
Hh	CBB	EST	NA	Idarraga et al. (2011)
Hh	CBB	Illumina (HiSeq2500)	SUB4491034	Noriega et al. (2019)
Hv	CLR	454 pyrosequencing	PRJNA188788	Cristancho et al. (2014)
Hv	CLR	454 pyrosequencing	NA	Fernandez et al. (2012)
Hv	CLR	Illumina (MiSeq)	NA	Florez et al. (2017)
Lc	CLM	EST	NA	Cardoso et al. (2014)

CBB: Coffee berry borer; CBD: coffee berry disease; Ck: *Colletotrichum kahawae*; CLM: coffee leaf miner; CLR: coffee leaf rust; Hh: *Hypothenemus hampei*; Hv: *Hemileia vastatrix*; Lc: *Leucoptera coffeella*; NA: not available; NP: not published.

4.4 Genetic Transformation, RNAi, and Genome Editing for Biotic Stress in Coffee

Despite the tremendous success in producing new varieties, the improvement in coffee and acquisition of a new cultivar based on traditional methodologies is a process that requires approximately 20–30 years (Melese 2016). It includes several steps, such as the selection of species or varieties to be used, their subsequent hybridization, and evaluation of the resulting progeny. In some cases, backcrosses and interspecific crosses are conducted (Orozco and Schieder 1982).

Although classical breeding is one of the main and most widely used strategies for obtaining improved cultivars, there are limitations in regard to the characteristics that can be added because most species of the genus *Coffea* are self-incompatible (Davis et al. 2006). Additionally, different ploidy of the allotetraploid *C. arabica* in relation to other *Coffea* species hinders the introduction of agronomic characteristics present in the *Coffea* genetic pool. Therefore, it is important to use techniques that can break the barriers between species, complement traditional genetic breeding programs, and the available genetic base.

Since the first commercial use of transgenic plants in 1996, genetic transformation has become an essential tool for the genetic improvement of crops (James 2018). Genetic transformation technology is considered an extension of conventional breeding technologies (Zhong 2001) and offers unique opportunities to overcome compatibility barriers between species and thus can develop phenotypes with desired traits that are not available in the germplasm of crop plants (Mishra and Slater 2012).

The main objectives of genetic improvement through the use of genetic engineering techniques in coffee are to introduce new traits into elite genotypes, develop new cultivars with desirable characteristics, such as resistance to pests and diseases, resistance to herbicides, tolerance to drought and frost, and increased cup quality (Mishra and Slater 2012). Additionally, genetic transformation can also be used as a tool for the functional validation of coffee genes and promoters (Brandalise et al. 2009; Cacas et al. 2011; Mishra and Slater 2012; Girotto et al. 2019).

The first report of genomic transformation in coffee was made by Spiral and Petiard (1991), wherein genomic transformation was mediated by the bacterium *Agrobacterium tumefaciens*. Later, Barton et al. (1991) used the indirect genomic editing methodology of electroporation, and van Boxtel et al. (1995) used particle bombardment, with successful regeneration of transformed explants with both methodologies. Transformation techniques are more efficient in *C. canephora* than in *C. arabica* and are associated with the difficulty in inducing embryogenic tissues and regeneration of explants in the latter (Kumar et al. 2006). Despite the constraints of *C. arabica* genetic transformation, successful transformation rates of up to 90% have been achieved with specific tissue culture conditions, such as a high auxin-to-cytokinin ratio associated with the use of specific callus phenotypes (Ribas et al. 2011).

Gene silencing using RNA interference (RNAi) strategies is an important tool for the control of biotic stresses (Cagliari et al. 2019). RNAi or post-transcriptional gene silencing (PTGS) is a mechanism in which small double-stranded RNA (dsRNA) regulate gene expression. Small pieces of RNA, called micro-RNA (miRNA) or small interfering RNA (siRNA), can shut down protein translation by binding to the messenger RNAs that code for these proteins. This was first described in *Caenorhabditis elegans,* but it is present in all eukaryotic organisms and has become a popular tool in functional genomics. RNAi technology can be used to control biotic stress with genetically modified plants with target mi or siRNA. Plants controlling western corn rootworm (*Diabrotica virgifera*) with RNAi have been commercially approved for use in Canada and USA. RNAi technology can also be used with non-transgenic methods, such as dsRNA sprayed products and could substitute chemical pesticides in the future.

Genomic editing by clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) has been successfully used as a tool for the elimination of genes of agronomic interest, paving the way for improvement of coffee cultivars (Breitler et al. 2018). This technique uses guide RNA (gRNA), which indicates that the Cas9 enzyme is a target region in the genome. This target region suffers a double break in the DNA strands, which can lead to non-homologous recombination, thereby causing gene silencing or homologous recombination. Therefore, it is possible to change or cleave a specific gene and/or insert a new gene (Ran et al. 2013; Sander and Joung 2014). With the availability of genome sequences of *C. canephora* and *C. arabica*, transformation and genome editing via CRISPR/Cas9 became possible.

A web program called "CRIP" (Coffee gRNA Identification Program) was developed (Breitler et al. 2018), which allows the identification of all target sequences of single guide RNA (sgRNA) in the genome of *C. canephora*, with the advantage of identifying non-target sequences for each leader sequence. This program was tested by choosing regions of three exons for genome editing of a phytoene desaturase (CcPDS), which produces an easily visible albino phenotype when mutated. For one exon, genome editing was observed in 30.2% of the plants regenerated, with a total of nine different mutations, whereas no mutants were obtained for the other exons. None of the plants showed a complete albino phenotype, but a range of phenotypic mutants were attained that included plants with small, lanceolate leaves with abnormal pigmentation (yellow, chlorotic), shorter internodes, and missing leaves, or in some cases, in greater numbers than normal (Breitler et al. 2018). Thus, genome editing using CRISPR-Cas9 has been shown to be an efficient and reliable way to inactivate genes of agronomic interest in *C. canephora.*

Despite the high potential of genetic engineering in the development of resistant *Coffea* cultivars, molecular information regarding the defense mechanisms against biotic stresses is still limited. Additionally, one of the obstacles encountered in the development of genetically modified organisms (GMOs) in coffee growth is consumer approval and acceptance, which could represent an obstacle for coffee commercialization. Despite the use of commercial GMOs for more than 25 years in other species, marketing strategies should be taken into consideration to ensure the

public understanding of genetically modified (GM) coffee. With proper information regarding the benefits and possible risks of GMOs, these improved crops could be progressively incorporated into the market. However, CRISPR and other genome editing technologies, called new plant breeding technologies (NPBTs) (Wolt et al. 2016), have been classified as non-GMOs in several coffee-producing countries, as well as in the main importing and consuming countries. Plants produced by NBTs can bypass the legal regulations imposed on GMOs, saving time and costs related to regulatory approval. Hence, genome editing could become an alternative for the development of novel coffee varieties that are resistant to different biotic and abiotic factors, thereby increasing production and cup quality.

4.5 Breeding for Biotic Stress Resistance in Coffee: Some Case Studies

4.5.1 Coffee Leaf Rust (CLR)

Hemileia vastatrix Berk. et Br, the causative agent of CLR, is one of the major threats to coffee production in almost every coffee-producing region. The pathogen is a biotrophic fungus that attacks only plants in the genus *Coffea.* When not controlled through application of fungicides, the disease can reduce productivity by more than 50%, both in *C. arabica* and *C. canephora* (Zambolim 2016; Zambolim and Caixeta 2021). The disease can be managed by integrating several measures, but the use of resistant cultivars is the best alternative because of farmer sustainability and environmental concerns (Sanders 2019).

To assist in cultivar development, some resistant sources for CLR have already been identified. At least nine dominant genes (S_H1 to S_H9) present in different coffee species have been characterized to date. S_H1, S_H2, S_H4, and S_H5 genes were identified in *C. arabica,* S_H3 in *C. liberica*, and S_H6, S_H7, S_H8, and S_H9 in *C. canephora* (Noronha-Wagner and Bettencourt 1967; Rodrigues et al. 1975). Other major and minor genes have been reported in interspecific hybrids (Varzea and Marques 2005).

Although resistance sources are available, obtaining a cultivar with durable resistance is a challenge for breeders because of the variability in *H. vastatrix*. More than 50 physiological races of *H. vastatrix* have been identified worldwide, which makes it difficult to manage disease resistance (Zambolim 2016; Zambolim and Caixeta 2021). The high adaptive potential of the pathogen, the emergence of new physiological races, and a corresponding breakdown of resistance has been observed in many coffee cultivars (Varzea and Marques 2005; Capucho et al. 2012). Therefore, breeding programs are an important strategy for disease control.

Among the resistance sources, the Timor Hybrid (HdT) germplasm stands out because it is composed of genotypes with substantial genetic variability, with different genes for resistance to CLR and other diseases, such as CBD, root knot nematode (*Meloidgyne exigua*), and bacteriosis (*Pseudomonas syringae* pv. *garcae*). HdT is a

natural hybrid between the species *C. canephora* and *C. arabica* (Bettencourt 1973), presenting the resistance provided by *C. canephora* and the sensory characteristics of arabica, which are widely used in breeding programs (van der Vossen 2009; Setotaw et al. 2020).

The CLR-resistant cultivars derived from HdT germplasm have been planted in Latin America and East Africa and were first developed by the Coffee Rusts Research Center (CIFC, Centro de Investigação das Ferrugens do Cafeeiro) in Portugal. Since 1955, CIFC has received and characterized coffee and rust germplasm and supplied breeding programs (Talhinhas et al. 2017). CIFC developed the hybrids HW26 (Caturra Vermelho × HdT CIFC 832/1 -Catimor), H46 (Caturra Vermelho × HdT CIFC 832/2-Catimor), H361 (Villa Sarchi × HdT CIFC 832/2-Sarchimor), H528 (CatuaíAmarelo × HW26/13), and H529 (CaturraAmarelo × H361/3) (Silva et al. 2006). Some selected F_1 and F_2 plants with resistance to all known races were spread to many institutions in coffee-growing countries and were incorporated into their breeding programs.

Other natural interspecific hybrids used as CLR resistance sources are Indian selections (*C. arabica* × *C. liberica)* S.288, S.333, S.353 4/5, and Series BA. This germplasm carries the resistance gene S_H3 (Alkimim et al. 2017; Silva et al. 2019). HdT and Indian selections, which are tetraploid materials, are used to facilitate introgression resistance genes from diploid species (*C. canephora* and *C. liberica*) in the tetraploid species *C. arabica.* The accumulation of different genes in a cultivar allows the production of coffee with more durable resistance compared to that of cultivars with a single race-specific resistance gene.

Molecular markers associated with these genes have been identified to assist in the introgression of CLR resistance genes. Molecular markers are an important tool for improving the efficiency of selection in coffee breeding programs (Sousa et al. 2019), especially when different genes are accumulated. In breeding programs, a segregating coffee population inoculated with *H. vastatrix* races can be easily screened for resistance. However, it is difficult to distinguish between plants with one or more resistance genes because of epistatic effects. Because molecular markers show no such epistatic effects, MAS can be an efficient alternative (Pestana et al. 2015). MAS also allows the identification of resistance in the absence of pathogens.

Markers associated with CLR resistance genes in *C. liberica* and HdT have been identified. Prakash et al. (2004), using a segregating population with susceptible (Matari) and resistant (S. 288) parents for *H. vastatrix* race VIII (*Vr* 2,3,5), identified 21 amplified fragment length polymorphism (AFLP) markers linked to the S_H3 gene. Later, Mahé et al. (2008) developed sequence-characterized amplified region (SCAR) markers based on AFLP and a library of bacterial artificial chromosome (BAC) clones. The developed SCAR and three simple sequence repeat (SSR) markers were used to construct a linkage map containing the S_H3 resistance gene. The markers closest to the S_H3 gene (BA-48-21-f, Sat244, BA124-12 K-f, and Sp-M16-SH3) have been used in MAS (Alkimim et al. 2017).

Furthermore, molecular markers linked to HdT resistance genes were obtained from an F_2 population (HdT UFV 427-15 × CatuaíAmarelo) segregating to a gene that confers resistance to *H. vastatrix* race II (*Vr5*) (Diola et al. 2011). In this study, a

genetic map with 25 AFLP markers was obtained, which enabled the development of a high-density map with six SCAR markers closely linked to the CRL-resistant gene. SCAR markers delimited a chromosomal region of 9.45 cM, flanking the resistance gene by 0.7 and 0.9 cM.

Also, markers associated with HdT resistance genes were identified in other accessions. Four SSR markers were linked to the CRL-resistant quantitative trait locus (QTL) in the HdT-derived genotype DI.200 (Herrera et al. 2009; Romero et al. 2014). Phenotypic CLR resistance data were obtained in the field and from controlled inoculation using a mixture of uredospores collected in Caturra. The markers flanked the QTL in a region of 2.5 cM. This locus was aligned to chromosome 4 of the *C. canephora* reference genome.

Pestana et al. (2015) identified markers flanking the quantitative trait loci (QTLs) present in HdT UFV 443-03. A linkage map was constructed and indicated that this HdT has at least two independent dominant loci conferring resistance to race II of *H. vastatrix* and four independent dominant loci for resistance to race I and pathotype 001. Markers flanking these QTLs were identified based on the genetic map and used an artificial neural network statistical approach (Silva et al. 2017). Based on these studies, molecular markers flanking seven different loci of resistance to CLR have been identified. These markers have great potential to increase selection efficiency and allow the pyramidation of different resistance genes in new cultivars.

Coffee breeding programs with the goal of coffee resistance to CLR rely not only on molecular marker technology but also on structural and functional genomics to increase their efficiency and competitiveness. Studies of the transcriptional profile of the interaction between *H. vastatrix* and coffee allow the identification of pathogen virulence genes and host genes involved in the defense mechanism (specific and non-specific pathogens). The information obtained in these studies is useful in the identification of new genes to be incorporated into the cultivars, development of new markers, and incorporation of new directions into breeding programs. Additionally, they can be targets for genetic manipulation, such as for the constitutive activation of disease resistance signaling routes. Information regarding genes involved in pathogen infection and its interaction with coffee can also be used to identify new methods of disease control.

To identify *C. arabica* genes involved in a hypersensitive reaction (HR), Fernandez et al. (2004) analyzed expressed sequence tags (ESTs) obtained by suppression subtractive hybridization (SSH). HR is the most common expression of incompatibility interactions (resistance) and has been previously identified in the early stages of *H. vastatrix* infection (Silva et al. 2002). To differentiate the transcripts strictly involved in the defense response, two cDNA libraries from incompatible and compatible interactions were used in the SSH approach. Incompatible interactions were observed in the *C. arabica* Caturra (S_H5) inoculated with race VI and S4 Agaro (S_H4; S_H5) inoculated with *H. vastatrix* race II. Compatible interactions were performed with Caturra inoculated with race II and S4 Agaro with race XIV. The transcripts used to build a catalog of non-redundant ESTs represent genes with expression of early resistance mechanisms, because they were identified in coffee samples at 12, 24, and 48 h after inoculation (hai) with the fungus. Silva et al. (2002;

2008) used a microscopic approach to demonstrate that spore germination and development of the appressorium occurs 12–17 hai, hypha penetrates through the stomata at approximately 24 hai, and haustorial mother cells develop at 36–48 hai. Of the obtained ESTs, 13% may represent genes involved in plant defense reactions and 13% in signaling processes. The predicted proteins encoded by the ESTs had homologies with disease resistance proteins, stress- and defense-proteins, and components of cell signaling pathways. Using SSH, 28 genes were found to be involved in resistance mechanisms. Expression analysis of a subset of 13 candidate genes revealed some HR-upregulation with homology to proteins, such as chitinases, cytochrome P450, heat shock proteins (HSP), non-race-specific disease resistance (NDR1) protein, ionic channel (CNGC2/DND1) involved in the resistance to *Pseudomonas syringae*, kinases, and transcription factors.

In another transcriptome study using a differential analysis performed by combined cDNA-AFLP and bulk segregant analysis (BSA), coffee resistance gene candidates were identified in incompatible interactions (Diola et al. 2013). F_2 population plants (HdT UFV 427-15 × Catuaí Amarelo IAC 30) were inoculated with *H. vastatrix* race II, and leaves were collected at 48 and 72 hai. From these samples, 108 transcript-derived fragments (TDFs) were differentially expressed in resistant plants and sequenced. Among the sequenced TDFs, 20 and 22% were related to signaling and defense genes, respectively. Twenty-one genes were selected for validation using Quantitative reverse transcription PCR (RT-qPCR) at 0, 12, 24, 48, and 72 hai. The highest levels of transcription of the signaling and defense genes were observed at 24 and 72 hai, respectively. The *NBS-LRR*, *RGH1A*, *MEK*, *MAPK2*, *CDPK*, and *β-Zip* genes had the highest levels of transcript increase in the resistant genotypes in relation to susceptibility. Among the defense genes, a pathogen-related, *PR5*, was found. The results of this study reinforced the hypothesis that the transcription of signaling and defense genes have different time profiles and a greater transcription of signaling genes is capable of upregulating coffee defense genes (Diola et al. 2013).

The first transcriptome study using NGS technology was performed by Fernandez et al. (2012). In this study, data were collected 21 days after-inoculation (dai) with *H. vastatrix*, a stage when a large number of haustoria and hyphae were observed in infected *C. arabica* leaves. This allowed the identification of an exhaustive repertoire of genes expressed during the infection of both the plant (61% of the contigs) and the fungus (30%). Two proteins were identified as potential effectors active in the infection process: a flax rust haustorial expressed secreted protein (HESP) and bean rust transferred protein 1 (RTP1). Regarding the plant, 13 new WRKY transcription factors in coffee where identified. Additionally, contigs responsible for encoding pathogenesis-related (PR) proteins (1,3-b-glucanases, PR1b, PR-5 of the thaumatin-like protein family, and chitinases) were most abundant. The identification of effectors and polymorphisms in these regions has applicability in studies of genetic diversity, which can reveal the mechanisms underlying the dynamics of adaptation of the populations of the pathogen against the resistance of the plant. Additionally, the polymorphisms identified in the effectors could be used as molecular tools for the characterization and diagnosis of *H. vastatrix*.

Florez et al. (2017) performed RNA sequencing (RNAseq) to compare gene expression profiles and obtain a global overview of the transcriptome in both compatible and incompatible interactions of *C. arabica* and *H. vastatrix*. The genotypes Caturra CIFC 19\1 (susceptible) and HdT CIFC 832/1 (resistant) were inoculated with *H. vastatrix* race XXXIII. A database of 43,159 transcripts was obtained using bioinformatics tools. HdT responded to *H. vastatrix* infection with a larger number of upregulated genes than Caturra during the early infection response (12 and 24 hai). These data suggest that the infection of *H. vastatrix* to HdT, one of the most important CLR resistance sources, is related to pre-haustorial resistance. The identified genes were involved in receptor-like kinases, response ion fluxes, production of reactive oxygen species, protein phosphorylation, ethylene biosynthesis, and callose deposition. These genes are closely involved in the recognition of PAMPs and induction of PTI (Boller and Felix 2009). The resistant and susceptible genotypes also showed upregulation of genes associated with programmed cell death at 12 and 24 hai, respectively. According to the authors, this may infer that programmed cell death during the early response is efficient in containing the pathogen. A subset of candidate genes upregulated HdT-exclusive was analyzed by RT-qPCR, and most confirmed the higher expression in HdT at the early stage of infection. These genes include the putative basic helix-loop-helix bHLH DNA-binding superfamily protein, ethylene-responsive transcription factor 1 B, putative disease resistance protein RGA1, putative disease resistance response (dirigent-like protein) family protein, and premnaspiridione oxygenase genes. Interestingly, Capucho et al. (2012) reported a decrease in the resistance of cultivars with introgression of HdT (CIFC 832/1) infected with rust XXXIII. Thus, the results obtained in this work, based on the dynamics between the plant-pathogen interaction, could aid in the elucidation of the supplantation of the plant's resistance to specific races of the fungus and development of new strategies to control CLR.

Also, through the sequence information of a HdTCIFC 832/2 BAC library (Cação et al. 2013; Barka et al. 2020; Almeida et al. 2021) cloned two coffee resistance genes in HdT. They share conserved sequences with others S_H genes and displays a characteristic polymorphic allele conferring different resistance phenotypes. Two resistance gene analogs (RGAs) containing the motif of leucine-rich repeat-like kinase (LRR-RLK) were identified and were highly expressed during both the compatible and incompatible coffee-*H. vastatrix* interactions.

To gain a more comprehensive understanding of the *C. arabica*-CLR interaction, Porto et al. (2019) sequenced the genome of *H. vastatrix* race XXXIII using the Pacific Biosciences PacBio RS II and Illumina HiSeq 2500 platforms and assembled all reads to obtain a high-quality reference genome. The obtained genome comprised 547 Mb, with 13,364 predicted genes that encode 13,034 putative proteins with transcriptomic support. Putative secretome was analyzed with 615 proteins and 111 effector candidates specific to *H. vastatrix* were identified. Of these, 17 were analyzed by RT-qPCR over time during the infection process. During the pre-haustorial phase (24 hai) of an incompatible interaction (HdT CIFC 832/1 inoculated with *H. vastatrix* race XXXIII), five effector genes were significantly induced. This illustrated the probable role of these effectors in the recognition of *C. arabica* resistance genes.

Another nine genes were significantly induced after haustorium formation (48 and 72 hai) in a compatible reaction (Caturra CIFC 19/1 inoculated with race XXXIII). The authors inferred that these effectors were likely to be translocated to host cells via haustories.

Through genetic engineering, Cacas et al. (2011) isolated and validated the coffee *non-race-specific disease resistance NDR1*, previously identified as a participant in the defense mechanism against *H. vastatrix*. The coffee *NDR1* gene was expressed in the Arabidopsis knock-out null mutant ndr1-1. The *ArabidopsisNDR1* ortholog is a well-known master regulator of the hypersensitive response that is dependent on R proteins. Upon challenge with *Pseudomonas syringae*, coffee *NDR1* was able to restore the resistance phenotype in the mutant genetic background. They showed that the *NDR1* is a key regulator initiating hypersensitive signaling pathways and that there is an *NDR1*-dependent defense mechanism conservation between Arabidopsis and coffee plants. Thus, the authors proposed that the coffee *NDR1* gene might be a main target for genetic manipulation of the coffee innate immune system and achieve broad-spectrum resistance to *H. vastatrix* races. Additionally, they also provided a methodology for isolating and validating other genes for resistance to *H. vastatrix*.

Genomic and transcriptomic studies related to CLR have provided tools to accelerate coffee breeding programs. With the reference genomes of C*offea*, as well the pathogen genome, our knowledge of this plant-pathogen interaction will increase, and we will have diverse opportunities to control this disease, such as the use of RNAi for key rust genes or genome editing of susceptible genes in coffee plants (Cui et al. 2020).

4.5.2 Coffee Berry Disease (CBD)

CBD is caused by the hemibiotrophic fungus *Colletotrichum kahawae* Waller and Bridge and can quickly destroy 50–80% of developing berries (6–16 weeks after anthesis) in susceptible arabica cultivars during prolonged wet and cool weather conditions (van der Vossen and Walyaro 2009). The pathogen can infect all organs of the host, but the outbreak of the disease with visible symptoms occurs during the expanding stage of berry development, producing sunken, black, anthracnose-like lesions on the green pulp (Hindorf and Omondi 2011). Although the application of fungicides can provide adequate control, the use of coffee resistant varieties is the most appropriate and sustainable management strategy against this disease. Despite being restricted to Africa, it is a concern for other coffee-producing regions, such as Latin America and Asia, where preventive breeding studies for CBD are in progress (Diniz et al. 2017).

Studies based on inheritance have reported three CBD resistance genes, the dominant *T* and *R* genes and the recessive *k* gene. The *T* gene comes from HdT, the *R* from *C. arabica* (var Rume Sudan), and the *k* from Rume Sudan and *C. arabica* K7 (van der Vossen and Walyaro 1980; Gimase et al. 2020).

Gichuru et al. (2008) characterized the *T* gene (Ck-1) through a genetic mapping approach from segregating populations for resistance to CBD. In that study, two SSRs and eight AFLP markers were strongly linked to the *T* gene. In another study, through a GWAS approach, using an F_2 population from the parents Rume Sudan (resistant) and SL28 (susceptible), two SNPs were associated with resistance conferred by the *R* gene (Gimase et al. 2020). These two associated SNPs, Ck-2 and Ck-3, explained 12.5 and 11% of total phenotypic variation, respectively. Additionally, these SNPs are linked to genes involved in the resistance mechanism of plants against pathogens, such as the response to abscisic acid (ABA) and cell wall metabolism. These three markers, Ck-1, Ck-2, and Ck-3, were validated and used in progenies with introgression of resistance to CBD and displayed their essential role in reducing the time required to develop resistant cultivars (Alkimin et al. 2017; Gimase et al. 2021).

Understanding the molecular basis governing coffee resistance to *C. kahawae* is fundamental to gain insights into the distinctive processes underlying the plant resistance response and aid breeding efforts. Therefore, the expression of two defense-related genes (*RLK* and *PR-10*) was analyzed by RT-qPCR in *C. arabica*-*C. kahawae* compatible and incompatible interactions (Figueiredo et al. 2013). For this, two coffee genotypes, Catimor 88 (resistant) and Caturra (susceptible) were infected by *C. kahawae*, and the samples were collected at 12, 48, and 72 h after inoculation (hai). The expression of the *RLK* gene, which is predicted to encode a receptor-like kinase, increased in the resistant genotype with the highest transcriptional level at 48 h post-inoculation. *PR10*, which appears to be related to the jasmonic acid-dependent resistance pathway and to accumulate in host cells in incompatible interactions, increased during the inoculation time-course in the resistant genotype. Additionally, 10 genes (*S24*, *14-3-3*, *RPL7*, *GAPDH*, *UBQ9*, *VATP16*, *SAND*, *UQCC*, *IDE*, and *β-Tub9*) were selected as reference genes for interaction studies in the pathosystem that allowed the precise quantification of gene expression of resistance to CBD.

In another study, to elucidate the involvement of phytohormone pathways in coffee resistance and susceptibility to *C. kahawae*, Diniz et al. (2017) evaluated the expression by RT-qPCR of salicylic acid, jasmonic acid, and ethylene biosynthesis, reception, and responsive-related genes. Again, the genotypes Catimor 88 and Caturra after *C. kahawae* challenge were used and samples were collected at time points during the infection process based on a cytological analysis of fungal growth and associated host responses (6, 12, 24, 48, and 72 hai). The expression patterns of the salicylic acid pathway-related genes were quite similar for both coffee varieties, suggesting that this hormone is less relevant in this pathosystem. Conversely, some genes related to jasmonic acid pathway, such as *12-oxoplytodienotae reductase 1-like* (*OPR3*) and *pathogenesis-related 10* (*PR10*), showed earlier and greater magnitude in the resistant genotypes. According to the authors, this evidence supported the role of the jasmonic acid pathway activation in defense responses and inhibition of fungal growth. In relation to the genes involved in the ethylene pathway, the stronger activation of the *ethylene-responsive factor 1* (*ERF1*) gene at the beginning of the necrotrophic phase in the susceptible variety suggested the involvement of this pathway in tissue senescence.

4.5.3 Bacterial Halo Blight (BHB)

Bacterial halo blight (BHB), caused by the bacteria *Pseudomonas syringae* pv. *garcae* (Amaral et al. 1956), affects coffee production mainly at high altitudes and in mild temperature regions where cold winds impact the plants, favoring the incidence of the disease (Patrício and de Oliveira 2013). This disease has already been reported in Brazil, Kenya, Ethiopia, Uganda, and China (Ramos and Shavdia 1976; Chen 2002; Zoccoli et al. 2011; Xuehui et al. 2013; Adugna et al. 2012). BHB is also a major problem in nurseries, where density and excessive moisture on the surfaces of the leaves provide an ideal environment for pathogen development. The lack of control, in this case, can result in 100% damage (Zoccoli et al. 2011). Control is achieved using copper and antibiotics, which increases production costs and is frequently inefficient in the field, in addition to its possible environmental impacts (Zambolim et al. 2005; Patrício and de Oliveira 2013). In this limited scenario, cultivars resistant to BHB are a sustainable alternative and can reduce costs.

In a *C. arabica* wild type collection (FAO 1968), substantial genetic variability for BHB resistance was identified. The accessions of this collection have levels of resistance to BHB that vary between highly resistant, resistant, moderately resistant, moderately susceptible, susceptible, and highly susceptible (Mohan et al. 1978). Using this information, GWAS for resistance to BHB identified five SNPs associated with resistance (Ariyoshi et al. 2019). The genes linked to the SNPs identified in this study, *serine/threonine-kinase* and *NB-LRR*, have pathogen-specific recognition functions and trigger defense responses, such as cell death (Zong et al. 2008; Coll et al. 2011). These genomic regions of resistance have a high potential for application in MAS and as a target for gene editing.

A genetic diversity study and pathogenicity test among 25 strains of *P. syringae* pv. *garcae* were performed using enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) and Repetitive element sequence-based-PCR (REP-PCR) techniques. For the pathogenicity tests, the strains were inoculated in the leaves of the susceptible coffee cultivar Mundo Novo (Maciel et al. 2018). In the combined analysis of ERIC- and REP-PCR, the Brazilian strains obtained from 1958 to 1978 grouped separately from the remaining strains and the Kenyan strains grouped separately from the Brazilian strains. Regarding the pathogenicity tests, the strains could be divided into four aggressiveness classes (highly aggressive, aggressive, moderately aggressive, and less aggressive). These results could contribute to the development of rapid and reliable methods for early detection of the pathogen to minimize their negative impacts on coffee production.

4.5.4 Nematodes

Root-knot nematodes (RKN), which belong to the genus *Meloidogyne*, are one of the most limiting factors in coffee cultivation. Because of its wide distribution, combined

with its high reproductive capacity and aggressiveness, this group of nematodes could be responsible for 15% of the total reduction in relation to the 20% of losses caused by all phytonematodes (Amorim et al. 2016). Among the 19 nematode species reported in association with the crop (Aribi et al. 2018; Villain et al. 2018), the most common, harmful, and well-known species are *Meloidogyne exigua, Meloidogyne coffeicola, Meloidogyne incognita*, and *Melodoigyne paranaensis*. Together, *M. incognita* and *M. paranaensis* can destroy up to 80% of the roots of *C. arabica* (Bertrand and Anthony 2008). They are distributed worldwide and are considered the most important parasitic nematodes in coffee (Aribi et al. 2018), causing yield losses of approximately 36% worldwide (Fatobene et al. 2017).

The use of resistant cultivars and clones is considered a key strategy in the management of nematodes in *Coffea*, because once the area is infested, the management of nematode populations can be costly and have low sustainability. Resistance to *M. incognita* has been found in *C. arabica, C. canephora*, and *C. congensis* (Gonçalves et al. 1988, 1996; Sera et al. 2006; Fatobene et al. 2017). However, the sources of resistance to *M. paranaensis* are scarce but have been observed in *C. arabica* and *C. canephora* (Anthony et al. 2003; Sera et al. 2006; Fatobene et al. 2017). Nevertheless, cultivars such as IPR 100 originated by crossing Catuaí and the hybrid Catuaí x BA-10 coffee and IPR 106 (Icatu), demonstrated resistance to *M. incognita* and *M. paranaensis* (Ito et al. 2008; Sera et al. 2017; Shigueoka et al. 2017). Breeding programs are also exploring Ethiopia's wild accessions as a source of resistance to nematodes because of their greater genetic diversity than that of breeding materials (Silvestrini et al. 2007). Resistance to *M. incognita, M. exigua* and *M. paranaensis* (Fatobene et al. 2017; Holderbaum et al. 2020) has been detected in selected accessions.

Sources with high resistance to *M. exigua* have been found in several *Coffea* species, including *C. arabica, C. canephora, C. congensis, C. dewevrei, C. liberica, C. racemosa, C. kapakata, C. eugenioides, C. salvatrix, C. stenophylla*, and *C. sessiliflora* (Curi et al. 1970; Fazuoli and Lordello 1977, 1978; Anthony et al. 2003; Fatobene et al. 2017). Cultivars with an HdT background, such as IAPAR 59 and IAC 125-RN Acauã, MGS, Catiguá, and Paraiso MG H491-1 are highly resistant to *M. exigua* (Muniz et al. 2009; Pereira and Baião 2015). Furthermore, *C. canephora* Apoatã IAC-2258 is recommended as a rootstock and is resistant to the three most important coffee nematodes (Salgado et al. 2005; Sera et al. 2006; Andreazi et al. 2015).

Nematodes use molecular tools that are successful in infection and plant parasitism. They synthesize proteins that make up a complex parasitism methodology, inducing cell wall modifications and even control of host gene expression (Maluf et al. 2008). In this sense, one of the key points in the development of a new resistant cultivar is the selection of R genes capable of overcoming sophisticated nematode parasitism. The search for R genes in coffee pathosystems is one of the priorities of breeding programs. *Mex-1* was the first and only nematode resistance gene identified in *C. arabica*, providing HR-type responses to *Meloidogyne* infection (Noir et al. 2003; Anthony et al. 2005). Additionally, 14 AFLP markers were identified and associated with resistance to *M. exigua*, from which a genetic map of the *Mex-1*

locus was prepared, with markers for the selection of resistant genotypes (Noir et al. 2003). The *Mex-1* locus is located on chromosome 3, which houses the S_H3 gene that confers resistance to coffee rust (Saucet et al. 2016).

Despite reports of sources of resistance to *Meloidogyne*, the transcriptome of these pathosystems with coffee plant interactions has barely been studied. Albuquerque et al. (2017) evaluated the initial expression (4, 5 and 6 days after-inoculation of 88 differentially expressed genes between resistant and susceptible coffee to *M. incognita*. This study reports the overexpression of genes encoding miraculin (defense), *RGLG1* (protein degradation), *SENA* (cell death associated with senescence), *NLR* (immunity signaling), *CaWRK11* (transcription factor), and *OBP* (wall modification). by analyzing *C. arabica* resistance to *M. incognita* and *M. paranaensis*, recent studies from our group suggest that the response is different and may be caused by differences in nematode aggressiveness, which can result in different molecular patterns and effectors secreted by these nematodes. The response of resistant plants is mostly earlier, occurring at 5 dai with *M. incognita*, and the response to *M. paranaensis* was mostly later, at 14 dai, but it was higher than that triggered by *M. incognita.* These studies increase our knowledge of coffee resistance responses to *M. incognita* and *M. paranaensis* and provide new genes to improve plant resistance.

Although some genes have already been shown to participate in the process of coffee resistance to RKN, many other studies must be conducted, because the plant-nematode interaction is complex and encompasses many unknown genes. Genetic transformation of coffee with resistance genes can not only produce resistant cultivars, but also help to understand how the resistance process is developed by plants.

4.5.5 Coffee Leaf Miner (CLM)

Coffee leaf miner (CLM), caused by *Leucoptera coffeella* (Guérin-Mèneville 1842), is considered one of the most important pests of coffee plantations, causing serious economic damage in the main coffee-producing regions. During their life span, the moth lays its eggs in the adaxial part of the leaves. The larvae that hatch form mines in the mesophile during their feeding, which can lead to leaf fall and consequently a reduction of up to 75% in photosynthetic rates, with productivity losses from 30 to 80%, depending on the infestation level (Dantas et al. 2020). The use of insecticides has been widely used to control CLM, however, it is not completely efficient. Chemical control also increases production costs and can affect natural predators of *L. coffeella.*

Traditional breeding for CLM control has been a challenge because there are no sources of resistance in *C. arabica* or *C. canephora.* However, introgression of *C. arabica* with resistant *C. racemosa* originated from the Aramosa hybrids, which have been incorporated in breeding programs to select for CLM resistance. From these hybrids, the cultivar *C. arabica* Siriema has been released to coffee farmers, presenting resistance to CLM (Matiello et al. 2015).

Alternatively, search for R genes in other species could contribute to the genetic improvement of the coffee tree. One of these genes, which is widely known to play an important role against pests in several species, is the *Cry* gene identified in *Bacillus thuringiensis* (Bt). This gene has the potential to protect coffee trees against the miner bug and has already been used in the genetic transformation of *C. arabica* and *C. canephora*. The *cry1Ac* gene encodes proteins CryIA (c), CryIB, and CryIE, whereas *cry10Aa*, present in the *B. thuringiensis* serovar *israelensis* (Bti), translates the protein Cry10Aa, which is highly toxic to insects (Guerreiro Filho et al. 1998; Méndez-López et al. 2003).

In addition to *Cry* genes, Mondego et al. (2005) isolated and characterized genes associated with resistance during the infestation of the miner bug in progeny resulting from a cross between *C. canephora* and *C. racemosa*. Five short repetitions in tandem (STRs) or microsatellites were characterized as a starting point to unravel the mechanisms of resistance to CLM. Additionally, Mondego et al. (2005) reported the participation of the *CoMir* gene, which codes for proteinase-inhibiting proteins and may be involved in the process of resistance to minor bugs.

Through a database of EST sequences, Cardoso et al. (2014) validated 18 genes differentially expressed in resistant and susceptible plants to miner bugs. These authors reported that positively regulated genes include those responsible for defense mechanisms, hypersensitivity responses, and genes involved in cell function and maintenance, suggesting that resistance to miner bugs is not triggered by a larger resistance gene. Additionally, they found that the differential expression between resistant and susceptible genotypes was observed in the absence of miner bugs, indicating that the defense already exists in resistant plants.

In a pioneering study on the genetic transformation of *C. canephora*, Leroy et al. (2000) produced plants genetically transformed with *cry1Ac* genes. More than 50 transformation events were obtained, showing different levels of Cry protein production, as well as tolerance resistance to the miner bug in greenhouse tests (Leroy et al. 2000). In field tests, several events showed stable resistance during 4 years of experimentation, demonstrating the great potential of using *Cry* genes in the control of CLM (Perthuis et al. 2005).

4.5.6 Coffee Berry Borer (CBB)

Among the different pests that can affect coffee production, coffee berry borer (CBB), *Hypothenemus hampei* (Ferrari 1867) (Coleoptera: Curculionidae), is the most important pest for coffee farmers. Both green and ripe berries are susceptible to attack by *H. hampei*. Damaged fruits suffer seed rot, which affects maturation and early fall on the ground as well. Additionally, there is a loss of quality with depreciation of the product because the number of coffee grains damaged by CBB is considered a defect for coffee quality. The insect feeds and reproduces within the coffee seeds inside the coffee berry, which makes control by the application of insecticides inefficient. For several years, farmers have used organochlorides to control

CBB, but because of safety, health, and environmental reasons, this practice is now forbidden in the main coffee-producing regions. Strategies of biocontrol, such as the use of the parasitoids C*ephalonomia stephanoderis*, *Phymastichus coffea*, and *Prorops nasuta*, did not produce acceptable control of CBB. Biological control of CBB using the fungus *Beauveria bassiana* (Balsamo) has been effective using natural and industrial conidial suspensions, which are sprayed on the fruits. However, the efficiency of *B. bassiana* in controlling CBB depends on environmental conditions, such as high humidity during application. Furthermore, *B. bassiana* CBB control is still hampered by the slow infection process that allows the adult insects to live for enough time to damage the coffee berry, and by the challenges and costs for conidial industrial-scale production (Infante 2018).

Genetic resistance to CBB is not easily available in the coffee gene pool or is difficult to incorporate using conventional breeding. Neither *C. arabica* nor *C. canephora* exhibited natural resistance to *H. hampei*. Despite some reports on CBB resistance in *C. eugenioides* and *C. kapakata* (Sera et al. 2010), the differences in ploidy levels between the allotetraploid *C. arabica* and other diploid *Coffea* species, as well as their incompatibility, are also major limitations associated with conventional coffee breeding for CBB. Therefore, genetic engineering is one of the main strategies employed to transfer CBB resistance to *Coffea.*

De Guglielmo-Cróquer et al. (2010) transformed *C. arabica* (Catimor) by introducing the *cry1Ac* gene again through microjet bombardment methodology (biobalistics). The presence of the gene has been reported in regenerated explants, without the need for selection genes and reporters. Of the 60 regenerated explants, seven had the *cry1Ac* gene in their genome, as confirmed by PCR. However, subsequent studies to verify the performance of the transgenes have not been conducted at the field level.

In the search for alternatives to CBB control using *cry* genes, the high toxicity of *B. thuringiensis* serovar *israelensis* against first instar larvae of CBB has been demonstrated. Bioassays against first-instar *H. hampei* from *cry10Aa* and *cyt1Aa* provided estimated larval mortality rates of 20% and 50%, respectively. Additionally, a combination of *cry10Aa* and *cyt1Aa* resulted in 100% larval mortality. Therefore, the development of coffee varieties resistant to CBB using transgenic technology with these genes may represent an alternative to manage this important pest (Villalta-Villalobos et al. 2016). More recent work by Valencia-Lozano et al. (2019) developed a protocol for obtaining transgenic *C. arabica* plants using biobalistics. The authors introduced the *Cry10Aa* gene into the coffee genome, which presents CBB toxicity, and regenerated explants expressed the protein in a stable manner.

The first characteristic of agronomic importance in the transformation of *C. canephora* coffee trees (clone 126) was the introduction of the *cry1Ac* gene, which is responsible for the production of CryIA (c), CryIB, and CryIE, which are toxic to the CLM (Leroy et al. 2000). Despite the plants being produced for CLM resistance, these plants could also be tested for their potential toxicity against CBB and to stack resistance genes in *Coffea.*

Transgenic genotypes of *C. arabica* (Catuaí Vermelho) were also produced to develop a CBB-resistant cultivar. The *αAI-1* and *αAI-Pc* genes, originating from beans

and responsible for inhibiting α-amylases, enzymes important in starch degradation and digestion in *H. hampei*, were introduced into the coffee tree using a biobalistic methodology. The assays generated 15 transformed plants, of which six genotypes presented a single copy of the *αAI-1* gene and a seventh genotype presented two copies (Barbosa et al. 2010). Despite the potential for plant protection, subsequent experiments to ascertain the potential of genotypes in the interaction with *H. hampeii* in the field have not been conducted.

In contrast, the CBB transcriptome was performed at three different stages of the development of *H. hampei* infesting coffee (Noriega et al. 2019). They identified genes that encode cuticular proteins (CPs) and enzymes involved in cuticle metabolism, which play important roles in physiological processes, such as protection from insecticide penetration, physical injuries, pathogens, and dehydration (Willis 2010). Additionally, genes encoding OBPs, which are proteins with chemosensory functions, play key roles in the copulation and reproduction of the species. The genes encoding CPs and OBPs are potential candidates for the development of silencing methodologies for gene expression based on RNAi (Antony et al. 2018).

The genome of *H. hampei* has been sequenced (Vega et al. 2015). The *Rdl* gene was identified as conferring resistance to insecticides of the two cyclodiene classes, as well as the genes of the carboxyl esterase family (CE), previously identified as being responsible for promoting resistance to other species of insects (*Drosophila melanogaster*, *Aedes aegypti*, *Anopheles gambiae*, and *Tetranychus urticae*) to insecticides of the class of two organophosphates, as well as genes of the *CYP* and *GST* families (Ranson et al. 2002; Claudianos et al. 2006; Strode et al. 2008; Grbić et al. 2011; Oakeshott 2003). Other genes related to antimicrobial activity were also identified. This information can contribute to the development of management strategies associated with chemical and biological control.

4.6 Final Remarks

In any crop plantation, biotic stresses will always need attention because of the natural co-evolution between the host and pathogens. The surge of new pathogen races, as well as the development of insect resistance to pesticides will always require the dedication of coffee farmers and researchers. Preventive breeding using modern genetic tools can help produce cultivars with multiple pathogen resistance. Therefore, the need to conserve and explore the coffee genetic pool is important to increase the necessary arsenal to combat diseases and pests. Molecular tools can rapidly characterize new R genes and identify markers for quick incorporation into elite cultivars by breeders. Extensive use of MAS or genome editing to shutdown susceptible genes in coffee plants can lead to faster development of new cultivars to better handle biotic stresses. These improved cultivars are fundamental to avoiding outbreaks, decreasing the use of pesticides, and helping farmers in sustainable coffee production.

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Chapter 5
Disease Resistance in Cotton

Jinfa Zhang and N. Manikanda Boopathi

Abstract Though cotton has been adapted to subtropical and tropical regions of the world as a long season crop, it experiences a large array of diseases triggered by parasitic nematodes, fungi, bacteria and virus. Such diseases have severe impact on cotton growth and development starting from seed germination, plant growth and reproduction which ultimately lead to significant losses in fiber yield and quality. In this review chapter, the top most major diseases of cotton viz., bacterial blight, Fusarium wilt and Verticillium wilt are elaborated. Information on other cotton diseases also is referred to wherever appropriate. The objective of this chapter is to provide a comprehensive synthesis on the research progress made in the area of breeding, genetics, mapping of resistance genes or quantitative trait loci (QTLs) and marker-assisted selection for disease resistance in cotton. Considerable progress has been made in the resistance aspects of cotton's tissue structure, physiological and biochemical features, R-gene-mediated and hormone-mediated resistance through different signal pathways and their interactions. Availability of cotton whole-genome sequences and high-throughput molecular markers, offer new avenues in precisely pinpoint the site of resistant genes. As the developments in genetically modified cotton breeding germplasm is slow and problems associated to evolve a cultivar that adapts to different ecological environments still persists, modern genomics tools need to be integrated with traditional breeding methods to accelerate the process of disease resistance cotton breeding in the future.

Keywords Cotton · Diseases · Molecular breeding · Resistance sources · Marker-assisted selection

J. Zhang
Department of Plant and Environmental Sciences, New Mexico State University, Las Cruces, NM 80003, USA
e-mail: jinzhang@nmsu.edu

N. Manikanda Boopathi (✉)
Department of Plant Biotechnology, CPMB&B, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu 641003, India
e-mail: nmboopathi@tnau.ac.in

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Abbreviations

AM	Association Mapping
Avr	Avirulence Gene
BB	Bacterial Blight
BC	Back Cross
BG	Bollgard
BILs	Backcross Inbred Lines
cM	CentiMorgan
CRICPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-Associated Protein 9
CSSLs	Chromosome Segment Substitution Lines
FCF	Fungus Culture Filtrate
FOV	*Fusarium oxysporum* f. sp. *Vasinfectum*
FW	Fusarium wilt
GBS	Genotyping-By-Sequencing
GWAS	Genome-Wide Association Study
LG	Linkage Group
MAGIC	Multiparent Advanced Generation Intercross
MAS	Marker-Assisted Selection
QTL	Quantitative Trait Loci
RFLP	Restriction Fragment Length Polymorphism
RILs	Recombinant Inbred Lines
RKN	Root Knot Nematode
RR	Roundup-Ready
SNPs	Single Nucleotide Polymorphisms
SSR	Simple Sequence Repeat
VIGS	Virus Induced Gene Silencing
VW	Verticillium Wilt
Xcm	*Xanthomonas citri* pv. *malvacearum*
ZFN	Zinc Finger Nuclease

5.1 Introduction

Cotton (*Gossypium* spp.), an important economic fiber and oilseed crop with a production value of US$50 billion, is being cultivated on 2.5% of the world's arable land that spreads in more than 80 countries (http://cottonanalytics.com/cotton-in-the-world-economy/). It has been estimated that nearly two third of the global cotton production is chiefly contributed by India, China and the United States though Brazil, Pakistan, Uzbekistan, Turkey, Greece, Mexico and Argentina also contributing in significant level (https://www.statista.com/statistics/263055/cotton-production-worldwide-by-top-countries/). *Gossypium* genus has so far described with 45 diploid ($2n = 2x = 26$) and seven allotetraploid species ($2n = 4x = 52$) (Wendel and Cronn

2003). With respect to the pairing affinities of chromosomes, all the diploid species are grouped into eight genome groups viz., A to G and K (Stewart 1994).

Among the 49 *Gossypium* spp. the cultivated species are two Asiatic diploids (*G. herbaceum*, A1 and *G. arboreum*, A2 genome; $2n = 2x = 26$) and two tetraploids (*G. hirsutum*, AD1 and *G. barbadense*, AD2 genome; $2n = 4x = 52$). However, *G. hirsutum*, often referred as Upland cotton (originated in Mexico), occupies more than 95% of the global cotton production and *G. barbadense*, often called as Pima, Egyptian, or Sea-island cotton (originated in Peru) accounts for the remaining 5% of the world's raw cotton production. Asiatic cottons are cultivated on certain pockets of India and Pakistan.

It has been theorized that tetraploid cotton, including Upland and Pima, might have originated from a natural hybridization that occurred few million years ago between an Asiatic cotton and a wild D-genome species such as *G. raimondii* (Wendel and Cronn 2003). Consequently, all the seven tetraploid cottons are cross compatible and they form the primary gene pool. Species from the genomes such as A, B (dispersed in Africa) and F (found in Arabia) as well as D (distributed in the America continent) constitutes the secondary gene pool, as genes from these genomes can be transferred to the tetraploid in interspecific hybridization. On the other hand, perfect hybridization between the A and D subgenomes as well as among tertiary gene pools (such as the C (dispersed in Australia), E (spread in Arabia), G (distributed in Australia), and K (disseminated in Australia) genomes) have been found to be very poor (Stewart 1994).

Owing to its long season nature in subtropical and tropical climates, cotton is vulnerable to many diseases caused by nematodes, fungi, bacteria and virus. Cotton diseases are widespread and they limit seed germination, plant growth and reproduction and ultimately results into severe losses in final fiber yield and quality. For example, the estimated cotton yield losses due to diseases in the US alone were 11.8–12.6% in 2016–2017; even higher percentages of yield losses were noticed in some of the other states. Among the diseases, nematode complex (root knot—*Meloidogyne* spp. and reniform—*Rotylenchulus reniformis* and other nematodes) triggered the highest yield loss at estimated 4.3–4.7%, followed by boll rot (caused by pathogens such as *Rhizopus* spp.), seedling diseases (owing to complex fungal pathogens such as *Rhizoctonia solani, Pythium* spp., *Fusarium* spp. and bacterial pathogens) and Fusarium (caused by *Fusarium oxysporum* f. sp. *vasinfectum*, FOV) and Verticillium (caused by *Verticillium dahliae*) wilt. In addition to these diseases, when favourable weather conditions prevail, diseases such as root rot (*Phymatotrichopsis omnivora*), Ascochyta blight (*Ascochyta*gossypii) and leaf spots (*Alternaria* spp.) also infect cotton plants. Though cotton leaf curl virus emerges as a disease in certain pockets of Asian countries, it has been rarely reported in other cotton growing areas.

Cotton diseases are controlled by employing chemical, biological and cultural practices and the use of resistant cultivars. Among them, evolving and utilizing disease resistant cultivars is considered as affordable and efficient strategy of controlling cotton diseases. Conventional breeding for genetic improvement of cotton with improved disease resistance has been reported as early as 1910s; however, resistant

cultivars for bacterial blight was reported during 1930s in Sudan, and later in USA. Subsequently, several resistant cultivars were developed and simple management strategies were promoted (such as acid-delinting) to control this disease.

In order to combat the worldwide fibre damage caused by Fusarium and Verticillium wilt, attention was focused to evolve novel cotton cultivars with improved resistance to these wilts in 1950s. Similarly, as the incidence of root-knot nematode gradually increased in 1990s, genetic and breeding efforts on root-knot nematode resistance have been taken in USA. Such efforts were subsequently helped to identify two major resistance genes and they were used in commercial conventional and transgenic breeding programs in recent years.

This chapter highlights the progress made in the lines of genetics, breeding, mapping of resistance genes or quantitative trait loci (QTLs) and marker-assisted selection in three major cotton diseases viz., bacterial blight, Fusarium wilt and Verticillium wilt. Information on other cotton diseases have also been provided but with little details as the information are scarce on those diseases.

5.2 Bacterial Blight

5.2.1 Causal Agent and Significance

Almost all the cotton growing regions in the world are affected by Bacterial blight (BB) caused by *Xanthomonas citri* pv. *malvacearum* (*Xcm*), a gram-negative bacterium. All the aerial parts of the cotton, throughout its growth phases are vulnerable to *Xcm* infections. Water-soaked and angular-shaped lesions develops on the leaves and other vegetative parts of cotton and the nearby lesions amalgamate into bigger block spot which subsequently result into defoliation in susceptible plants (Fig. 5.1). *Xcm* infection has also been found in immature bolls, which eventually lead to boll rot (Fig. 5.1). A simple, effective and affordable management strategy of BB is use of resistant cultivars and planting with acid delinted cotton seeds. Using 11

Fig. 5.1 Symptoms of bacterial blight in cotton **a** Angular leaf spot, **b** Vein necrosis and **c** Boll rot

host differential cotton accessions, 19 *Xcm* races has been so far recognized (Hillocks 1992; Delannoy et al. 2005) and among them, race 18 is the most virulent in the USA and elsewhere in the world where cotton is cultivated (Allen and West 1991; Zhang et al. 2020b).

5.2.2 Resistant Germplasm

Cotton germplasm can be screened for BB resistance using several kinds of inoculation and evaluation methods (Thaxton and El-Zik 1993). In general, such methods should allow BB pathogen to enter into the leaves through open stomata or wounds to initiate infection (Delannoy et al. 2005). To this end, a low-pressure sprayer with organosilicone non-ion surfactants (Wheeler et al. 2007) or scratching (wounding) the lower surface of cotyledons/leaves with a toothpick (Bird 1982) or a combination of both wounding and spraying (Bourland 2018) are frequently used. Such artificial inoculations helped to evaluate in large scale cotton accessions with less labour and time. Alternatively, under laboratory conditions, syringe or vacuum infiltration have also been attempted to infect cotton seedlings with BB pathogen (Cox et al. 2019). Irrespective of the methods, the main factors that limit the development of the BB symptoms are temperature and humidity (Wheeler et al. 2007; Jalloul et al. 2015; Cox et al. 2019).

It has been shown that quantitative resistance as well as various levels of qualitative resistance mediated by major resistance genes are hard to evaluate using toothpick scratching method. Actually, during initial studies, several numbers of major BB resistance genes were identified by employing sprayer inoculation (Zhang et al. 2020b). Though variation in BB resistance level have been documented within the genus *Gossypium*, it has been invariably found that cultivars of the diploids (such as *G. arboreum* and *G. herbaceum*) and the wild diploid (e.g., *G. anomalum*) have found to be highly resistant or immune to BB (Hunter et al. 1968; Wallace and El-Zik 1989; Hillocks 1992; Zhang et al. 2020a, b, c, d, e, f). On the other hand, tetraploids (such as *G. hirsutum*) have exhibited large variations in responses from extremely susceptible to highly resistance to BB. Nevertheless, another tetraploid species, *G. barbadense*, has always displayed susceptibility to BB (Delannoy et al. 2005; Jallou 2015), including the current commercial cultivars. Screening the cotton pre-breeding materials for BB resistance is a routine process in USA (for example, University of Arkansas regularly conducts screening program for upland and elite public breeding lines for BB resistance (Bourland 2018); Similarly, in the Texas High Plains there is an annual cotton screening program to *Xcm* race 18 under field conditions (Wheeler and Dever 2020); at New Mexico, Jinfa Zhang established a genetic and genomic research program for BB resistance. In a massive screening program, Hanan et al. evaluated more than 330 obsolete upland cotton cultivars and germplasm lines and identified several BB resistant lines. Such efforts have clearly demonstrated that resistance to *Xcm* race 18 is qualitative and can be easily fixed in breeding without a selection pressure. Further, pedigree investigation indicated that there could be

different sources of BB resistance, which can be used as potential donors in cotton breeding program to improve BB resistance.

5.2.3 Genetics of Resistance

In response to tireless efforts made in Sudan during 1930s and 1950s, nearly 10 BB resistance genes were identified (including B_1, B_2, and B_7 from Upland, B_2, B_3 and B_{10K} from *G. hirsutum* race *punctatum*, B_4 and B_6 from *G. arboreum*, B_5 from a perennial *G. barbadense*, recessive gene b_8 from *G. anomalum*, and B_{9K} from *G. herbaceum*) and were introgressed into Egyptian (*G. barbadense* L.) Sakel and other commercial Upland cotton cultivars that were cultivated in Sudan (Knight and Clouston 1939; Knight 1963).

In addition to these genes, several other BB resistance genes such as B_{9L} and B_{10L} from Upland cotton, B_{11} and another unnamed resistance gene from *G. herbaceum* which was grown in Africa, were also reported. On the other hand, such BB resistance genes were scouted only from upland cotton in USA during 1950s and 1960s and those studies were lead to identify major BB resistance genes such as B_7, B_{12}, B_{In}, B_N and B_S; besides polygene modifiers or complexes have also been reported from upland cotton (Zhang et al. 2020a, b, c, d, e, f). Though there were numerous unnamed BB resistance genes, their allelic relationships have not yet been clearly unraveled.

Bachelier et al. (1992) reported nil reciprocal effects in BB resistance as tetraploid upland cotton share a cytoplasm as that of its cytoplasm donors, *G. herbaceum* and *G. arboreum.* Nevertheless, exotic cytoplasm introgressed from distant *Gossypium* species will have its impact on cotton growth as well as responses to abiotic and biotic stresses. Despite of this fact, it has been noticed that nearly 12% of increased BB resistance in upland cotton when the *G. harknessii* cytoplasm was transferred (Mahill and Davis 1978).

The genetic basis of BB resistance was quantified using disease resistance rating scale by employing quantitative genetic designs such as F_2 and parents, generation-mean analysis (with parents and their F_1, F_2, and/or F_3, BC_1P_1 (i.e., $F_1 \times P_1$) and BC_1P_2 (i.e., $F_1 \times P_2$)) and diallel analysis (with F_1 and/or F_2) that were exposed to infections with *Xcm* 1, 2, 4, 7, 18 or 20, or a mixture of two or more races (Zhang et al. 2020a, b, c, d, e, f). It has been invariably found that resistant parents were harbouring one or more major B genes, whereas susceptible parents did not harbour such genes. Further, the heritability estimates of these studies were moderate to high (0.45–0.97; depending on crosses and studies). In general, additive effects were predominant; however, dominant effects were also reported. Consequent to the existence of residual heterozygosity in certain parents and non-uniform distribution of *Xcm*, heritability estimates of as low as 0.24 was reported for BB resistance under field conditions (Bachelier et al. 1992).

5.2.4 Resistance Breeding

Pyramiding of several BB resistance genes into the commercial *G. hirsutum* and *G. barbadense* were taken up during 1930–1950s in Sudan by Knight and his coworkers (Innes 1961, 1963, 1965, 1974, 1983; Zhang et al. 2020a, b, c, d, e, f). Similarly, during 1950–1970s, American cotton breeders, more specifically, scientists from Texas and New Mexico, have involved in evolving elite cultivars carrying one or more BB resistance genes and such cultivars have developed little or no disease symptoms during *Xcm* incidences. Thus, employing resistant cultivars with known BB resistance genes (such as BB resistant upland cultivars with the TAMCOT prefix and germplasm lines that were released from Texas A&M University (Bird 1976, 1979, 1982, 1986; El-Zik and Thaxton 1995, 1996, 1997; Thaxton and El-Zik 2004) and planting of acid delinted seeds were shown to be effective to control BB incidence during 1970s.

In the same way, during 1950s, cotton breeding for BB resistance were taken up at New Mexico State University (Zhang 2018a) and seven BB resistant cultivars (Acala 1517BR, Acala 1517BR-1, Acala 1517BR-2, Acala 1517-70, Acala 1517-77BR, Acala 1517-88, Acala 1517-95 and Acala 1517-99) were developed. The alphabets "BR" in the variety name indicates BB resistance). Both the cultivars, Acala 1517BR and its derivative Acala 1517BR-1, received their BB resistance genes from the donor parent Stoneville 20 (carrying B_7); however, they were resistant to BB race 1 but susceptible to race 2. On the other hand, Acala 1517BR-2 was resistant to both races and the resistance gene(s) to race 2 was/were donated by NM8738, which has Arizona Long Staple 120 (*G. barbadense*) genome in its background.

Acala 9136 was evolved from *G. barbadense* cv. Tanguis and it was used to evolve Acala 1517-70, which was resistant to races 1, 2, and 10. Interestingly, single plant selection in Acala 1517-70 have served to evolve several BB resistant lines. For example, Acala 1517-77BR and its cross-breeding line Acala 1517-88 were resistant to BB races 1, 2, and 10. It is also worth to mention that Acala 1517-95 was resistant to BB races 2 and 10 and Acala 1517-99 was resistant to race 1, 2, and 10, both of them have evolved their resistance from Acala 9136.

Owing to the widespread cultivation of second generation transgenic cotton since 2010, resurgence of BB was noticed in the US cotton belt. In order to combat this issue, the first generation of transgenic upland cotton cultivars with Bollgard (BG) and/or Roundup-Ready (RR) traits (for insect and herbicide resistance, respectively) were developed by backcrossing with BB-resistant conventional cultivars as recurrent parents. Despite of this effort, the disease turns out to be an issue, when replacing BG and RR cultivars with Bollgard II (BGII) and/or Roundup-Flex (RF) cultivars during 2007–2010 (Wheeler 2018). Consequently, BB triggered substantial losses in the southern US Cotton Belt (prominently in Arkansas and Mississippi, where Deltapine and Stoneville cultivars that were susceptible to BB were widely grown (Phillips et al. 2017). Therefore, almost all transgenic commercial cotton seed companies have recently designed their breeding program to incorporate BB resistance as

an important trait (Wheeler 2020). It has been reported that field screening with artificial inoculations has indicated 50–79% of the transgenic lines were BB resistant, depending on seed companies (Wheeler and Dever 2020). It has also been confirmed that 21 of 62 commercial upland cultivars from five seed companies, 11 of 150 elite breeding lines from New Mexico State University and 22 of 66 superior breeding lines from public cotton breeding programs have shown to contain different levels of BB resistance.

5.2.5 *Molecular Mapping of BB Resistance Genes*

The first report on quantitative trait locus (QTL) mapping of BB resistant genes B_2, B_3, b_6 and B_{12} was with restriction fragment length polymorphism (RFLP) markers using four F_2 populations derived by crossing susceptible *G. barbadense* Pima S-7 with four resistant upland lines: Empire B2, Empire B3, and Empire B2b6 and S295 (Wright et al. 1998). Totally seven QTLs were identified: QTLs corresponding to B_2 and B_3 on (chromosome) c20, B_{12} on chromosome c14, and four additional QTLs corresponding to the recessive b_6 alleles (b_{6a} on linkage group (LG) D02 (formerly LGU01), b_{6b} on c5, b_{6c} on c20, and b_{6d} on c14). It was indicated that the genomic region near the RFLP marker, pAR1-28 on c5, was mapped to homoeologous region, B_2 on c20. It was also speculated that this regions may be correspond to the BB resistance gene B_4 (that was identified in the diploid A genome species, *G. arboreum*) and assigned to c5 using cytological stocks.

A genome-wide association analysis using 330 US upland germplasm accessions and 26,345 single nucleotide polymorphisms (SNPs) derived from CottonSNP63K array was used to identify SNPs associated with BB race 18 resistance (Elassbli et al. 2021a, b). Among them, c5, c14 and c24 had the highest number of SNPs associated with BB resistance and those QTLs on c5, c14, and c20 were likely to be the QTLs reported by Wright et al. (1998). Thus, such studies have confirmed the consistent QTLs linked to the BB resistance in cotton germplasm.

Though it was originally hypothesized that CS50, an Australian resistant upland cultivar, possessed the $B_2B_3B_7$ and B_{Sm} genes on c20, later it was shown by making interspecific cross with susceptible Pima S-7 that the BB resistance was due to single dominant locus. It was also observed that such resistance locus was co-segregated with a RFLP marker on c14, which was associated with B_{12}, an African cotton cultivar's gene that confirms broad-spectrum resistance (Rungis et al. 2002).

Structural position and validation of B_{12} on c14 was also demonstrated by developing intraspecific upland cross consisting 285 $F_{4:5}$ families derived from Australian Delta Opal (which was resistant to BB race 18) and susceptible DP 388 (Xiao et al. 2010). Such effort has identified closely linked simple sequence repeat (SSR) markers (viz., CIR246, BNL1403, BNL3545, and BNL 3644), which had 5.6 cM distance to the resistance gene, B_{12}. Subsequently, this genomic region was further fine mapped with SNP markers (viz., NG0207069, NG0207155, NG0210142, and NG0207159) which had 3.4 cM to B_{12}.

Another study in Brazil has employed bulk segregant analysis using 127 F_2 population obtained from Delta Opal × BRAS ITA 90 and revealed that 80-bp SSR marker, amplified by BNL 2643, was linked to BB resistance in Delta Opal (Marangoni et al. 2013). Similarly, Silva et al. (2014) demonstrated that a 146-bp SSR marker amplified by CIR246 was associated with B_{12} in S295 and Delta Opal; besides it was also shown to be linked with other genes such as B_2B_3 (in 101-102B) and $B_{9L}B_{10L}$ (in Guazuncho-2). Accordingly, it can be concluded that B_{12} in S295 may be intimately associated with B_2B_3 locus, which in turn might be homologous to or co-segregates with $B_{9L}B_{10L}$. Therefore it was resolved that though CIR246 may be useful to identify resistance to BB race 18, but it cannot be used to differentiate gene(s) within the same chromosomal region that confirms BB resistance.

In an attempt to identify the candidate genes around the B_{12} region, Yang et al. (2015) described a 354-kb region containing 73 putative plant disease resistance genes by employing an interspecific F_2 population derived from S295 × Pima S-7 and the genome sequence of *G. raimondii*. Another similar attempt has delineated B_{12} gene and described few putative disease resistance genes using 550 multi-parent advanced generation intercross (MAGIC) lines and 500,000+ genotyping-by-sequencing based SNPs (Thyssen et al. 2019; Zhang et al. 2020a, b, c, d, e, f).

5.2.6 Marker-Assisted Selection (MAS)

As outlined above, CIR246, a breeder friendly SSR marker, reported by Xiao et al. (2010) has found to be helpful in cotton molecular breeding to evolve BB resistance cultivars using MAS. Xiao et al. (2010) have clearly showed that among the three alleles (146, 156, and 166-bp) amplified by CIR246, the cotton lines with the homozygous 146-bp alleles were found to be resistant; cotton genotypes with heterozygous alleles, 146-bp and 156-bp or 146-bp and 166-bp were found to be segregating for BB resistance; and homozygous cotton accessions with 156-bp allele (including Acala Maxxa) or 166-bp allele or heterozygous 156-bp and 166-bp alleles were found to be susceptible. It has also been demonstrated that the above resistant 146-bp allele was consistent with the SNP haplotype (ACTT) in the nine resistant lines. On the other hand, the susceptible allele, 155-bp or 165-bp, was consistent with the SNP haplotype (GGCA) in the nine susceptible lines.

Consistent of this SSR marker for its association with BB resistance has also further confirmed by Silva et al. (2014): CIR246 amplified the 146-bp allele in BB race 18 resistant lines S295 (carrying B_{12}), Delta Opal (carrying B_{12}), 101-102B (carrying $B_2B_3B_{sm}$) and Guazuncho-2 (carrying $B_{9L}B_{10L}$); alternatively 156-bp allele was amplified in BB race 18 susceptible lines Memane B1 (carrying B_2B_{sm}) and ST 2B-S9 (carrying B_{sm}). Similarly, 166-bp allele was amplified in susceptible Acala 44, deficient of known *B* genes. It was hypothesized that owing to outcrossing over generations and artificial development of advanced breeding lines as well as commercial cultivars, those alleles were no longer be homozygous for resistance.

Use of SSR marker CIR 246 (1.8 cM from B_{12}) and SNP marker NG0207155 (0.6 cM from B_{12}) for screening three Tanzania and four Brazilian Upland cultivars has revealed a differential allele frequency and resistance and showed that the cultivars were not homozygous at B_{12} (Faustine et al. 2015). Presence of 8–15% of water-soaked symptoms in the seedlings of resistant cultivars (such as PHY 375WRF, FM 1830GLT and FM 2484B2F) has also been described under greenhouse screening conditions. Thus, a vigilant inference on use of markers in evolving BB resistant cotton cultivars is also warranted while doing the MAS.

5.3 Verticillium Wilt

5.3.1 Causal Agent and Significance

A soil born fungal pathogen, *Verticillium dahliae* Kleb., causes a serious disease called Verticillium wilt (VW) in several cotton production countries and resulted drastic yield reduction. The first report on VW was noticed in Virginia during 1914 (Carpenter 1914) and now it is being reported in almost all the global cotton cultivating zones. With respect to its impact on defoliation, the strains of *V. dahliae* is grouped as two pathotypes: defoliating and non-defoliating (Schnathorst and Mathre 1966). It has also been grouped in to three categories with respect to vegetative compatibility based on complementation with auxotrophic nitrate non-utilizing (*nit*) mutants: VCG 1, 2 and 4 (Daayf et al. 1995). Besides, few reports such as Hu et al. (2015) have categorized it as race 1 and 2 based on its virulence. It is also interesting to note that VCG 1 found to be defoliating pathotype and race 2; on the other hand, VCG 2 and VCG 4 are non-defoliating pathotypes and race 1 (Daayf et al. 1995; Hu et al. 2015).

Primarily VW disease happens during the vegetative stage of the cotton as microsclerotia of *V. dahliae* invade the roots profusely and dwell in root vascular system (Fig. 5.2). Such invasion in root system seriously upset the uptake of water and nutrients from the soil, which ultimately lead to xylem discoloration, leaf necrosis, chlorosis and shedding, plant wilting and stunted growth and finally death of the plant. Therefore, there would be a significant fiber yield loss. It has been reported that cotton yield reduction to the tune of 0.5–3.5% in USA (Blasingame and Patel 2013) and 7.9% in other countries (Karademir et al. 2012; Hu et al. 2015) was due to VW. If VW develops during reproductive and boll setting stage, the fibre quality traits such as 50% span length and micronaire were found to be largely affected (Bell 1992, 2001; Zhang et al. 2012).

In order to control VW, several tested strategies such as crop rotation, judicial application of fungicides and use of endophytic and arbuscular mycorrhizal fungi have been proposed. However, evolving VW resistant cotton cultivars continues to be the most efficient and affordable method.

Fig. 5.2 Symptoms of Verticillium Wilt in cotton **a** Interveinal chlorosis and necrosis, **b** Dark brown streaks in the stem

5.3.2 Screening Techniques and Resistance Sources

In general, the cotton field that have shown severe VW symptoms can be selected for screening of newly evolved cotton lines as they serve as natural reservoir of the VW pathogen, *V. dahliae*. Indeed, such strategy has long been used to identify and release VW resistant cultivars in USA (Bell 1992).

Another strategy would be to successive cultivation of VW susceptible cultivars in the field that has been ear marked for VW disease resistance screening. By doing so, the field will be enriched with the *V. dahliae* inoculum; besides the field inoculum can also be supplemented with artificial inoculation or applications of crop residues and gin trash from heavily infected cotton plants. It has also been suggested that addition of excess nitrogen fertilizer with more irrigation water fasten the occurrence of VW incidence.

Bell (1992) opined that VW develops effortlessly when the boll setting stage coincide with mid-summer as there would be increased soil temperature that favours disease development. Therefore, scoring of VW disease severity would be better, if it collected during boll development stage. It should also be noted that short season or early-maturing cotton genotypes which has the capacity to produce heavy bolls during summer may show increased susceptibility when compared with late-maturing cotton genotypes (as they have more vegetative growth and less fruiting during this season). Consequently, when VW resistance rating is practiced at boll harvest stage, there would be a confuse in separating the pre-mature boll opening due to VW infection and early maturation of bolls in those short duration cotton genotypes. Hence, it would be better to avoid screening the cotton genotypes for VW resistance when the plants are in maturity phase (Bell 1992).

Usually, field VW resistance in cotton is decided by several factors including resistance genes, environmental features, inoculum density and host–pathogen interactions. Invariably, it has been realized that difficulty in maintaining high and uniform

level of pathogen inoculum in the field greatly limits the reliable screening for disease resistance. Further, changes in environmental factors and weather across the cotton growing seasons and locations affects VW screening procedure. In addition, genotype responses to the VW infection also varies. For example, individuals of the given genotypes, including susceptible cotton accessions, may produce different morphological features ranging from no symptom to severe disease symptoms. Such variations have also been reported even after artificial inoculation of *V. dahliae.* Consequently, it is suggested that field screening of VW resistance may be carried out on case by case i.e., on a population basis.

As expected, irrespective of cotton genotypes or pathogen strains, all the genetic evaluations reports indicated the quantitative inheritance of VW resistance. Thus, it is essential to test the cotton accessions, that were selected for field VW resistance, under replicated field trials and confirm its VW resistance across the seasons and locations. Therefore, it would be desirable to screen multiple entries of the genotype of the germplasm under field conditions across the season and locations with two or more replications in order to reduce the experimental error. Such effort would increase the reliability of process that favours the selection of germplasm line with sufficient VW resistance under field conditions. Besides, this will also facilitate the cataloguing of the germplasm lines according to their response to VW.

On the other hand, VW resistance screening under field conditions possesses the following limitations: There would be different races of the pathogen with changing distribution pattern, which make the field extremely heterogenous. As larger area is required to screen large number of cotton accessions with minimum two replications at boll development stage or maturity (over a period of five to six months), the field heterogeneity further increases. Requirement of large field area also generate additional challenges during crop, soil, and irrigation management practices, which may ultimately affect the reliability of VW resistance screening procedure. To circumvent these issues, artificial inoculation of *V. dahliae* under controlled conditions (such as greenhouses) have been proposed for screening VW resistance in cotton.

Artificial screening under greenhouse conditions involves inoculation of cotton roots (when the plants are in two to four leaf stage) with *V. dahliae* by employing root-dipping or root-ball techniques (Bell 1992). Cotton can also be grown in bottomless plastic or paper pots on a soil bed and roots are wounded when they are in young seedlings stage; further *V. dahliae* spore suspension is also irrigated to the root zones. In order to make it as an effortless and inexpensive procedure, it has been proposed to wound the roots before or immediately after root inoculation. Nevertheless, damaging the roots may neutralize the first line of defenses against VW infection in cotton. Therefore, it would not mimic the natural field screening resistance.

Artificial screening under greenhouse conditions can be effectively and simply performed by supplying conidial suspensions directly onto the surface of potted soil and it does not require root wounding. By maintaining optimal temperature conditions, it may take ~15 days to develop VW symptoms. Though identifying number of plants infected with VW symptoms can be easily done, it may not provide the disease severity (and thus it will not be useful in categorizing the germplasm as VW resistance). Alternatively VW disease severity can be collected

by using arbitrary scale of 0–5 (0 = no visible leaf symptoms (healthy plant); 1 = <25% chlorotic/necrotic leaves (mild to moderate leaf symptoms); 2 = 25–50% chlorotic/necrotic leaves and little defoliation (severe leaf symptoms); 3 = 50–75% chlorotic/necrotic leaves or defoliation; 4 = >75% chlorotic/necrotic leaves or up to 90% defoliation/terminal dieback/stunted plant; 5 = complete defoliation/stems dying back to ground level). Replications-wise scores have to be collected from each investigated cotton germplasm lines, which can be used to calculate the average VW severity rate as sum of rating × number of plants divided by the total number of plants evaluated (Zhang et al. 2012; Fang et al. 2013). Relative disease index (%) can also be calculated from disease severity rating on a 0–100% scale by calculating the ratio between the average rating and the highest rating (5), multiplied by 100.

Few alternative screening methods have also been reported for VW evaluation. For example, the soil infested with *V. dahliae* that were grown on wheat bran and corn meal or with microsclerotia (@10^3 microsclerotia g^{-1} soil) can be used for VW resistance screening. However, they were shown to be less effective when compared with root-dipping method. Bell (1992) proposed a stem-puncture method to inoculate *V. dahliae* suspensions (@ 2–3 × 10^6 mL^{-1} per plant) under both greenhouse and field conditions. Though such effort was labourious and lengthy, it conciliates the resistance mechanisms provided by the root. Therefore, visual symptoms of VW are more obvious in stem-puncture method when compared with root inoculation technique.

Alternatively other screening strategies such as culturing cotton accessions in a hydroponics system or in tissue culture and then subsequently inoculating with *V. dahliae* conidial suspension (@10^7 spores mL^{-1}) by 40 min of root-dipping have also been reported (Peng et al. 2008). It has also been proposed to culture the cotton seedlings or callus tissues in a plant tissue culture medium containing toxin isolated from *V. dahliae*. Though such strategies offer accelerated identification of VW resistance lines within a month period, such bioassay techniques are not commonly reported in the literature.

Though *G. barbadense* has shown highest level resistance to VW when compared with other three cultivated cotton species (Zhang et al. 2012, 2014a, b), the resistance mechanism has not been efficiently introgressed into commercial Upland cotton (Zhang et al. 2012). As majority of upland cotton breeding programs were conducted under non-VW infected fields, the lines evolved from these programs were shown to be susceptible to VW. Hence, it is essential to identify resistant upland cotton lines by evaluating under field (natural infections) and/or greenhouse (artificial inoculation) conditions. Such screening is a routine program since 2000 in USA, China and in other countries and commercial cultivars are now released only after screening for VW resistance (Wheeler and Schuster 2006; Wheeler and Woodward 2016; Zhang et al. 2012; Zhou et al. 2014). Since 1980, several national and provincial level field screening facilities have been established in China to evaluate cultivars and advanced breeding lines for VW resistance (Zhang et al. 2014a).

5.3.3 Resistance Breeding

Efforts in evolving novel cotton lines with VW resistance has been tried in the farms of New Mexico and California (Smith et al. 1999). Pedigree selection was used to release the first VW resistance upland cotton during 1940s and reselections from the Acala 1517 (which was commercialized in 1939) have identified VW tolerant Acala 4-42 and Acala 1517WR, which were released in California and New Mexico, respectively during mid-1940s. Subsequently, in 1960 the first VW resistant line Acala 1517D was commercialized in New Mexico. Additionally, in 1967, with substantial introgression of genetic elements from *G. barbadense* into AxTE-1, a new improved VW resistance line Acala SJ-1 was released in California. Subsequently, Acala SJ-2 was evolved through pedigree selection from Acala SJ-1 in 1973.

After 1970, cross breeding has been used as a major breeding strategy to evolve VW resistance cotton lines and varieties such as Acala 1517 V, 1517-70, 1517-75, and 1517-91 were released using this method in New Mexico. When Acala 1517-70 (which has a parentage of Hopcala with Tanguis (*G. barbadense*)) crossed with NM 8874 (which was derived from 1517 V) resulted into VW resistant Acala 1517-91. During mid-1970's–1990, Mexico VW resistant lines were used in California crossing program to evolve VW resistant Acala cultivars such as Acala SJ-3, Acala SJ-4, Acala SJ-5, SJC-1, GC-510, and Acala Maxxa. Further, realizing the importance in VW resistance in cotton cultivars, several other VW resistant cultivars were commercialized (to name a few: Kings M-5, GC-356, Acala Prema, Acala Royale, and DL 6166). At the same time, few cultivars such as Deltapine 20, Deltapine 50, Deltapine 51, DP Acala 90, Stoneville 495, Delcot 344, Paymaster 147, Paymaster 303, Paymaster 404, and Paymaster HS 26 shown to possess moderate resistance (Bell 1992; Smith et al. 1999). Using the breeding history of VW resistance Acala cultivars, Bell (1992) hypothesized that VW resistance might be derived from *G. barbadense*.

After 1995, transgenic insect resistant and/or herbicide tolerant cotton become widespread in USA cotton belt and it gradually replaced conventional cotton cultivars in subsequent years. Field and greenhouse experiments conducted by Zhang et al. (2012) confirmed that most of the Acala cotton cultivars had moderate levels of VW resistance; besides few of the transgenic cultivars commercialized by key seed companies in the USA also shown similar levels of VW resistance. Thus, Bell (1992) and Zhang et al. (2012) concluded that cotton breeding has made significant achievement in increasing or maintaining the resistant level to VW. Zhang et al. (2012) have shown the first line of evidence that backcross breeding can be used to transfer VW resistance from Pima S-7 parent (*G. barbadense*) to upland cotton.

Though several other cotton producing countries have also pursued breeding for VW resistance in cotton, recent publication on these efforts were rarely available. A comprehensive review made by Ma and Chen indicated that selections under natural field conditions (showing VW symptoms) during 1950–1970 resulted into the identification of VW resistant/tolerant cultivars such as Zhong 8004, Zhong 7327, Zhong 3474, Liaomian 5, and Shan 1155 in China. Similarly, cross breeding has also

been used to release CCRI 12 in 1987 in China. It is a high-yielding, highly resistant to Fusarium wilt and tolerant to VW cultivar and in 1990, it occupied 20% of the China's cotton area. Subsequently, cultivars such as Yumian 19, 86-6, BD18, Chuan 737, and Chuan 2802 were also released which had VW resistance (Ma et al. 1997, 2002). Though the genetics of VW resistance in CCRI 12 and Chuan 2802 were found to be controlled by major genes, it was found that these cultivars were shown to contain moderate resistance and hence VW is a most serious disease in China in certain seasons.

5.3.4 Genetics of Resistance

As *V. dahliae* race 2 (defoliating pathotype) is more virulent when compared with *V. dahliae* race 1 (non-defoliating), screening cotton lines for VW resistance generally employs race 1 under field or greenhouse conditions. Detailed review on genetic studies using segregating populations of F_2 or BC_1F_1 derived from interspecific or intraspecific crosses involving *G. barbadense* and upland cotton lines have been shown that VW resistance was qualitatively inherited (Zhang et al. 2014a). On the other hand, so far no major VW resistance gene has been reported using Mendelian segregation or QTL mapping with the help of molecular marker. However, Guo et al. (2016) and Zhang et al. (2016a, b) have shown marker-assisted selection to genetically improve VW resistance in cotton.

As there is no direct connection between a disease resistance gene (R) in cotton and an avirulence gene (*Avr*) in VW has been reported, it is speculated that cotton may have a quantitative trait to response to VW infection. Therefore, conventional as well as modern genomics for quantitative genetic analysis have been chiefly used to unravel the genetic basis of VW resistance in cotton (Zhang et al. 2014a).

As indicated by majority of the studies that were conducted on interspecific crosses derived from *G. barbadense* and *G. hirsutum* (Pan et al. 1994; Ma et al. 2000), a dominant or partially dominant gene was involved in VW resistance and allelic nature of the resistance genes from different sources of *G. barbadense* have also been established (Ma et al. 2000). It has also been shown in intraspecific hybrids of upland cotton that inheritance of VW resistance is more complex.

Thus, there is no concrete evidence (whether VW resistance is a qualitative trait controlled by one or two major genes or a quantitative trait regulated by minor polygenes, and whether the resistance is dominant or recessive) that supports VW resistance in cotton. Different resistance sources and homozygosity of genes in VW resistance, virulence and inoculum levels of pathogen, evaluation methods, environmental factors (especially soil temperature and moisture), and plant maturity level at the time of evaluation were found to confound to draw a solid inference on inheritance of VW resistance in cotton.

So far reports have supported resistance to VW in cotton was both qualitative (governed by single dominant gene) (Mert et al. 2005) and quantitative (conditioned

by multiple genes) trait (Roberts and Staten 1972; Wang et al. 2005). Addtionally, there are reports that supports the VW resistance is controlled by two or more dominant genes (Zhang et al. 2000).

There are also studies that provided evidence to state that VW resistance in upland cotton was controlled by dominant gene and two additive genes and the additive effects were vital in imparting resistance to VW. It has been shown that the cotton lines with both additive genes were resistant, whereas lines with only one of the additive genes displayed tolerance and lines with neither of the two genes were susceptible. By employing the progenies derived from VW-resistant cultivar Zhongzhimian KV-3 and VW susceptible cotton line KV9-1, similar kind of additive gene actions has also been reported.

However, in another study conducted by Jiang et al. VW resistance in upland cotton lines was controlled by two dominant major genes with additive-dominance-epistatic effects. This study was conducted on F_2 and $F_{2:3}$ progenies derived from the resistant upland cotton line 60,182 and susceptible upland cultivar Junmian 1 by inoculating with individual *V. dahliae* isolates BP2 (non-defoliating), VD8 (defoliating), T9 (defoliating) and a mixture of these isolates.

Reports have also supported that VW resistance in cotton is race or pathotype specific. For example, Pan et al. (1994) shown that the VW resistance in the progenies derived from *G. barbadense* × *G. hirsutum* was conditioned by a major gene when inoculated with a single *V. dahliae* race or strain; on the other hand, VW resistance was controlled by quantitative genes when inoculated with mixed races or strains. Similarly, Mert et al. (2005) reported that the VW resistance in upland cotton to a defoliating (D) pathotype was governed by a single dominant gene. However, dominant alleles at two loci were involved in imparting resistance to a non-defoliating (ND) pathotype. Such variations in the genetic studies have been attributed to the vigor, development and maturity of cotton plants and temperature, virulence and inoculum level of the pathogen (Bell 1992) which can affect their responses to VW infection.

Other quantitative genetic studies that were conducted on diallel crosses among 10 selected lines of upland cotton (Verhalen et al. 1971) and half-diallel design using five cotton cultivars Aguado et al. have also reported that VW resistance was a quantitative trait, and additive genetic variance component was more predominant in governing the resistance trait.

In contrast, recessive gene mediated VW also reported in few cotton genetic studies. For example, Roberts and Staten (1972) has concluded the VW resistance was recessive based on their investigation on F_2 and F_3 populations derived from 8229 × Lankart 57, 8076 × Lankart 57, and 8861 × Lankart 57. It should also be noted that this study has reported heritability which was varied from 0 to 0.83 depending on exposure level, generation tested, and type of parental tolerance. Similarly, generation mean analysis conducted on seven generations of a crosses (derived from VW resistant cultivar Acala SJC-1 with the susceptible lines S5971, Acala 4-42, and Deltapine 70) have indicated that more than one gene were involved in controlling VW resistance and the resistance was recessive (Devey and Roose 1987).

5.3.5 *Mapping of Verticillium Wilt Resistance QTLs*

Zhang et al. (2015a) have documented a total of 193 QTLs linked to VW resistance in cotton from meta-analysis 13 published reports, which comprised seven early segregating populations (F_2, BC_1S_1 and $F_{2:3}$) and five recombinant inbred lines (RILs) and backcross inbred lines (BILs) including chromosome segment substitution lines (CSSLs) and one association mapping (AM) panel. Similar kind of meta-analysis has reported 201 QTLs for VW resistance (Abdelraheem et al. 2017).

Recent studies have actually reported additional QTLs (more than 400) associated with VW resistance. For example, Palanga et al. (2017) found 119 QTLs using 196 intra-upland RIL population that were tested in five environments. Similarly, Shi et al. (2016) found 48 QTLs using BC_1F_1, BC_1S_1 and BC_2F_1 derived from an interspecific *G. barbadense* × *G. hirsutum.* By employing early segregating biparental populations, main-effect QTLs and QTL hotspots for VW resistance have also been reported (Zhang et al. 2014a, 2015a; Abdelraheem et al. 2017; Zhao et al. 2018).

Several studies (Zhao et al. 2014, 2017, 2018; Baytar et al. 2017; Li et al. 2017; Sun et al. 2019) have also used AM panel that comprises different cotton germplasm lines to identify VW resistance genomic regions. Totally, 26 SSRs were found to be associated with VW resistance in a study conducted at Turkey that used a panel of 108 elite upland germplasm lines and 967 SSRs (Baytar et al. 2017). In another study conducted at China using a panel of 158 upland lines, Zhao et al. (2014) documented that 42 out of 212 SSRs were strongly linked to VW resistance. In an extended effort by the same crew, 192 SNPs were found to be linked to VW resistance out of 18,726 SNPs (Zhao et al. 2017). They have further shown that three clustered QTLs on D09 (c23) is likely to be the main-effect QTL VW-D09-1 reported by Ning et al. (2013).

Consistent QTL (on chromosome A10 (c10)) across three environments for VW resistance along with 16 other QTLs have been reported by Li et al. (2017) using genotyping-by-sequencing (GBS) based SNPs. They have also shown that the gene, TIR-NBS-LRR on A10 would be a likely candidate gene that confer VW resistance in cotton. Similarly, other candidate genes such as *GbTMEM214* and *GbCYP450* on chromosome D04 (c22) would be representing two main-effect VW resistance QTLs (Zhao et al. 2018). This study has employed QTL mapping that used a segregating population derived from a CSSL and its recurrent parent and virus induced gene silencing (VIGS). Though there are VW responsive genes were identified (Li et al. 2017; Shaban et al. 2018), they were not shown to have direct genetic relationship with any VW resistance genes or QTLs that have been reported so far.

Identification of consistent QTLs linked to VW resistance by testing same segregating plant materials across the environments and seasons have been reported (Fang et al. 2013, 2014; Zhang et al. 2014a; Li et al. 2017; Palanga et al. 2017; Zhao et al. 2017). Similarly, QTLs that were consistently identified across segregating populations that employed different parents were also documented (Zhang et al. 2015a; Abdelraheem et al. 2017). Despite of this effort, owing to use of relatively small mapping populations (usually 100–200 progeny) and a low number of markers (usually 200–300), QTL mapping resolution was low. Alternatively, it is proposed to

use high-throughput sequencing- or chip- based SNP markers and a large mapping population to fine map the identified QTL regions. Such effort will not only enable the location of candidate genes that governs the natural variation in VW resistance but also will help to clone VW resistance genes.

5.3.6 Marker-Assisted Selection for VW Resistance

The first report on MAS for introgression of VW resistance was by Du et al. and they used the SSR marker, BNL 3556, on c8 or A02, which explained 50% of the phenotypic variance for VW resistance. Nevertheless, this marker was withdrawn and a new SSR viz., BNL 3255_208 on the same chromosome was found to be linked with the VW resistance gene at a genetic distance of 13.7 cM. Further studies on this marker established that it was found in 85–89% of the interspecific F_2 or BC_1F_1 progenies that have shown VW resistance.

Among the 18 SSRs linked to VW resistance, Kong et al. (2010) reported that three SSRs (viz., BNL1721, BNL2733, and BNL3452) on c8 were useful to select cotton progenies with VW resistance. Similarly, Wang et al. documented two VW resistance QTLs on c16 and reported that two SSR markers (NAU 751 and BNL 195) were useful in increasing VW resistance; especially when both the markers were used, the resistance level was increased. Li et al. (2013) has surveyed 39 SSR markers linked to VW resistance among the progenies derived from a composite cross and its reciprocal cross made in upland cotton. Such effort has concluded that BNL 3241 (on c17), NAU 1225 (on c5 or c19), NAU 1230 (on c5 or c19), JESPR 153 (on c1 or c18), and BNL 3031 (on c9 or c23) were substantially enhanced VW resistance and selection of progenies that has two marker combinations, especially JESPR 153 and BNL 3031 were found to be superior in VW resistance. In another interesting study, Qi et al. shown that SSR markers NAU 1225, NAU 828, and NAU 1269 (all on D5) generated specific PCR products that can discriminate VW resistant and susceptible cultivars.

Thus, a large array of reports on QTLs linked to VW is available for MAS. However, MAS has not yet been shown as an effective procedure to introgress the VW resistance in routing cotton breeding program due to several limitations including validating the markers for their utility. For example, though c8 carries VW resistance, use of those flanking SSR markers in MAS provided variation in resistance level when it was used in interspecific and intraspecific crossings (Kong et al. 2010). Similarly, the usefulness of the two SSRs on c16 and other five SSRs reported by Li et al. (2013) yet to be validated for MAS towards genetic improvement of VW resistance.

5.4 Fusarium Wilt

5.4.1 Causal Agent and Significance

Another most serious disease in cotton caused by caused by *Fusarium oxysporum* f. sp. *vasinfectum* Atk. Sny & Hans (FOV) is Fusarium wilt (FW), which is a soil-borne fungal disease. This disease occurs almost in every cotton growing areas in the world and it attacks young roots. Especially, the vascular systems of stems and roots are heavily infected with fungus toxic compounds which limits the uptake of water and nutrients and lead to xylem discoloration, leaf necrosis/chlorosis/shedding (Fig. 5.3), stunting, wilting and finally heavy yield losses (Zhang et al. 2015a, b, c; Sanogo and Zhang 2016).

FW symptoms development is heavily dependent on genotype, the pathotype and inoculum load, environmental factors such as temperature, moisture and nematodes. For example, the FW pathotype, California isolate FOV4, develops symptoms independent of nematodes whereas other pathotypes need heavy fungal inoculum and nematode infection that cause root wounding. It has also been reported that crop management practices including planting date and application of fertilizers also influence the FW occurrence. In USA alone, annual cotton yield loss in cotton due FW was reported to be in the range of 0.19–1.36% (Blasingame and Patel 2013).

Globally eight pathogenic FOV races have been reported and among them races 1, 2, 3, 4 and 8 were identified in the cotton belts of USA (Kim et al. 2005; Holmes et al. 2009; Cianchetta et al. 2015). Other races were reported in other parts of global cotton growing regions: race 3 in Egypt and Israel, race 4 in India, race 5 in Sudan, race 6 in Brazil, and races 7 and 8 in China (Armstrong and Armstrong 1960; Hillocks 1992; Davis et al. 2006; Sanogo and Zhang 2016; Bell et al. 2017). As the recent molecular evidences cannot distinguish the races 3 and 5 as well as races 4 and 7, the FOV race classifications were recently revised (Davis et al. 2006; Halpern et al.

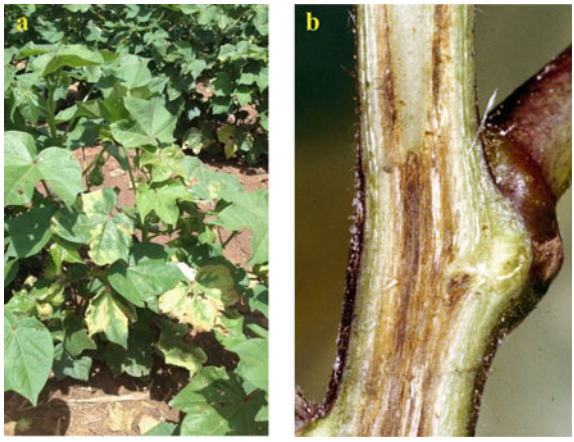

Fig. 5.3 Symptoms of Fusarium Wilt in cotton **a** Plants with yellowing leaves and **b** Stem showing browning symptoms in vascular system of the cotton stem

2020). During the recent past decade, it has been found that FOV race 4 has become a serious threat to cotton productivity in the west and southwest US Cotton Belt (Halpern et al. 2018; Zhu et al. 2020a, b).

5.4.2 Sources of Resistance

As that of VW, screening for FW resistance in cotton can be conducted using both greenhouse and field experiments. The cotton fields showing heavy infestation of FOV due to continuous cropping of susceptible cotton cultivars can be employed to select cotton accessions having FW resistance. Such kind of field has been effectively established since 1952 in the National Fusarium Wilt Nursery in Auburn University at Tallassee, AL. In order to increase the inoculum load, the crop residues of heavily infected cotton plants may be ploughed back in to the soil. Employing such kind of natural field conditions have the advantages of subjecting the testing cotton accessions under a set of physical and biological environments that favours the FW. However, it will be difficult to maintain uniform inoculum load throughout the field and hence each investigated cotton line may be exposed to different levels of FOV inoculum. Screening under natural field conditions may also experiences the other limitations such as presence of mixed FW pathotypes, plant infection with other soil pathogens, and variable soil and crop conditions which may lead to experimental errors.

In order to assess the stability of the genotype for its resistance to FOV, each genotype has to be tested across the locations and seasons with multiple replications. Evaluation of the genotypes under controlled greenhouse conditions offers unique advantages such as replicated screening of large number of genotypes within limited space and effective control on specific inoculum type and dose, method of inoculation, and phenological stage of the plant.

First report on FOV infection was documented by Atkinson in USA and it was reported that variation in response to FW (specifically to race 1) among the cotton cultivars. Such variations were invariably found in both upland and pima cotton and hence elaborate FW resistance breeding efforts were taken up. As a result, FW resistance in the newly evolved cultivars were documented but with poor yield. For example, though Auburn 56, Auburn M, and Dixie King II were shown resistance to FOV consequently for four years, they possess poor yield. Similarly, though Coker 310 and Stoneville 603 were high yielding with good fiber quality, they have moderate resistance to FW. Results on the field screening at the National Fusarium Wilt Nursery in Auburn University at Tallassee, AL for FOV-RKN disease complex have been published by Verticillium Wilt and Fusarium Wilt Committee in various issues of the Proceedings of Beltwide Cotton Conference or special reports from the Alabama Agricultural Experiment Station.

Report summarized by Kappelman indicated that among 2208 cotton cultivars and lines evaluated in the National Fusarium Wilt Screening Test, Tallassee, AL, between 1967 and 1974, few of them were promising. Interestingly, Auburn 623

RNR, Auburn BR1 and Auburn BR2 were found to be resistant consequently for six years of experiments. Similarly, Dixie King II, Auburn 56, Auburn M and Rex Smoothleaf-66 exhibited FW resistance for eight successive years, and McNair 511 and Delcot 277 were found to be tolerant to FW for four years. However, few of them have exhibited intermediate tolerance (for example, Deltapine 25, Coker 312, Coker 310 and Dixie King III) during two to five years of investigation.

In general, lines developed after 1965 had higher FW resistance than the previously released cultivars and further genetic improvement of FW resistance was made in breeding lines after 1975. Kappelman documented that Reba B-50, a commonly grown cultivar in Paraguay, was highly resistant to FW. Another report by Kappelman indicated that Deltapine 55, McNair 220, Delcot 311, Rex 713 and Stoneville 603 were highly tolerant to the FOV-RKN complex, but none was as resistant as the Auburn BR2 check. Both under field and greenhouse conditions, several commercial and experimental cotton materials were screened for FOV race 4 in California, New Mexico and California (Hutmacher et al. 2013; Zhang et al. 2020a, b, c, d, e, f).

Extensive screening of large set of cotton germplasm lines (over 3700 accessions) against FOV race 7 have been conducted in China and reported that 0.62% were immune, 4.42% highly resistant, and 5.04% resistant. It is generally believed that *G. arboreum* was extremely resistant to FOV. However, few upland cotton cultivars that have shown their resistance against FW were collected from naturally infected fields and used as donors in cotton biotic stress resistance breeding.

In another report, which published after a decade, it was highlighted that among the 1211 cotton accessions tested for FOV race 7 resistance, 0.25% were immune and 9.66% were highly resistant. It has also been shown that primitive landraces of upland cotton found to be resistant to race 1 and race 7. After 1980, screening the cotton prebreeding materials at national and provincial level against FW as well as VW have become a regular activity.

Variations in FW resistance among cultivated diploid Asiatic cottons have also been documented. Evaluation of two cultivated diploid species (*G. arboreum* and *G. herbaceum*) in India for FW (race 4) resistance has indicated that though *G. arboreum* was not shown immunity, LD 212, LD 224, LD 231, LD 252, LD 254 and LD 258 were exhibited resistance with lesser (2.2 to 7.3%) disease incidences. In another report by Leena and Kshirsagar, it has been indicated that among the 12,983 diploid genotypes (*G. arboreum* and *G. herbaceum*) screened at the AICCIP center at Pune, India, 979 genotypes were found to be resistant with 0.01–10.00% disease incidence and 1,205 genotypes exhibited moderate resistance with 10.01–30.00% disease incidence.

Similar kinds of large-scale screening for FW resistance have been conducted in other global regions including the former Soviet Union, Uganda, Sudan, Tanzania, Egypt, Peru, Brazil, Argentina, Australia (Hillocks 1992). *G. barbadense* lines including Bahtim 185, and Giza 67 were found to be resistant to FOV (race 3). In Sudan, screening has indicated that few upland cultivars exhibited no resistance to race 1; however, they were almost immune to race 5. Similarly, totally 10 *G. barbadense* were found to be resistant to race 1 and they have also shown mixed

reactions to race 5 (i.e., response was ranged from highly susceptible to highly resistant).

Experiments with cotton wild species indicated that *G. australe* Mueller and *G. sturtianum* Willis have also shown mixed responses to a highly virulent FOV strain (race not classified). They have also shown that none of the cotton lines have shown resistance to this FOV strain.

5.4.3 Resistance Breeding

Cotton accessions that have shown FW resistance when they had grown in FOV—infested soils were selected and they formed the earliest FW resistant cotton cultivars. Later, in 1920–1930's several other resistant cultivars including Cook 307-6, Wilds, and Coker Clevewilt were evolved and they occupied huge cotton growing zones of Southeast USA. When Wilds was crossed with Coker 100-Wilt, it resulted into Coker 413 and its reselection Coker 421. In Central African Republic, several FW resistant cotton cultivars were developed using Coker 100-Wilt as FW resistance source. Similarly, Cook 307-6 was used as resistance source to evolve Empire, Delcot 277, Hartsville, and McNair 220. Auburn 56 was developed by crossing Cook 307-6 and Coker Clevewilt and further reselections resulted into release of Auburn M, McNair 511, and McNair 1032. Interestingly, Auburn 56 has also been employed to develop RKN resistant cultivars such as Auburn 634RNR in USA and FW resistant cultivars in Brazil. As infection by RKN enhance the chance of FW development, breeding for RKN resistance in cotton would increase the possibility of developing FW resistance or at least reduce the FW disease severity.

Despite of these efforts, earlier attempts in evolving FW resistant cultivars usually have poor agronomic trait expressions and low yield in the fields that has shown no FOV inoculum. At this juncture, the first cotton line that has both wilt resistance and good agronomic traits was Coker 100 Wilt which was commercialized in 1942.

Though it is cumbersome to combine both wilt resistance and desirable agronomic properties, there was a slow progress in genetic improvement cotton for improved FW resistant, several cotton cultivars with FW resistant and good quality have been developed since 1967. Later, host plant resistance mechanisms were utilized in development of cotton for FW resistance in public cotton breeding programs conducted in Arkansas, Louisiana, Alabama, New Mexico and Texas (Bourland 2018).

Commercial cotton seed companies always focus in introgressing FW resistance in their cultivars or hybrids and it can be exemplified with current commercial cultivars. As new virulent strains are evolving recently, such as FOV race 4 in California, New Mexico and Texas, molecular breeding has addressed this problem and released FOV race 4 resistant transgenic Pima cotton cultivars. On the other hand, available current commercial upland cultivars are susceptible to FOV race 4.

China has the most successful and extensive breeding programs for FW resistance. Upland cotton was first introduced from the USA for direct commercial production in the early 1900s until 1950s. In the 1950s, selection for FW resistance in

heavily FOV-infested production fields resulted in the development of highly resistant Chuan 52-128 from Delfos 531 and Chuan 57-681 from Deltapine 15 in Shichuan Province. Chuan 52-128, Chuan 57-681 and introgression lines with resistance from *G. arboreum* have become the major sources of FW resistance for the development of more than one hundred of FW-resistant cultivars in China (Feng et al. 1996b). In the 1960s, of the 13 FW resistant cultivars released, the most notable was the development of many FW resistant cultivars including Shaanmian 4, Shaanmian 112, Shaanmian 401, Shaanmian 5245, Shaanmian 3563, and Shaanmian 65-141 by using these two resistant sources in cross breeding in Shaanxi Province. These in turn gave rise to many wilt resistant cultivars including 86-1 and Lukang 1 in the 1970s (of a total of 29 FW resistant cultivars released) and Jimian 7 (of 48 wilt resistant ones released) in the 1980s. In the 1980s, however, the most significant progress was the release of Zhongmian (CRI) 12 in 1986, derived from a Uganda cotton × Xingtai 6871, which in turn gave rise to more than 40 cultivars in the 1990s. The Uganda cotton was first introduced from the U.S. in the early twentieth century with origin from Allen and Sunflower. Selections and breeding produced the long staple Uganda Bukalasa Pedigree Albar (BPA) in production and short to medium staple Serere Albar Type Uganda (SATU). Xingtai 6871 was a selection from Xuzhou 1818 which was also selection from Xuzhou 209, and Xuzhou 209 was directly selected from Stoneville 2B. Therefore, CRI 12 and Chuan 57-681 (from Deltapine 15) shared Stoneville 2B and Allen in their origins, so both understandably shared the same FW resistance gene *Fw2* based on an allelic test (Feng et al. 1998). Since CRI 12 combined high yield with high resistance to FW, tolerance to VW and wide adaptations, it continued to be the single most dominant commercial cultivar until the mid-1990s when more than 20% of cotton acreage in China was grown to this cultivar. In the 1990s, more than 100 wilt resistant cultivars were released including Zhongmian 24, 27, 35, 36 and 41, and Yumian 19. In the 2000s, numerous FW resistant cultivars and hybrids have been released for commercial production.

In China, improvement of FW resistance is one of the breeding objectives in almost all the cotton breeding programs. Presently, most released cultivars carry FW resistance. The cotton acreage in China grown with FW resistant cultivars was increased from 44% in the early 1990s to more than 80% in the early 2000s. FW is no longer a problem in cotton production in China. Based on the National Cotton Variety Tests on FW and VW resistance over 16 years (1973–88), 74 wilt-resistant cultivars were identified and 24 of them were released for commercial production. However, a pedigree analysis indicated that majority of the FW resistant sources were from Delfos 531, Deltapine 15, *G. arboreum* and Uganda cotton (Feng et al. 1996b). The narrow genetic basis was confirmed by Wen et al. and Wang et al. based on SSR and AFLP markers on 54 Chinese germplasm with FW and VW resistance.

Many other cotton-growing countries such as Argentina, Australia, Brazil, Egypt, Israel, Sudan, Tanzania, and Uzbekstan also have had successful breeding programs for enhancing resistance to FW in Upland and *G. barbadense*. For example, FW was a serious problem in India in *G. arboreum* and *G. herbaceum*, but it is controlled by the release and use of many FW resistant cultivars including Jirila, H420, Suyog, G. Cot 11, and G Cot 15.

5.4.4 Genetics of Resistance

Studies on genetic analysis of FW resistance using Mendelian and quantitative genetic approaches have shown that it was both a qualitative and a quantitative trait (Zhang et al. 2015a). Initial attempts on genetic studies in response to FOV were conducted under natural field conditions as there is no proper screening techniques under greenhouse conditions. In 1930–40s, it has been established in India that resistance in *G. arboreum* to race 4 was controlled by two complementary dominant genes and a third inhibitory gene; however, in *G. herbaceum* it was by one dominant gene. Fahmy indicated that in Egyptian *G. barbadense,* the resistance to race 3 was governed by one dominant major gene with few minor genes. In another study conducted in segregating populations of a cross derived from susceptible Pima S-5 (*G. barbadense*) and the resistant upland cultivar Acala SJ-2 at Israel disclosed that the resistance to race 3 was determined by a dominant gene. Similarly, Gridi-Papp et al. and Zhang et al. (2015a, b, c) have shown in Brazil that field resistance to race 6 in IAC 17 cultivar was due to a major gene which was derived from Auburn 56.

Studies in F_2 and BC_1F_1 derived from resistant upland cultivars Empire, Delfos 425 and Cook 307–6 in USA highlighted that a major dominant or recessive gene involvement in resistance development to race 1 or 2. In Sea-Island Seabrook (*G. barbadense*), it was established that the resistance to race 1 or 2 was determined by two dominant genes with an additive effect. A major dominant gene (*FOV1*) which provide resistance to race 1 in Pima S-7 (*G. barbadense*) has been identified under greenhouse conditions and mapped to c16 or D07 using SSRs (Wang and Roberts 2006; Ulloa et al. 2011).

Similarly in another study by Ulloa et al. (2013), a major resistance gene (*FOV4*) to race 4 was identified in Pima S-6 and mapped to c14 or D02 using SSRs. Though wwo major RKN resistance genes were mapped on c14 or D02 (Niu et al. 2007; He et al. 2014), it is not yet revealed that whether *FOV4* and one of the two RKN resistance genes are the same or different (Wubben et al. 2019).

Feng et al. (1996a) in China have employed several upland cotton germplasms to study the genetics of FW resistance to race 7 and reported that a major dominant resistance gene was involved in four resistant lines including CRI 12. Additionally, it has further been shown that the resistance gene (named as Fw_1) in Chuan 52–128 was allelic or same as that of Shaan 1155 and 86–1 resistance gene; similarly, the resistance gene (named as Fw_2) in Chuan 57–681 was allelic or same as that of CRI 12 (Feng et al. 1998).

Another effort in China using two $F_{2:3}$ populations derived from resistant CRI 35 and Sumian 10 have shown that a dominant resistance gene (Fw^R) on c17 or D03 was involved in FW resistance (Wang et al. 2009). However, the inheritance of this gene and the other two genes (Fw_1 and Fw_2) reported by Feng et al. (1998) is yet to be established. SSRs were also used to map a single dominant resistance gene on c21 or D11 using progenies derived from susceptible Xinhai 21 and highly resistant HK 237. Hence, it can be concluded that the two major resistance genes for race 1 (mapped on D07 from Pima S-7) and race 4 (located on D02 from Pima S-6) might

be dissimilar to two major resistance genes identified for race 7 (which were mapped as one each on D03 from Upland and on D11 from *G. barbadense*). Du et al. (2018) have reported a main-effect QTL on c11 or A11 for FOV7 resistance using GWAS that employed 215 Chinese *G. arboreum* accessions. They also identified a candidate gene *Ga11G2353* encoding for a glutathione S-transferase in that region.

Apart from these efforts, quantitative inheritance of FW resistance in cotton has also been shown in several occasions. In general, FW resistance was halfway in F_1 when compared with parents and displayed a continuous variation among the progenies irrespective of F_2, F_3, and BC_1 populations. FW resistance to FOV race 1 or 2, it has been shown that there may 2–3 major resistance genes. Additive effects with greater degree were usually reported than those of dominance or epistasis. Wang et al. have confirmed by employing generation-mean analysis that significant additive effect to FOV7 resistance was exist, whereas dominance and epistatic effects were also found in a few crosses.

Nan has shown the additive variance of 0.348 (i.e., narrow-sense heritability) for phenotypic variation of FW disease index caused by FOV7 using a RIL population from a cross of HS46 × MARCABUCAG8US-1-8. Additive effects on FOV resistance have also been investigated in *G. arboreum* (to race 4) in India, *G. barbadense* (to race 3) in Egypt, and *G. hirsutum* (to race 7) in China (Xiao 1985, 1988, 1992; Zhang et al. 1994, 1995; Feng et al. 1996a, b, c, 1998). These studies were consistently shown that additive variances (general combining ability) were greater than dominance variances (specific combining ability) in several investigations; however, narrow-sense heritability estimates were varied from 0.2 to 0.9 which were due to different diallel crosses and evaluation conditions used in those studies.

5.4.5 Molecular Mapping of FW Resistance Quantitative Trait Loci

Both biparental and association mapping strategies were applied to identify QTLs linked to the FW resistance developed by FOV races 1, 4 and 7 as well as Australian FOV (for a review, see Zhang et al. 2015a). In another attempt, Zhang et al. (2015b) have utilized meta-analysis of six different mapping studies that were conducted with different types of segregating populations including RILs and declared 33 QTLs associated with FW. Abdelraheem et al. (2017) also employed meta-analysis and reported 47 QTLs related to FW resistance in cotton. They have noticed that most of those QTLs were found on five chromosomes viz., c6 (A06), c14 (D02), c17 (D03), c22 (D04), and c25 (D06).

Wang et al. (2018) used 367 SSRs to genotype RILs derived from an interspecific cross (Pima S-7 (resistant to FOV race 1 and susceptible to FOV race 4) × Acala 991 NemX (susceptible to FOV race 1 and tolerant to FOV race 4)) and located six major QTLs on c1 (A01), c2 (A02), c12 (A12), c15 (D01), and c21 (D11) linked to

resistant to FOV race 1 and two major QTL on c14 (D02) and c17 (D03) associated with resistant to FOV race 4.

Abdelraheem et al. conducted GWAS in USA upland cotton germplasms using 25,677 SNPs derived from CottonSNP63K array and reported 13 QTLs for FOV race 4 resistance on six chromosomes viz., c8 (A08), c14 (D02), c17 (D03), c19 (D05), c16 (D07), and D13. In another study, the same team has utilized 163 RILs derived from FOV race 4-resistant *G. barbadense* Pima S-6 and susceptible 89,590 using 403 SSRs and identified seven QTLs for FW resistance (c14 (D02), c17 (D03), c19 (D05), c25 (D06), c24 (D08) and c21 (D11) under greenhouse conditions.

Thus, it has been concluded that though there were different QTLs identified for various races (1, 4, and 7) and an Australian strain of FOV, the major QTLs for FW resistance were found to be localized in only few chromosomes such as c4 (A04) and c20 (D10) (Zhang et al. 2015a, b; Abdelraheem et al. 2017).

It is obvious that the cotton lines that are resistant to different FOV races might be susceptible to the Australia FOV strains. However, there are multiple sources for the FW resistance such as MCU-5. It has been shown that the major FW resistance gene(s) in MCU-5 would be different from those resistant genes found in upland cottons of China and *G. barbadense* (which were resistant to race 7), American Pima S-6 (resistant to race 4) and Pima S-7 (resistant to race 1).

Though common and consistent QTLs across the biparental mapping populations, environments/locations and seasons were reported (Zhang et al. 2015a, b; Abdelraheem et al. 2017), such QTL detections were hindered by the genetic variation contributed by the two parents used in the mapping population development. Further, use of small numbers of molecular markers (100–300) result into poor genome coverage and thereby led to lower resolution in QTL detection process. Though recent developments in sequencing- or chip- based SNP markers have increased the availability of large numbers of SNPs, better increased size of mapping population (such as >500) is required to identify the finely mapped QTL (which has flanking markers with a distance of <0.1 cM. Identification of such fine mapped QTLs allows documentation of candidate genes underlying the natural variation in FW resistance as well as the cloning of FW resistance genes.

5.4.6 Molecular Breeding Techniques

Biochemical assays have helped to identify secondary metabolites as well as enzymatic activities that were involved in FW resistance. For example, electrophoretic mobility of polyphenol oxidase (or catechol oxidase) obtained from germinating seeds of upland cotton cultivars that differ for FW and VW resistance showed that cultivars that has higher level of resistance to FW and VW possess more and stronger polyphenol oxidase bands than susceptible lines and they have shown that selection of breeding materials based on this biochemical assay actually speed up the selection process with lowest cost.

Plant tissue culture technique also favours the selection of FW resistant cultivars as shown by Wang et al. They indicated that the growth of resistant cultivars was less affected by the presence of crude extracts of FOV and resistant lines developed more proficient calli even after several incremental increase of the FOV inoculum over generations of subculture. In another attempt, the FOV toxin (fusaric acid) was employed to screen calli from FOV treated explants of Coker 201, and identified FW tolerant cell lines which was then completely regenerated into cotton plants (Zhang et al. 1994). Ganesan and Jayabalan regenerated plantlets from somatic embryos that were cultured on medium mixed with 40% FOV fungus culture filtrate (FCF), which were isolated from embryogenic calli cultured on 5–50% FCF callus induction medium. Such effort has helped to identify regenerated plants with FW tolerance when those plants were grown in pots inoculated with 1×10^5 spores mL^{-1} of FOV.

In spite of the successful progress in identifying DNA markers linked to FW resistant genes or QTLs with major effects, utilization of these markers in MAS has not been reported in cotton biotic stress resistance breeding. On the other hand, genetic engineering approaches have been tried to impart FW resistance in cotton. Exogenous DNA was introduced into susceptible cotton cultivar via called pollen tube pathway and generated FW resistance. Specifically, single resistance gene from the resistant cultivar Chuan 52-128 was transferred into the ovaries of the FW susceptible upland cultivars Jiangsu 1 and Jiangsu 3 and two promising resistant lines viz., 3049 (from Jiangsu 3) and 3072 (from Jiangsu 1), were generated.

Similarly, Cheng et al. transformed cotton with genes that code for *chitinase* and *β-1,3-glucanase* (which are involved in the plant defense system with synergistic effects) through the pollen-tube pathway method. Screening of the transgenic plants under greenhouse and wilt-infected field nurseries in successive four years helped to identify transgenic cotton lines with improved FW and VW resistance. Ganesan and Jayabalan also highlighted that transfer of chitinase resulted into enhanced resistance to FW as well as to *Alternaria macrospora.* Further transgenic development in upland cotton cultivar SVPR2 with *chitinase* II under the control of the CaMV 35S promoter exhibited resistant to FW and Alternaria leaf spot. It has also been shown by Emani et al. that transgenic cotton developed with cDNA that encode 42 kDa *endochitinase* from the mycoparasitic fungus, *Trichoderma virens*, has exhibited substantial resistance against a soil-borne pathogen, *Rhizoctonia solani* and a foliar pathogen, *Alternaria alternata.* However, its responses to FW were not documented.

Non-expressor of pathogenesis-related (*NPR*) genes usually involved in plant defense system. Parkhi et al. obtained *Arabidopsis thaliana NPR1* gene and transferred to cotton and documented the transgenic cotton's resistance against FW, VW, *Rhizoctonia solani*, *Alternaria alternata*, and reniform nematodes. Similarly, Lei et al. documented that the transfer of *SNC1* (*suppressor of npr1-1, constitutive 1*) from *A. thaliana* into upland cottons, Zhongmian (CRI) 35 and Junmian 1, has enhanced FW resistance when the transgenics were exposed to FOV by the root-dipping method. Gaspar et al. has reported the effectiveness of the plant defensin *NaD1*, from *Nicotiana alata*, against FW and VW in transgenic cotton.

Kristyanne et al. indicated that the antifungal activity of magainin 2 (obtained from the skin of the clawed frog *Xenopus* laevis) on five species including FOV.

Rajasekaran et al. shown that expression of synthetic *D4E1* in transgenic upland cotton plants inhibited FW and VW development. Besides, these transgenic seedlings also developed lesser disease symptoms of black root caused by *Thielaviopsis basicola,* synonym. *Chalara elegans.*

5.5 Future Prospects

Primary and secondary metabolites produced by cotton against environmental stresses including disease causing pathogens helps them to combat the ill-effects caused by these pathogens. Production of these metabolites are usually regulated by gene expression especially at transcriptional and post-transcriptional level. Identification and characterization of these genes and their alleles through genomics assisted allele mining strategies will be useful to unravel the molecular mechanisms involved in cotton disease resistance. Besides such efforts would be used to conserve the cotton biotic stress resistant germplasm resources. Ultimately these efforts would be useful to design a sound molecular breeding strategy that focus the genetic improvement of cotton for improved disease resistance.

Recent developments have indicated even the complex agronomic traits can be manipulated by simply generating point mutations or single base changes in a certain gene. Such small nucleotide changes can be easily executed with gene editing technology which uses artificial engineered and modified nucleases (such as ZFN and CRICPR/Cas9). Genome editing has shown to possess wider applications in cotton as this tool is simple to operate with high efficiency and less cost. Even though realizing the impact of genome editing in cotton for disease resistance is currently limited, its applications in wheat, rice and corn provide strong evidences for its role in cotton disease resistance breeding. Though considerable progress has been made in the aspects of cotton's tissue structure resistance, physiological and biochemical resistance, R-gene-mediated resistance, hormone-mediated disease resistance signal pathways and their interactions, still we don't have clear understanding on the complete disease resistance mechanisms in cotton, which warrants further research in this direction. Availability of cotton whole-genome sequences and high-throughput molecular markers (Boopathi 2020), offer new avenues in finely pinpoint the site of resistant genes. As the developments in genetically modified cotton breeding germplasm is slow and problems associated to evolve a line that adapts to different ecological environments, modern genomics tools need to be integrated with traditional breeding methods to accelerate the process of disease resistance cotton breeding in the future.

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Chapter 6
Conventional and Molecular Interventions for Biotic Stress Resistance in Floricultural Crops

Vipasha Verma, Akhil Kumar, Jyoti Verma, Priti, and Bhavya Bhargava

Abstract Biotic stresses are one of the major problems for ornamental crop loss worldwide. In general, the spreading of biotic stresses is controlled by the spray of chemicals or by other means. But the residue of some chemical is retained in the soil and hence cause groundwater contaminations. Also, the chemicals used for disease control adversely affect pollinators and humans. Despite this conventional and molecular breeding approaches have been applied for disease improvement in several ornamental crops. Genetic engineering also offers an attractive approach for creating biotic stress resistance in ornamentals. But the limited availability of the transformation and tissue culture protocol, genome complexity, lack of gene pool information, high heterozygosity or genetic variability level and limited genomic information of the ornamental crops restricts the development of biotic stress resistant varieties. Nevertheless, the currently available sequencing technology along with newly emerging genome editing tools will definitely help in unravelling the molecular and genetic basis of disease development and herbivory. This understanding will ultimately assist for the improvement of ornamentals against disease and insect pest attack. This book chapter gives a comprehensive look over the diseases and pests affecting the economically important ornamental crops and also highlight the recent progress in developing biotic stress resistance in ornamental crops through conventional breeding, molecular breeding, genetic engineering, RNAi, and genome editing tools.

Keywords Breeding · Floriculture · Pathogen attack · Insect pest · CRISPR Cas9

6.1 Introduction

Floriculture deals with the cultivation of ornamental crops with the objective of development of new varieties, cultivation, marketing, and value-added products. The business of this industry is growing at a very high pace globally with the growth rate

V. Verma · A. Kumar · J. Verma · Priti · B. Bhargava (✉)
CSIR-Institute of Himalayan Bioresource Technology, P.O. Box 6, Palampur, Himachal Pradesh 176061, India
e-mail: bhavya@ihbt.res.in

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of 6–10% per annum. In floriculture, the estimated revenue is approximately 300 billion USD. The cut flower market individually holds a promising global market (Chandler and Sanchez 2012). India has a total of 0.61% share in global floriculture trade, thus ranks 18th in floriculture trade (Vahoniya et al. 2018). Cultivation of flowers is usually carried out in both open and protected conditions. Ranges of important cut flowers like rose, gerbera, bird of paradise, carnation, tuberose, gladiolus, etc. have been cultivated for their use in flower arrangements, bouquet making, garden display and in landscaping. However, loose flowers like marigold are used in making garlands, rangoli decoration, temple offerings, etc. Due to global warming, and climate change abnormalities, like any other crop, floriculture crops are also encountering number of abiotic and biotic stresses that directly and indirectly affect the growth and development of crops, thus result in bad quality flower, poor yield, and low income. Both yield and flower quality get affected by abiotic and biotic stresses which subsequently cause huge loss to farmers.

6.2 Biotic Stress in Floriculture Crops

Market value of cut flowers depends upon their phenotype and quality. Therefore, any visible symptoms of disease on floriculture crop can lead to major impact on its quality, market value and productivity. Most common diseases in flower crops is generally caused by fungi followed by virus and bacteria (Matthews 2019). Among fungal pathogens *Fusarium, Phytophthora, Pythium, Rhizoctonia,* and *Sclerotinia* are the common enemies of ornamental production. Several control methods based on chemical use have been used for controlling fungal diseases, but compared to fungal diseases, it is more challenging task to control diseases associated with viruses and bacteria. Upon the assessment of losses that occur due to diseases caused by virus and bacteria, it was found that about 10–100% loss usually occurs during growth and distribution of floriculture crops. The major obstacle in the cultivation and export of ornamental crops is their susceptibility to multiple viruses (Loebenstein et al. 1995). It is therefore become very important to use quality planting material (disease free/virus free) for the control of diseases among floriculture crops. Looking at the importance of quality and market value of ornamental crops, it is must to develop resistance against various insect, pest and various diseases for effective biotic stress management using conventional and molecular approaches (Tables 6.2 and 6.3).

Table 6.1 Biotic stress in floricultural crops

S. No	Crop	Major diseases	Major pests
1	Rose	Black spot, powdery mildew, botrytis blight, crown gall other disease like stem canker, cercospora leaf spot, and rust etc.	Aphids, two spotted spider mite, scale, thrips, beetles, and leaf cutter
2	Gerbera	Root rot, crown rot, powdery mildew, and botrytis blight	White fly, leaf miner, thrips, red scale, and leaf eating caterpillar
3	Carnation	Fusarium wilt, foot rot, basal rot, leaf spot, and botrytis blight	Thrips, mites, and bud caterpillar
4	Chrysanthemum	Septoria leaf spot, powdery mildew, and blossom blight	Aphids, thrips, mites, and leaf miner
5	Gladiolus	Fusarium wilt, and cucumber mosaic virus	Thrips, and spike caterpillar
6	Orchid	Crown rot, anthracnose, petal blight, and bacterial soft rot	Scale, aphids, thrips, shoot borer, mealy bug, red spider mite, and snail
7	Anthurium	Anthracnose, bacterial blight, and root rot	White fly and thrips
8	Lily	Bulb and scalar rot, foot rot, and gray rot	Tulip breaking virus, tobacco rattle virus, leaf bulb mites and aphids

6.3 Different Approaches to Alleviate Biotic Stress in Ornamentals

The floriculture trade is in full boom globally. Therefore, the major challenge in commercial floriculture is to produce quality planting material of international standards. Disease and pests' management strategies aim to prevent the establishment of diseases and pests in greenhouse, as well as to minimize the development and spread of the same. Control measures against virus and bacteria include detection at earlier stage, making production areas free from infected planting material and other precautionary methods to control the insect vectors are being used presently in floriculture (Powell and Lindquist 1992). Moreover, to improve disease management, other measures which are urgently needed include, preliminary screening of greenhouses, selection of Disease free (virus and bacteria) planting stock from the cultivation areas, weed removal, appropriate surveillance of insect-pest inhabitants and sensible use of pesticides. Moreover, the use of resistant varieties can be used as effective strategy for the alleviating biotic stress in floricultural crops. Conventional breeding strategies also play pivotal role in providing resistance against diseases. However, looking at the diversity of ornamentals and their susceptibility to variety of pathogens, the most challenging task is the identification of sources of disease resistance genes. Above that, it is very rare to find germplasm in ornamental plants with disease resistance. In case a germplasm with disease resistance gets identified

then it may require back-crossing generations for multiple times for the gene flow or transfer of a single gene into the desired selected floricultural crop. This practice is not very feasible and may also cause reduction of the resistance to disease as compared to the source material. Therefore, to achieve biotic stress resistance effectively, genetic engineering and recently emerging tools like gene editing can be used to modify plant defence against diseases. Moreover, this will minimize the use of pesticides/insecticides and other agro-chemicals for the insect pest and disease management and will improve both quality and quantity of flower crops in an eco-friendly manner.

6.3.1 Conventional Breeding

Most of the floricultural crops are domesticated and the most desired wish of ornamental plant breeders is improved disease resistance. As the cultivation of ornamental crops rely on regular breeding programs for biotic stress resistance, and such breeding programmes involves monocultures at large scale which ultimately make crop susceptible towards several diseases (Hayes et al. 1955). For improving crop against diseases, breeding has a vital role in selection of parents with desirable characteristics and producing offspring with desirable combinations. General steps in crop improvement involves creation of variation, selection, evaluation and release of new cultivar. In conventional breeding, the selection of methods and approaches for improving crop against biotic stresses primarily relies on the presence of sources that carry resistance to diseases. And more importantly it also depends upon reproduction system of crops (self-pollinating and cross pollinating). For self-pollinated species, breeding methods involves mass selection, pure line selection, population bulking, pedigree analysis, backcrossing, single seed descent, multiline, composite and heterosis, while cross pollinating species involves mass selection, recurrent selection and heterosis etc. As the whole marketability of ornamental crops depends upon their cosmetic appearance, so it is very important to control disease and pests in ornamental crops Mutation breeding as one of the conventional breeding method can also lead to the development of biotic stress resistance. This technique can be exploited when gene of interest/variability is not present in existing population. As chemical control is not always economical and long lasting, therefore breeding resistant varieties offer durable economical way out. But for the successful breeding program genetic variation in the form of wild germplasm or variation at species level should be available, so that it can be its successfully transferred to the next generation.

6.3.1.1 Heterosis

Heterosis is a common phenomenon among plants. It is the superiority of F_1 over its parents for growth, yield and others characters yield, earliness, growth vigor and stress tolerance in many plants. This term was first coined by Shull (1914). Nowadays

heterosis studies in ornamental crops are mainly resolute on yield and stress tolerance. Hydrangea is a temperate ornamental plant known for its large flower heads. The main reason for its yield loss is powdery mildew (PM) disease. However, resistance to PM disease was achieved in F1 hybrid resulted from cross between *Hygrangea angustifolia* and *Hydrangea macrophylla* (Kardos et al. 2009). Anthurium is a well-known cut flower valued for its foliage and coloured spathe. The major hindrance in its cultivation is bacterial blight disease which is caused by *Xanthomonas axonopodis* pv. *Dieffenbachiae* (Anais et al. 1998). But an interspecific cross between *A. andreanum* and *A. antioquiense.* resulted in germplasm of anthurium with improved resistance against bacterial blight (Anais et al. 1998; Elibox and Umaharan 2010). Likewise, Fusarium wilt is a major disease in carnation caused by *Fusarium oxysporum* f.sp. *dianthi*. Around 500 varieties were collected and crosses were made to finally obtain wilt resistant carnation plants (Mitteau 1987). Chrysanthemum, (*Chrysanthemum* × *morifolium*) as an important cut flower also suffers losses in the form of quality and yield due the disease named white rust, caused by *Puccinia horiana.* In a study, few resistant cultivars of chrysanthemum cultivars showed no macroscopic lesions upon infection with white rust. The resistance in such cultivars was regulated by a single dominant gene (De Jong and Rademaker 1986). So finding and identifying genes that are sources of resistance to pathogens can solve the problem to some extent. Gladiolus corm production is majorly hampered by Fusarium wilt. A resistant variety Dheeraj against Fusarium was developed by crossing between Watermelon Pink × Lady John. Similarly, Arka Shringar developed by cross between Mexican Single × 'Pearl' Double is found resistant against root-knot nematode which is a devastating pest.

6.3.1.2 Mutation Breeding

Conventional breeding involves developing resistant varieties against various stresses by simply crossing different varieties. But this in only possible when resistance is present in the germplasm but in the absence only way out to create variability is going for mutation. Use of mutation to create new variability in a population for a particular cause like against biotic stress is known as mutation breeding. Mutation breeding can be more advantageous in floricultural crops as it may change other important phenotypic floral attributes like flower color, shape and size, flower structure etc. (Ibrahim et al. 2018). Mutation is sudden heritable changes in phenotype of an individual or change in the DNA base pair. This term was first time used by Hugo de vries in 1900 by seeing changes in primrose. Individual showing such changes are known as mutant. Generally mutant genes are generally recessive to their wild or normal type. Mutations are generally lethal and very few are beneficial i.e. frequency is as low as 0.1% (Patil and Patil 2009). For mutation breeding, two types of mutagenic agents are used, that includes physical mutagens (gamma radiation, UV ray and X-ray irradiation) and chemical mutagens (colchicine, EMS, MMS, etc.). For physical mutagens LD_{50} (lethal dose 50) is calculated or standardize beforehand, it is the dose that cause 50% lethality. Therefore, in order to get more desirable results less than

LD_{50} dose should be used. Mutagens are used on seed, seedling, cutting or any other plant or in vitro plantlets. X-rays is most common mutagen used in case of ornamental plants. Mutation caused by using different types of agent is called as induced mutation and mutations occurring naturally in are known as spontaneous mutations. Mutation can occur in cell, tissue or organism. The frequency and types of mutations depends upon the amount of mutagen and duartion of exposure to the mutagen rather than its type. The key in mutation breeding is to identify offspring with desirable changes. Mutant confirmation is a process of re-evaluation of putative mutants under controlled and replicated trails (Oladosu et al. 2016). Mutation breeding offers time saving in breeding program and it is mainly used to create new flower color and shape. In lilium, Fusarium rot occurs at early bulblet stage or in scales. Resistance against Fusarium has been reported in oriental lily with higher ploidy level using colchicine and mutant with high saponin content (Liu et al. 2011). In camation, two mutants named 'MaieIla-lonchabi' and 'Galatee-lonvego' generated after gamma-ray irradiation showed high level of resistance to Fusarium wilt. Similarly, another gamma ray mutant Loncerda verified to be tolerant against Fusarium (Mitteau and Silvy 1983). Moreover, disease resistance against Alternaria was found in X-ray induced mutant lines of carnation cv. Mystere (CasseIls et al. 1993). Another successful example of mutation breeding involves improved resistance to mildew in a mutant named "Herloom" of *Begonia elatior* hybrids cv. Schwabenland Pink, generated through fast neutron induced mutation (Mikkelsen 1975). James (1983) has reported a gamma ray mutant 'Pink Hat', in Floribunda rose which was found to be resistant to mildew. Nambisan et al. (1980) has also developed leafspot-resistant mutants of *Jasminum grandifiorum.*

6.3.1.3 Recurrent Selection

In a perspective of huge biotic stresses, there is pressing demand for plant cultivars with favourable gene frequency that are adapted to such conditions. To achieve this demand recurrent selection serves its role. As defined by its name recurrent selection involves re-selection generation after generation. This has significant role in maintaining genetic variability in the population. This is a cyclic selection for enhancing the chances of desirable/favourable genes' frequency in a breeding population (Ramalho et al. 2005) or it is a variant of backcross where selection for better type is exercised in consecutive segregating progeny. This method is most suited to cross pollinated crops. This method was first time used in maize (Bolanos and Edmeades 1993). The concept of recurrent selection was first introduced by Hayes and Garber in 1919, and East and Jones in 1920 and in 1940, Jenkins named scientist described the method of recurrent selection. Later on, the term recurrent selection was coined by Hull in 1945. This method can be used for both Intra and inter-populations to advance the combining ability. Recurrent selection can be utilized to greater extent in case of heterozygous base population and cytoplasmic male sterile (CMS) population. Crosses between accession of *Lilium dauricum* (resistant) and *Lilium longiflorum* (susceptible) resulted in resistant offspring (Löffler et al. 1994). *Magnolia* ×

brooklynensis var 'Yellow Bird' has been developed by cross between *M. acuminata* var. *subcordata* and *M.* × *brooklyensis var. vamaria* found resistant against the pest known as Japanese long scale (https://planthealthportal.defra.gov.uk/). Similarly this method has been used in many other agricultural crops viz., for developing bacterial disease resistant lines in alfalfa (Barnes et al. 1971). Recurrent selection is most suitable for quantitative traits, or controlled by many genes where each gene has its own little effect on the expression of a particular character. Therefore, such breeding approach can be used against devastating disease like bacterial wilt whose resistance is usually governed by multiple genes.

6.3.1.4 Multiple Lines Breeding Approaches

Multiline varieties are the mixture of several isogenic lines having similar agronomic characteristics but having different genes for disease resistance. To make a variety commercially successful disease resistant variety is alone not sufficient along with resistance characters all other agronomical features (high yield, agronomic performance, and vase life) should also be present. Therefore, multiline breeding is a useful tool to develop composite varieties. But this method is still unutilized in case of ornamental plants (Table 6.2).

6.3.2 Molecular Approaches

6.3.2.1 Molecular Breeding

Ornamental plants are one of the important for commercial purposes as well as for improving human living environment. Previously these crops have been improved for various traits like color, flower size, scent, biotic and abiotic resistance. Biotic stresses (virus, insects, fungi and bacteria) are one of the main problems leading to the losses in ornamental crops globally. Therefore, different breeding strategies have been applied to develop new disease resistant cultivars of economically important ornamental crops. However, complex genome, lack of gene pools and limited genetic variation within gene pool are the major drawbacks which limits the application of conventional breeding for creating biotic stress resistance in floriculture crops. Being an economically important flower crop, Rose is used widely for various purposes such as medicinal, and cosmetics industry (Debener and Byrne 2014). In One of the most devastating disease of Rose is Powdery mildew which is caused by *Podosphaera pannosa*, spreads most commonly in the green houses. It has been found that the loss of the function of the *MLO* gene (susceptible Mildew Locus) can confer disease resistance against powdery mildew (Buschges et al. 1997) In rose, upon analysis of the allelic variants of *RhMLO* genes, about 10 alleles per gene were found. This analysis further demonstrates that out of 19 *RhMLO* genes, *RhMLO1* has the maximum number of sequences (23) whereas *RhMLO4* has lowermost number

Table 6.2 Status of biotic stress management through conventional approaches

Crop	Disease/Pest	Resistant/Tolerant variety	References
Rose	Black Spot	Hybrid Tea: Pride N Joy Floribunda: Sexy Rexy, Rainbow Sorbet, Julia Child Grandiflora: Prima Donna	Hutabarat (2012)
	Cercospora leaf Spot	*Rosa whichuriana*, carefree Wonder Nozomi, Hansa	Hutabarat (2012)
	Spider Mites	Parfait, Lady Rose and Hildalgo	Grzeszkiewicz and Wiltaszek (1994)
		Rajhans, Apsara and Rose evening	Hole and Solunkhe (2005)
		Tinike, Skyline, Confittee, Grand Gala and First Red	Dhananjaya Kumar (2007)
	Thrips	Landora	Gahukar (2003)
Carnation	Bacterial Wilt	Super Gold × *Dianthus capitatus* line 91BO4-2	Onozaki et al. (1998)
	Fusarium Wilt	Arbel and Scarlette	Ben et al. (1997)
	Stem Rot	William and Vermillion Protuding	Guba and Ames (1953)
Gerbera	Foot Rot	H229/3, H134/8 and H147/5	Maria and Chis (2006)
	Powdery Mildew	Figaro, Marinila and Palmira	Kumar et al. (2013)
	Mites	Sirtaki, Rondena, fame and Blanca	Krips et al. (2001)
Gladiolus	Fusarium Wilt	Novalux and White prosperity	Dallavalle et al. (2002)
China Aster	Fusarium Wilt	Heart of France, Crego, Ostrich, Feather, stardust, American Branching, Roment, Powderpuff and Lapplator	Kratka and Duskova (1991)
	Nematode	IIHR-2 (highly resistant), Shashank, AST-5, IIHR-17 and IIHR-21 (resistant), Poornima (Moderately resistant)	Nagesh et al. (1999)
Marigold	Alternaria leaf spot	Golden Guardian and Doubloon	Beckerman and Lopez (2009)

(continued)

Table 6.2 (continued)

Crop	Disease/Pest	Resistant/Tolerant variety	References
Pansy	Cercospora leaf spot resistance	Bingo Red, Bingo Yellow, Crown Blue, Crown Golden, Crystal Bowl Supreme Yellow, Crystal Bowl True Blue, Dynamite Red, Dynamite Yellow, Majestic Giants Yellow, Sorbet Blackberry Cream	Beckerman and Lopez (2009)

of sequences (6). Moreover, in *RhMLO1*, *RhMLO2* and *RhMLO3* around 271, 161 and 337 single nucleotide polymorphisms (SNPs) were detected respectively. Thus, this study concluded that *RhMLO1 and RhMLO2* are the potential susceptible targets for creating resistance against powdery mildew disease (Fang et al. 2021). Another important candidate gene (*Rpp1*) has been identified in rose which exhibits race specific resistance against powdery mildew. The molecular mapping of this dominant resistance gene (*Rpp1*) has been performed by using sequence characterized amplified region (SCAR) markers (Linde et al. 2004). Similarly, in roses, quantitative trait loci (QTLs) in genomic regions involved in the pathotype specific resistance were identified by utilizing combinations of 20 amplified fragment length polymorphism (AFLP) and 43 simple sequence repeat (SSR) primers. This may help in the understanding of the underlying molecular mechanisms involved in disease development and resistance in roses (Moghaddam et al. 2009, 2012). Gerbera is another commercially important and most popular cut flower among the ornamental plants. Due to its high demand, this crop need to be transported at long distances. High humidity during transportation at distant places leads to high chances of infection with fungus *Botrytis cinerea* in gerbera, ultimately result in the loss of quality of cutflower (Elad et al. 2016). However, in Gerbera, a genetic map using SNP markers developed from the expressed sequence tags was constructed. A total of 20 QTLs were observed in the parental maps which help in the understanding of the complex molecular mechanism of *Botrytis cinerea* (Fu et al. 2017). Another study confirmed that root-rot disease in gerbera lead to high expression of the tyrosine metabolism pathway genes such as *GhTAT*, *GhAAT*, *GhHPD*, *GhHGD* and *GhFAH*, thus suggested their possible role in infection (Munir et al. 2019). Among bulbous crops, lilies hold an important place. However, *Fusarium oxysporum* and *Lily mottle virus* (LMoV) are two major problems faced during lily's bulb production. However, some of the Asiatic lily hybrids were found be resistant against the *Fusarium oxysporum* and *Lily mottle virus*. Using three different molecular marker systems (DArT, AFLP and NBS profiling) in an intraspecific Asiatic lily backcross population, a genetic map was constructed. QTL mapping using AFLP Markers showed four linakge groups for *Fusarium oxysporum* and one locus for LMoV. Thus such loci can be used for molecular breeding of lilies against fusarium wilt and viral disease (Van et al. 2001; Shahin et al. 2009). Similarly, about six putative QTLs in the genetic map of AA

population of lilium was identified, which conferred resistance against the *Fusarium oxysporum* (Shahin et al. 2011). Most of the pathogenesis related genes (PR) get upregulated by different stimuli such as fungal/ bacteria/virus. It has been found that the plant hormones such as salicylic acid (SA), methyl jasmonate (MeJA), gibberellic acid (GA_3), abscisic acid (ABA), and ethylene (ET) could induce the expression of the *PR10* (Pathogenesis related 10) gene family (Borsics and Lados 2002; Liu et al. 2003). In addition, compared with the susceptible variety of lilium, the expression of the *LrPR10* gene family including *LrPR10-2*, *LrPR10-4*, *LrPR10-5*, *LrPR10-6*, *LrPR10-7*, and *LrPR10-9* was strongly increased by *Fusarium oxysporum* in resistant lilium variety (He et al. 2014). In carnation, a genetic linkage map using RAPD and SSR markers was constructed and a quantitative trait loci (QTL) for resistance against bacterial wilt caused by *Burkholderia caryophylli* was mapped This will further help in improvement in carnation breeding program (Yagi et al. 2013).

6.3.2.2 Genetic Engineering

Genetic mapping for biotic stress resistance is relatively unusual due to the heterozygosity, complex genome and high ploidy level present in the ornamental plants. Nevertheless, recent advancements in the genome sequencing have provided information for further molecular breeding program for creating biotic stress resistance in the floriculture crops. Genetic engineering is a powerful tool that enables the improvement of the ornamental plants in response to various biotic and abiotic stresses. Moreover, introduction of gene of interest which are not existing naturally in the target plant is a key advantage of genetic engineering over conventional breeding (Azadi et al. 2016). Among floriculture crops, chrysanthemum is one of the economically important floriculture crops used as cut flower, pot flower, garden plant, also used as medicinal tea and cosmetic industry (Mekapogu et al. 2020). Chrysanthemums are severely affected by wide variety of biotic stresses such as fungal (leaf spots, gray mold, rusts, and powdery mildew) and insect (lepidopteran larvae). However, the use of large quantity of chemicals to control theses diseases results in environment pollution, health problem and increase in the cost of production. Several efforts have been paid to make disease resistant varieties of chrysanthemum through genetic engineering. Previously a transgenic chrysanthemum was developed by transferring the *cry1Ab* gene from *Bacillus thuringiensis* var. *kurstaki HD-1* (mcbt) and a modified gene sarcotoxin 1A from sarcophagi peregrine (*msar*) accumulate *cry1Ab* soluble protein which confer strong resistance against the white rust and four species of lepidopteran larvae (Ichikawa et al. 2015). Similary, *Agrobacterium* mediated transformed heterogeneously expressed *hpaGXoo* gene from *Xanthomonas oryzae* pv. *oryzae* confer resistance to Alternaria leaf spot and also accelerate the chrysanthemum development (Xu et al. 2010). In another case overexpression of rice chitinase gene in chrysanthemum exhibited increased resistance against *Septoria obese* and *Botrytis cinerea* (Takatsu et al. 1999; Sen et al. 2013). The N-methyltrasferase involved in the biosynthesis of caffeine stimulates the production of salicylates which results in the activation of several defense mechanisms in biotic stresses. In chrysanthemum,

overexpression of the N- methyltrasferase genes such as *CaXMT1*, *CaMXMT1*, and *CaDXMT1* results in enhanced resistance against grey mold (*B. cinerea*) (Kim et al. 2011). Beside this overexpression of the linalool synthase gene *FaNES1* enhanced resistance against western flower thrips (WFT) in transgenic chrysathemum (Yang et al. 2013). In case of rose, petals are main source of essential oil used in cosmetics, medicine, rose tea and perfume industry. However, its cultivation is affected by various pathogens and insect pests such as powdery mildew, black spots, botrytis blight, downy mildew, rust, *Rose mosaic virus*, Cercospora leaf spot, aphids, spider mites and thrips occurring widely and adversely affecting the yield and quality (Feng et al. 2010). In *Rosa hybrida* cv. carefree beauty, transformation of an antimicrobial protein gene *Ace-AMP1* by *Agrobacterium* showed increased resistance to powdery mildew (Li et al. 2003). Similarly the overexpression of rice chitinase gene in rose confer resistance against blackspot disease (Marchant et al. 1998). In another case overexpression of genes of particular antifungal proteins results in increased resistance to blackspot in transgenic rose (Dohm et al. 2001). In case of carnation, transformation of jasmonate methyl transferase gene through *Agrobacterium* the transgenic lines show the increase in resistance against Fusarium wilt (Ahn et al. 2004). Besides, the overexpression of the rice chitnase gene also confers the resistance against Fusarium wilt (Brugliera et al. 2000). In case gladiolous, overexpression of the chloroperoxidase gene from *Pseudomonas pyrrocinia*, and an exochitinase and endochitinase from *Fusarium venetanum* results in the resistance to *Fusarium oxysporum* f. sp. *gladioli* (Kamo et al. 2016). Transformation of a defective replicase or coat protein subgroup II gene from *Cucumber mosaic virus* through *Agrobacterium* in gladiolus shows enhanced resistance against *Cucumber mosaic virus* in transgenic lines (Kamo et al. 2010). Similarly, overexpression of the bacterial chloroperoxidase or fungal chitinase genes in gladiolus confers increased resistance against *Fusarium oxysporum* f. sp. *gladioli* (Kamo et al. 2015). In another study transgenic gladiolus containing synthetic antimicrobial peptide, *D4E1* gene exhibited enhanced resistance to *Fusarium oxysporum* f. sp. *gladioli* (Kamo et al. 2015). Genetic manipulation in the ornamental plants is challenged by the transformation protocol, transformation system, and regeneration of plant tissue. Although introducing foreign gene into ornamental plants through *Agrobacterium* is simple and efficient.

6.3.2.3 RNAi/Antisense

RNA interference (RNAi) is a sequence specific gene silencing which involves utilization of RNA for the sequence-specific suppression or degradation of the gene expression by double stranded RNA. The mechanism of RNA-silencing initiated with the cleavage of dsRNA by RNaseIII-like enzyme or dicer into short 21–24 nucleotides referred as small interfering (siRNA) or microRNA (miRNA) duplex. Then this double stranded siRNA binds with RNA-induced silencing complex (RISC) containing argonaute (AGO) protein that has siRNA binding domain and have endonuclease activity for cleavage of target RNA strand. The unwinding of RISC from the siRNA results in the formation of sense and antisense strand in an ATP

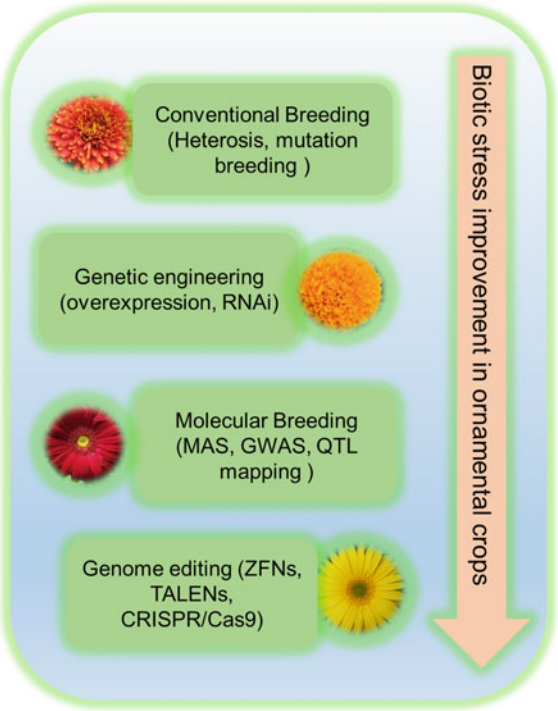

Fig. 6.1 Different approaches used for biotic stress improvement in ornamental crops

dependent reaction. While sense strand undergoes degradation, RISC containing the antisense strand undergoes binding by complementary base pairing with mRNA strand therefore degrade the mRNA and distrupt protein biosynthesis or translation (Vaucheret 2006; Liu and Paroo 2010). In chrysanthemum (*Chrysanthemum morifolium* Ramat cv. *White Snowdon*) transformation of *Chrysanthemum virus B* (*CVB*) coat protein gene sequence in sense, antisense and double sense orientation was conducted in transformation vector for induction of RNA interference. The resulted transgenic chrysanthemum plants obtained through *Agrobacterium* mediated transformation confer the resistance against *Chrysanthemum virus B* (Mitiouchkina et al. 2018) (Fig. 6.1).

6.3.2.4 Gene Editing

Due to ethical issues raised against the genetically modified (GM) crops such as horizontal gene transfer, allergic, carcinogenic, infertility in cattle etc. the crops modified through genetic engineering are not widely accepted. Genome editing is one of the emerging tools in modern biology which is rapidly expanding its possibilities and opening wide opportunities to introduce biotic stress resistance in ornamental crops. Due to high efficiency, high specificity and low off targeting makes it's globally

acceptance over the existing available transgenic technologies. Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated 9 (Cas9) is a gRNA dependent DNA cleavage through restriction endonuclease (Cas9) mechanism in which cleave at target site in DNA by recognizing palindromic sequence (PAM) (Doudna and Charpentier 2014). The cleavage in the double strand of DNA undergoes repair mechanism through homologous and non-homologous manner, which results in the introduction of insertion or deletion at target site (Li et al. 2013). The first genome editing in chrysanthemum using CRISPR/Cas9 system was utilized to introduce mutation in the transgenic plant expressing the yellow–green fluorescence protein gene from *Chiridius poppei* (*CpYGFP*) (Kaboshi et al. 2017). Previously genome wide transcriptome analysis of *Lilium longiflorum* showed upregulation of about 38 *WRKY* genes, which suggested its role in providingresistance against biotic stress under both in vivo and in vitro conditions. Beside this, five genes *LlWRKY* such as *LlWRKY3, LlWRKY4, LlWRKY5, LlWRKY10,* and *LlWRKY12* have been identified which can offer strong resistance against leaf blight caused by *Botrytis elliptica.* This study set a background that *LlWRKY* genes can be potential target to create resistance to *Botrytis elliptica* in *Lilium longiflorum* through CRISPR Cas9 mediated gene editing (Kumari et al. 2021). Similarly, in case of rose RNA-Seq of rose petal analysis revealed that 188 transcription factor (TFs) such as *ERF*, *WRKY*, *bHLH*, *MYB*, *NAC*, *bZIP*, *TGA*, *HSF*, *GTE*, *MADS-box*, *MYC*, *zinc-finger*, and *NFYC* family members are upregulated in response to *B. cinerea.* Out of 188 TFs the *ERF* family, genes show highly upregulation in response to *B. cinerea.* This molecular knowledge about ERF gene family will also help to create target mutagenesis to develop resistance mechanism against *B. cinerea* (Li et al. 2020). Similarly in other ornamental crops such as carnation (Jo et al. 2015), *Lagerstroemia indica* (Wang et al. 2015), Tulip (Miao et al. 2016; He et al. 2019), orchid (Niu et al. 2016), rose (Moghaddam et al. 2014; Diaz-Lara et al. 2020; Chandran et al. 2021), gerbera (Fu et al. 2016; Bhattarai et al. 2020) etc. the availability of genomic and transcriptomic information provide base for the genome editing through CRISPR/Cas9 to create disease resistance varieties. Although availability of few tissue culture protocols for ornamental crops and regeneration protocol remain drawbacks for the CRISPR/Cas9 mediated gene editing (Table 6.3).

6.4 Conclusion

Commercial floriculture has immense significance in the diversification of agriculture and national economy. With the global boom in floriculture trade, production of quality flowers of international standards has become a major challenge in commercial floriculture. Biotic stresses (fungal, bacterial, insects and viruses) are one of the main factors for ornamental crop loss. It is therefore, need to develop such new varieties which are resistant to biotic stresses. However, development of new varieties through convention breeding is time consuming and are not genetically stable. Recent advancement in the next generation sequencing technology help us to obtain whole

Table 6.3 Status of biotic stress management through molecular approaches

S. No	Crop name	Targeted resistance	Target gene (s)	Molecular approach	References
1	Chrysanthemum	White rust and lepidopteran larvae	*cry1Ab*	Overexpression	Ichikawa et al. (2015)
		Puccinia horiana	*CmWRKY15-1*	Overexpression and RNAi	Bi et al. (2021)
		Chrysanthemum virus B	*CVB*	RNAi	Mitiouchkina et al. (2018)
		Septoria obese and *Botrytis cinerea*	*Rice chitinase* gene	Overexpression	Sen et al. (2013)
		Western flower thrips	*FaNES1*	Overexpression	Yang et al. (2013)
2	Rose	Powdery mildew (*Sphaerotheca pannosa*)	*Ace-AMP1*	Expression	Li et al. (2003)
		Botrytis cinerea	*RcERF099*	RNAi	Li et al. (2020)
		Powdery mildew	*RhMLO1*	RNAi	Qiu et al. (2015)
3	Petunia	Powdery mildew	*MLO1*	Overexpression	Jiang et al. (2016)
			chitinase gene	Overexpression	Khan et al. (2012)
		Cucumber mosaic virus (CMV)	*PhERF2*	RNAi	Sun et al. (2016)
		Pseudomonas syringae	*MKS1*	VIGS	Gargul et al. (2015)
4	Lilium	*Botrytis cinerea*	*RCH10 chitinase gene*	Overexpression	Gonzalez et al. (2015)
		Botrytis elliptica	*lre-miR159a*	Overexpression	Gao et al. (2020)
5	Sunflower	*Sclerotinia sclerotiorum*	*Oxalate oxidase (OXO)*	Overexpression	Hu et al. (2003)
		Verticillium dahliae and *Sclerotinia sclerotiorum*	*glucanase* and *chitinase*	Overexpression	Radonic et al. (2008)
6	Geranium	*Botrytis cinerea*	*Mannitol dehydrogenase (MTD)*	Overexpression	Williamson et al. (2013)
7	Carnation	Bacterial Wilt	*Oat Thionin* Gene *(Asthi l)*	Expression	Seo et al. (2002)

genome sequence information which can be further utilized for the identification of genes and understanding of molecular mechanism related to biotic stresses among floriculture crops. Moreover, the identified genes involved in biotic stress can be used to produce transgenic varieties through genetic engineering. Recently, genome editing technologies have progressed rapidly and become one of the most important genetic tools in the implementation of pathogen resistance in plants. Therefore, this genome editing tool has potential to create desirable mutation at a particular location to create disease resistance ornamental crop varieties.

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Chapter 7
Genomics for Biotic Stress Tolerance in Jute

Pratik Satya, Soham Ray, B. S. Gotyal, Kunal Mandal, and Suman Roy

Abstract Jute (*Corchorus capsularis* and *C. olitorius*), valued for its industrial applications and environmental benefits, is attacked by selected pests and diseases that hamper its growth and development, reduce fibre yield up to 40% and negatively affect the quality of the fibre. As the fibre crop is predominantly grown in the Indian subcontinent, the changing subtropical climate would invariably modulate the pest and disease dynamics as well as the interaction of jute and the biotic stress causing organisms. To develop effective biotic stress mitigation strategies in jute, it is essential to understand both the nature of resistance in jute and interaction with the pest/pathogen at genetic, physiological and molecular level. This chapter first provides an outline of the major pest and disease of jute and their management strategies, genetics of resistance and traditional breeding approaches to combat the pests and disease of jute. Thence, standing on the backbone of traditional genetics and breeding, we scanned the recent developments in molecular genetics and genomics researches in jute that have helped to identify and exploit resistance genes, including their use in evolution, phylogeny and population structure analysis, molecular mapping of resistance loci and identification of QTLs. We then mined the genomic resources to identify the genes involved in host–pathogen interaction, particularly against *Macrophomina phaseolina*, the most dreadful pathogen of jute. Transgenic development for resistance is also gaining momentum in jute in recent years, although recalcitrance of jute is a challenging issue in developing stable transgenic system. Unraveling the resistance mechanisms in jute, a crop not preferred by many pests and pathogens, can help to devise novel resistance breeding strategies in other crops.

Keywords Biotic stress · Genomics · *Corchorus* · QTL · Resistance · Transgenics

P. Satya (✉) · S. Ray · B. S. Gotyal · K. Mandal · S. Roy
ICAR-Central Research Institute for Jute and Allied Fibres, Barrackpore, Kolkata 700121, India
e-mail: pscrijaf@gmail.com

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7.1 Introduction

7.1.1 Economic Importance

Jute is the most important industrial crop for production of non-textile fiber products. Jute fiber is used to make sacks, hessian, yarn, carpet backings and other jute diversified products. The fiber is obtained from two Malvaceous species, *Corchorus olitorius* L. and *C. capsularis* L., of which *C. olitorius* or '*tossa* jute' is presently the dominant cultivated species (Satya et al. 2014). India and Bangladesh produce about 95% of the jute fibre, followed by China. During the twenty-first century, the area under jute has dwindled around 1.4 million ha, while the production was around 32–34 million tons of fiber per year (Fig. 7.1). While the production of jute in India has gradually declined from 67 to 51% during 2001–2019 particularly due to introduction of competing crops like maize, banana and sesame, the contribution of Bangladesh has increased from 30 to 48% during the same period. However, jute production in China has declined during this period from 2.1 to 0.8%.

As an industrial crop, the production of finished products (bags, sacks, hessian and diversified products) is equally important indicator of the jute crop economy. During 2018–19, India imported jute and jute products valued at about 160 million USD and exported jute products valued at 325 million USD (Source: National Jute Board, India; https://www.jute.com/). While the primary use of jute is as non-textile fiber, jute has a number of diversified end uses, which include both diversified use of fiber, the primary economic product as well as other non-fiber parts and use of whole biomass. Jute fibers are used to produce jute diversified decorative products, geotextiles, fiber-reinforced composites and can be used as source of cellulose and lignin. As health-promoting vegetable, jute leaf is popular in many Asian and African countries. The young seedlings of jute are consumed as vegetable in many African, Asian and Latin American countries for high nutritive values (Zeghichi et al. 2003; Ndlovu and Afolayan 2008). The cultivated and wild *Corchorus* species also have high ethnomedicinal value for treatment of cystitis, diarrhea, fever, and cold (Oboh et al. 2009).

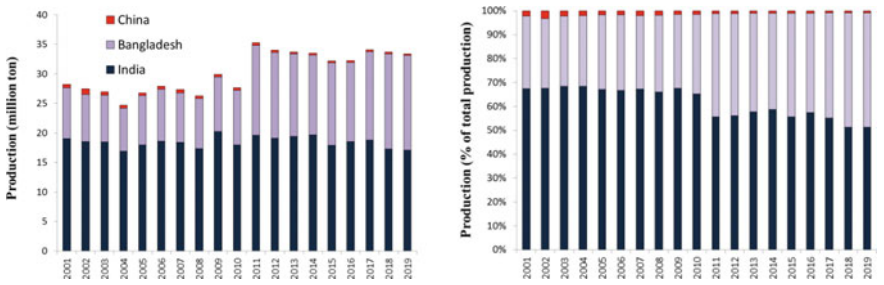


Fig. 7.1 Comparative jute production status in India, Bangladesh and China. *Data source* FAOstat (http://www.fao.org/faostat/). Accessed on 30.07.2021

7.1.2 Reduction in Yield and Quality Due to Biotic Stresses

Biotic stresses are one of the major reasons for reduction in yield potential of jute. Jute is grown in a hot and humid subtropical environment that also favors development of insect-pests and diseases. Consequently, the crop is attacked by many biological organisms including virus, bacteria, fungi, nematode, insects and non-insect pests, leading to a loss of about 15–20% in fiber yield. Pest problem is one of the major constraints responsible for low productivity of jute because the crop is prone to damage from seeding stage to harvest stage. For example, Yellow mite (*Polyphagotarsonemus latus* Banks), a major pest of jute, causes severe damage during early plant growth. As these mites are microscopic, their damage on jute often goes unnoticed in the initial phase. Fiber yield loss to the tune of 60% has been reported from the attack of yellow mite in jute (Kamruzzaman et al. 2013). The jute semilooper, a major lepidopteran pest of jute, causes 20–25% crop damage (Babu et al. 2020). Among the diseases, stem rot (c.o. *Macrophomina phaseolina*) causes the most serious damage to stem, reducing fiber recovery as well as fiber quality (Islam et al. 2012). It can attack any part of the plant at any growth stage and accounts for average yield loss of about 10% which, in severe conditions, may go up to 35–40% (Roy et al. 2008). Although fungicide application is an effective measure of controlling this disease, host resistance is much more preferable for the resource poor jute farming community as the pathogen can survive on several alternate hosts making it almost impossible to eradicate by chemical means (Islam et al. 2012).

7.1.3 Growing Importance in the Face of Climate Change

The service of jute crop to the restoration of the climate is extraordinary. When a jute crop is grown in an area of one hectare for four months, it produces over 50 tons of green biomass, thereby purifying more than 15 tons of CO_2 from atmosphere (Palit and Meshram 2008). The jute fiber is being grown primarily in the Gangetic delta of the Indian subcontinent over the past 250 years. Till the last phase of the twentieth century, jute was a monopoly crop of the Indian subcontinent, which contributed 97% of the total jute production of the World. Attempts to grow jute as fiber crop in other parts of the World, except China, did not met with much success due to the unique climatic conditions required for jute. The rising global warming and environmental concern of synthetic fibers have opened up new scope for industrial and environmental applications of jute in the recent decades.

Climate change has a profound effect on the dynamics of insect-pests and diseases of crops. Although the responses of the insect-pests to global climate change are complex, it is predicted that for 41% of the insect-pests, response to climate change would lead to more damage to the crop (Lehmann et al. 2020). However, studies on the effect of climate change on the insect-pests and diseases of jute are limited. Species distribution modeling of these pests under predictable climates would not

only help reduce the pest problem but also would help in devising pest/disease specific breeding programs. For example, the optimum temperature for development of the yellow mite is estimated as 30 °C (Luypaert et al. 2014). Therefore a temperature range around 28–32 °C with high humidity would favor the growth of the mite. An increase in the temperature and intermittent rainfall during May–June (active growth stage of jute) would be more conducive to growth and development of the mite causing more damage to jute crop. Temperature and rainfall also plays key role in incidence of the jute semilooper. Among the major pathogens, *M. phaseolina*, which can withstand a higher temperature (up to 40 °C) by forming microsclerotia (Pandey and Basandrai 2021), is expected to remain a major threat for not only jute but for other crops like legumes, vegetables and oilseed crops.

7.1.4 Limitations of Traditional Breeding and Rationale of Genome Designing

The principal source of genetic diversity is most of the crops is natural selection that act upon variation created through mutation and recombination. Jute is a sexually reproduced self-pollinated species, though cross pollination may go up to 17% in *C. olitorius* (Satya et al. 2014). But the unique nature of jute cultivation does not allow the plant to enter into reproductive phase, as the whole plant is harvested during active vegetative growth. Consequently, natural mutations that accumulate during the vegetative period are not transmitted to the progeny and any chance of recombination is not possible. The only variability permitted in jute lies in the wild and weedy plants surviving in the nature. Moreover, the two species show high sexual incompatibility with each other and with other *Corchorus* species, further limiting the chance of introgression of genes from wild relatives. Consequently, genetic diversity in jute is extremely low, which is evident from low molecular marker polymorphism and diversity indices (Benor et al. 2012; Satya et al. 2014).

Although *C. olitorius* jute originated in Africa, the fiber type jute was possibly domesticated in Central and Northern India (Sarkar et al. 2019). While jute is being grown in the Indian subcontinents sporadically for about 2000 years, large-scale cultivation started only about 250 years ago. Traditional breeding started in jute with selection of local types by Finlow and Burkill during early twentieth century, resulting in release of two cultivars, D-154 of *C. capsularis* and Chinsurah Green of *C. olitorius*, which dominated the Indian subcontinent for about 50 years, gradually replacing the local landraces. During the latter half of the twentieth century these long duration cultivars were considered unfit for jute-rice cropping system that became very popular in the Indo–Gangetic plain after the spread of the photo-insensitive rice cultivars. This had to two major impacts, improvement in quality of the seed and loss of genetic diversity. Earlier, jute farmers saved their own seed by allowing a few lines to maturity for seed harvest, as they did not grow rice in the same field; rather they cultivated winter crops like potato, mustard or vegetables. As a consequence of

jute-rice cropping system, a seed production industry flourished that cultivated jute only for seed at Central and Southern India and supplied the seed for fiber crop in the Indo–Gangetic plain in India as well as Bangladesh. Replacement of farmers' saved seed ensured seed quality and varietal replacement, but the local genotypes were completely lost within a period of 10–20 years from both the countries, further limiting the existing gene pool of jute. The main variability in jute is thus limited to the germplasm collections of a few countries, breeding populations and some wild jute sporadically distributed in nature (Nasim et al. 2017). Many of the germplasm collections may have duplicate entries, as most of the germplasm collection was taken up by the International Jute Organization and was distributed to different jute growing countries. Since traditional breeding is principally dependent on variability available in the species, low genetic variability is the primary bottleneck of jute breeding. The novel genomic and genetic engineering tools that can transfer genes beyond the sexual compatibility barrier, and create new variations through targeted mutations can have tremendous impact on generating new variability in jute. However, research on genetic transformation, genomics and genome editing are still limited in jute, halting the progress of jute breeding.

7.2 Description on Different Biotic Stresses

Pest problem is one of the major constraints responsible for low productivity of jute because the crop is inflicting to damage by more than 40 species of pests including insects and mites from seedling stage to harvest of the crop. Bihar hairy caterpillar (*Spilarctia obliqua* Walker), jute semilooper (*Anomis sabulifera* Guenée), yellow mite (*Polyphagotarsonemus latus* Banks), stem girdler (*Nupserha bicolor* Thoms.), indigo caterpillar (*Spodoptera exigua* Hübner), red mite (*Oligonychus coffeae* Nietner), stem weevil (*Apion corchori* Marshall) and gray weevil (*Myllocerus discolor* Boheman) are considered as the major pests of jute (Rahman and Khan 2012a). *Meloidogyne incognita* Chitwood is an important nematode pest of jute. Besides, gram caterpillar (*Helicoverpa armigera* Hübner), safflower caterpillar, *Condica capensis* Guenée, green semilooper, *Amyna octa* Guenée, leaf webber, *Homona* sp. Walker and leaf miner, *Trachys pacifica* Kerr are also emerging as insect-pests of jute in the recent past (Selvaraj et al. 2016; Gotyal et al. 2019). The list of major insect-pests, damage stage, nature of damage and distribution is provided in Table 7.1. For a detailed discussion on the insect-pests of jute, please refer to Selvaraj et al. (2016).

Table 7.1 List of insect and mite pests' scenario in jute crop

Name of the insect-pests	Damage stage	Nature of damage	Distribution
Major pests			
Indigo caterpillar, *Spodoptera exigua*	Larva	The leaves are skeletonized; the older caterpillars often devour the entire lamina and causes defoliation. Typical damage is noticed in young seedlings, which are cut on the ground surface by the larvae causing reduction in plant stand	China, Indonesia, India, Japan, and Malaysia
Jute stem weevil, *Apion corchori*	Grub, adult	The female makes one or more punctures at the top nodes, where knot is formed and there are corresponding numbers of grubs seen inside the plant. Damage of apical meristem checks the vertical growth and makes multiple branching. The tissues damaged by the grub that binds the fiber together; which breaks at these points during fiber extraction and results in '*knotty fiber*'	Jute tracts of India and Bangladesh
Jute semilooper, *Anomis sabulifera*	Larva	Approximately 95% of the damage is restricted to nine fully opened top leaves of the crop. The edges of the tender leaves are eaten, serrated, diagonal cuts seen in apical leaves In seed crop, scooping of terminal stem causes drooping of the plants and larvae damage seed pods by making holes that affects the quality of seed inside	Jute tracts of India, Bangladesh, Myanmar, Sri Lanka and in parts of Africa

(continued)

Table 7.1 (continued)

Name of the insect-pests	Damage stage	Nature of damage	Distribution
Yellow mite, *Polyphagotarsonemus latus*	Nymph, adult	Both nymphs and adults suck the sap from the ventral surface of young leaves. The infested leaves turn deep green with coppery-brown shades with typical inverted boat like shape and drop prematurely. The vertical vegetative growth of the crop is arrested, and significant yield loss occurs	Australia, Asia, Africa, North America, South America and the Pacific Islands
Bihar hairy caterpillar, *Spilarctia obliqua*	Larva	Young larvae feed gregariously and scrap the chlorophyll content and completely skeletonize the plant. The damaged leaves of the plant gives an appearance of net or web and under severe condition complete defoliation may occur. Damage seen during June and continued till mid-September coinciding with 60–100 day old crop	India, South-eastern Afghanistan, northern Pakistan, Bhutan, Bangladesh and Myanmar
Mealybug, *Phenacoccus solenopsis*	Nymph, adult	The damage is mostly caused by the immature stages of mealybug which suck the sap. Infested plants exhibits symptoms of distorted and bushy shoots, crinkled and/or twisted bunchy leaves, and plants become stunted and dry completely in severe cases. The vertical growth reduces and gives bushy appearance. Repeated attacks on the stem cause the development of crust due to which fiber bundles resist separation at the time of retting, resulting in the formation of '*barky fiber*'	India, Indonesia, Japan, Malaysia, Philippines, Sri lanka, Taiwan, Australasia, and Pacific islands

7.2.1 Major Insect-Pests of Jute and Their Management

7.2.1.1 Indigo Caterpillar, *Spodoptera Exigua* (Noctuidae: Lepidoptera)

Once considered as minor pest, indigo caterpillar has recently becoming important for its regular occurrence in *C. olitorius*. Although average yield loss is estimated around 20%, its infestation in the early stage of the crop may cause complete crop failure requiring re-sowing of the crop. It mostly infests the seedling stage of the early sown crop (Fig. 7.2). During day time, the caterpillars defoliate the plants and hide in the bottom of the plant in the cracks and crevices. It is a highly polyphagous pest sporadically assumes destructive nature in the early sown jute crop. The young larvae after hatching feed on tender leaves in groups. The feeding activity of grown up larva is generally confined to a few hours early in the morning and late evening. March to April month is the peak period of infestation.

Integrated management

- Early infestation can be spotted by monitoring of the insect underside the clods and the base of the plants prior to the damage and initiation of spray.
- Destruction of egg masses/gregarious larvae by inspecting the field in the early hours when they are active on plant parts can reduce the damage to great extent.
- The early instar larvae can be controlled by spraying neem seed kernel extract 5% along with suitable sticker.
- In case of severe infestation, application of chlorpyriphos 20 EC @ 2.5 ml/L or synthetic pyrethroids such as cypermethrin 25 EC @ 0.5 ml/L, or lambda cyhalothrin 2.5 EC @ 1 ml/L should be done.

Fig. 7.2 Major insect-pests of jute and their damage symptoms

7.2.1.2 Stem Weevil, *Apion Corchori* (Curculionodae: Coleoptera)

It's an internal feeder causing damage in all the jute growing tracts of India. The infestation by the weevil adversely affects the quality and yield of fiber. The grub tunnels and feeds inside the stem restricting the vertical growth and encouraging multiple branching. In the affected nodes mucilaginous substances accumulates, hardens which produces 'knotty fibers'. It attacks *C. capsularis* more than *C. olitorius.* The early season crop is more susceptible to weevil infestation. Yield loss in white jute is estimated to the extent of 18% (Datt 1958).

Integrated management

- Removal and destruction of stubbles and self-sown plants avoid the carry of the pest and reduce the infestation.
- Sowing of *tossa* and *white* jute during end of April considerably reduces its incidence while delayed sowing in late March to early April increases the risk of weevil infestation.
- Balanced application of nitrogenous, phosphatic and potassic fertilizers reduces the pest attack.
- In endemic areas preventive soil application of carbofuran (1 kg ai/ha) is effective in reducing the pest pressure. Need based foliar spray of cypermethrin 25 EC @ 0.5 ml/lit in early hours can control the damage caused by stem weevil.

7.2.1.3 Jute Semilooper, *Anomis Sabulifera* (Noctuiidae: Lepidoptera)

Semilooper is one of the most important foliage feeding insects of jute, which occurs regularly in all the jute growing areas of the country (Rahman and Khan 2012a). Slender, light green semiloopers initiate the damage by feeding the young unopened leaves, later it spreads to fully opened leaves (Fig. 7.2). They remain in clusters up to 3rd instar, mainly feeding on the lower epidermis of leaves, hence often are difficult to find. From the 4th instar the larvae disperse in different plants, chewing the leaves leaving only ribs, which is a characteristic damage sign of this insect. In majority of the cases the 7–9 leaves of upper part of the standing crop are damaged (Datt 1958). Upon repeated infestation, crop growth reduces drastically and profuse branching is observed resulting in loss of fiber yield. It is a cosmopolitan pest, being distributed in wide geographical area and can damage other crops like pulses, groundnut, soybean and many vegetables.

Integrated management

- Balanced use of fertilizers is the key to reduce semilooper infestation. Plough the infested fields after harvest to expose and kill the pupae.
- *Bacillus thuringiensis* is an effective biocontrol agent against jute semilooper. Foliar spray of *Bt* formulation may be recommended @ 1 kg/ha.
- Individual economic injury level (EIL) for semilooper is 10% plant damage at 55 DAS. Whenever the damage by semilooper reaches 15% then any contact

insecticide such as profenophos 50 EC @ 2 ml/lit, fenvalerate 20 EC @ 2.5 ml/lit or cypermethrin 25 EC @ 0.5 ml/lit may be applied.
- The insecticidal sprays need to be targeted towards the apical portion of the plant rather than covering the whole plant from top to bottom as the infestation of the pest is confined to the top leaves.

7.2.1.4 Yellow Mite, *Polyphagotarsonemus Latus* (Tarsonemidae: Acari)

Yellow mite is the most destructive sucking pest of jute. The mite affected leaves curl down, become coppery-brown, dry and fall off (Fig. 7.2). The yield loss varied from 20 to 50% depending on the level of infestation and stage of the plant (Keka et al. 2008). High humidity and morning temperature enhances the rate of multiplication and damage by mite. *Tossa* jute is more susceptible to yellow mite than the white jute.

Integrated management

- *C. olitorius* jute varieties, JRO-204 and JROG-1 are comparatively more tolerant to mite.
- Early sown jute crop suffers more from mite infestation. Instead of March, the crop sown in April escapes the damage of mite to greater extent. Foliar spray of mineral oil @ 3 ml/lit + neem oil @ 3 ml/lit twice at 35 and 50 days after sowing (DAS) may be applied for management of yellow mite.
- Two sprays of spiromesifen 240 SC @ 0.7 ml/L, at 36 and 46 DAS may be applied for protecting the jute crop from yellow mite. Need based spray of abamectin 1.8 EC @ 0.8 ml/L or fenazaquin 10 EC @ 1.5 ml/L, alternatively at fortnightly interval is quite effective for mite management.

7.2.1.5 Bihar Hairy Caterpillar, *Spilarctia Obliqua* (Arctiidae: Lepidoptera)

Bihar hairy caterpillar (Fig. 7.2) has become a serious pest of jute in West Bengal, Bihar and some parts of Assam in India. In field, the initial damage can be spotted by seeing whitish jute leaves. High humidity, rains with intermittent sunny days with high temperature is the congenial condition for hairy caterpillar infestation.

Integrated management

- Regular monitoring to spot early oviposition and egg masses in the early stage, when the caterpillars remain gregarious on leaf, it is easy to destroy them after plucking such infested leaves and then dipping them in insecticidal solution.
- When caterpillars disperse, their control is achieved by insecticidal spraying of lambda cyhalothrin 2.5 EC @ 1.0 ml/L or indoxacarb 14.5SC @ 1.0 ml/L to reduce the pest population to a greater extent.

- Early instar larvae are more vulnerable to the larval parasitoid, *Protapanteles obliquae.* In case of greater activity of parasitoids, insecticidal interference may be avoided.

7.2.1.6 Mealybug, *Phenacoccus Solenopsis* (Pseudococcidae: Hemiptera)

Cotton mealybug, *P. solenopsis* (Fig. 7.2) has been reported to be a new pest of jute crop in 2012 in South Bengal. (Satpathy et al. 2016). It is highly polyphagous and occurs in many economically important crops. Earlier, three species of mealybug i.e. *Maconellicoccus hirsutus, Ferrisia virgata* and *Pseudococcus filamentosus* had been reported to infest jute. High temperature and stretches of dry period and less number of rainy days favor its infestation.

Integrated management

- Preventive seed treatment with thiamethoxam (70 WS @ 5 g/kg seed) or clothianidin (50 WDG @ 3 g/kg seed) is very effective against mealybug.
- The systemic insecticides are more effective against mealybug crawlers. Foliar spray of profenophos 50 EC @ 2 ml/lit or imidacloprid 17.8 SL @ 100-125 ml/ha or thiamethoxam 25 SG @ 200 g/ha is recommended for management of mealybug.
- The control of ants which help the mealybug colonies to grow and spread by soil application of chlorpyriphos 20 EC @ 2 ml/litre or malathion dust 5% @ 25 kg/ha restricts the mealybug crawlers to spread to non-infested plants.

7.2.1.7 Root Knot Nematode, *Meloidogyne Incognita* and *M. Javanica*

The nematodes colonize in the root zone and produce small knotty galls, which interfere with nutrient uptake. The infected plants show stunted growth, wilt and finally die. The population of nematode can increase rapidly in the soil. Rahman and Khan (2012a) observed >460% rise in the population of nematode in soil during 120 days crop growth period, resulting in about 15% plant loss.

Integrated management

- Soil amendment with lime, potash, sulphur, mustard oil cake and jute seed powder can reduce root knot nematode infestation.
- Cultural practices followed by crop rotation are effective with rice and wheat for two years reduced the *M. incognita* and *M. javanica* population in jute.
- Sunnhemp is a suitable trap crop for controlling nematode population in jute.

7.2.2 Diseases of Jute

The most severe pathogen that attacks jute is *Macrophomina phaseolina*, causing stem and root rot. Other serious diseases are anthracnose (*Colletotrichum corchori* and *C. gloeosporioides*), black band (*Lasiodiplodia theobromae*), soft rot (*Sclerotium rolfsii*), jute mosaic (Begomovirus) and Hooghly wilt (Table 7.2).

7.2.2.1 Stem Rot

M. phaseolina is a necrotrophic fungus that infects more than 500 plant species and causes several diseases like damping off, seedling blight, collar rot, stem rot, charcoal rot, basal stem rot and root rot in many plant species including major agricultural crops. It is affecting both *C, capsularis* and *C. olitorius* species. It is prevalent in all jute growing regions. Its incidence makes significant reduction in yield and quality of fiber (Fig. 7.3). The fiber yield loss is generally about 10%, but can be as high as 40% in severe infestation (Roy et al. 2008). Attack at early stage even leads to the death of the plant resulting total crop failure, while infection at the latter stage damages the quality of the bast fiber. The sclerotia can survive in soil and crop residue over four years, starting a new cycle of infection under favorable condition by invading

Table 7.2 List of important diseases in jute crop

Name of the disease	Causal organism	Damage symptom	Distribution in India
Stem rot	*Macrophomina phaseolina*	Damping off, seedling blight, leaf blight, collar rot	Assam, West Bengal, Bihar and Odisha of India, Bangladesh
Anthracnose	*Colletotrichum corchori* and *C. gloeosporioides*	Small lesions with round, gray margins on leaves; sunken spots on stem	Assam, Bihar, UP and North Bengal of India, Bangladesh, southeastern China
Black band	*Lasiodiplodia theobromae*	Small dark brown to black lesions, enlarges to girdle the stem	Assam, West Bengal, Bihar and Odisha of India, Bangladesh
Soft rot	*Sclerotium rolfsii*	Blackish brown lesions or depressions on the stem	Sporadic in India and Bangladesh
Jute mosaic	Begomovirus	Yellow flecks on leaf lamina Yellow mosaic appearance	Sporadic in India and Bangladesh
Hooghly wilt	*Ralstonia solanacearum, M. phaseolina and Fusarium solani*	Plants droops, hang down, turn brown, and ultimately dies within a day or two	Hooghly, Howrah, North-24 Parganas and Nadia of India, Bangladesh

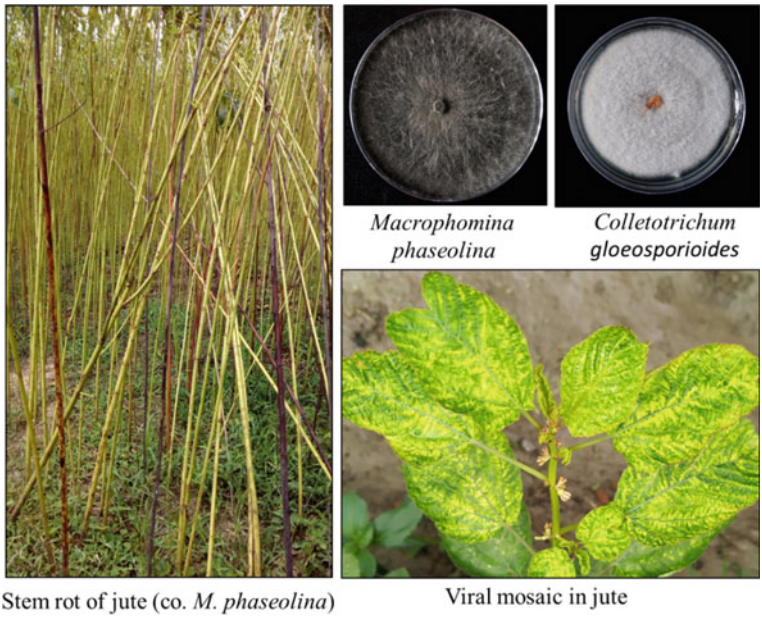


Fig. 7.3 Symptoms and pathogens of major diseases of jute

the plant cell wall mechanically or by releasing cell wall degrading enzymes (Islam et al. 2012).

Integrated management

- Medium to high land with well drained sandy loam soil is very good for jute crop.
- In field experiment with different dates of sowing of jute variety JRO-8432, early sown crop suffered from more stem rot than late sown crops.
- Sowing in the month of April reduce the incidence of stem tot than March sowing (De 2013). Stem rot disease showed declining trend in later sown crop.
- The infected seeds are the major source of the infection; seeds must be treated before sowing with carbendazim 50 WP @ 2 g/kg. When the disease incidence is 2% or more spraying of carbendazim 50 WP @ 2 g/L or copper oxychloride 50 WP @ 4 g/L or tebuconazole 25.9 EC @ 1.5 ml/L is recommended. If the infection is high, 3–4 sprays at 15–20 days interval may be used.

7.2.2.2 Anthracnose

It is a regular disease of capsularis belt of India. The disease entered India during 1930s along with jute germplasm from South-East Asia, particularly from Taiwan. Continuous rain, high humidity and temperature around 35 °C are congenial for anthracnose. It is widespread in southeastern China and is considered to be the most damaging disease of capsularis jute in China (Niu et al. 2016). It reduces both fibre

yield and quality, resulting in development of knotty fibre. It is a seed borne fungi, thus transmission from infected seed is a silent threat to the jute growers.

Integrated management

- Seed treatment with carbendazim 50 WP @ 2 g/kg or captan @ 5 g/kg and spraying of carbendazim 50 WP @ 2 g/L or captan @ 5 g/L or mancozeb @ 5 g/L check the disease spread.
- Seeds having 15% or more infection should not be used even after treatment. Removal of affected plants and clean cultivation reduce the disease (Sarkar and Gawande 2016).

7.2.2.3 Black Band

Once it was considered as minor disease but gradually it is spreading. Its incidence is seen both in *C. capsularis* and *C. olitorius* and causes serious damage to the older crop from July onwards, from which neither fiber nor seeds can be obtained. It first appears as small blackish brown lesions which gradually enlarge and encircles stem resulting in withering of apical shoot. On rubbing the stem surface, unlike stem rot profuse black sooty mass of spores adhere to the fingers. Crops grown from infected seeds show seedling blight.

Management

- Destruction and removal of diseased plants is recommended. Before sowing treat seed with carbendazim 50 WP @ 2 g/kg. Foliar application of carbendazim 50 WP@ 2 g/L, mancozeb @ 4–5 g/L or copper oxychloride @ 4 g/L water is recommended.

7.2.2.4 Hooghly Wilt

This disease is mostly prevalent where *tossa* jute is followed by potato or other Solanaceous crop. During 1970s and 1980s, causing 30–34% crop loss in Hooghly district of India, particularly when the preceding crop was potato (Ghosh 1983). During late eighties and early nineties, 5–37% disease was recorded in Hooghly district and 2–20% in some areas of Nadia and North 24 Paraganas (Mandal and Mishra 2001). Presently this disease is not a serious concern. The disease is caused by infection of a microbial complex involving *Ralstonia solanacearum*, *M. phaseolina* and *Fusarium solani* (Ghosh 1983).

Management

- Solanaceous crops such as potato and tomato should not be grown before jute in the same field.
- Seed treatment with carbendazim 50WP @ 2 g/ kg and spraying the same fungicide @ 2 g/L of water reduce root rot incidence which favors the incidence of wilt.

7.2.2.5 Jute Mosaic

The disease is caused by a begomovirus and is transmitted by whitefly (Ghosh et al. 2007). It is also called as leaf mosaic, yellow mosaic or jute golden mosaic, as small yellow spots appear on leaf that spread and coalesce to form patches. It is reported in capsularis jute from different jute growing belts of India and Bangladesh. In India, the disease is reported in capsularis jute from West Bengal (Ghosh et al. 2007) and from Assam. The incidence of the disease has increased from 20 to 40% (Ghosh et al. 2007).

Management

- Use of seeds from mosaic-free plants, rouging of diseased plant and field sanitation are recommended.
- Spraying of imidacloprid 17.8 SL @ 100–125 ml/ha could prevent the spread of the disease through vector control.

7.3 Traditional Breeding Methods

7.3.1 Intraspecific Hybridization

7.3.1.1 Pedigree Breeding

Pedigree breeding involving two diverse parents, often advanced breeding lines is the major approach in new cultivar development in most crops including jute. Screening for insect-pest and disease resistance is often performed at late generations at F_7-F_8 during station trials. Following the station trials the materials are tested in national evaluation trials, where extensive screening for field resistance to various pests and diseases are performed. Most of the jute varieties released in India are having field tolerance to major insect-pests and diseases of jute, which provides considerable resistance to the field level damages caused by various insect-pests and diseases. For example, the jute variety JRO 204 exhibits considerable resistance to stem rot with a disease incidence of 4.2% (Mandal et al. 2021). However, no resistant variety has yet been developed for viral diseases of jute. Although the damage caused by the viral diseases in jute is negligible, in the changing climate, viruses may be a major threat to jute production as observed in case of mesta, where yellow mosaic virus is becoming a potential threat in recent years.

7.3.1.2 Backcross Breeding

Only a few reports are available on breeding efforts made for incorporating resistance to diseases in jute using backcross breeding. A resistant parent OIN 154 and

popular high yielding cultivar JRO 204 were crossed in a backcross breeding program at ICAR-Central Research Institute for Jute and Allied Fibres at Kolkata, India (Satpathy et al. 2019). The F_1 was backcrossed to the recurrent parent JRO 204 and selections were made in BC_1F_4. A total of 200 recombinant backcross inbred lines (BC-RILs) were advanced to BC_1F_4 generations and screened using artificial stem inoculation. A total of 20 resistant lines were identified and utilized in subsequent resistant breeding programs.

7.3.2 *Interspecific Hybridization*

Wild crop relatives are major source of plant defense related traits. In jute, Palve et al. (2006) evaluated 84 accessions against stem rot disease and stem weevil insect-pest. Out of them, 66 were from six wild *Corchorus* species, and 18 belonged to the two cultivated *Corchorus* species. They observed that the wild *Corchorus* species *C. fascicularis*, *C. pseudo-olitorius* and *C. tridens* were resistant, but some accessions of *C. aestuans* and *C. trilocularis* were susceptible to stem rot infection. On the other hand, *C. fascicularis*, *C. pseudo-olitorius*, *C. urticifolius* and *C. tridens* exhibited high resistance to stem weevil infestation. Recently, wild species like *C. aestuans* have been found to be an important source of resistance to *M. phaseolina* in jute. Through interspecific hybridization between *C. aestuans* and *C. olitorius*, a resistant genotype RS-6 has been developed. This genotype showed considerable resistance to stem rot infection under sick plot (2.6% infection), stem inoculation (lesion length 5.9 cm) and screening in growth chamber (15.3% infection) (Mandal et al. 2021).

7.3.3 *Limitations of Classical Genetics and Breeding in Developing Resistant Cultivars*

Only a few sources of resistance have been found in the existing gene pool of jute (see Sect. 7.4). Combined with low genetic diversity, absence of genetic polymorphism for the resistance to the biotic stresses in the parental lines has hindered the resistance breeding efforts in jute. Till date, sporadic attempts have been made to decipher the genetics of insect-pest and disease resistance in jute. As genetic analysis requires distinct resistant and susceptible lines, clearly defined artificial screening systems and scoring methodologies for distinguishing the resistant lines from the susceptible lines are necessary. Various screening techniques have been used for identification of stem rot resistance including evaluation under sick plot and stem inoculation (Mandal et al. 2021), but a standard screening system for evaluating stem rot resistance is not yet available. For example, Kamruzzaman et al. (2013) observed that at a later growth stages, the lignified stem tissues are not preferred by the yellow mite, thus the mite damage is more severe at early crop growth stage. Considering this, they suggested to

screen for tolerance to yellow mite at early crop growth stage (<90 days). Standard evaluation systems (SESs) for insect-pest/disease resistance have been rigorously developed only in few major crops, such as rice and maize. Resistance breeding and molecular analysis of resistance in many crops will remain elusive until and unless SES for these traits are well-established. Non-availability of good screening system has limited the power of classical genetic analysis as well as identification of linked molecular markers and high-effect quantitative trait loci (QTLs) for marker assisted resistance breeding (MARB) in several crops, including jute.

7.4 Genetic Resources of Resistance Genes

Development of cultivars exhibiting heritable resistance to biotic stresses is a safer long-term solution over chemical control. Often, such resistance sources are found in weedy and wild landraces, germplasm collection or wild crop relatives. Unfortunately for jute, no wild relative can be found in its primary gene pool, as none of the wild *Corchorus* species readily hybridizes with cultivated jute. Even the two cultivated species *C. olitorius* and *C. capsularis* are not easily crossable. Therefore the primary gene pool of jute is principally within-species. The unique nature of the cultivation of jute as fiber crop, where the plants are harvested long before induction of flowering, ensures non-survival of any natural mutant. Untapped genetic potential can be harvested from African countries like Ethiopia and Sudan, the center of origin and diversity of many *Corchorus* species. It is noteworthy to mention that jute is consumed as minor vegetable in many African and South-East Asian countries, and often used as an ethnomedicinal plant for treating of various ailments by African *shamans*. While a good number of African collections have been evaluated for genetic variability (Benor et al. 2012), very few landraces have been screened for resistance to insect-pests and diseases. Mir et al. (2011) evaluated 12 *C. capsularis* genotypes under field condition and identified a resistant line CIM-036 showing 6% disease incidence. In contrast, the popular cultivar JRC 321 exhibited 19.4% disease incidence, while another susceptible genotype JRC 412 exhibited 22% disease incidence. Meena et al. (2015) evaluated 13 *C. olitorius* landraces at two locations in India for two consecutive years and identified six accessions (OIN-125, OIN-154, OIN-467, OIN-651, OIN-853 and OEX-27) to exhibit moderate resistance against stem rot. Another 40 germplasm accessions were screened by Nasim et al. (2017) against various diseases including stem rot, die back, soft rot, root rot, black band, anthracnose, leaf mosaic, leaf curl and root knot at Bangladesh and reported that Acc. Numbers 1045, 1050, 1060, 1062, 1065, 1143, 1261, 1338, 3711, 3724, 4178, 5009 and variety O-72, were resistant to majority of the diseases. Conversely, Sharmin et al. (2012) reported that the cultivar O-72 was susceptible to stem rot disease. At ICAR-Central Research Institute for Jute and Allied Fibers, India, over 500 lines have been evaluated for resistance to major pests and diseases in natural conditions during the past decades. These studies identified a number of indigenous and exotic lines of jute as donors for resistance to insect-pests and diseases, particularly

under hot-spot conditions. For example, lines OIN-07, OIN-27, OIN-121, OIN-125 and CIM-07 exhibited lower stem rot incidence than the susceptible check variety JRC-412 (AINPJAF Annual Report 2020). Similarly, OIJ-08 recorded about 50% lower infestation than cultivar JRO-2407. Another landrace OIN-154 collected from Madhya Pradesh, India exhibited good resistance to root knot nematode infestation as well as to stem rot, and has been utilized in resistance breeding programme to develop elite breeding lines. The wild relatives of jute are good sources of resistance to major biotic stresses. However, a *C. trilocularis* accession was noted to be resistant to stem rot disease by Sharmin et al. (2012). They identified two xyloglucan endotransglycosylase/hydrolase (XTH) genes (*CoXTH1* from *C. olitorius* and *CtXTH1* from *C. trilocularis*) that expressed differentially upon challenged inoculation with *M. phaseolina*. The expression of *CtXTH1* gradually amplified over time while *CoXTH1* was found to be downregulated. Since XTH is a key player in cell wall development, enhanced expression of *XTH1* in the wild species is indicative of XTH polymerization that may provide resistance to stem rot disease. De and Mandal (2012) identified eight accessions, OIN-107, OIN-125, OIN-154, OIN-157, OIN-221, OIN-651, OIN-853 and OIJ-084 as moderately resistant to stem rot. Gotyal et al. (2014) screened jute germplasm against stem rot for two years and identified OIN-431 as a resistant germplasm. They reported that the *C. capsularis* germplasm exhibited more susceptibility than the *C. olitorius* germplasm. A list of important germplasm accessions showing good resistance to various biotic stresses are presented in Table 7.3.

Little information is available for resistance to insect-pests, which are more difficult to screen as unlike fungi, insects move around to cause damage in different parts of the plant. In addition, plants have different mechanisms like antibiosis, antixenosis or tolerance, which are difficult to score. For example, yellow mite, one of the most serious insect-pests of jute can damage the plant at any crop growth stage, move from one leaf to another and can spread from one plant to another plant. Since artificial screening against yellow mite is troublesome, natural field reaction is considered the most appropriate approach for identifying tolerant lines. It was observed that the *C. capsularis* cultivars are less preferred than the *C. olitorius* cultivars by yellow mite

Table 7.3 Sources of insect-pest resistance in the primary and secondary gene pool

Common name of the pest/disease	Resistant source	References
Hairy caterpillar	*C. aestuans* (WCIN-179)	Gotyal et al. (2014)
Jute semilooper	OIN-87, OIN-88, OIN-89, OIN-92, OIN-94	AINPJAF Annual Report (2017)
Jute stem weevil	OIN-95, OIN-114, OIN-121, OIN-100, OIN-110	
Yellow mite	OIN-91, OIN-96, OIN-97, OIN-98, OIN-103	
Stem rot	*C. aestuans* WCIN-136–1	Germplasm reg. no. INGR21036

(Rahman and Khan 2012b). However, significant variation was observed among *C. capsularis* genotypes under field screening. Kamruzzaman et al. (2013) observed that infestation of female yellow mite was lower (2.2/cm^2) in moderately tolerant cultivars BJC-83 and CVL-1, but damage of yellow mite caused yield loss of 54%. In contrast, BJC-7370 with higher female mite infestation (3/cm^2) exhibited 60% yield loss. In addition to cultivars the incidence of yellow mite is dependent on environmental conditions including temperature, relative humidity, plant canopy structure and the age of the plant (Islam et al. 2020). The population of yellow mite was found to concentrate more on the five apical leaves, while the lower leaves have less population. Moreover, the population is more at noon than morning and afternoon. A standard screening system for yellow mite, thus, has to give proper weightage to these factors for identifying effective resistant lines.

7.5 Resistance Gene-Based Marker Development and Utilization

Resistance gene analogs (RGA) are genomic sequences sharing conserved regions of plant resistance genes. The RGAs are often part of R genes, or may be tightly linked with R genes. RGA markers are a group of functional DNA markers that reveal variability in these RGAs, which is used for genetic diversity analysis, evolutionary studies and plant genetic resource characterization (Satya and Chakraborty 2015). Molecular markers based on RGAs are functionally related to plant defense response, thus these have unique advantages for studying genetic diversity of isolated populations adapted under different environmental conditions (Satya and Chakraborty 2015). Genetic polymorphism of RGA sequences or resistance gene analog polymorphism (RGAP) is a unique functional marker system, which has been used in many crop species for genetic mapping of R genes, genetic polymorphism analysis of population, evolution of plant resistance, population ecology and plant genetic resource characterization. Such information is desirable not only for devising strategies for genetic improvement in a breeding program or to transfer genes from sexually compatible species, but also to gain insight in the evolutionary processes of plant defense related genes (Michelmore and Meyers 1998). The evolution of RGAs is influenced by several factors such as host–pathogen co-evolution, geographical isolation, adaptation of a crop to a particular ecology, and extent of cultivation as a crop. Currently, two models are suggested for the origin of multiple R genes. One model considers that recombination and unequal crossing overs at intergenic regions have led to divergence of a single gene into large multi-gene families (Richter and Ronald 2000). The other hypothesizes that the plant R genes have evolved through a birth and death process involving recombination and gene conversion that alter the structures of the functional domains followed by divergent selection (Michelmore and Meyers 1998). The first process results in a concerted evolution with fixation of highly homogeneous populations with high inter-population diversity. In contrast,

the birth and death based divergent selection would allow more diversity within each population and more possibilities for inter-population genetic exchange.

7.5.1 Utilization of Resistance Gene-Based Genic Markers for Domestication and Population Genetic Analyses in Jute

As a dominant marker system, RGAs have certain advantages for genome mapping and phylogenetic analysis. RGAs are dispersed throughout the genome, making these markers suitable for genetic diversity studies at genome level. RGAs are principally associated with functional regions of genome, being distributed in tandem array of repeated gene families (Poczai et al. 2013). Many of these genes may be functionally inactive at certain reference points (pseudogenes), but may express under different disease scenario or environmental conditions. RGA markers thus may be more suitable than neutral markers for study of geographically isolated populations domesticated under different agro-ecological conditions. Genetic diversity of 80 accessions of the cultivated and the wild *Corchorus* species when evaluated using RGA markers, amplified a total of 182 fragments in the Indian *C. olitorius* group, of which 76.4% were polymorphic. In the African group 164 RGA fragments were amplified exhibiting 84.8% polymorphism (unpublished data, P. Satya).

7.5.2 Use in Phylogenetic Analysis

Markers associated with stress evolve with adaptation, which makes them useful for identification of geographically isolated groups (Satya et al. 2014). RGA-based phylogenetic analysis of 80 *Corchorus* accessions following weighted neighbor-joining method identified four major genetic groups (Fig. 4a). The first group was a mixture of African wild jute and Indian fiber type cultivars along with mutants that were mostly derived from these cultivars. The second group consisted of only Indian jute landraces. The third group was heterogeneous (25 accessions), comprising of African, Indian jute accessions and wild relatives. Similar results were also observed using principal coordinate analysis (Fig. 4b). As the African *C. olitorius* genotypes have broader genetic base than the Indian *C. olitorius* genotypes for RGA loci, a differential adaptation for RGA loci in African and Indian jute can be envisaged. Of particular interest was *C. aestuans*, which was distantly placed from other *Corchorus* species, indicating it might harbor different resistant genes. Indeed, *C. aestuans* is being used in resistance breeding to develop stem rot resistant genotypes (Mandal et al. 2021).

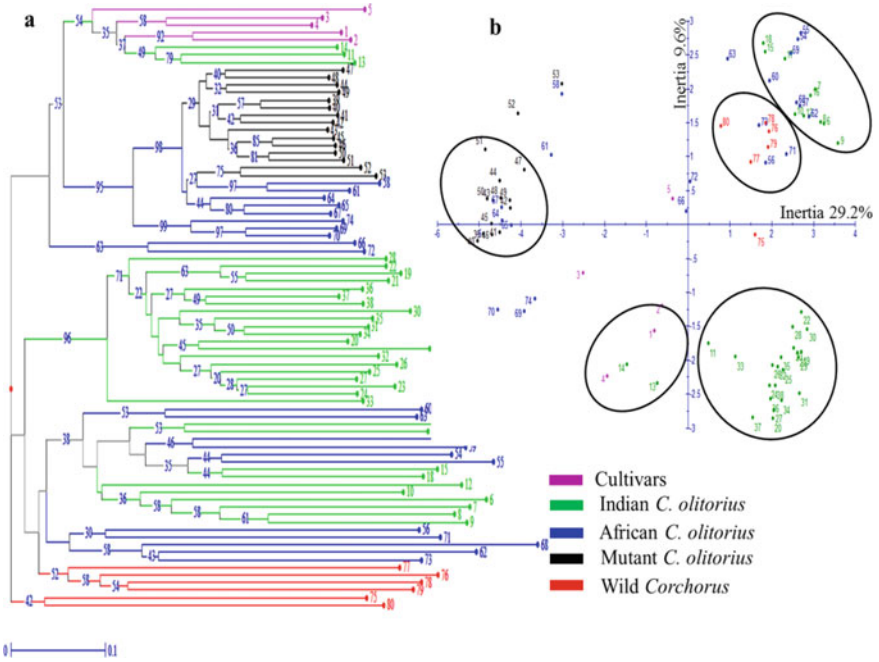


Fig. 7.4 Phylogenetic analysis of jute germplasm using RGA markers. **a** Nearest-neighbor phylogenetic tree of 80 *Corchorus* accessions (color-coded). **b** Principal coordinate analysis of 80 *Corchorus* accession

7.5.3 Use of RGA in Population Structure Analysis

RGA-based population structure analysis following Bayesian approach resulted in clear differentiation of genetic structures (unpublished data, P. Satya). At $K = 2$, the proportion of two genetic clusters under non-admixture model were 61.82% and 38.18% (Fig. 7.5). The first cluster comprised all the wild species, 43.5% Indian *C. olitorius* accessions, 60% of African *C. olitorius* accessions and 72% of *C. olitorius* accessions from other countries. All the fiber type cultivars and mutant genotypes of *C. olitorius* and fiber type cultivars of *C. capsularis* from India were classified under cluster II. At $K = 3$, cluster I remained largely unchanged (60.9%), while cluster II was subdivided into two clusters, having 15.45% (cluster II) and 23.64% (cluster III) accessions, respectively. Cluster III comprised the 15 mutant genotypes of *C. olitorius*, three accessions from Kenya, two accessions from Nigeria, three accessions from Russia, two accessions from Myanmar and one accession from Thailand. The results of admixture model closely followed the outputs from non-admixture model (Fig. 7.5). The African and India *C. olitorius* also shared high genetic relatedness for RGA loci, which indicates tossa jute might have migrated during early civilization periods in India, most possibly through land as an herbal medicinal or vegetable plant. In another study, Satya et al. (2014) observed clear

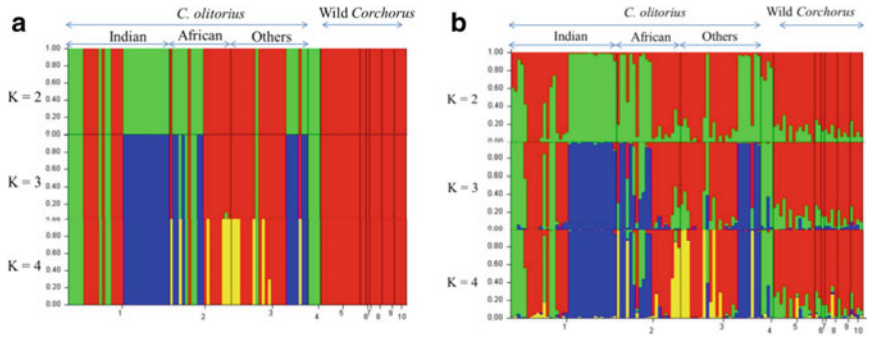


Fig. 7.5 Optimal population structure of *Corchorus* based on RGAP under non-admixture (**a**) and admixture (**b**) models. Each vertical line represents one population, each color represents one cluster. The length of the colored segment represents estimated proportion of membership of each cluster in the population

population structure difference between Indian and African jute genotypes using neutral simple sequence repeat (SSR) and functional peroxidase gene-based markers, which also indicate early domestication and introduction of jute in India.

7.6 Genomics-Aided Breeding for Resistance Traits

7.6.1 Genomics to Decipher Plant-Pathogen Interaction Pathways in Jute

Plants manifest their defense mechanism against numerous biotic agents by expressing diverse genes associated with resistance. Several genes are involved in recognition of pathogen/pest and development of cascades of signal transduction pathways. A good number of genes are involved in the KEGG plant-pathogen interaction pathway (KO04626) that expressed in hypocotyl (earliest growth stage) even in absence of any biotic stress (Table 7.4) indicating that jute expresses an array of pathogen recognition and resistance genes.

Analysis of hypocotyl transcriptome (GCNR00000000) and bast transcriptome (GBSD00000000) of white jute (*C. capsularis*) cv. JRC-212 was performed in order to identify genes which can potentially be involved in stem rot resistance. Jute expresses an array of biotic stress resistance related genes both in hypocotyl and bast tissues (Fig. 7.6). Classification of these genes in different categories depicts enrichment of two different classes—(i) General biotic stress-related genes and (ii) Pathogenesis-related (PR) proteins (Fig. 7.6). General biotic stress-related genes are a mixed class of genes having diverse functionalities, but in general, are upregulated during all kind of biotic assault. On the other hand, PR-proteins are a group of toxic plant proteins which are also structurally diverse in nature. These are constitutively

Table 7.4 Jute genes expressing in KEGG pathway KO04626 (Plant-Pathogen interaction) at early developmental stage

KEGG orthology	Gene name	Jute genes mapped in KEGG pathway
K00864	Glycerol kinase	unigene_S3_18878, unigene_S3_22035, unigene_S3_29038
K02183	Calmodulin	unigene_S3_13054, unigene_S3_34974, unigene_S3_35082, unigene_S3_35105, unigene_S3_37730, unigene_S3_37911
KO4079	Molecular chaperone HtpG	unigene_S3_8998, unigene_S3_13558, unigene_S3_31587
KO4368	Mitogen-activated protein kinase kinase 1	unigene_S3_19240
KO5391	Cyclic nucleotide gated channel, plant	unigene_S3_5842, unigene_S3_5843, unigene_S3_8206, unigene_S3_8207, unigene_S3_8208, unigene_S3_8436, unigene_S3_31190, unigene_S3_31281, unigene_S3_31336, unigene_S3_31457, unigene_S3_31677, unigene_S3_31776, unigene_S3_42931, unigene_S3_42937
K09487	Heat shock protein 90 kDa beta	unigene_S3_16535, unigene_S3_31076, unigene_S3_32140
K12795	Suppressor of G2 allele of SKP1	unigene_S3_24963, unigene_S3_30641, unigene_S3_44523
K13412	Calcium-dependent protein kinase	unigene_S3_6046, unigene_S3_6047, unigene_S3_8069, unigene_S3_9373, unigene_S3_10367, unigene_S3_10787, unigene_S3_11019, unigene_S3_11616, unigene_S3_13721, unigene_S3_21586, unigene_S3_30827, unigene_S3_31104, unigene_S3_31448, unigene_S3_32948, unigene_S3_33750, unigene_S3_33780
K13413	Mitogen-activated protein kinase kinase 4/5	unigene_S3_11687
K13414	Mitogen-activated protein kinase kinasekinase 1	unigene_S3_31308, unigene_S3_31359
K13416	Brassinosteroid insensitive 1-associated receptor kinase 1	unigene_S3_16444
K13420	Leucine rich repeat (LRR) receptor-like serine/threonine-protein kinase FLS2	unigene_S3_17046, unigene_S3_44483
K13424	WRKY transcription factor 33	unigene_S3_33360, unigene_S3_33982

(continued)

Table 7.4 (continued)

KEGG orthology	Gene name	Jute genes mapped in KEGG pathway
K13425	WRKY transcription factor 22	unigene_S3_39301
K13427	Nitric-oxide synthase	unigene_S3_30358
K13429	Chitin elicitor receptor kinase 1	unigene_S3_2478, unigene_S3_2479, unigene_S3_6662, unigene_S3_6663, unigene_S3_6664, unigene_S3_19454, unigene_S3_40624
K13430	Serine/threonine-protein kinase PBS1	unigene_S3_12240, unigene_S3_30782
K13434	Pathogenesis-related genes transcriptional activator PTI6	unigene_S3_11274
K13436	*pto*-interacting protein 1	unigene_S3_18586, unigene_S3_29132, unigene_S3_33171
K13447	Respiratory burst oxidase	unigene_S3_6079, unigene_S3_6615, unigene_S3_25996, unigene_S3_32011, unigene_S3_39638
K13448	Calcium-binding protein CML	unigene_S3_8981, unigene_S3_12622, unigene_S3_13244, unigene_S3_13853, unigene_S3_14800, unigene_S3_19509, unigene_S3_28107, unigene_S3_29343, unigene_S3_30322, unigene_S3_30611, unigene_S3_30637, unigene_S3_33699, unigene_S3_34923, unigene_S3_34949, unigene_S3_35760, unigene_S3_35842, unigene_S3_38062, unigene_S3_38224, unigene_S3_38517, unigene_S3_39026, unigene_S3_39356, unigene_S3_40103
K13456	*RPM1*-interacting protein 4	unigene_S3_29227
K13457	Disease resistance protein RPM1	unigene_S3_11841, unigene_S3_12078, unigene_S3_12156, unigene_S3_12201, unigene_S3_31985
K13458	Disease resistance protein RAR1	unigene_S3_40708
K13459	Disease resistance protein RPS2	unigene_S3_16907, unigene_S3_31867, unigene_S3_32694
K18834	WRKY transcription factor 1	unigene_S3_6366
K18835	WRKY transcription factor 2	unigene_S3_10459, unigene_S3_31188
K18875	Enhanced disease susceptibility 1 protein	unigene_S3_33706

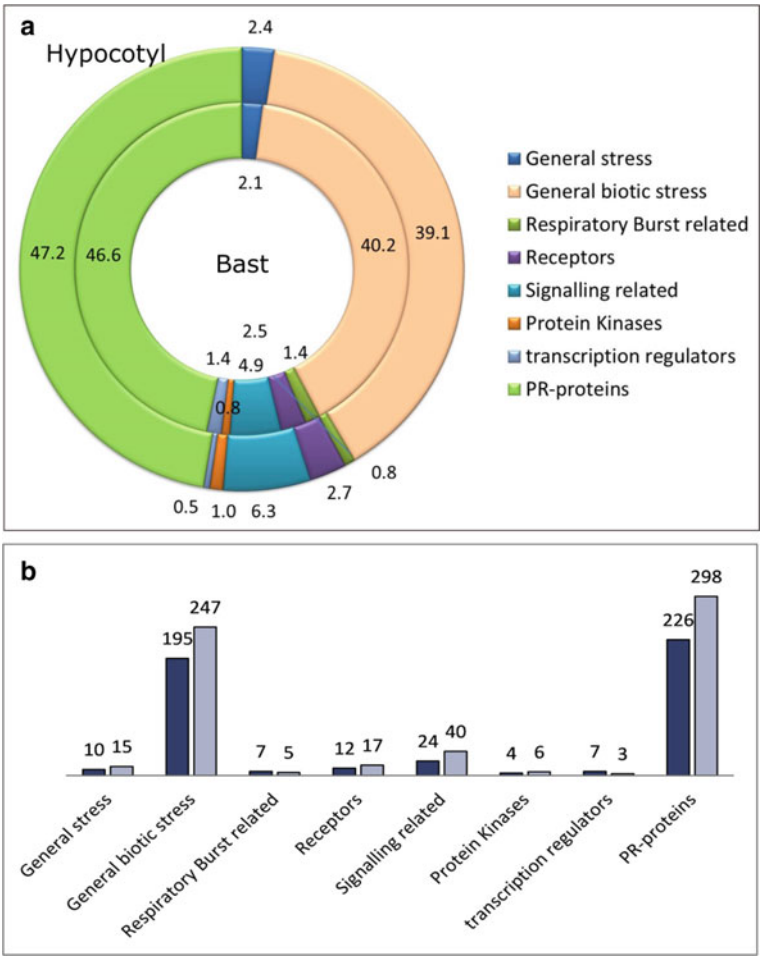
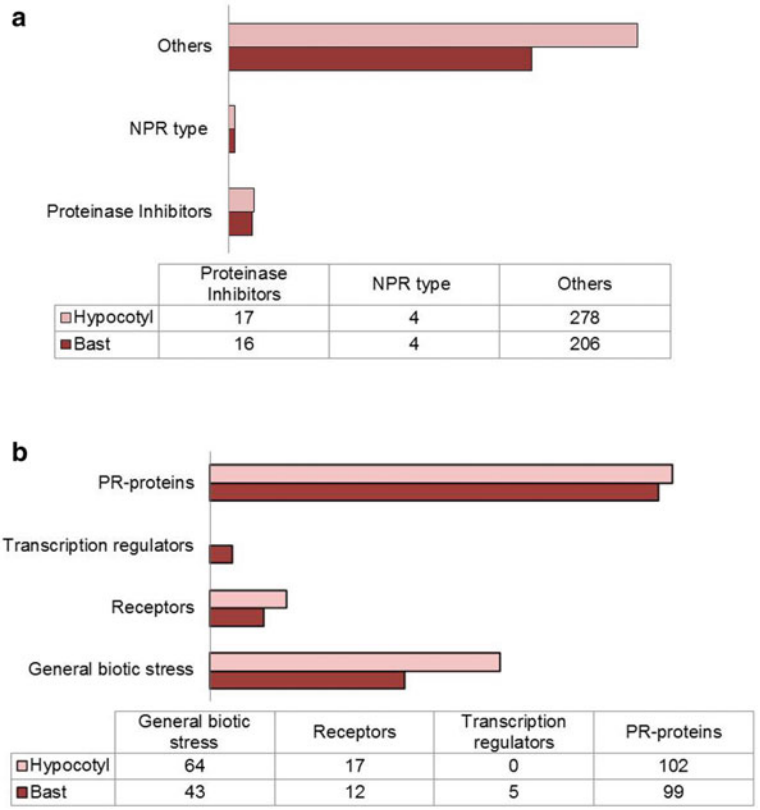


Fig. 7.6 Categorization of biotic stress-related genes obtained from hypocotyl and bast transcriptomes of jute. **a** Percentage contribution of each group of genes to the total biotic stress-related genes expressed, presented in doughnut charts. **b** Vertical bar chart representing the absolute number of gene expressed under each category. Each pair of bars indicates genes expressed in bast (first bar) and hypocotyl (second bar)

expressed at a low level in normal condition but are upregulated by several-folds in response to invading fungal pathogens (Van Loon 1997).

PR-proteins can get accumulated at intracellular and intercellular spaces and variation of the type- and content- of PR-proteins also depend upon the type of the tissue. Cell wall is one of the major sites of PR protein accumulation (Agrios 2005). Among the expressed PR-proteins of hypocotyl and bast, we could identify two specialized classes—non-expressor of pathogenicity related (NPR) proteins and proteinase inhibitors (PI) (Fig. 7.7a).



	Proteinase Inhibitors	NPR type	Others
Hypocotyl	17	4	278
Bast	16	4	206

	General biotic stress	Receptors	Transcription regulators	PR-proteins
Hypocotyl	64	17	0	102
Bast	43	12	5	99

Fig. 7.7 Classification of PR-Proteins (**a**) and Leucine rich repeat (LRR)-motif containing proteins (**b**) into functional categories. The horizontal bar chart depicts relative abundance of each group and the table below each graph represents absolute number of expressed gene in two different tissue-types

7.6.2 Genomics for Identifying Genes Involved in Resistance to Stem Rot Disease

Among all the diseases of jute, stem rot caused by *M. phaseolina* is the most prominent and destructive one. There are reports of some well-known basic pathways which tend to be upregulated or activated during defense response in general, like cell wall biosynthesis, production of reactive oxygen species, programmed cell death, synthesis of gaseous phytohormones such as ethylene, jasmonic acid, (JA), salicylic acid (SA) and abscisic acid (ABA) and hypersensitive response (HR) (Biswas et al. 2014). They also identified 1715 contigs in disease inoculated plants. Of these, 158 were expressed in response to abiotic or biotic stress, about 22% being involved in biotic stress tolerance. Gene ontology analysis revealed that the majority of these

genes were hydrolases, transferases, protein binding factors and molecular transporters. These genes were involved in several gene ontology pathways, namely, "hydrolase", "oxidoreductase", "secondary metabolic process", "cellulose and pectin containing cell wall" and "lyases", being overexpressed in disease inoculated plants. The study also identified 22 miRNAs that could be master regulators of the systemic acquired resistance (SAR) pathways. A number of disease resistance genes from jute could be placed on the KEGG 'plant-pathogen interaction pathway' (Table 7.3). Additionally, we also classified the LRR containing proteins, a well-recognized class of proteins implicated in plant disease resistance (McHale et al. 2006, Lee and Yeom 2015), into different functional categories (Fig. 7.7b) and observed that most of the LRR proteins belong to PR-proteins. Kabir et al. (2021) identified 119 APETALA2/Ethylene-Responsive Factors (*AP2*/ERF) genes. They observed that a group of different transcription factors, namely *CoERF*-01, *CoERF*-39, *CoDREB*-18, *CoDREB*-23 and *CoDREB*-13 were downregulated in stem rot infected tissues, which indicate possible involvement of these genes in disease signal transduction pathway. Three genes, *CoDREB*-01, *CoDREB*-28 and *CoDREB*-30 were upregulated, which might be involved in response to pathogen induced stress.

7.6.3 Genomics for Deciphering Systemic Acquired Resistance

Up-regulation of a large number of PR-proteins in a cohort manner is a key characteristic feature of SAR, a well-established defense strategy of plants against necrotrophs, which does not involve HR mediated cell death (Durrant and Dong 2004). There is an intricate balance of plant defense system maintained by interplay of NPRs and PIs. Plants have two distinct defense mechanisms, one against biotrophs (pathogens that requires live host for nutrient acquisition) and the other against necrotrophs (pathogens that acquires nutrition form dead host). Defense against biotrophs is mediated by SA signaling where NPR1 functions as master regulator and the process is culminated by HR-mediated death of infected cell. While on the other hand, defense against necrotrophs is regulated by JA signaling which is culminated by up-regulation of PI-I and PI-II group of proteins, which do not induce cell death but owing to their antimicrobial activity make the cellular environment of the host inhospitable for the necrotrophic pathogen (Rahman et al. 2012). The intricate balance between two defense mechanisms is maintained by transcription factor *TGA1* which is, in turn, regulated by *NPR1* master regulator and suppresses JA dependent defense signaling during biotrophic infection (Rahman et al. 2012). While another transcription factor *SNC1*, which we found to be expressed specifically in bast tissue in our transcriptome data, is reported induce *TGA1* mediated, but *NPR1* independent, upregulation of defense signaling (Rahman et al. 2012) and hence, might be associated with defense against necrotrophs.

7.6.4 Deciphering Role of Chitinase in Resistance

Chitinases are well-annotated class of genes implicated in defense against fungal pathogens. These are the group of protein which disintegrates chitin—the prime polysaccharide component of fungal cell wall (60% w/w of the cell wall)—by enzymatic hydrolysis and limits the fungal infection (Pusztahelyi 2018). In fact, four (PR-3, PR-4, PR-8 and PR-11) out of 17 defined families of PR-proteins contain different types of chitinases (Moosa et al. 2018). The hypocotyl transcriptomes of jute cv. JRC 212 contains 12 chitinases. Evidence of up-regulation of plant chitinases in response to *M. phaseolina* infection has also been reported in several plants (Saima and Wu 2019).

7.7 Brief Account of Molecular Mapping of Resistance Genes and QTLs

Genetic mapping of resistance to fungal pathogens using molecular markers is a well-established approach to identify additive and dominance components of genetic effects and also to identify molecular markers linked to the trait in concern to aid MARB. Since *C. olitorius* is the principal cultivated jute species, development of resistant cultivar is crucial in *C. olitorius* to avert major economic loss from stem rot infection. The genetic basis of resistance to this disease is not well investigated, and only a few resistant genetic stocks have been identified till date (De and Mandal 2012). A study in *C. olitorius* reported quantitative inheritance exhibiting presence of both additive and dominant gene actions (De and Kaiser 1991). Quantitative inheritance of resistance to *Macrophomina* is observed in many crop species. This approach has been a major strategy to combat *Macrophomina* in several crop species including cowpea, sorghum and bean. Only a few reports of genetic mapping and quantitative trait loci (QTL) identification are available in jute. A few linkage maps have been created in *C. capsularis*, using randomly amplified polymorphic DNA (RAPD), sequence-related amplified polymorphisms (SRAP), inter-simple sequence repeats (Chen et al. 2014) and single nucleotide polymorphism markers (Biswas et al. 2015), but no QTL was placed on these maps. QTLs for fiber yield and associated characters on SSR and restriction-site-associated DNA (RAD) based linkage map have been identified in *C. olitorius* (Das et al. 2012; Topdar et al. 2013; Kundu et al. 2015).

Plants harbor a large array of defense related genes including pathogen specific active resistance (R) genes, RGAs as well as genes involved in HR including synthesis of pathogen inducible proteins (PR-proteins) and modulation of oxidative burst. Among these, two groups of genes are present in large families (superfamilies), the R gene families sharing common nucleotide binding site-Leucine rich repeat (NBS-LRR) motif encoding sequences and the peroxidase gene superfamily that comprise of a large number of plant peroxidase genes (POGs). These genes are often distributed

as gene clusters in particular genomic regions in plant genomes (Chen et al. 2015). Many of the candidate R genes and RGAs have been mapped and found to be linked with resistance phenotype against a number of pathogens (Marone et al. 2013; Chen et al. 2015).

The first linkage map for locating resistance to stem rot disease was developed by Mir et al. (2011) using 69 F_2 from a cross of CIM-036 (resistant) and JRC-412 (susceptible). They used RAPD and SCAR (sequence characterized amplified region) markers from SSR loci and identified two linkage groups LG1 (seven markers) and LG2 (two markers). In our study (Satya et al. 2016), *C. olitorius*, OIN 154 was used as donor for resistance by backcrossing to high yielding cultivar JRO 204 as a recurrent parent. A backcross mapping population was constituted comprising of 120 BC_1F_2 genotypes. Under a project funded by Department of Biotechnology, Government of India, a total of 88 SRAP primer combinations were screened for polymorphism among the parents. Likewise, 110 SSR, 20 RGA and 12 POG markers were also screened for polymorphism. A genetic map was constructed carrying 66 SRAP, 11 SSR, 6 RGA and 6 POG markers (Fig. 7.8). The number of markers in different linkage groups (LGs) ranged from 3 (LG7) to 21 (LG1). Chromosome-wide marker interval varied from 3.3 cM (LG2) to 19.2 cM (LG7), with a genome-wide marker interval of 8.3 cM. The distribution of SRAP markers was random over all the LGs, but LG4 comprised of only SRAP markers. The distribution of SSR markers was also random, though no SSR marker could be mapped on LG4. However, the distribution of RGA and POG markers was non-random, showing clustering of RGA and POG loci on certain LGs. The RGA markers were present on LG2, LG3 and LG5. Similarly, the POG markers were also distributed on LG2, LG3 and LG5, showing clustered distribution on LG5 (Satya et al. 2016).

Based on multiple QTL mapping, a total of three QTLs were identified on LG3 and LG5. Of these, *qRM-3-1* was a major disease resistance QTL, explaining 29.4% of the phenotypic variance with a log of odd's ratio (LOD) peak of 11.04 on LG3. A second QTL, *qRM-3-2* was mapped on LG3 with LOD values of 2.0, explaining

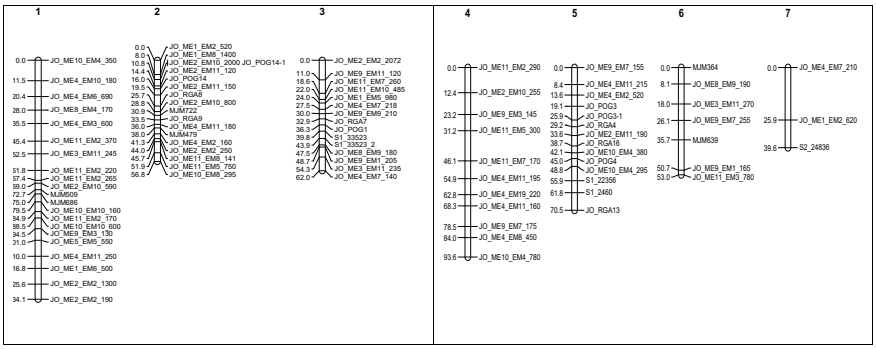


Fig. 7.8 Genetic linkage map of seven LGs of *C. olitorius* constructed with SRAP, RGA, POG and SSR markers. The genetic distances (cM) from top to bottom are indicated to the left of the LGs

4.4% of the phenotypic variance, respectively. A third QTL, *qRM-5-1* was identified on LG5, with a peak at 45.0 cM. Large effect QTLs for resistance to *Macrophomina* was also reported by Reddy et al. (2008) explaining up to 19.29% of the phenotypic variation.

While marker development for disease resistance in jute has made a moderate progress, little effort has been directed towards identification of markers lined with insect-pest resistance. Ghosh et al. (2010) developed a linkage map by crossing *C. olitorius* variety O-7/95, tolerant to yellow mite attack and var. O-72, sensitive to mite attack. They genotyped 150 F_2 plants using 88 SSR markers, phenotyped using a mite tolerability index (MTI) and constructed a genetic map comprising of 21 SSR markers distributed over five LGs and proposed three markers to be linked with MTI based on chi-square test. However, the trait was not mapped on any of the LGs and is consequently of little further use.

7.8 Brief on Genetic Engineering for Resistance Traits

Development of transgenic organisms has become a routine practice in the field of plant molecular biology for the purpose of gene functional validation. Apart from this, commercial transgenic crops have also shown promise worldwide in the direction of food and livelihood security. In India, although transgenic food crop has still not obtained approval for commercial cultivation, but a lone fiber crop—transgenic Bt-cotton—has shown its worth by propelling the country to become a major cotton producer in the world. But transgenics have not flourished much in case of jute—the second-most important commercial plant fiber grown in the world. The major obstacle for producing transgenic jute lies in its extreme recalcitrance to tissue culture mediated plant regeneration (Saha and Sen 1992; Sarker et al. 2008; Saha et al. 2014). Nevertheless, efforts have been strengthened in recent times to optimize transgenic protocol in jute and also transfer desirable traits. The first report of jute transformation dates back to the year 2008 when two different protocol of jute transformation were proposed by groups working at University of Dhaka, Bangladesh. One of these two studies reports *Agrobacterium tumefaciens*-mediated transformation of petiole-attached cotyledons and mature embryo explants obtained from two different varieties of white jute (Sarker et al. 2008). However, this study validated positive transformants only by GUS (*β-glucuronidase*) expression and polymerase chain reaction. Sajib et al. (2008) developed a tissue culture independent method of transforming *C. olitorius* utilizing *in planta* transformation technique. Juvenile jute plants were subjected to transformation by pricking with fine needle at the shoot apical meristem followed by agroinfiltration (Sajib et al. 2008). Production of transgenic jute harboring artificial microRNA (amiRNA) and hairpinRNA (hpRNA) targeting downregulation of monolignol biosynthetic genes were achieved using this protocol (Shafrin et al. 2015, 2017). Two different transformation protocols for *C. capsularis*) were reported from Indian Institute of Technology, Kharagpur, India. Both of these protocols reported transformation of popular white jute variety JRC 321. One of these

two protocols reported production of a transgenic hairy root system carrying *gusA* reporter gene in jute through *A. rhizogenes*-mediated root infection, which can further be used as a continuous source of explant for obtaining transgenic plants (Chattopadhyay et al. 2011). While the other protocol reported successful stable transformation of jute with bialaphos resistance gene (*bar*) by using particle bombardment of apical meristematic tissues of one day-old germinated seedlings (Bhattacharyya et al. 2015). Stable transformation technique for *C. capsularis* var. JRC 321 through *A. tumefaciens*-mediated shoot tip transformation is also available (Saha et al. 2014). This protocol was used to introduce Cry1Ab/Ac δ-Endotoxin (Majumder et al. 2018a), rice *chitinase11* (*OsChi11*) and *Phosphinothricin N-acetyltransferase* (*bar*) genes in jute (Majumder et al. 2018b) for resistance to lepidopteron pests, stem rot disease and herbicide (Phosphinothricin), respectively. The major genetic transformation efforts for introducing biotic stress tolerance in jute ARE presented in Table 7.5.

An important trait which has been incorporated in transgenic jute is resistance to lepidopteran insect-pests. Transformation of white jute with synthetically fused *cry1Ab/Ac* gene of *Bacillus thuringiensis* (Bt) resulted in increased protection to jute- semilooper (*Anomissa bulifera* Guenee), hairy caterpillar (*Spilarctia obliqua* Walker) and indigo caterpillar (*Spodoptera exigua* Hubner). The transgenic lines expressed Cry1Ab/Ac endotoxin in the range of 0.16 to 0.35 ng/mg of leaf which resulted in high insect mortality (66–100% in case of semilooper and hairy caterpillar, while 87.50% in case of indigo caterpillar) in detached leaf assay (Majumder et al. 2018a). Apart from this, transgenic expression of rice chitinase (*Chi11*) and *Phosphinothricin N-acetyltransferase* (*bar*) genes in white jute has shown promising results in controlling *M. phaseolina* infection as well as imparting herbicide tolerance in jute. The transgenic plant harboring duel-gene construct of *Chi11* and *bar* not only demonstrated high chitinase induced antifungal activity against *M. phaseolina* in detached leaf assay but also successfully withstood 10 mg/L glufosinate ammonium in culture media as well as glufosinate herbicide (0.25%) (Majumder et al. 2018b). In this study, a 473 bp long cDNA of rice chitinase 11 (X54367) was cloned downstream of constitutive promoter CaMV35S and shoot tips of jute was transformed through *Agrobacterium* mediated transformation method. Crude protein extract obtained from T_2 transgenic plants were found to degrade chitin much more effectively than control non-transgenic plants both in gel diffusion assay and in in-solution assay. Results of whole plant antifungal bioassay carried out in transgenic- and non-transgenic-plants at about 80–90 days after sowing also demonstrated much reduced lesion length (typical to stem rot) in transgenic plants compared to non-transgenic strategies. Finally, better quality of fiber was also observed after retting from transgenic plants compared to non-transgenic controls.

7.9 Future Perspectives

Despite presence of numerous pests and diseases causing economic damage of jute crop at various growth stages, progress in genomic research on the biotic stress

Table 7.5 Summary of the jute transformation techniques developed

Transformed species	Variety used	Transformation technique	Explant	Gene transferred	References
C. capsularis	CVL-1 and CVE-3	*Agrobacterium tumefaciens*-mediated	Petiole-attached cotyledons and mature embryo	*β-glucuronidase* (*gusA*)	Sarker et al. (2008)
C. olitorius	O-72	*Agroinfiltration*	Shoot apical meristematic region	*β-glucuronidase* (*gusA*)	Sajib et al. (2008)
C. olitorius	O-9897	*Agroinfiltration*	Shoot apical meristematic region	5H–amiRNA and C3H–amiRNA	Shafrin et al. (2015)
C. olitorius	O-9897	*Agroinfiltration*	Shoot apical meristematic region	COMT-hpRNA and C4H-hpRNA	Shafrin et al. (2017)
C. capsularis	JRC 321	*A, rhizogenes* mediated	Root tip	*β-glucuronidase* (*gusA*)	Chattopadhyay et al. (2011)
C. capsularis	JRC 321	Particle bombardment	Apical meristematic tissues	Bialaphos resistance gene (*bar*)	Bhattacharyya et al. (2015)
C. capsularis	JRC 321	*A. tumefaciens*-mediated	Shoot tip	*β-glucuronidase* (*gusA*)	Saha et al. (2014)
C. capsularis	JRC 321	*A. tumefaciens*-mediated	Shoot tip	Cry1Ab/Ac δ-Endotoxin	Majumder et al. (2018a)
C. capsularis	JRC 321	*A. tumefaciens*-mediated	Shoot tip	*OsChi11* and *bar*	Majumder et al. (2018b)

tolerance in jute is limited. However, a number of genes associated with resistance to various biotic stresses have been identified recently in jute. Future research should focus on development of markers linked to the resistance genes and use of these markers in breeding for pest and disease resistance. However, as jute is not preferred by many insects and diseases and have inherent high-phenolics and flavonoid content, the molecular mechanism of non-preference of jute as a host by major pests and pathogens can be an interesting research area to identify novel R genes. For example, jute is not attacked by most of the mold fungi, and is little affected by leaf spot causing pathogens. Functional characterization of such unique R genes from jute can open up new avenues for genomics assisted improvement in biotic stress resistance in other crops. Furthermore, due to its unique geographical adaptation and restrictions in interspecific genetic exchange, jute can be an ideal system for studying host–pathogen coevolution in a relatively isolated environment.

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Chapter 8
Genomic Designing for Biotic Stress Resistance in Mulberry

K. Vijayan, G. S. Arunakumar, B. N. Gnanesh, Prashanth A. Sangannavar, A. Ramesha, and W. Zhao

Abstract Mulberry has several economic uses and all parts of the plant have one or another use and the leaves are used as a sole food for monophagous silkworm (*Bombyx mori* L.). Production of premium silk in the sericulture industry is directly associated with quality mulberry leaves production. However, mulberry is affected by various pathogens and pests like fungi, bacteria, virus, nematodes and cause considerable crop loss. Mulberry genetic improvement through traditional breeding relies on the availability of compatible genetic resources carrying the genes of interest. Mulberry being highly heterozygous and with a long generation gap and also due to genetic drag, it is difficult to introgress genes from wild germplasm to cultivars through recurrent back crosses. Nonetheless, significant works have been made to develop disease resistant lines/varieties through germplasm screening and identification of suitable parents for breeding. To speed up the breeding programme, DNA markers tightly linked to the trait of interest are used for early and reliable selection of desirable genotypes through the process of Marker Assisted Selection (MAS). The major limitations MAS include the sparse distribution of markers, large genetic intervals between the markers and the trait genes, many QTLs identified as minor QTLs that show a small effect on phenotypic variations and the low success rate in validating identified QTLs in different genotypes and environments. Further, to develop high resolution maps to identify markers with the tight association, more abundantly available markers like Single nucleotide polymorphisms (SNPs) have to be developed. Such effort is currently in progress at different research organizations

K. Vijayan (✉) · P. A. Sangannavar
Central Silk Board, BTM Layout, Madiwala, Bengaluru, Karnataka 560068, India
e-mail: kvijayan01@yahoo.com

G. S. Arunakumar · B. N. Gnanesh
Central Sericultural Research and Training Institute, Manandavadi Road, Srirampura, Mysuru, Karnataka 570008, India

A. Ramesha
Seribiotech Research Laboratory, Kodathi, Carmelram Post, Bengaluru, Karnataka 560035, India

W. Zhao
Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang 212018, Jiangsu, China
e-mail: wgzsri@126.com

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across the globe. This chapter deals with current initiatives of genomic designing such as gene editing, and recent concepts and strategies developed for biotic stress resistance in mulberry.

Keywords Mulberry · Biotic stress · Genomics · Transcriptomics · Marker

8.1 Introduction

Mulberry is a fast-growing deciduous, deep rooted, woody perennial plant that originated in the sub-Himalayan tract and afterward spread across the world (Le Houerou 1980; Rodríguez et al. 1994). Currently, mulberry is seen growing in Asia, Europe, Africa, and America (Sanchez 2000a, b). Taxonomically *Morus* L. belonged to the family Moraceae under the order Urticales; molecular phylogenic studies placed Moraceaeunder the order Rosales (Ii 2003). The species delimitations of the genus *Morus* are yet to be solved as the morphological traits vary considerably under different growing conditions and stages of development and also due to uncontrolled natural hybridization among the species most of the accessions being maintained by different institutes are of natural hybrids. The greater success rate with cross-species reproduction indicates that these species have comparable genetic relationships and therefore, 'species' status needs to be studied additionally (Wang and Tanksley 1989). Zeng et al. (2015) used the complete ITS data for reconstruction of a phylogenetic tree and proposed that the *Morus* genus should be classified into eight species, including *M. alba, M. celtidifolia, M. insignis, M. mesozygia, M. nigra, M. notabilis, M. rubra* and *M. serrata.* This observation is further supported by an investigation with genome-wide SNP markers, which shows that utilizing morphological data may not be sufficient to envisage taxonomic inferences in mulberry varieties (Muhonja et al. 2020). Mulberry grows luxuriously in a flat land with fertile, well drained, deep, and clayey to loamy, porous soil with good moisture holding capacity and a pH ranging from 6.5 to 6.8. Mulberry is reported to have a moderate level of tolerance to salinity and drought (abiotic stresses). Since it is a plant of temperate origin, during the winter months it shed the leaves and the buds remain in dormant conditions. Morphologically, the leaves are alternate, simple, petiolate, stipulate, entire or lobed and inflorescences are catkin with drooping or pendent peduncle bearing unisexual flowers develop from axillary dormant buds immediately after the colder months. The long male catkins bear loosely arranged florets but the short female catkin bears compactly arranged florets with bifid feathery stigma and ovary is one-celled. Pollination is anemophilous and the fruit is a sorosis and the colour of the fruit diverges from white to lavender to black depending on the species. Seed is brown or light yellow, at the micropylar region, it is a nearly flat surface with oval shaped. In general, under room temperature seeds keep viability only for a few weeks, but if kept under controlled humidity and temperature, the viability may be extended for 3–6 months. The ideal temperature for germination is 28–30 °C. Since mulberry is propagated mainly through vegetative means, plants in different ploidy

such as haploid (*M. notabilis:* 2n = 14), diploid (*M. indica and M. alba:* 2n = 28), triploid (*M. bombysis:* 2*n* = 42), tetraploid (*M. boninensis, M. cathayana* and *M. laevigata:* 2*n* = 56), hexaploid (*M. serrata* and *M. tilaefolia:* 2*n* = 84), octoploid (*M. cathayana:* 2*n* = 112) and decasoploid (*M. nigra:* 2n = 308) are present in nature (Vijayan et al. 2009). However, diploids and triploids are preferred for cultivation, especially for sericulture purposes.

8.1.1 Economic Significance

Mulberry has several economic uses and all parts of the plant have one or another use. However, the most priced one is the leaf, which is used for feeding the monophagous silkworm *Bombyx mori* to produce silk. Mulberry leaf is also used as cattle feed as it is highly nutritious, palatable and digestible (Uribe and Sanchez 2001). The fruit is a berry that has numerous medicinal compounds like vitamins, minerals, aminoacids, carotenoids, flavonoids, anthocyanins, resveratrol, zeaxanthin, lutein, carotenes are (alpha and beta), apigenin, morin, quercetin, luteolin, caffeic acid, rutin, umbelliferone, gallic acid, chlorogenic acid cyanidin-3-O-β-D-glucopyranoside and kaempferol etc. and is consumed in raw and also processed into jelly, juice, fruit powder, sauce, tea, cakes, Mouro, wine, etc. (Asano et al. 2001; Hassimotto et al. 2007; Ercisli and Orhan 2007; Singhal et al. 2009; Yigit et al. 2010; Yang et al. 2010; Arfan et al. 2012). It is used as a worming agent, as a treatment for dysentery, and as an expectorant, hypoglycaemic, laxative, odontalgic, anthelmintic and emetic (Kang et al. 2006; Chen et al. 2006). Keeping these properties of mulberry fruit is consumed fresh. The barks of the root and stem are a good source of phenolic compounds such as morin, rutin, maclurin, resveratrol, isoquercitrin and also used for anthelmintic, purgative and astringent purposes (Chang et al. 2011). Mulberry wood is very hardy and smooth; hence, it is exploited for manufacturing sports articles, house buildings, turnery items, agricultural implements, spokes, furniture, poles and carts. In Europe and the United States, Mulberry is used for landscaping (Tipton 1994) as it is a perennial crop with good foliage, root-spread it provides green cover and it helps to soil conservation.

8.1.2 Effect of Biotic Stress on Yield and Quality

Mulberry is affected by various pathogens and pests like fungi, bacteria, virus, nematodes and cause considerable crop loss. About 300 pests species of insect and non-insect are known to occur on mulberry (Kotikal 1982). The important fungal diseases of mulberry are the leaf spot caused by *Cercospora moricola*, powdery mildew by *Phyllactinea corylea*, leaf black rust by *Ceroteliumfici*, red rust by *Aecidium mori*, twig blight by *Fusarium pallidoroseum*, root rot by *Resellinianecatrix* and *Helicobasidiummompa*, stem canker and die back by *Botryodiplodia theobromae*, stem rot

by *Polyporushispidus* and *Ganoderma applanatum*, collar rot by *Phomamororum*, stem blight by *Phomaexigua* and bud blight by *Fusarium lateritium* are well known from different parts of the world. These pathogens inflict heavy crop loss in the form of mortality of plants; reduction in the quantity and quality of leaf and fruit yield. Since the quality mulberry leaf is required for better silkworm growth, the leaf with poor nutritive values impairs the cocoon yield and quality of the silk (Vijaya Kumari 2014) as detailed elsewhere.

8.1.3 Increasing Population and Climate Change Scenario

The change in the climate caused by an increase in the average temperature of the atmosphere is defined as global warming due to the increase in the concentration of carbon dioxide (CO_2) because of the industrialization, urbanisation, burning of fossil fuel, deforestation and so on. Global warming affects the ecosystem, agriculture and human beings to a considerable degree. It is estimated that the atmospheric temperature increased at a rate of 0.3% per decade or 5 °C in 170 years but the increase may double by the end of the twenty-first century affecting agricultural production and food security. Global warming can result in severe weather conditions such as drought, unseasonal rain, prolonged winter, the emergence of new pests and diseases etc. and these jointly affect plant growth and development (Jiang et al. 2016). The changes in air temperature can alter the availability of moisture in the soil as well as the atmosphere affecting the growth and reproductive cycle of the crop. The survival, distribution and host preference of pest and other pathogens may also undergo drastic changes necessitating the adoption of new crop management practices (Krupa 2003). Further, the exploding population and loss of arable lands through urbanization and salinization necessitate the utilization of hereto unutilized and underutilized lands for agriculture. Mulberry cultivation, thus, has to be expanded to such areas.

8.1.4 Logical of Genome Designing and Bottlenecks of Traditional Breeding

Mulberry genetic improvement through traditional breeding relies on the availability of compatible genetic resources carrying the genes of interest. Mulberry being highly heterozygous and with a long generation gap and also due to genetic drag, it is difficult to introgress genes from wild germplasm to cultivars through recurrent back crosses. Nonetheless, efforts have been made to develop disease resistant varieties through germplasm screening and identification of suitable parents for breeding. Maji et al. (2009) screened 85 germplasm lines for resistance to powdery mildew and found *M. multicaulis, M. australis*, Italian and Thailand lobed were highly resistant, another nine lines resistant, yet another 43 lines were moderately resistant to

the disease complex. Likewise, Prabhakar et al. (2015) screened 100 accessions and identified 82 genotypes showing immunity to thrips. Among them, three accessions were resistant to mealy bugs *Meconellicoccus hirsutus* Green and 20 were thrips *Pseudodendro thrips mori*. In another effort, twenty mulberry cultivars screened for disease response to *M. phaseolina* and found all are susceptible (Chowdary 2006), though Hongthongdaeng (1987) reported in Thailand, mulberry cultivar Pai and F_1 hybrids Pai × Noi (6, 18, 33 and 36) displayed resistance to root rot disease indicating root rot-resistant genes in mulberry. Therefore, it is necessary to collect as many germplasm as possible from different countries and screen them for resistance. But screening of huge germplasm accessions for biotic resistance is resource demanding and time consuming. Thus, baring a few successes such as the development of "Shimgang" resistance to popcorn disease (Sung et al. 2016) not much success could be obtained in developing disease resistant mulberry through traditional breeding (Vijayan et al. 2010). Further, to develop and release a mulberry variety employing traditional breeding methods take not less than 15 years. Another major bottle neck for the development of desired mulberry varieties is the lack of detail on the genetic control of most of the disease resistance. Therefore, it is intricate to transfer the resistance from other genotypes through introgression (Vijayan et al. 1997, 2008). To resolve these troubles proper understandings of (a) the chromosomal location of these loci, (b) the number of genetic factors (loci) influencing the expression of the traits, (c) pleiotropic effects, (d) the relative size of the contribution of individual loci, (e) variation of expression of individual factors in different environments and (f) epistatic interactions among genetic factors, are essential. Only detailed genomic and transcriptomic analyses can provide such information.

8.2 Description of Different Pathogens Causing Biotic Stress in Mulberry

The biotic stress on plants is caused by living organisms that include mycoplasma, virus, bacteria, fungi, nematodes, insects and other animals that feed on different plant parts causing damage to the plant. The major diseases and pests that cause considerable damage to mulberry are described here under.

8.2.1 Major Diseases in Mulberry

8.2.1.1 Fungal Diseases

Leaf Spot

This is one of the major foliar diseases established during the rainy and winter seasons. It affects considerable losses in vegetative yield and degrades leaf quality (Philip et al. 1991; Peris et al. 2012). The disease causes a direct leaf yield loss due to defoliation is 5–10% and the destruction of leaf area is of 20–25% as additional loss (Sukumar and Ramalingam 1989; Srikantaswamy et al. 1996). The disease adversely affects moisture, protein, chlorophyll and total sugar contents in the leaves (Srikantaswamy et al. 1996). Hence, the economy of sericulture is severely affected by foliar diseases. Further, feeding the silkworm with diseased leaves affects the commercial characters of the cocoons (Nomani et al. 1970; Sullia and Padma 1987). There are many fungal pathogens causing leaf spot of mulberry and they are discussed below.

Cercospora moricola

The most common symptoms of leaf spot caused by *C. moricola* are the appearance of small brownish, irregular spots on the leaves in the initial stages which gradually increase in size and turn dark brown. As in severe cases, the dead tissues from the spot fall off resulting in the formation of a shot hole lining yellow circle around it. Severely affected leaves become yellowish and fall off prematurely (Fig. 8.1a). The disease is very common in the rainy season (Siddaramaiah et al. 1978) and causes a leaf yield loss of 10–20% besides making the leaf nutritionally poor due to less moisture, proteins and sugars. Rearing with infected leaves affects the health of silkworms and in turn quality and quantity of cocoons (Sikdar and Krishnaswami 1980).

Setosphaeria rostrata

Setosphaeria rostrata caused by *S. rostrata* appearing as specks at initial stages and the spot enlarges into irregular shape with brownish center surrounded by a yellow halo as the disease becomes severe. The spot size ranges from 0.4 to 1.5 cm. These spots are inter-veinal in character and a few of the spots starts from the leaf margins, expand and combine, leading to blightened appearance (Fig. 8.1b). Rigorously affected leaves turn out to be yellowish and fall prematurely (Arunakumar et al. 2019a).

Fig. 8.1 Different types of leaf spot in mulberry. **a** Leaf spot caused by *Cercospora moricola* (arrows). **b** Leaf spot caused by *Setosphaeria rostrata* (arrows). **c** The shot hole symptoms caused by *Nigrospora sphaerica*

Nigrospora sphaerica

The shot hole symptoms caused by *N. sphaerica* appears specks initially (2 to 4 mm), characterized by circular or irregular shape with a brownish center surrounded by a yellow halo. These spots began from the leaf margin and gradually enlarged and coalesced to form large lesions (Fig. 8.1c). Later, the larger lesion dries and fell out and appears as a shot hole (Arunakumar et al. 2019b).

Powdery Mildew

Powdery mildew disease caused by *Phyllactinia corylea* is the most common and widespread disease, causes a direct leaf yield loss due to defoliation is 5–10% and by the destruction of leaf area is of 20–25% as additional loss (Sukumar and Ramalingam 1989; Teotia and Sen 1994). It is an obligate, biotrophic parasite of the phylum Ascomycota of Kingdom Fungi and belongs to the order Erysiphales. The conidia of fungus spread through wind, when it lands on the leaf surface, it germinates and mycelium is observed as a white mat on the abaxial surface of the leaf. (Fig. 8.2a). In addition to the loss of leaf yield, the quality of the mulberry leaf is also affected, when such low quality leaves are fed to silkworms, cocoon productivity and quality reduces. (Manimegalai and Chandramohan 2007). It is also observed that feeding of mildew affected leaves to silkworm adversely affects silkworm growth and development resulting in poor cocoon yield and silk quality. Infection of disease reduces leaf yield qualitatively and quantitatively and feeding of infected leaves to silkworm prolongs larval duration (Qadri et al. 1999). Spraying of Dianocap 0.2% can control the disease with a safe period of 10 days for silkworm feeding (Gunasekhar and Govindaiah Datta 1994).

Leaf Rust

It is caused by *Ceroteliumfici* (Cast). Arthur and also known as *Peridiospora mori* Barclay (Prasad et al. 1993). Leaf rust is also called black rust belongs to the family Uredinaceae under the order Uredinales in the class Imperfect fungi. The pathogen produces numerous pin-head-sized circular to oval, brownish to black eruptive lesions/spots on the surface of the leaves (Fig. 8.2b). The affected leaves turn yellowish, under severe disease conditions, the leaves wither off prematurely. The disease appears on mature leaves and can cause a crop loss of 5–10% and also affects the quality of the leaf reducing moisture, crude protein, sugars and total sugars in the infected leaves (Sengupta et al. 1990; Philip and Govindaiah 1994). Out of fourteen fungicides evaluated, Ametoctradin 27% + Dimethomorph 20.27% SC, a combi-product was found highly effective at all the concentrations tested and showed the least spore germination (0.62%) at 0.1% concentration. Similarly, chlorothalonil at 0.3%, a non-systemic fungicide that is currently recommended for management of leaf rust of mulberry also found on par with other effective fungicides and showed

Fig. 8.2 Different types of foliar diseases in mulberry. **a** Powdery mildew caused by *Phyllactinia corylea.* **b** Leaf rust caused by *Cerotelium fici*. **c** Twig blight caused by *Fusarium lateritium*

2.06% spore germination. The effective novel fungicide molecule could be used for the management of leaf rust after evaluation in field condition and bio-assay with silkworm. It is the combination of both systemic and contact mode of action, that becomes an alternative to the existing fungicide in leaf rust disease management (Poojashree et al. 2021).

Red Rust

Red rust is caused by *Aecidium mori* Barclay. This disease causes up to 15% leaf loss (Teotia and Sen 1994; Prateesh Kumar et al. 2000). It belongs to a family Pucciniaceae under the order Puccinales in the class Pucciniomycetes. Upon infection, numerous round shiny spots appear on both surfaces of the leaf which later protrude gradually into yellow. The affected young shoots become swollen and curl up abnormally with densely and slightly protruded yellow spots on the malformed buds. The disease can be controlled by applying 0.25% wettable powder of 5% zinc or 0.75 solutions of 5% wettable powder of nitrite are reported effective for controlling the disease with a safe period of 7 days (Maji 2003).

Twig Blight

It is caused by *Fusarium lateritium* that belongs to the order Moniliales of Class Deuteromycetes. The diseased plants show bushy appearance with profuse growth of auxiliary branches, leaves show marginal browning/blackening at the beginning and complete burning in the later stages resulting in severe defoliation. Affected, branches have black longitudinal lesions which later lead to the splitting and drying of branches (Fig. 8.2c). The disease management can be taken by application of Foltaf 80 W and Dithane M-45 as a foliar spray.

Root Rot

It is a major limitation in mulberry farming due to its epidemic nature and potential to kill plants completely, resulting in leaf yield loss of up to 31.5% (Chowdary and Govindaiah 2009). A range of root rot such as dry root rot caused by *Fusarium solani* (Mart.) Sacc., *F. oxysporum* Schlecht. (Manomohan and Govindaiah 2012), black root rot—*Botryodiplodia theobromae* Pat. [syn. *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl.] (Sowmya et al. 2018) and charcoal root rot—*Macrophomina phaseolina* (Tassi.) Goid. [syn. *Rhizoctonia bataticola* (Taub.) Butler] (Muthuswami et al. 2011) have been reported (Sharma et al. 2003). All the root rot diseases can be controlled by the application of *Rot-fix* a target specific plant based formulation (Pratheesh Kumar 2019). The various types of root rot symptoms are discussed below (Fig. 8.3a–d).

Black Root Rot

It is caused by *Lasiodiplodia theobromae* Pat. (Syn: *Botryodiplodia theobromae* (Pat.) Griff. and Maubl.). These fungi cause black root rot disease and incidences were reported in the southern Indian states like Tamil Nadu and Karnataka (Radhakrishnan et al. 1995; Sukumar and Padma 1999). Once the mulberry plants are weak

Fig. 8.3 Symptoms of root rot in mulberry. **a** Partial wilting of mulberry shoots. **b** Root rot affected dried shoots. **c** Complete dried plants affected. **d** Rotted roots

to infection, fungus control inside the roots rapidly multiplies in the cortical tissues and reaches up to the pith. It enters the xylem vessels and causes the death of the plants (Radhakrishnan et al. 1995; Sukumar and Padma 1999; Sharma and Gupta 2005). As the disease advances, it is observed that vascular browning and subsequent death of the plants (Sharma and Gupta 2005). The survey reports the incidence of black root rot disease in major southern sericulture states of India. Disease incidence maximum recorded in the districts of Tamil Nadu followed by Andhra Pradesh and Karnataka. The root dip method of inoculation was highly effective for germplasm screening against *L. theobromae* (Arunakumar and Gnanesh 2022).

Rhizopus Rot

It is caused by *Rhizopus oryzae,* initial symptoms of Rhizopus rot in mulberry are the roots turning to black colour and tissues becoming fragile. Infested plant leaves are characterized by yellowing wilting and defoliation, followed by decaying of roots. (Yoshida et al. 2001; Fang et al. 2011; Gnanesh et al. 2020). In severe conditions, white and cottony fungal hyphae were observed on the affected root tissues.

Dry Root Rot

In India, earlier dry root rot has been reported as violet and white root rot in mulberry (Rangaswami et al. 1976) but various surveys indicate that the disease is dry root rot caused by *Fusarium solani* and *F. oxysporum* (Philip et al. 1995c; 1997). There are reports on *Fusarium* species causing root rot disease in China and Philipines which is restricted to certain areas and occurring occasionally (Luo and Zhaoxuan 1989; Telan and Gonzales 1999). The dry root rot causing *F. solani* and *F. oxysporum* is considered to be one of the most devastating fungal pathogens, which causes root rot disease on more than 500 plant species worldwide. It is difficult to control the fungus due to its thick-walled resistant sclerotia which persist in the soil and plant debris. The initial symptoms can be seen as root bark turn black in colour due to the presence of fungal spores/mycelium and decaying of root cortex. On severity, the entire root system gets decayed and plants die. The affected plants after pruning, either fail to sprout or plant sprouted bears small and pale yellow leaves with a rough surface. Colonized sorghum grain was found to be the best method for germplasm screening against *F. solani,* and *F. oxysporum* (Arunakumar and Gnanesh 2022).

Charcoal Root Rot

Macrophomina phaseolina is the most widespread pathogen in the South Indian sericulture belt (Sowmya et al. 2018; Yadav et al. 2011). Most mulberry cultivars are prone to charcoal rot disease and can cause up to 35% leaf yield loss, reduction in leaf size, deterioration of leaf quality, and plant mortality (Chowdary 2006). These in turn adversely affect profitability in sericulture (Philip et al. 2009).

8.2.1.2 Bacterial Diseases

Bacterial Blight

It is caused by *Pseudomonas syringae* pv. *mori* that accounts for 5–10% leaf yield loss during the rainy season (Sinha and Saxena 1966). Numerous irregular water-soaked patches appear on the lower surface of leaves. In severe conditions, the leaves become curled, rotten and turn brownish black. The disease can be controlled by

uprooting and burning of the affected plants and also by application of phytobacteriomycin (0.05 to 0.1%) containing glycerine, streptomycin sulphate, streptocycline and streptopenicillin.

Bacterial Leaf Spot

This disease is caused by *Xanthomonas campestris* pv. *Mori* (Choi et al. 1989). Numerous water soaked spots initially appear on the lower surface of the leaf as soon as the rainy season starts. These spots grow gradually into brown to brownish black surrounded by a yellow halo around the spot. The application of agromycin may control the disease to a certain extent.

8.2.1.3 Nematode Disease

Root-Knot Nematode

The primary root-knot nematode parasitizing mulberry is *Meloidogyne incognita* (Kofoid and White) Chitwood which belongs to class Nematoda, order Tylenchida of family Heteroderidaeand it was reported on mulberry for the first time by Narayanan et al. (1966) from Mysuru, Karnataka, India. In India, RKN infestation is widespread and more prevalent in red sandy soils followed by red loamy soils. The severity of RKN increases with the age of the garden and the estimated leaf yield loss is up to 20%, besides affecting leaf quality (Devi and Kumari 2014). The RKN control is very difficult, because of its wide host range and its ability to survive in the soil for several years. Four races of *M. incognita* have been identified and reported across the world (Hartman and Sasser 1985). Among them, race-2 has been reported to infect the mulberry in India (Govindaiah Sharma et al. 1993). The stunted growth with marginal chlorosis and necrosis of leaves is the common symptoms of severely infected mulberry plants (Fig. 8.4a). In the root system of susceptible plants, the formation of knots/galls which are spherical or vary in size, younger galls are small and yellowish, while older galls are big and blackish brown (Fig. 8.4b, c) (Arunakumar et al. 2018). It can be managed by the application of Neemahari—a plant based product for the management of root-knot nematode in mulberry (Sharma et al. 2013).

8.2.1.4 Molecular Characterization

Classifying the fungal species just based on morphological features is not sufficient and for this purpose DNA sequence-based approaches have been widely recommended (Crous and Groenewald 2005; Bautista-Cruz et al. 2019). Precise identification of *L. theobromae* can be achieved by using the combination of two or more

Fig. 8.4 Root-knot nematode symptoms of mulberry. **a** Above ground symptoms of interveinal chlorosis. **b**, **c** Below ground symptoms showing galls on the roots of mulberry

genes, like internal transcribed spacer (ITS), β-tubulin (TUB), and translation elongation factor 1-α (TEF1) genes (Chen et al. 2013, 2021; Marques et al. 2013; Rosado et al. 2016).

For many years, *L. theobromae* was treated as a monotypic genus within the Botryosphaeriaceae (Larignon et al. 2001; Slippers et al. 2013). However, the application of DNA meta barcoding for phylogenetic analysis evidenced the existence of many additional species (de Silva et al. 2019; Rosado et al. 2016; Santos et al. 2020). Hence, it is likely that many previous findings might be inappropriately classified, in

addition, literature suggests the occurrence of hybridization between various species of *Lasiodiplodia* spp. (Cruywagen et al. 2017). For example, *L. viticola* is described as a hybrid species produced by hybridization of *L. theobromae* and *L. mediterranea* (Úrbez-Torres 2011). Transport of plant material that hosts *Lasiodiplodia* spp. to new fields/soil with autochthonous strains can stimulate the formation of new hybrid species. This implies that the taxa reported so far are not stable and highlights the need of considering multiple genes in analyzing phylogeny, along with referencing the type strains directly. This strategy can avoid misidentifications (Cruywagen et al. 2017; de Silva et al. 2019).

Several research investigations reported new species of *Lasiodiplodia* and an increasing number of first reports (Rosado et al. 2016; de Silva et al. 2019) indicating the extension of its host range highlights the need of prospecting novel *Lasiodiplodia* species associated with mulberry. There are limited data on molecular characterization of mulberry pathogens in India, and the previous characterization was based on morphology. Sowmya et al. (2018) used RAPD and SSRs to study the genetic variability, among the ten isolates of *L. theobromae* causing Black root rot of mulberry, similarly, Pappachan et al. (2020) characterized only one isolate of *L. theobromae* (Table 8.1).

8.2.2 Major Insect and Pests in Mulberry

8.2.2.1 Sap Suckers

Pink Mealybug

Tukra is the name of the malformation of the leaves and shoots of mulberry caused by the Mealybug (*Maconellicoccus hirsutus* (Green)). The main symptoms of the disease are retardation of the growth of shoot shows and appearance of dark green wrinkled leaves initially and later turn into pale yellow. Due to the stunted growth, the shoot and leaf form a hard and compact structure that cannot be opened without breaking away the crisp leaves (Fig. 8.5a, b). Because of the stunted growth, the leaf yield is tremendously reduced and the leaves become nutritionally very poor. The pink mealy bugs are one of the major pests of mulberry, causing severe damage and recurring loss in leaf yield of about 3000–6000 kg/ha/year (Kumar et al. 1989). Tukra appears mostly during the summer months and can be controlled to a certain extent by removing and burning the affected shoots, spraying chemicals like DDVP (Nuvan) prepared in detergent solution and releasing of the natural enemies like *Cryptolaemus montrouzieri* @ 250 beetles/acre and *Scymnuscoccivora* @ 500 beetles/acre (Table 8.2).

Table 8.1 List of mulberry fungal pathogens identified using gene specific markers

Pathogen	Disease	Marker	Geographic region	References
Setosphaeria rostrata	Leaf spot	ITS	CSRTI-Mysore, Karnataka, India	Arunakumar et al. (2019a)
Nigrospora sphaerica	Shot hole Disease	ITS	Santai County, Sichuan Province, China	Chen et al. (2018)
Nigrospora sphaerica	Shot hole leaf spot	ITS	CSRTI-Mysore, Karnataka, India	Arunakumar et al. (2019b)
Rhizopus oryzae	Rhizopus root rot	ITS, ACT, TEF	South India	Gnanesh et al. (2020)
Lasiodiplodia theobromae	Root Rot	ITS, β-tubulin	Kolasib, Mizoram, India	Pappachan et al. (2020); Gnanesh et al. (Unpublished)
Lasiodiplodia theobromae	Root Rot	ITS, EF1-α	Guangxi Province, China	Xie et al. 2014
Phyllactinia corylea	Powdery mildew	ITS	CSRTI-Mysore, Karnataka, India	Arunakumar et al. (Unpublished)
Ceroteliumfici	Leaf rust	LSU & ITS		
Lasiodiplodia theobromae	Leaf spot/blight	ITS		
Fusarium equiseti	Leaf blight	ITS, TEF, β-tubulin		
Meloidogyne incognita	Root-knot Nematode	SCAR	Karnataka, Andhra Pradesh and Tamil Nadu, India	Manojkumar et al. (Unpublished)
Meloidogyne enterolobii	Root-knot Nematode	rDNA-IGS2	Hainan provinces of China	Sun et al. (2019)

Thrips

Pseudodendrothrips mori Niwa, (Thysanoptera: Tripiidae) are tiny, slender insects that feed on mulberry causing deformation and quality deterioration of leaves (Ye and Gu 1990). The infested leaves gradually become brittle, dry and assume a stippled or silver flecked appearance (Lewis 1997). The infested leaf generally records a loss 8.0–10% of leaf moisture, 10–15% of protein content and 5–10% of total sugar content, which makes the leaf qualitatively poor for silkworm rearing (Fig. 8.5c). The sprinkling of water on the infested leaf, spraying of 0.02% DDVP twice at weekly interval with a safe period of 7 days and application of Quinalphos (0.2%) with no adverse effect on the rearing of silkworms, may reduce the infestation (Misra 2003).

Fig. 8.5 Pests of mulberry. **a** Lower leaf affected by mealy bugs. **b** Mealybug infestation on stems. **c** Thrips infestation on young twigs. **d** Adult white flies on lower surface of the leaf

Whitefly

The white flies attacking mulberry plants belong to the species *Dialeuropora decempuncta* (Quaintanceand Baker) and *Aleurodicus dispersus* Russell (David 1993). It is a highly polyphagous pest causing heavy leaf damage to the plant by sucking the plant sap and secreting honeydew which acts as a substrate for the growth of the sooty mold *Capnodium* sp. to interfere with the process of photosynthesis (Fig. 8.5d).

Table 8.2 Important predators and parasitoids used to control mulberry pests

Name of the insect pest	Name of the biocontrol agent	Numbers to be released/acre/crop
Pink mealy bug *Maconellicoccus hirsutus*	Predators A. *Cryptolaemus montrouzieri* B. *Scymnus coccivora*	250 adults 500 adults
Thrips *Pseudodendrothrips mori*	Predator *Chrysoperla* spp.	4000–8000 eggs
Spiraling whitefly *Aleurodicus dispersus*	Predators A. *Axinoscymnus puttarudriahi* B. *Scymnus coccivora*	250 adults 250 adults
Papaya mealy bug *Paracoccus marginatus*	Parasitoids A. *Acerophagus papayae* B. *Pseudleptomastix mexicana* C. *Anagyrus loecki*	50–100 adults
Leaf Webber *Diaphania pulverulentalis*	Parasitoids A. *Trichogramma dchilonis*—egg B. *Bracon brevicomis*—larval C. *Tetrastichus howardii*—pupal	4 tricho cards per acre 200 adults 1 lakh adults in 3 splits

Source Sakthivel et al. (2019)

Both nymphs and adults remain in colonies under the surface of leaves and suck the sap which results in chlorosis, falling of leaf and reduction of plant growth sooty mold fungus. When whitefly infestation is >50% of leaf area, loss of leaf yield goes up to 28.09%, cocoon yield reduces by 48.09%. Use of triazophos 40EC at 0.06%, dimethoate 30 EC at 0.05%, neem oil and cotton seed oil at 0.01% is found effective in controlling the pest. Further, several parasitoids like the aphelinid parasitoids *Encarsiahaitiensis* Dozier and *E. meritoria* Gahan are also effective in managing the pests.

Jassids

Jassid, *Empoasca flavescens* F. (Homoptera: Cicadellidae) called leaf hoppers is the major sucking pest of mulberry. Both adult and nymphs suck the sap of the leaf giving rise to "hopper burn" symptoms (Fig. 8.6a, b). Initially, symptoms appear as triangular brown spots at the tip of the leaf and gradually, the affected leaves become brick red or brown, crinkled, curled and ultimately the plant shows stunted growth. Attack of jassids not only affects the leaf yield quantitatively but also reduces the quality. The infestation occurs more during February (20.36%) and September (21.80%) months in India. Mahadeva and Shree (2007) reported that free amino acids, total soluble proteins, reducing sugars, soluble sugar, phenol and photosynthetic pigments (total chlorophyll, chlorophyll-a, chlorophyll-b, chlorophyll-a/b ratio and carotenoids) affected considerably the jassid attack. Setting light traps for attracting

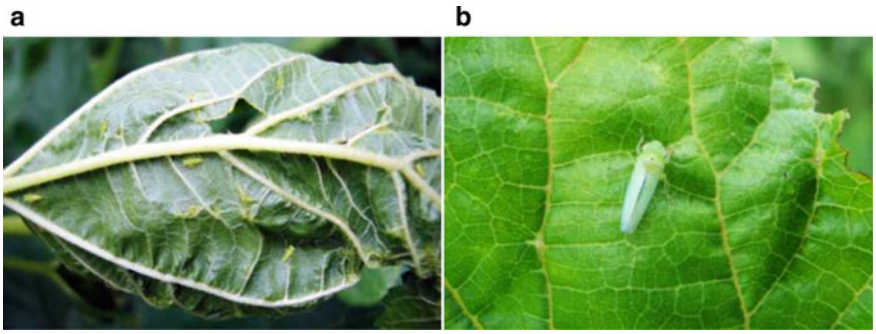

Fig. 8.6 Jassid **a** Nymph on a lower surface of the leaf. **b** Adult on a lower surface of the leaf (Sakthivel et al. 2019)

Fig. 8.7 Leaf roller infestation of mulberry. **a** Webbing of tender leaves. **b** Young larvae on tender leaves

and trapping adults and spraying 0.1% Dimethoate (Rogar) or 0.05% DDVP (Nuvan) with a safe period of 11 days can be adopted to manage Jassid infestation.

8.2.2.2 Defoliators

Leaf Roller

Diaphania pulverulentalis (Lepidoptera: Pyralidae) infestation on mulberry apical shoot after webbing the tender leaves (Fig. 8.7a, b) together and inhibit the growth of plants and causes a leaf loss of 12.8% with an average incidence of 21.77%

Fig. 8.8 May June beetle *Holotrichia serrate* Fabricius infestation on mulberry during the night time

resulting in economic loss to sericulturists (Siddegowda et al. 1995). Infestation also affects biochemically as protein percentage reduces up to 29.08%, carbohydrates were 24.92%, phenols 13.7% and chlorophyll 13.6%. Being seasonal, its infestation starts with the onset of monsoon, remains up to February, but the maximum infestation is observed from September to November. Spraying of 0.076% DDVP (76% EC) for 12–15 days after pruning (1 ml in 1 L water) with a safe period of 10 days can control the pest.

May–June Beetle

Holotrichia serrate Fabricius (Coleoptera: Scarabaeidae) infestation coincides with the onset of monsoon and an occasional pest to mulberry crop in south India. During night time, adult beetles enter into mulberry fields in swarms and feed voraciously on the foliage, leaving only the stem (Fig. 8.8a, b). After the first monsoon keep a vigil for adult beetles in mulberry field (Sakthivel et al. 2019). Collect adult beetles and destroy them by keeping them in Kerosene solution. Spray 0.2% DDVP 76% EC (2.5 ml/lit) with a safe period of 15 days preferably during evening hours. Drench the soil with 0.2% Chloropyriphos 20% EC to kill the grubs. Plowing just before the monsoon helps in the exposure of various stages of the pest to natural enemies.

8.3 Germplasm Resources for Disease Resistance

The complete alleles present in a population are called gene pool. The conservation strategies used for mulberry plants encompass a wide spectrum of activities ranging from the establishment of protected areas to building of DNA libraries (Tikader et al. 2009). However, the most widely adopted one is the ex situ field gene bank established by planting saplings/stem cuttings/bud grafting on ideal rootstocks. Considering the importance of collecting and conserving as many genetic resources to have a good gene pool, countries across the world have collected and being maintained a good number of accessions in the ex situ germplasm. These germplasm resources have been well characterized for different traits including responses to different pests and diseases (Akioet al. 2002; Tzenov 2002; Pan 2003; Tikader and Dandin 2006). Accurate detection of pathogens is very essential for the development of proper management approaches, moreover the use of highly pathogenic or aggressive isolates is necessary for inoculation trials for selecting genotypes with broader resistance (Oliveira et al. 2016, 2021). Also, there is an immediate need to identify resistant sources to transfer resistance genes into elite backgrounds of mulberry. In this direction, there are different germplasm resources resistant to different diseases have been identified in mulberry (Table 8.3).

8.3.1 Primary Gene Pool

As the primary gene pool (GP-1) is the gene reservoir for crop improvement mulberry genetic resources containing commercial stocks and landraces, wild gene pool comprising possible ancestors and closely connected species with a reasonable percent of fertile relationships with domesticated ones (Allem et al. 2001). The primary gene pool of mulberry has been evaluated for biotic stress caused by pests and diseases. For instance, Maji et al. (2009) screened 85 mulberry accession collected from different countries were screened for powdery mildew caused by *Phyllactinia corylea* (Pers), Myrithecium leaf spot caused by *Myrothecium roridum* Tode Ex. Fr. Pseudocercospora leaf spot caused by *Pseudocercospora moricola* (Hara), bacterial leaf spot caused by *Xanthomonas campestris* pv. *mori* and sooty mold caused by Acetomycetes and Deutromycetes fungi and identified eight accessions resistant to powdery mildew, 78 to *Myrothecium*, six to *Pseudocercospora* leaf spot, and 44 to sooty mold. Later, Banerjee et al. (2009) screened another 82 mulberry germplasm accessions for stable resistance to Bacterial leaf spot (BLS) pathogen *Xanthomonas campestris pv.mori* and identified the accessions *M. rotundiloba* and *MS-8* resistant, which could be utilized for breeding. Likewise, the accession Surat is highly susceptible to the disease. Bacterial leaf spot caused by *Xanthomonas campestris Pv. Mori* is a major foliar disease in mulberry causing foliage loss of up to 15%. It was observed that resistant and susceptible germplasm showed a high positive correlation between disease severity index and stomata frequency and a

Table 8.3 Resistant sources identified for different diseases of mulberry

Disease	Resistant sources	References
Leaf spot	Kanva-2, S-54, C-799, Shrim-2, Shrim-8, MR-2, Assambola, AB × Phill, Mouli, Mizusuwa, English black, K2 × Kosen, China peaking, Cattaneo, Sujanpur × Kokuso-13, Calabresa, Miruso, Acc-153, Acc-152, Acc-151, Acc-150, Acc-128, Acc-135, Acc-106, Acc-109, Acc-112, Acc-114, Acc-115, Acc-116, Acc-117, Acc-119, Acc-121, Acc-123, Acc-124, OPH-1, OPH-3, MS-2, MS-5, Acc-210, Paraguay, RFS-135, RFS-175, S-146, S-523, MS-7, MS-8, Acc-125, Acc-128, K2 × Kokuso, S-1531, LF-1, Almora local, S-31 and S-1096	Govindaiah Sharma et al. (1989)
	S 54, MR 2, S 36, Dharwar, Kajali, VS Z, Kosen, Prutppine, Itaiiai, papua, J,4l lenlbang	Philip et al. (1995a)
	Kajali, MS2	Philip et al. (1995b)
	Belidevalaya, Kaliakuttai	Philip et al. (1996)
	S-30 × ber C 776, K 2 × Kosen, Miz x RFS 135, Miz × S41, ACC 155 × Ber S 799, S-30 × ber S 799, S54	Philip et al. (1996)
	Kajali, Jatinuni. *Morus cathayana*, Almora local, Bogura-1, Meergund-6, Fernodias, Punjab local, *M. tiliaefolia*, Sultanpur, Golaghat, Bush malda-A and Sujanpur	Pratheesh Kumar et al. (2003)
	M. rotundiloba, MS-8	Banerjee et al. (2009)
	BM-4, BM-11	Ahmed et al. (2016)
	Morus multicaulis	Arunakumar et al. (2019a)
Brown leaf spot	Kanva-2	Peris et al. (2012)
Die-back	Fukayuk, Yakshinogi	Philip et al. (1995a)
Bacterial blight	Bulgaria-24, Butgaria-3, Galicea, Tbilisuri, Minarnisakar, Hatesakari, Husang, Chine, Shine, Ichinose, Gumji, Ichihie, Mysore Local, Berhamporelocaj, S-36, S-41, S-54, Goshoerami and Kosen	Philip et al. (1995a)

(continued)

Table 8.3 (continued)

Disease	Resistant sources	References
Leaf spot and powdery mildew	Goshoerami, Ichinose, Rokokuyaso	Mir et al. (2013)
Powdery mildew	Mandalaya, Cattaneo, Chinawhite, Jodhpur, Calabresa, Mizsuwa, Acc-123, OPH-3, S-523, Punjab Local, Shrim-2, Himachal Local, S-796, S-1531, S-1096, S-31, Almora local	Govindaiah et al. (1989)
	Buriram-60, Betidevalaya, kaliakutta, S54, K2	Philip et al. (1995a)
	M. laevigata and *M. serrata*	Babu et al. (2002)
	BM-11, BM-8, Black	Ahmed et al. (2016)
	Morus multicaulis and Kalimpong	Arunakumar et al. (Unpublished)
Leaf rust	MS-6, MS-2, Cattaneo	Philip et al. (1991)
	China Peking, Cattaneo	Philip and Govindaiah (1994)
	Kokuso-27, Kajryonezumigaesh, Cattaneo, China Peking	Philip et al. (1995a)
	Acc. 12, Rajouri, Acc. 148 and Acc. 9	Arunakumar et al. (2022)
Root-rot	Negumigaesi, Russkaya, Adrcnelli 03 and 02, Griyaus, Ghrusnl l, Sh-2 and Grusia X 020	Philip et al. (1995a)
	M. cathayana, M. multicualis (ME-0006 and ME-0168), Thai Pecah, Hazzaz, S-799, RFS-135, Acc. 106, T-36, UP-22, ERRC-103, ERRC-73, Acc. 8, Seekupari, Moulai, Pillighat, Kollihills-1, Kota-4, Jalalgarah-3, G2	Pinto et al. (2018)
Black root rot	*M. multicualis* (ME-0006 and ME-0168), Philippines, Australia, LF-1, C-18, Vadapuram and Meghamalai-1	Gnanesh et al. (Unpublished)
Root-knot nematode	Calabresa	Campos et al. (1974)
	S30, MR2 and RFS 135, Shrim 5, AB x Phillippines and K2 x Kokuso, Himachal Local	Philip et al. (1995a)
	RFS-135	Gnanaprakash et al. (2016)

(continued)

Table 8.3 (continued)

Disease	Resistant sources	References
	BR-8, Karanjtoli-1, Hosur-C8, Nagalur Estate, Tippu, Calabresa, Thai Pecah and SRDC-3	Arunakumar et al. (2021)

negative correlation of DSI with leaf thickness. Two unique RAPD primers with the fragment of 500 bp and 450 bp for the resistant and susceptible progenies respectively were identified (Banerjee et al. 2011). Under temperate conditions, Mir et al. (2013) screened seven germplasm resources for resistance to powdery mildew caused by *P. corylea* and leaf spot caused by *Cercospora moricola* (Cooke), found that the mulberry accession Kairyo-nezumigaeshi (KNG) is moderately resistant to both the pathogens. Pinto et al. (2018) screened 214 mulberry accessions for resistance to *M. phaseolina* charcoal root rot through simulated inoculation and identified twenty accessions with <26% root rot. Some accessions like *M. cathayana* (Hybrid) with 9.85%, *M. multicualis* Perr., with 12.03% G-4, with 35.91% infection were selected as resistant to root rot. Evaluation of worldwide collection of mulberry germplasm accessions for leaf spot (235), powdery mildew (14) and leaf rust (235) was undertaken during 2015–19 under natural epiphytotics. It was found that none of the accessions found resistant and twenty accessions showed moderate resistance to leaf spot. Four accessions namely Acc. 12, Rajouri, Acc. 148 and Acc. 9 were found resistant to leaf rust. *Morus multicaulis* and Kalimpong were identified as resistant to powdery mildew at CSR&TI, Mysuru, Karnataka (Arunakumar et al. 2021). A total of 415 different indigenous and exotic germplasm accessions were screened under glasshouse conditions and found mulberry accessions with 48 resistant and 21 immune. Further, 30 accessions were screened at 4 locations based on rooting ability with infested soil. Finally, 8 germplasm accessions viz., BR-8, Hosur-C8, Karanjtoli-1, Tippu, Nagalur Estate, Thai Pecah, Calabresa and SRDC-3 were identified as possible genetic resources for root knot resistance breeding programme and rootstock establishment of mulberry garden (Arunakumar et al. 2021).

8.3.2 Secondary Gene Pool

The species differentiation in mulberry is very thin as natural cross hybridization is very common among these species. However, there are a few species of mulberry that show very poor hybridization with other species. Prominent among them are *M. serrate* Roxb, *M. cathayana* Hemsl., *M. laevigata* Wall., *M. nigra* Linn., and *M. mongolica* Schneid., *M. wittiorum* Hand-Mazz., and these species are considered to be the secondary gene (GP-2) pool of mulberry as, though, the species can cross with other species but produce fewer seeds and sterile hybrids as most of them are polyploids (Weiguo et al. 2007). Most of these secondary gene pools have several unique traits which can be exploited effectively for crop improvement. For instance,

M. serrata is known to have several agronomical importance traits such as greater leaf moisture content, moisture retention, higher leaf thickness and resistance to biotic and abiotic stress. The highest numbers of root-knot nematode resistant accessions were found in *M. alba* (Arunakumar et al. 2021).

8.3.3 Tertiary Gene Pool

The tertiary gene pool (GP-3) consists of distantly related species of the primary gene pool and the crossing between these two is difficult and gets only sterile hybrids. Paper mulberry (*Broussonetiapapyrifera*) may be one of the geniuses which could be considered as the tertiary gene pool of mulberry as it belongs to the family Moraceae and has variable-shaped leaves that are rough to touch the plant looks like a hybrid that originated from a cross between mulberry and Osage-orange.

8.3.4 Artificially Induced/Incorporated Traits/Genes

Plants use different strategies and mechanisms to overcome numerous beneficial and harmful organisms (pathogens) in the environment (Kozjak and Meglič 2012). Initially, plants employ physical and mechanical barriers to prevent the pathogen entry into the plants through structural and anatomical modifications. If the pathogens overcome these barriers, plant receptors initiate the expression of the resistance genes (R genes). In a specific gene-for-gene fashion, *R* genes code for proteins that recognize specific pathogen effectors known as avirulence proteins. For a century, plant breeders have genetically characterized and used *R* genes to manage the loss due to diseases. However, recently to provide broader spectrum control and improved durability transgenic approaches have been adopted. Although mulberry gene pool has enough genetic variation and mulberry is highly heterozygous, efforts have been made to generate variations through various means such as plant tissue culture, mutation, polyploidy, and genetic engineering to explore the possibility of creating *de nov* variations. In genotype RFS-135, induction of mutations with EMS resulted in the isolation of varieties with wide economic importance in sericulture (Anil Kumar et al. 2012, 2013); it was brought to the notice that 0.1% and 0.3% of EMS treatment effective for changing the morphometric characters, phytochemical constituents such as proteins, minerals, reducing sugars, biomass yield and moisture content. Mutation induced through gamma rays irradiation, two mutants resistant to die-back disease. Similarly, the somaclonal variant (SV1) developed from S1 (*M. alba*) gave increased branching, higher leaf yield and tolerance to drought (Chakraborti et al. 1999). New plant varieties have also been developed through the induction of polyploidy (Chakraborti et al. 1998) to develop triploids as triploids are known to be more resistant than diploids.

8.4 Overview on Classical Genetics and Traditional Breeding

Traditionally, mulberry is developed through hybridization and selection. The whole breeding process in mulberry starts with the evaluation of germplasm using morphological, biochemical, physiological characters, and suitable parental germplasm are chosen and controlled hybridization is imposed. Ripened fruits from those formed by natural hybridization and controlled hybridization in selected mother plants are collected to take out seeds, further seeds will be sown in nursery beds to raise seedlings.

8.4.1 Traditional Breeding Methods

Screening and selection of hybrids initially based on a few important characters like growth, leaf texture, branching and disease susceptibility are done in progeny row trials (PRT). Due to more or less all mulberry accessions being highly heterozygous and poses a longer gestation period, conventional breeding methodologies mainly carried on the production of the F1 hybrid (Das 1984). Hybrids with advantageous characters, identified through the PRT, are additionally evaluated in primary yield trial (PYT) for agronomic, biochemical and silkworm bioassay. From the PYT, 5–10% of top performing hybrids are further chosen through the final yield trial (FYT) for the detailed assessment using 25–49 plants per replication with 3–5 replication. In FYT, plants are put to thorough assessment for rooting ability, leaf quality, leaf yield, response to agronomic practices, adaptation, susceptibility to pests and diseases, and silkworm bioassay. Once a hybrid is found to have almost all the desired traits, hybrid is chosen and vegetatively mass multiplied and tested under multilocation trials (MLT) at various Seri regions. In general, 8–9 hybrids are selected for MLT studies. Hybrids that exhibit consistently good in all the locations, seasons and years are further selected tested in All India Coordinated Experimental Trial (AICEM) to assess hybrids performance in different agro-climatic conditions across India, AICEM will be carried at least for four years. The current AICEM test is carried out at 24 test centers lying from south to north and west to east of India. The best performing hybrids under the AICEM are authorized and released for commercial utilization by the Seri-farmers.

8.4.2 Breeding Objectives: Positive and Negative Selection

As mulberry leaves are the primary plant part for silkworm rearing in most of the Asian nations, the breeding was aimed at the development of varieties with wider adaptive and higher leaf yield potential. Mulberry growth and leaf production depend

on several factors and associated traits, thus, the breeding process always relies on certain markers/traits which contribute considerably to the growth and development of the plant. The section process may be of two types, positive and negative. The selection based on traits or markers that confer a selective advantage for the plant is called positive selection while those confer a disadvantage is called negative selection. Positive (Darwinian) selection is in which genes/traits/variants that have a selective advantage increase in number and spread until they fix in the relevant population. On the other hand, the negative selection also called purifying selection, is a purging process wherein disadvantageous or deleterious alleles/genes/traits get eliminated from the population. The strength of selection varies between locus/genes. In the case of strong negative selection on a locus, the purging of deleterious variants will result in the occasional removal of linked variation, producing a decrease in the level of variation surrounding the locus under selection. In mulberry, a number of traits have been identified which have a strong and positive correlation with the survival and leaf yield of mulberry under stress conditions and also a set of characters that have negative correlations (Vijayan et al. 2010). It has been found that the character association changes with the intensity of the stress imparted by salinity. Under normal condition, leaf yield is significantly and positively correlated with leaf size, root length, shoot length, protein content of the leaf and the photosynthesis of the plant. However, under a stress caused by 1.00% NaCl (ECe 19 dSm^{-1}) the leaf yield has highly significant correlation with plant height, leaf size, shoot weight, root weight, root length, protein, NRase activity and WUE of the plant. Likewise, under normal cultural conditions the leaf fresh, leaf moisture and dry weights showed a non-significant negative correlation with leaf yield. Thus, the selection strategy for different traits is made based on the breeding objectives.

8.4.3 Achievements of Conventional Breeding (Quality, Stress Resistance, Yield etc.)

Through conventional breeding, several mulberry lines/varieties have been developed across the world. For instance, India has developed 27 mulberry varieties and China has developed 31 mulberry varieties. The leaf productivity of these varieties increased considerably from 8–10 MT/ha/yr in traditional varieties to 60–65 MT/ha/year in the newly developed varieties. Similarly, a few varieties with stress resistance have been developed. AR-12 for alkalinity tolerance and C776 for salinity stress are examples of it.

8.4.4 Constrains of Conventional Breeding and Basis for Molecular Breeding

The biggest limitation of mulberry breeding is the long periods required for the development of varieties. It takes almost 15–20 years to develop varieties as mulberry has a long juvenile period as it takes a minimum of 2–3 years to get the plant ready for developing the next generation. Further, the lack of inbreds and the high heterozygosity associated with the accessions make the genetic improvement through conventional breeding is highly laborious. Hence, in most of the breeding plans, crosses between selected parents are made to develop F_1 hybrids and the F_1 hybrid is further used for screening and selection of promising ones to put into evaluation for variety development. Thus, trait specific improvement does not have much scope in this type of breeding programme as recurrent hybridization and selection take decades to be completed. Additionally, stress tolerance is a difficult phenomenon including morphological, physiological, biochemical, and developmental changes in plants (Hirayama and Shinozaki 2010; Gill and Tuteja 2010). Stress tolerance selection in the field is not a suitable method as the intensity of stress imposed by both drought and salinity in the field can differ depending on soil depth and season. Plants also interact with other numerous environmental factors which involve the intensity of stress tolerance. Thus, screening of plants has to be under controlled environmental conditions to assist the true appearance of their natural capacity to tolerate biotic and abiotic stresses. However, a large number of F_1 hybrids screening for stress tolerance by imparting stress is near prohibitive. Therefore, it is highly desirable to use molecular biology tools such as Marker-Assisted Selection and genetic modification through genetic engineering.

8.4.5 Classical Mapping Efforts and Its Limitations and Utility of Molecular Mapping If Any

As mulberry is a highly heterozygous plant with an extended juvenile period, high genetic load, no inbred lines could be developed to work out the genetic basis of traits. Since it is not easy to develop segregation populations no efforts have so far been made to construct a genetic map of mulberry. Further, the expression of most of the important traits is highly influenced by environmental factors and stages of development. A certain set of characters would appear under a given set of climatic conditions in a particular stage of development and another set under another set of conditions and growth stages. Thus, no systematic efforts have been made to use the classical genetical approach to elucidate the genetic base of the character of mulberry. The molecular markers on the other hand are present in abundance, stable across the developmental stages, least influenced by environmental factors, devoid of the pleiotropic and epistatic effects. Thus, molecular markers were found much better than the phenotypic markers in assessing the genetic diversity, identification of

parents, and evaluation and selection of hybrids. Depending on the techniques used, these markers can be broadly classified as hybridization based markers and polymerase chain reaction (PCR) based markers. In hybridization based marker systems like restriction fragment polymorphism (RFLP), the DNA profiles are visualized by hybridizing the DNA with restriction enzyme digestion and blotted against a solid membrane with a labeled probe. PCR based marker method, amplification of desired DNA sequences are carried out with the help of arbitrarily or specifically chosen primers using thermostable enzyme, called *Taq* polymerase under in vitro condition. The amplified fragments are aligned using an electrophoresis system on agarose or polyacrylamide gels and banding patterns (amplicons) are identified either by autoradiography or staining. Some of the important PCR based marker systems are amplified fragment polymorphism (AFLP), random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), simple sequence repeats (SSR), expressed sequence tag (EST). Each of these marker system has its own merits and demerits. Markers like SSR, RFLP and EST are co-dominant, therefore, have ability to detect genetic variability at allelic level. Though, the development and utilization of these marker systems are costly, laborious and time taking. Thus, in mulberry, Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), and Inter Simple Sequence Repeat markers were used for genetic divergence and molecular characterization of germplasm (Vijayan et al. 2004a, 2005, 2006; Zhao et al. 2006; Vijayan 2010; Sangannavar and Vijayan 2020). However, all the above stated markers had several inherent limitations including, dominant nature, reproducibility, anonymity etc. The advent of next-generation sequencing and the drastic reduction in their cost of sequencing have enabled designing and utilization of more robust, reproducible and informative molecular markers such as SSR and SNPs. Using SSR markers, many QTL maps have been developed in mulberry for different traits (Sarkar et al. 2017). With the advent of NGS, efforts are underway to develop SNP panels for their utilization in mulberry.

8.4.6 Use of Morphological Markers

Plenty of research has been done on characterization and evaluation of parental materials, screening of hybrids and evaluation of selected genotypes using morphological markers such as leaf shape and size, lenticels ferequency, stem colour, flower color, stigma length and nature, fruit shape and colour, seed colour and size, plant height, stem length, leaf retention capacity, number of branches, moisture content and retention capacity, nodal length, leaf yield, biomass production, etc. (Bindroo et al. 1990; Sahu et al. 1995; Vijayan et al. 1997; Tikader et al. 2009) along with others traits like adaptability to different climatic conditions, resistance to biotic stresses like diseases and pests, tolerance to abiotic stresses like salinity, cold and drought, other abilities like higher vegetative propagation, better leaf quality, and better coppicing (Vijayan et al. 2009). Plant defense mechanism against pathogen attack is not simple, with many local and systemic aspects (Felle et al. 2004). Further, resistance to biotic

stress has been correlated with a few morphological markers such as leaf cuticular thickness, quality of wax and cuticle that cover the epidermal cells, frequency of leaf hairs, cystolith, structure of epidermal cell walls, shape, size and location of lenticels and stomata. Other than these, thick walled cells tissues also avoid the progress of the pathogen. Morphological characteristics (significantly higher thickness of epidermis cum cuticle, more number of Palisade layers, nature of palisade and cuticle tissue, relatively thinner spongy parenchyma and significantly higher palisade proportion) are ideal to act as physical or structural barriers against the diffusion and incursion by different pathogens (Sonibare et al. 2006). However, with the high heterozygosity, long juvenile period of the plant, multigenic and multifarious nature of these morphological markers, their application often becomes difficult (Vijayan et al. 2006). Further, testing of a large number of progenies for pest and disease resistance requires huge space and resources, which often act as the major impediments.

8.4.7 Limitations and Prospect of Genomic Designing

For the identification of genetic variability in mulberry, several molecular marker techniques have been successfully utilized in mulberry crop improvement. These markers are RAPD (Xiang et al.1995; Bhattacharya and Ranade 2001; Chatterjee et al. 2004; Srivastava et al. 2004; Zhao et al. 2009; Orhan et al. 2007), AFLP (Sharma et al. 2000; Wang and Yu 2001; Huang et al. 2009; Pinto et al. 2018), ISSR (Vijayan and Chatterjee 2003; Awasthi et al. 2004; Vijayan 2004; Vijayan et al. 2004a, b, c, 2005, 2006; Zhao et al. 2006, 2007; Kar et al. 2008) and SRAP (Zhao et al. 2009) and DAMD (Bhattacharya and Ranade 2001; Bhattacharya et al. 2005). However, the dominant markers could not yield much desired information. Thus, genetic variation among the mulberry genotypes was measured by using codominant markers such as SSR markers extensively for identification of potential parents and progenies (Aggarwal and Udaykumar 2004; Zhao et al. 2005; Pinto et al. 2018; Garcia-Gómez et al. 2019; Orhan et al. 2020). A total of 247 mulberry specific SSR markers have been deposited in NCBI (www.ncbi.nlm.nih.gov) by various groups and are available for molecular genetic analysis. Consequent to genome sequencing of *M. notabilis* (He et al. 2013), over 2.17 lakh SSR motifs have been mined and hosted in an online mulberry microsatellite marker database (MulSatDB, Krishnan et al. 2014). The evaluation of molecular variation using polymorphic SSRs among the resistant and susceptible genetic resources is crucial for their effective and efficient utilization in plant breeding (Arunakumar et al. 2021; Shinde et al. 2021). Recent developments in next-generation sequencing, bioinformatics and consequent attempt to sequence the whole genome of mulberry and annotate the sequences have opened a plethora of opportunities to develop more densely distributed SNP markers for their application in mulberry genome analysis.

8.5 Diversity Analysis in Brief

8.5.1 Diversity Analysis Based on Phenotype

Evaluation of germplasm and grouping of the accessions based on diversity among them is required for selecting suitable parents for breeding. In the earlier days in the absence of molecular markers, germplasm was evaluated using phenotypic traits such as morphological traits and agronomical traits such as drought tolerance, alkaline and saline stresses, winter hardiness, or early sprouting, pests and diseases resistance (Tikader and Kamble 2007). Tikader et al. (1995) explored the variability in the expression of sex in mulberry in 301 genotypes from diverse geographical origins, and found that nearly 16% was male, 53% female, 17% monoecious and 13% were bisexual. Significant variation was noted in the flowering time, anthesis and floral characteristics. Variability was also observed in pollen grain viability, fruit morphology and seed setting %. Vijayan et al. (1999) estimated the genetic diversity among the 62 mulberry accessions indigenous to India, irrespective of their species and ploidy status. Significant genetic divergence was observed among these indigenous mulberry accessions based on the leaf yield traits. Tikader and Kamble (2008) evaluated the genetic diversity of 50 mulberry germplasm accessions using eight agronomically important traits and found a high amount of genetic diversity among the accessions. Banerjee et al. (2011) evaluated twenty two morphological traits in twenty-five Indian mulberry accessions belonging to the five species of *Morus*. Six principal components were identified explaining >88% of the total variation. Among the 18 major variables included for the analysis, shoot and root traits such as longest shoot length, leaf area, intermodal distance, green and dry leaf weight, lamina length, lamina weight, root volume, and fresh and dry root weight were identified as important variables. Chang et al. (2014) from seven *Morus* spp., assessed the genetic diversity of 27 mulberry accessions using 20 vegetative traits, chilling requirements, and reproductive traits. Based on the study, a classification system was suggested with three clusters: (1) *M. laevigata*; (2) *M. atropurpurea*, *M. bombycis*, *M. australis* and *M. formosensis*; (3) *M. alba* and *M. latifolia*. The study also showed that *M. atropurpurea*, often regarded as a member of *M. alba*, is closed to *M. bombycis*, *M. australis* and *M. formosensis*. Peris et al. (2014) assessed the genetic diversity among five mulberry accessions being maintained in Kenya which include the accessions Embu, Thika, Thailand (*M. alba*), Kanva-2 and S41 (*M. indica*) using twelve phenotypic traits recorded from two localities (Nairobi and Eldoret). Leaf lamina width and petiole length, petiole width and growth height, internodes distance and the number of branches showed significantly and using Duncan's Multiple Range Test (DMRT) the accessions were clustered into four groups. Efforts were also made to evaluate the germplasm for variability in stress tolerance. Hossain et al. (1991) evaluated 10 mulberry genotypes under tissue culture conditions to screen out the tolerant genotypes. Vijayan and Chatterjee (2003) under in vitro conditions evaluated 63 mulberry genotypes and selected 5 genotypes with a higher tolerance level. Likewise, Tewary et al. (2000) evaluated mulberry genotypes for osmotic stress tolerance by using

the medium with 1.0–10% polyethylene glycol (PEG) and observed considerable genetic diversity among the genotypes. These studies clearly showed that considerable genetic diversity is present in mulberry for stress tolerance. However, incorporation of the specific trait through conventional breeding has several bottlenecks important among them is the difficulty in introgressing a trait to a recurrent parent from a donor parent through repeated backcrossing and selection, because of the dioecy of the plant and prolonged juvenile period (Vijayan 2010).

8.5.2 Diversity Analysis Based on Genotype, Molecular Markers Applied

Since diversity analysis using morphological characters in plants are not very reliable due to the evolutionary dynamics, influenced by the growing conditions and development stage, information from non-morphological characters such as biochemical molecules and nucleic acids is increasingly being used for genetic resource management and utilization. Among all non-morphological markers, molecular based markers are suitable for genetic characterization of mulberry germplasm resources as they are widely polymorphic, multiallelic, codominant, non-epistatic, insensitive and neutral to environment control (Xiang et al. 1995; Vijayan et al. 2004a, b, c). Although several DNA markers such as Amplified fragment length polymorphism (AFLP) (Sharma et al. 2000), Random Amplified Polymorphic DNA (Zhou et al. 2014), Inter simple sequence repeats (ISSR) (Vijayan and Chatterjee 2003; Vijayan 2004; Vijayan et al. 2004a, b, c, 2005, 2006; Zhao et al. 2006, 2007; Sangannavar and Vijayan 2020) have been developed and used for genetic diversity analysis of mulberry. However, considering the reproducibility, robustness, and information generating ability, simple sequence repeats (SSR), and single nucleotide polymorphism (SNP) markers are considered the most suitable molecular markers for genetic diversity analysis in mulberry. Simple sequence repeats (SSR) or microsatellite or short tandem repeat (STR) or simple sequence length polymorphism (SSLP) are tandem repeats of short (2–6 base pair) DNA fragments scattered throughout the genome that lies between conserved sequences (Litt and Luty 1989). The three mechanisms that create a new allele at SSR loci are (a) replication slippage (b) unequal crossing-over and (c) genetic recombination. Replication slippage is considered to be a major factor affecting the repeat number for STR sequences, whereas unequal crossing-over is thought to result in a very large number of alleles for long tandem repeat arrays (Huang et al. 2002). However, the major disadvantage of SSR was the need genomic information to develop primers, which was expensive and time consuming, but with the introduction of Next generation sequencing technique, the cost has come down heavily and now it is possible to sequence any plant genome at a reasonable cost. Regarding SNPs, they are the most abundantly present DNA marker in any genome with a frequency of >1% in a population (Collins et al. 1997; Halushka et al. 1999). The frequency of SNPs is roughly estimated to be one in

every thousand nucleotides in the human genome, and one in 60–120 bp in maize (Ching et al. 2002). However, to date, no attempt was made to discover SNPs in mulberry. Nevertheless, considering the tremendous progress made on low-cost and high-throughput SNP genotyping in other crops, and the current pace of genomic research in mulberry genome, it is certain that within a short time SNPs become the commonly used molecular markers in mulberry. A large number of ESTs from mulberry genome have been deposited in the data bank (Lal et al. 2009; Zhao 2008). Attempts should, therefore, be made to identify potential SNPs from these ESTs, which can also be used for identifying causal polymorphism. Likewise, SNPs can also be developed through locus specific amplification (LSA) and comparative re-sequencing from multiple individuals (Rieder et al. 1998) by utilizing the information available from the genomic sequences deposited from markers like RAPD and ISSR that are linked with important phenotypic traits.

8.5.3 Relationship with Other Cultivated Species and Wild Relatives

Usage of crop wild relatives (CWRs) in cultivation and breeding is the best way to harness natural trait variation in genetic improvement programs. Wild relatives often have unique alleles for specific traits like resistance to biotic and abiotic stresses. Thus, it is desirable to understand the relationship between domestic and wild species to execute the crop improvement programs effectively. The phylogenetic relationship among different genera of the family Moraceae was generated with information from nuclear and chloroplast DNA sequence variations of thirteen species of *Morus* distributed in Asia, Africa, Europe, and North, Central, and South America. The study revealed that the genus *Morus*, as currently circumscribed, is non-monophyletic as the species *M. mesozygia* and *M. insignis* are placed outside the other domestic species. Thus, a further detailed investigation is required to clarify natural generic relationships of the family Moraceae (Nepal and Ferguson 2012). Vijayan et al. (2004d) used inter-simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) markers to find out the relationship among five species viz., *M. latifolia*, *M. bombycis M. alba* and *M. laevigata* and found that *M. laevigata*is different from other species. Population analysis further stressed the wild nature of *M. laevigata* as it showed considerably low gene flow (Nm) with other species. Likewise, Weiguo et al. (2007) using ISSR and SSR markers investigated the genetic diversity among 27 mulberry accessions including nineteen cultivated accessions (three *M. alba*, two *M. bombycis*, six *M. multicaulis*, two *M. atropurpurea*, two *M. rotundiloba*, one *M. australis*, one *M. alba* var. *pendula*, one *M. alba* var. *venose* and one *M. alba* var. *macrophylla*) and eight wild accessions (two *M. laevigata*, two *M. cathayana*, two *M. wittiorum*, one *M. mongolica* and one *M. nigra*). It has been found that prolonged cultivation caused loss of genetic diversity in domestic species.

Recently, Jiao et al. (2020) stated a high-quality, chromosome level domesticated mulberry genome (*M. alba*) and they confirmed with 28 chromosomes with *M. alba* and it is a diploid (2n = 2x = 28).

8.5.4 Association with Geographical Distribution

Although not much information is available of mulberry on the relationship between genetic diversity and geographic distribution, Vijayan and Chatterjee (2003) analysed the relationship between geographic distribution and genetic diversity among 11 cultivars, which are being widely cultivated in different parts of India. The study revealed that geographic groupings of the cultivar were based on their geographic origin. Chatterjee et al. (2004) found a strong relationship between geographic distribution and genetic diversity of *M. laevigata* (Vijayan et al. 2005) assessed the genetic diversity among 34 Indian mulberry accession and found that the accessions resolved into groups based on their geographic relationships. Efforts to find out the relationship among cultivars originated in India and Japan also showed clear and distinct grouping based on their geographic origin (Vijayan 2004). Jiao et al. (2020) used population genomic analysis by resequencing 132 mulberry accessions split into three geographical groups, namely, northern and southwestern China, Taihu Basin of southeastern China (Hu mulberry) and Japan. Among these Hu, mulberry exhibits the lowest nucleotide diversity and established apparent signatures of selection, representing environmental adaptation. Thus, mulberry varieties and cultivars from different geographic regions show high genetic diversity.

8.5.5 Scope of Genetic Diversity

The scope of genetic diversity within a species was investigated in *M. alba* and *M. serrata* with RAPD and ISSR markers. It is found that the genetic similarity among 11 mulberry genotypes of *M. albaviz*., Limoncina, Schinichinose, Kattaneo, Obawasa, Rangoon, China white, China black, Canton china, Almora local, Punjab local and Sujanpur-2 estimated based on Nei and Li (1979) varied from 0.644 between Rangoon and Punjab local to 0.943, between Sujanpur-2 and Almora local, with an average genetic similarity of 0.793 for data generated by ISSR markers. The same was in the range of 0.738 for China white and Kattaneo to 0.909 for Sujanpur-2 and Punjab local with an average of 0.834 in RAPD analysis. When the RAPD and ISSR data were combined to analyse with more markers the genetic similarity among the genotypes varied from 0.733 between China white and Kattaneo to 0.888 between Sujanpur-2 and Punjab local with a mean coefficient of 0.819. Genotypes collected from different climatic conditions such as temperate, tropical and sub-tropical from various countries show substantial amount of genetic similarity.

The genetic similarity coefficients revealed substantial amount of genetic similarity among the genotypes, though the genotypes were collected from different countries of much varied climatic conditions such as tropical, temperate and subtropical. Genotypes from similar geographic regions showed closer genetic similarity than those from geo graphically distant region. The correlation coefficient among the matrices as tested by Mantel's (1967) Z-statistics, revealed high correlations ($r = 0.4$; $p = 0.000$ between ISSR and RAPD; $r = 0.976$, $p = 0.000$ between ISSR and Pooled matrices, $r = 0.982$; $p = 0.000$ between RAPD and Pooled data matrices) (Srivastava et al. 2004). The genetic diversity evaluation of 16 populations of *M. serrata* Roxb., revealed presence of significant genetic diversity among the populations on morphological and anatomical as well as DNA markers. The average genetic distance, estimated from the ISSR markers was 0.165 (Vijayan et al. 2006). Thus, there is a great amount of inter and intra species genetic diversity in mulberry which can be used for crop development.

8.6 Association Mapping Studies

8.6.1 Genome Wide LD Studies

Gene scan surveys and genome wide association (GWA) mapping helps in identifying the genetic variation existed in the whole genome to locate genes or narrow regions that have important statistical connections with numerous complex traits. Since to conduct a genome wide association analysis, an enormous number of densely distributed markers is required, whole genome scan is usually carried out using the most frequent genetic variants available in the genome is SNPs. Generally, thousands of SNP markers are required for a whole genome scan for crops with high haplotype diversity and low LD. Recently, Pinto et al. (2018) used 214 accessions germplasm panels to spot out markers associated with root rot resistance (charcoal) and identified 5 AFLP markers allied with root rot resistance. These markers accounted for allele frequency of 0.132–0.401 and 9.6–12.7% of the total phenotypic variation in the trait (R^2). Similalry, Zhang et al. (2016) used a germplasm panel of 93 mulberry accessions of diverse origin was to identify markers for a few important fruit traits. A total of 24 markers associated with fruit traits were identified. Thus, very scanty work only has been done in mulberry on LD mapping.

8.6.2 Future Potential for the Application of Association Studies for Germplasm Enhancement

LD mapping is very useful for identification trait-marker associations in species where biparental mapping has limitations, especially crops like mulberry where

inbreds are hardly available. Mulberry being a perennial species, association mapping is very appealing as it is a reservoir of natural genetic variations in the form of wild species, weedy species, land races and evolved cultivars, which are originated from a number of historical genetic recombination events in response to different climatic conditions. (Tikader and Vijayan 2017). Exploitation of these genetic variability in the ex situ conserved genetic resources is vital to overcome future problems associated with narrowness of genetic base of modern cultivars as strong genetic diversity means diverse morphological traits and a higher potential to develop varieties for varied cultural and agronomic conditions (Abdurakhmonov and Abdukarimov 2008). Since LD analysis has the potential to identify a single polymorphic locus within a gene that is responsible for a difference in phenotype and to predict the best haplotype across one or multiple genes for optimum expression of the target trait, it can be used to determine the best donor parents for crop improvement programs. The current efforts to sequence the genome of diploid mulberry species in India and to identify SNPs would help perform more association mapping as biallelic codominant type of markers like single nucleotide polymorphisms (SNPs) is perfectly suitable for the quantification methodology of LD. LD quantification using dominant markers such as RAPD, AFLP, ISSR is poorly explored and usually subject to wrong perception and interpretation. Another important factor that determines the success of LD mapping is the choice of germplasm or population (Yu et al. 2006) as the false positives generated by population structure may make a marker allele that occurs at high frequency in a preferentially sampled subpopulation associated with a trait of interest even though it is not linked to a real QTL (Pritchard et al. 2000). In sort to overcome these interruptions, several methods such as structured association, mixed model approach, genomic control and principle component approach have been developed (Devlin and Roeder 1999; Pritchard et al. 2000; Yu et al. 2006). Thus, the true potential of LD mapping is yet to be harnessed in mulberry.

8.7 Map-Based Cloning of Resistance/Tolerance Genes

8.7.1 Traits and Genes

Genomic technologies such as genome sequencing and transcriptome analysis generated valuable information on functional and structural aspects of genes involved in various processes of stress responses, growth, and development, in a variety of plant species. Mulberry has the potential features to consider as a perennial tree model system. Limited genomic studies have been conducted in mulberry with transcriptomes, proteome, metabolome, and degradome approaches to elucidate comparative gene expression in response to stresses, variation between tissues and genotypes in *Morus* species. (Dhanyalakshmi and Nataraja 2018). Phytoplasma is a devastating pathogen causing yellow dwarf disease in mulberry. The molecular mechanism of phytoplasm pathogenicity is poorly understood due to the inability to culture In-vitro

(Wei et al. 2013). To understand the molecular mechanism of pathogenicity, differentially expressed miRNAs from phloem sap were analyzed. A total of 30 conserved miRNA and 13 novel miRNAs were differentially expressed upon phytoplasm infections were identified. It was suggested that Mul-miR482a-5p might negatively regulate resistance to phytoplasm infection in mulberry. Mul-miR482a-5p predicted to target the RCC1 gene, which is the guanine nucleotide exchange factor for the nuclear GTP binding Ran and it may act as a positive regulator of defense responses. Therefore, upon phytoplasm infection, Mul-miR482a-5p expression level increases that may repress the RCC1 gene and reduce host resistance to phytoplasma (Gai et al. 2018).

8.8 Genomics-Assisted Breeding for Resistance/Tolerance Traits

8.8.1 Functional and Structural Genomic Resources Developed

Some work has been done in identifying and characterizing downstream genes involved in defense response such as Pathogen-related proteins (PR), lectins, phenylpropanoid and Proanthocyanidins biosynthetic pathways. Pathogen-related proteins are a group of family genes that are induced in response to pathogen attack. The transcription and translation level of PR1 significantly increased in response to pathogens and therefore considered as marker proteins for the establishment of systemic acquired resistance (SAR) in plants (Ali et al. 2018). To explore the possibility of utilizing PR1 in mulberry breeding through genetic engineering, muPR1 was isolated from *M. multicaulis*. The isolated muPR1 expressed constitutively in selected tissues and induced by pathogens, methyl jasmonate, salicylic acid and GA3 phytohormones. Further, the involvement of muPR1 in disease resistance was shown by overexpression in transgenic Arabidopsis that ensued in increased resistance to *Botrytis Cinerea* and Pst. DC3000. It was also shown that muPR1 may have roles in the rate of reactive oxygen species formation and detoxification (Fang et al. 2019). Lectins are proteins that contain at least one non-catalytic sugar-binding domain and are synthesized in response to abiotic and defense response (Lannoo and Van Damme 2010). In *M. notabilis* 197 genes belonging to 12 distinct gene families have been identified. Expression analysis identified 4 lectin genes as upregulated under Jasmonic acid and salicylic acid treatments reminiscent of biotic stress conditions (Saeed et al. 2015). Proanthocyanidins are abundant polyphenolic compounds and have a role in disease resistance in plants additionally can improve human health. Proanthocyanidins are polymers of flavan-3-ols, primary catechin, and epicatechin where leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) catalyzes the synthesis of catechin and epicatechin respectively.TheMnANR and MnLAR transcripts increased in response to *B. cinerea* infection and methyl

Jasmonate stress conditions. The ectopic expression of MNANR and MnLAR in tobacco increased the disease resistance against the *B. cinerea* infection (Xin et al. 2020).

8.8.2 Genome Sequencing, Assembly and Annotation

The first whole genome sequencing of any mulberry species was done by He et al. (2013), they used *M. notabilis* with seven distinct pairs of chromosomes (2n = 14) for genome sequencing. The genome size of *M. notabilis* was estimated to be 357.4 Mb predicted to code for 29,338 genes and contains 128 Mb repetitive sequences. It is found that nearly half of the mulberry genome is composed of repetitive elements and nearly 50% of these sequences were belonging to *Gypsy*-like (6.58%) and *Copia*-like (6.84%) long-terminal repeat retrotransposons. A total of 27,085 protein-coding loci with complete gene structures were predicted using 21 Gb RNA-seq data from five tissues and unique ESTs. In another effort, Jiao et al. (2020) sequenced the genome of a diploid mulberry species *M. alba* L 28 chromosome (2n = 2x = 28) using combined three different technologies, the Oxford Nanopore, Illumina HiSeq, and high-throughput chromatin conformation capture (Hi-C) platforms and found the genome size as 328.3 Mb. A total of 180.11 Mb of non-redundant repetitive sequences by a combination of homology-based approaches and de novo, accounted for 52.85% of the assembled genome. A total of 22,767 protein coding genes were explained with an average gene length of 3209 base pairs (bp). In addition to these, the chloroplast of mulberry has been sequenced using a combination of long PCR and shotgun approaches. The chloroplast genome has circular double-stranded DNA of size of 158,484 bp containing two identical inverted repeats of 25,678 bp each, separated by a large (87,386 bp) and small (19,742 bp) single copy regions. From this sequence, 83 protein-coding genes, eight ribosomal RNA genes and 37 tRNA genes were identified. In another attempt, Chen et al. (2016) sequenced the chloroplast of *M. notabilis* and found that the circular genome is 158,680 bp in size, and comprises a pair of inverted repeat (IR) regions of 25,717 bp each, a large single-copy (LSC) region of 87,470 bp and a small single-copy (SSC) region of 19,776 bp. The chloroplast genome contains 129 genes, including 84 protein-coding genes (PCGs), eight ribosomal RNA (rRNA) genes and 37 transfer RNA (tRNA) genes. The maximum likelihood (ML) phylogenetic analysis revealed that *M. notabilis* was more related to its congeners than to the others. Later, chloroplast sequences from five other species of mulberry were generated (Kong and Yang 2016, 2017).

8.8.3 Impact on Gene Discovery and Germplasm Characterization

The whole genome sequencing has a significant impact on the characterization of germplasm as illustrated with 134 mulberry accessions by Jiao et al. (2020). Using the newly identified 14,273,912 high-quality SNPs, the phylogenetic relationship among 132 cultivars using 2 wild mulberry genotypes was assessed and found that the phylogenetic tree was based on whole-genomic SNPs was not good with consistent with the traditional delimitations of mulberry species. The cultivars from Chinese grouped into two viz., Hu mulberry (HU), from Taihu Basin, and non-Hu mulberry (NH), from the rest of China. This latter group could be further divided into two subgroups, East and West. Further, it was noticed a lower level of heterozygosity with high linkage disequilibrium decay. Likewise, Muhonja et al. (2020) worked out the genetic relationship among 54 mulberry accessions from seven species (*M. indica, M. alba, M. bombycis, M. latifolia, M. acidosa, M. rotundiloba and M. kagayamae*) using genome-wide 2229 SNPs. The phylogenetic analysis resulted in the construction of only 3 clear monophyletic clades viz, *M. acidosa* and *M. kagayamae* from different geographically isolated islands, two Japanese native species and a Thai species, *M. rotundiloba*, and other species were found non-monophyletic. It is also interesting to note that no clear monophyletic clades could form by varieties from *M. alba* and *M. latifolia* indicating admixture among them through natural hybridizations. These studies suggest the classification of the genus *Morus* is not an easy task even with genome-wide DNA markers. A similar type of results was obtained earlier with ISSR and ITS markers (Muhonja et al. 2020; Zhao 2005). Besides the inefficiency of the current species delimitations of the genus *Morus,* these studies also brought out the usefulness of the whole genome sequencing and DNA markers for germplasm characterization and crop improvement. The SNPs markers developed in the studies can be used for making SNP panels for automation of the mulberry germplasm to identify suitable parents for breeding programs. The genes identified from these studies can be used for further studies by gene knock out, overexpression and gene editing to develop varieties with desirable traits. Mulberry fruit is of economic value because of its high nutrition and presence of potential pharmacological active compounds beneficial to human health. Mulberry Sclerotiniose is caused by *Ciboria shiraiana*, which affects the quality of the fruit. To gain insight into the molecular mechanisms to provide direction to molecular breeding, diseased fruit was investigated using a transcriptome and metabolome approach. Differential expression analysis between healthy and diseased fruits revealed, genes related to plant hormone signal transduction, transcription factors and phenylpropanoid biosynthesis may play an essential role in response to Sclerotiniose pathogen infection (Bao et al. 2020).

8.9 Recent Concepts and Strategies Developed

8.9.1 Gene Editing

Abiotic stress is a complex trait controlled by many genes and their products that involved in signaling, regulatory and metabolic pathways, thus, just a modificationin a single gene may not produce any desired results. Therefore, more advanced and effective techniques that affect several genes simultaneously need to be applied. Gene manipulation with CRISPR-Cas 9 is one such technique. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR-associated (Cas) genes are essential components of a bacterial adaptive immune system to acquire resistance against invading virus. CRISPR/Cas9 uses a protein-RNA complex to target and cleavage the target sequence using a short guide RNA (Pennisi 2013). Scientists have found a great many utilities for this system in gene manipulation such as the introduction of single point mutations, deletions, insertions, inversions, translocations etc. in a particular target gene. In general, gene manipulation of monogenic traits is always easy than those controlled by many genes. However, abiotic stress is a trait controlled by many genes involved in gene regulations, signaling and several metabolic pathways. Thus, simultaneous manipulation of many of these genes is required to get the desired results. CRISPR-Case 9 can target several genes simultaneously due to the easiness of designing the high efficiency of sgRNAs. Multiplex genome editing has been successfully implemented in model and crop plants (Li et al. 2013; Mao et al. 2013; Zhou et al. 2014). CRISPR system is a powerful tool for genetic screen to identify gene function at genome wide level through generating point mutations and gene knock out. Guide RNA can be targeted to almost all the genes present in the genome in almost all the plant species, where genome sequence information is available. In addition, potential multiplexing CRISPR system help to investigate function of the gene families resulting from gene duplication. The screening of CRISPR induced mutants displaying altered biotic stress response aid in identifying genes involved in the process and also to develop disease tolerant crop plants. The use of CRISPR-Cas9 system in genotyping natural variations to distinguish homozygous biallelic mutants from wild-type has been demonstrated (Kim et al. 2014). However, in mulberry CRISPR based technology has been used yet. Thus, with the advancement of the genomics of mulberry, it is expected that gene editing with CRISPR-Cas9 technology would be applied in mulberry soon.

8.10 Brief on Genetic Engineering for Resistance/Tolerance Traits

Genetic engineering consists of isolation of a gene of interest, ligating it on a vector to transfer it into a plant genome to meet a purpose. The most important advantage of genetic engineering is the ability to manipulate gene expression as desired. In plant

breeding, the breeders can work only with plants that are cross-fertile but with genetic engineering genes from any organism including micro organisms can be inserted into the plant. However, the biggest challenges are the development of a robust, reproducible plant regeneration protocol and a genetic transformation method. In mulberry, such an efficient protocol for direct plant regeneration from leaf explants is still to be developed, though direct plant regeneration from hypocotyls has become an easy task (Vijayan et al. 2011).

8.10.1 Target Traits and Alien Genes

In mulberry, the most important trait is the leaf yield which is under the cumulative contribution of a number of associated traits such as plant height, leaf weight, number of branches, leaf retention capacity, nodal length, root length (Vijayan et al. 1997). However, under saline conditions, a change in correction was observed and the leaf yield had a significant correlation with plant height, leaf size, shoot weight, root weight, root length, protein, NRase activity and WUE of the plant. Similarly, the plant height was also found changing its correlation with most of the characters studied. This clearly shows that under different salinity levels the selection criteria for plants should be changed (Vijayan et al. 2009). It has also been observed that mulberry possess certain traits to confer higher tolerance to stress conditions. Some of these traits are elongated roots, thicker epicuticular wax, synthesis and accumulation of osmolytes like Proline, glycine beatain, etc. (Vijayan et al. 2005). Plants have progressed several mechanisms like thicker epicuticular wax, para heliotropic movements, salt-secreting hairs, elongated roots, synthesis and accumulation of osmolytes, etc. to tolerate the stress to facilitate retention and/or acquisition of water, protect chloroplast functions and maintain ion homeostasis (Vijayan et al. 2008). The genes involved in these pathways and mechanisms need to be incorporated either through conventional breeding or through genetic engineering. Since transfer of genes and traits into mulberry is very difficult due to breeding behaviour of the plant the easiest method is through genetic engineering. In genetic engineering, genes may be knocked out, over expressed, or modify the expression and product through gene editing. Over expression of *DREB*s (dehydration responsive elements binding proteins), *ERF* (Ets-2 Repressor Factor), *MYB* (myeloblastosis), *bZIP* and *WRKY* transcription factor families have shown promising results in several plant species (Jung et al. 2007). Further, Lu et al. (2008) have identified a low temperature encourage gene *WAP25* from Mongol mulberry, one of the wild species of genus *Morus* that cloned the gene and grows in cold regions (GenBank accession N0. DQ104333) into expression vector pIG121/*Wap*25 and transformed *Petunia hybrid* Vilm via *Agrobacterium.* This study, possesses the scope of genetic improvement of other mulberry species such as *M. indica, M. alba, M. latifolia* which are being used for silkworm rearing and are highly susceptible to cold and other stresses. Disease arises from compatible interaction between the host plant and pathogen. There are some genes in the plant which facilitate infection and further proliferation of pathogen upon entry, they

are referred to as susceptibility (S) genes. Therefore, mutation or loss of function of the S gene can provide resistance to different strains of the pathogen and long lasting protection. The best example for utilization of S genes in providing field resistance is Mildew Resistance Locus O (MLO) genes involved in powdery mildew resistance in barley (Acevedo-Garcia et al. 2014). To identify a susceptible gene, MLO was involved in powdery mildew in mulberry bioinformatics analysis using Arabidopsis MLO genes and MLO domain search was undertaken in *M. notabilis* genome. A total of 16 MLO genes were identified and their characteristic motifs were also determined. To identify MLO gene involved in powdery mildew susceptibility in mulberry, various criteria were applied such as phylogenetic analysis to identify clade V specific genes, protein motifs that are exclusively present in the functionally characterized MLO proteins and MLOs gene induction in response to powdery mildew infection identified MLO2 and MLO6A as candidate genes (Ramesha et al. 2020). For future work, the identified candidate genes may be screened for presence of non-functional mutants in the resistant germplasm or employ novel genome editing technologies to knock down the genes to impart powdery mildew resistance in mulberry.

8.11 Future Perspectives

Mulberry host plant is a perennial tree with prolonged generation times, high heterozygosity, out crossing breeding behavior, poor juvenile-mature trait correlations, polygenic nature of most of the vital traits posed many challenges to conventional breeders. Thus, marker-assisted selection (MAS) is considered a tool to accelerate breeding through early selection, especially for abiotic stresses. MAS depends on identifying DNA markers that are tightly linked to the trait of interest. As stated above, although a few genetic linkage maps have been developed using biparental mating with pseudo-test cross strategy, non of these genetic maps and subsequent efforts were able to identify validated QTLs to be used in breeding program in mulberry. The major limitations of the above efforts include the sparse distribution of markers, lack of tightly linked markers to the traits, minor QTLs and the low success rate in validating QTL in different genetic backgrounds and environments. Further, to develop high resolution maps to identify markers with a tight association, more abundantly available markers like Single nucleotide polymorphisms (SNPs) have to be developed. Such effort is currently in progress at different research organizations across the globe.

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Chapter 9
Genomic Designing for Biotic Stress Resistance in Sugarcane

R. Viswanathan, N. Geetha, A. Anna Durai, P. T. Prathima, C. Appunu, B. Parameswari, K. Nithya, T. Ramasubramanian, and A. Selvi

Abstract Sugarcane (*Saccharum* spp hybrid) is grown across the continents, principally for white sugar and bioethanol. It is a C4 plant, generates highest amount of biomass among the cultivated crops, and meets nearly 80% of the global white sugar requirement. The modern cultivated sugarcane is a derivative of *Saccharum officinarum* (noble canes) and the wild relative, *S. spontaneum*. Worldwide, breeding

R. Viswanathan (✉)
Division of Crop Protection, ICAR-Sugarcane Breeding Institute, Coimbatore 641007, India
e-mail: R.Viswanathan@icar.gov.in; rasaviswanathan@yahoo.co.in

N. Geetha · T. Ramasubramanian
Entomology Section, Division of Crop Protection, ICAR-Sugarcane Breeding Institute, Coimbatore 641007, India
e-mail: Geetha.N@icar.gov.in

T. Ramasubramanian
e-mail: T.Ramasubramanian@icar.gov.in

A. Anna Durai · C. Appunu
Plant Breeding Section, Division of Crop Improvement, ICAR-Sugarcane Breeding Institute, Coimbatore 641007, India
e-mail: A.Annadurai@icar.gov.in

C. Appunu
e-mail: C.Appunu@icar.gov.in

P. T. Prathima · A. Selvi
Plant Biotechnology Section, Division of Crop Improvement, ICAR-Sugarcane Breeding Institute, Coimbatore 641007, India
e-mail: Prathima.Pt@icar.gov.in

A. Selvi
e-mail: A.Selvi@icar.gov.in

B. Parameswari
ICAR-National Bureau of Plant Genetic Resources Regional Station, Hyderabad 500030, India
e-mail: parameswari.b@icar.gov.in

K. Nithya
Plant Pathology Section, Division of Crop Protection, ICAR-Sugarcane Breeding Institute, Coimbatore 641007, India
e-mail: Nithya.K@icar.gov.in

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strategies have improved sugarcane yield till 1970s and later cane yield remained static across the countries. Many biotic constraints seriously affect productivity of the crop which is specific to cane growing countries. Among the diseases, smut, ratoon stunting, yellow leaf and mosaic are the major constraints in most of the countries. The diseases like red rot and wilt seriously affect cane production in South and South East Asian countries with many historic red rot epiphytotics causing huge crop losses in India. Similarly, the phytoplasma diseases, grassy shoot and white leaf are serious constraints in Asian region. Recently, the diseases like rusts, pokkah boeng, red stripe etc. emerged as major diseases in different countries. Among the insect pests, stalk borers are ubiquitous in nature with serious economic losses and each country or region has unique group of borer pests. Apart from the borer pests, many sucking pests and root grub are also of serious concern to sugarcane cultivation. Among the management strategies, host resistance is successfully exploited against various diseases and healthy seed, heat treatment, and chemicals are the other management strategies adopted in tandem. In case of insect pests, an integrated management is followed with more emphasis on biological control and chemicals depending on the pests and the location. Though remarkable gains were achieved through breeding strategies, complex polyploidy hinders genetic advancements for various traits in sugarcane. Recently, various genomic tools, especially transcriptomics were applied to understand gene functions and molecular markers are partially successful. Although, genetic transformation was successful in developing many transgenic lines against various biotic constraints, application of genome editing is in nascent stage due to multiple alleles. Overall, the various biotic constraints are managed through host resistance and other strategies in an integrated approach. Genomic applications have helped to understand genomes of the crop and pathogens/insects and, host resistance and genetic engineering supports trait improvement.

Keywords Sugarcane · Diseases · Insects · Stalk borers · Complex polyploidy · Genomic applications · Transgenics · Molecular markers

9.1 Introduction

9.1.1 Economic Importance of Sugarcane

Sugarcane (*Saccharum* spp. hybrid, Poaceae) a C4 tall perennial grass, is commercially cultivated in tropical and subtropical areas around the globe (Yadav et al. 2020). Though sugarcane cultivation dates back to 5000 BC in the Indian subcontinent, its cultivation expanded after it became an industrial crop during the last 100–120 years in the continents of Asia, Americas, Australia and Africa. Amongst C4 plants, the crop is highly efficient in converting solar energy and accumulates maximum yield in biomass (Henry 2010). Currently, sugarcane contributes >70% of total global sugar production, mostly consumed as refined sugar and to some extent as *khandsari*, *gur*

or other sweeteners in the Asian countries. Of late, the crop has received much attention as a bioenergy crop to produce bioethanol, which is the major renewable energy source to meet the increasing requirement for energy by decreasing greenhouse-gas releases, hence, it has stimulated a widespread attention on this crop (Souza et al. 2014). Globally, it also generates a high biomass of about 279 million tons annually of lignocellulosic biomass of leaves in the field and bagasse in the industry (Chandel et al. 2012). Apart from bioethanol, sugarcane supports electricity production in the sugar mills by burning bagasse, the fibrous part of stalk after juice extraction in different countries. Paper and pulp industries use bagasse as a raw material in to produce paper and newsprints. In addition, green leaves and tops of sugarcane are also used as animal feed and filter-cake (pressmud) from sugar industries is fortified as manure in different countries.

If we consider biomass production, sugarcane stands number one among the cultivated crops; positions amongst the top 10 commonly cultivated crops globally. In 2020, ~1.9 billion tons of sugarcane was produced worldwide from an area of 26.5 million ha grown in ~100 countries. Brazil occupies the numner one position in terms of production of cane and sugar. India follows Brazil and both the counties contribute to nearly 64.0% of the global production. China, Thailand, Mexico, Pakistan, the United States, Colombia, Australia, Cuba, and the Philippines are the other major sugarcane producing countries [http://www.fao.org/faostat (accessed on 24 December 2021)].

In the past five decades, global sugarcane production increased nearly threefold, largely due to the growing demand for sugar and bioethanol. Genetic advances in new sugarcane cultivars that suited to specific situations were attributed to the enhanced production. At the same time, improvements in agronomical measures also played a role in increasing cane productivity (De Morais et al. 2015). However, overall growth in cane output is mainly contributed by a drastic rise in cultivation area of the crop. For e.g. from 1973 to 2013 sugarcane cultivation in Brazil, Thailand, China and India witnessed increase by approximately 500, 286, 237 and 94%, respectively, whereas improved cane harvest per ha in the respective countries were only modest viz. 60, 11, 59 and 38% during the same period (Zhao and Li 2015a, b). Many countries are facing yield plateaus and incidence of pests and diseases, declining soil fertility and climatic conditions are attributed to the observed stagnation in cane yield (Yadav et al. 2020). Genetic enhancement of recent varieties is the continuous process to improve sugarcane productivity. In addition, there is a need to improve management practices of various biotic agents viz. diseases, insect pests, nematodes etc. to prevent crop losses. To address stagnant yield scenarios in sugarcane, strong breeding strategies are need to be combined with protection and production strategies. Although there are numerous issues intrinsic to the crop constrain breeding efforts, new avenues in biotechnology and molecular biology can complement realization of genetic improvement through breeding. Many biotic constraints affecting production and productivity of sugarcane can be resolved through a holistic approach of integrating conventional and modern scientific advancements. This chapter addresses major biotic constraints affecting sugarcane crop across the globe, strength of classical breeding to address them through host resistance, integrated management of

biotic constraints, newer applications in genetic engineering and genome editing to address the constraints and way forward to a sustainable sugarcane cultivation by effective management of all the major biotic constraints.

9.1.2 Reduction in Yield and Quality Due to Biotic Stresses

9.1.2.1 Fungal Diseases

In India, severe red rot epiphytotics occurred in almost all the preceding decades and due to breakdown of resistance several elite cultivars such as Co 213, Co 1148, Co 6304, CoC 671, CoJ 64, CoSe 95422, etc. were removed from cultivation. Presently, the popular cv Co 0238 is affected by a very severe epiphytotics in the subtropical India due to sudden failure in ~0.5 M ha in the region. The present crop losses were estimated to be 1.0–1.414 billion US$ and is considered as the largest crop losses recorded in sugarcane (Viswanathan et al. 2022a). Impact of red rot to sugarcane is also recorded in Pakistan, Bangladesh, Myanmar, Thailand, Nigeria, South Africa, Malaysia, Guatemala, Nicaragua and other countries (Viswanathan 2021a). Over a century, red rot epiphytotics followed 'boom' and 'bust' cycles regularly after adopting a particular cultivar over an extensive area in India and the recent red rot epiphytotics on Co 0238 became catastrophic due to adoption of the variety in more than 70% cane area in subtropical states (Ram and Hemaprabha 2020; Viswanathan et al. 2021a) and this has been found to mimic 'Vertifolia Effect" where a selection pressure for the pathogen has occurred for emergence of a highly virulent pathotype due to uniformity in the host variety under field conditions (Viswanathan et al. 2022a). For commercial release of a variety, red rot resistance along with high yield and quality is prescribed in India. Varietal breakdown in sugarcane posed by the new variants of the pathogen *Colletotrichum falcatum* is huge as we are unable to harness the benefit of elite varieties in the field for a long time (Viswanathan 2021b). This puts extra efforts on breeding group to come out with matching clones regularly.

C. falcatum infection causes rotting of stalk tissues and in most cases entire stalk rots and dries, becomes unfit for juice extraction. Further invertases produced by the pathogen cause inversion of sucrose into glucose and fructose and this biochemical changes results in poor sugar recovery. In general, diseased canes exhibit a significant loss in cane weight (29–83%) and juice extraction (24–90%) or total losses (Viswanathan 2010). Further, inversion of sucrose due to mixing of juice from infected and healthy canes during milling process affects sugar recovery. The disease affects the crop from germination stage onwards, till harvest. Most prominent symptoms are pronounced after cane formation as drying of canes in patches or throughout the field. During severe outbreak, the disease causes 100% crop losses in plant and ratoon crops (Viswanathan 2021a; Viswanathan et al. 2018a, 2022a). Hence the disease is of foremost importance for sugarcane cultivation in many Asian countries, most pertinent to Indian subcontinent.

Smut is another important fungal disease which occurs globally and impacts sugarcane significantly. The disease becomes more serious under favorable conditions and often complete crop failures occur in ratoon crops (Viswanathan 2012a, b). Besides direct loss in cane yield, *S. scitamineum* infection can cause a significant reduction in sucrose content, purity and other juice quality parameters (Kumar et al. 1989). Varied losses were reported in different varieties and climatic conditions viz. 10–30% cane yield and 3–20% sugar losses, 68–80% cane yield, 32% in juice quality and 62% in cane yield from Australia and India (Goyal et al. 1982; Solomon et al. 2000; Magarey et al. 2010a, b).

In India, wilt is another major disease affecting sugarcane production due to extensive drying of stalks, like red rot; hence huge economic losses were recorded (Viswanathan 2020). In 1970s, loss to cane yield of as high as 65% was estimated with severe disease incidences in ratoons (Sarma 1976). Further, wilt causes deterioration in juice quality and is primarily due to conversion of sucrose into reducing sugars and other biochemical changes (Singh and Waraitch 1981). Reductions of 14.6–25.8% and 3–20% in juice extraction and sugar recovery due to wilt were reported respectively (Gupta and Gupta 1976). Under field conditions, wilt affected canes recorded poor juice quality of 1.5–2.0 Brix as against 13–19.5 in the healthy canes (Viswanathan 2020). Reduced juice quality in the wilt-affected canes usually hampers sugar processing in the mills. It was estimated that wilt causes a loss of 3–6 tons of canes per ha and annually it is estimated to about 12.7–25.4 MT in various seasons, by which wilt caused losses of several million dollars in India. Apart from direct losses to the growers, the sugar mills encounter loss in terms of unrecoverable sugar every year (Viswanathan et al. 2006). Combined infections of red rot and wilt pathogens are very common in epidemic areas and such infections cause more severe crop losses than their separate infections (Viswanathan 2010, 2013a, b). Further, losses caused by wilt are largely ignored due to its recognition during the stages of crop maturity. In Bangladesh, wilt occurs throughout the country and causes significant losses to cane production (Hossain et al. 2017).

Earlier importance of pokkah boeng (PB), a fungal disease was ignored since it was a minor disease; however different states in India recently recorded severe outbreaks of the disease (Viswanathan 2018). PB affects cane yield to a tune of 40–60% in the susceptible varieties (Goswami et al. 2014). PB affected canes recorded a considerable decline in sugarcane production and sugar yield parameters (Dohare et al. 2003; Singh et al. 2006). The disease severity with 1–90% disease incidences on most of the commercial varieties were recorded during 2007–2013 in Uttar Pradesh state, which cultivates more than 50% of sugarcane in India (Vishwakarma et al. 2013). Further, the disease drastically reduces internodal elongation in the stalks (Viswanathan et al. 2014a). The disease severity forced the farmers to take up fungicidal sprays in different parts of Tamil Nadu state in India. In China, correlation analysis of disease severity with plant height, cane girth, single cane weight, yield, and Brix showed significant negative correlation (Wang et al. 2017a, b).

Orange rust was not a serious constraint before 2000 in Australia; however, later appearance of a new virulent race caused severe outbreaks of the disease. Breakdown of rust resistance severely affected the popular cv Q124 which was gown in 45% of

cane area in the country (Magarey et al. 2001a). The epidemics caused about $200 M losses to cane industry in Australia (Magarey et al. 2001b). Here, the affected crop suffered a substantial drop in sugar content. In Florida, USA, almost all the varieties under cultivation were found susceptible to brown or orange rusts during 2015–16 crop season (Raid et al. 2015). During the same time in Brazil, the popular cv RB 72454 was grown in 22.1% of the sugarcane area, however by 2010 the varietal area was reduced to 4.7%. Orange rust susceptibility was considered as one of the reasons for loss in area. After noticing orange rust in 2009, severe outbreaks of the disease were recorded in new areas within two seasons in the country (Sao Paulo State) and susceptible varieties incurred a loss of 15–30% in cane production (Barbasso et al. 2010; Klosowski et al. 2013; Daros et al. 2015; Gazaffi et al. 2016).

Brown rust severity reduced 33 and 31% in cane and sugar tonnage per hectare, respectively in Australia (Taylor et al. 1986). After a severe brown rust epidemic in a popular variety, which occupied 60% crop area in Cuba, a new policy of restricting cultivation of a variety below 20% was implemented in the region, to reduce the impact caused by rust outbreaks (La et al. 2018). In USA, yield losses to a tune of 10–50% were reported in many popular varieties due to brown rust. Breakdown of resistance to rust due to new virulent strains of the brown rust pathogen in many popular cvs CP 74-2005, CP 78-1628 and CP 72-1210 led to their withdrawal from cultivation in Florida. In addition, a sudden outbreak of brown rust in south Florida state during 1988 caused destruction of more than 50% visible dewlap leaves in the canopy causing ~40% losses in the cv CP 78-1247 and 20–25% on another popular cv CP 72-1210, which occupied 60% sugarcane area, causig a monetary loss of $40 million (Raid 1988; Comstock et al. 1992; Raid and Comstock 2006).

9.1.2.2 Bacterial Diseases

Ratoon stunting disease (RSD) caused severe yield losses in Australia, USA, India, Argentina, South Africa, China and other countries (Putra and Damayanti 2012; Taher-Khani et al. 2013; Li et al. 2014; Viswanathan 2001a, 2016; Magarey et al. 2021). RSD incidence increased with the ratoon number and sugarcane in dryland areas were more severely affected than those in waterlogged areas. Further, RSD has a significant impact on sugarcane yield, usually reducing sugarcane production by 12%–37% however during drought stress, the yield reduction increases to 60% (Wei et al. 2019). RSD causes ~10–15% losses in cane yield, however, losses in cane harvest can go up to 50% in disease-susceptible cultivars, under drought conditions (Benda and Ricaud 1977). Magarey et al. (2021) made an impact analysis on RSD to Australian sugar industry and suggested $25 M loss in the 2019 crop.

The leaf scald disease (LSD) bacterium may cause severe losses in susceptible varieties by death of entire stools and impaired juice quality (Viswanathan 2012a, b). Red stripe caused by *Acidovorax avenae* subsp *avenae* (Aaa) was considered as a minor disease earlier. However, increased severity of the disease was recorded in different countries. In Louisiana, Aaa caused significant effects on sugarcane yields and studies suggested careful management strategies to prevent losses (Johnson et al.

2016). The following factors like changes in climatic conditions, promoting susceptible cultivars in a large area and development of new Aaa stains with high virulence were found associated with the disease outbreaks (Fontana et al. 2013; Grisham and Johnson 2014; Ovalle and Viswanathan 2020; Viswanathan 2012a, b).

9.1.2.3 Virus and Phytoplasma Diseases

All the viruses systemically infect sugarcane and virus titre increases over the vegetative generations, hence severe expression of the disease occur in the ratoons and where healthy seed nursery programs are not adopted. In Florida, Brazil, India and Reunion Island, the major sugarcane growing countries recorded severe occurrences of yellow leaf disease (YLD) up to 100% incidences (Comstock et al. 2001; Rassaby et al. 2004; Vega et al. 1997; Viswanathan 2002). The virus infection adversely affects various growth parameters in various sugarcane cultivars. Viswanathan et al. (2014b) estimated losses of 44–57% in photosynthetic rate, 47–48% in stomatal conductance, 36–47% in transpiration rate, 30–34% in chlorophyll concentration and 31–33% in leaf area index. By this, photosynthate movement from source to sink is hampered in sugarcane (Yan et al. 2009). Further, all the symptomatic leaves recorded increased sucrose content due to prevention of photosynthates in virus-infected canes (Izaguirre-Mayoral et al. 2002). Such physiological malfunction leads to reduced cane growth in YLD-affected crop (Lehrer and Komor 2008). In Thailand, 30% cane yield reductions were recorded (Lehrer et al. 2008). In India, YLD-symptomatic plants of the susceptible cvs Co 86032, CoC 671 and CoPant 84211 recorded a loss in the range of 38.9–42.3% in cane yield; similarly, ~34.15% loss in juice yield due to the disease was recorded (Viswanathan et al. 2014b). Similarly, drastic reductions in cane yield and cane juice quality in YLD affected crops were recorded in China and Brazil (Vega et al. 1997; Yan et al. 2009).

Studies conducted during 1970s in Brazil revealed that tolerant varieties with 100% mosaic showed 18% losses whereas, up to 75% losses were recorded with only 25% mosaic in the susceptible varieties (Matsuoka and Costa 1974). Impacts of the disease on crop growth and growth parameters were estimated on popular varieties cultivated in tropical and subtropical regions like CoC 671, Co 740, CoS 767, CoLk 8102, CoPant 90223. The study evidently revealed significant reductions in CO_2 assimilation rate, number of millable canes, sugarcane growth traits like stalk thickness, number of nodes and cane yield and cane quality traits and sucrose and reducing sugars metabolism (Bhargava et al. 1971; Singh et al. 2003; Viswanathan and Balamuralikrishnan 2005). Recently, Putra et al. (2014) observed mosaic in ~30% of surveyed sugarcane fields in Java, Indonesia, indicating widespread occurrence of the disease in the country.

In mosaic affected sugarcane plants, due to destruction of chlorophyll and weakening of photosynthesis growth is significantly repressed (Bagyalakshmi et al. 2019a) and this causes in shorter internodes, lesser millable canes, poor root growth, and a considerably lower sett germination and lower cane yield (Singh et al. 1997, 2003). Sugarcane mosaic has become ubiquitous in its occurrence in many countries like

Argentina, Australia, Brazil, Cuba, China, India, USA, Indonesia, Thailand, Puerto Rico, etc causing huge economic losses (Lu et al. 2021; Wu et al. 2012). Unfortunately, the impact caused by the mosaic viruses is not realized by the sugarcane farmers and sugar industries. Although Sugarcane bacilliform virus (SCBV) symptoms were clearly described in different countries, its impact to cane growth is not reported except a few. SCBV infection caused reductions in cane weight, juice recovery and sucrose level in juice in China (Li et al. 2010). In India also, SCBV infected clones exhibited severe stunting and poor growth in germplasm whereas the hybrid varieties shown extensive discolouration followed by drying of leaf lamina under field conditions (Viswanathan and Premachandran 1998; Viswanathan et al. 2019a).

Sugarcane white leaf (SCWL) disease is highly destructive in Thailand, Vietnam, Taiwan, Sri Lanka and Iran and severe yield losses were reported. In India, sugarcane grassy shoot (SCGS) phytoplasma caused 5–70% and complete crop losses in plant and ratoon crops, respectively, in popular cultivars in different states (Nasare et al. 2007; Tiwari et al. 2012; Viswanathan et al. 2011b). Impact caused by SCWL to sugarcane in Thailand revealed a loss of over 30 million US dollars to Thai sugarcane industry each year. Such severe economic losses due to SCWL were reported from Taiwan, Vietnam and Sri Lanka (Kumarasinghe and Jones 2001; Hoat et al. 2012; Wongkaew 2012).

9.1.2.4 Other Diseases

In Australia, Magarey et al. (2013) reported pachymetra root rot infection in 50% or more farms in nine of the 12 surveyed areas; however some areas had more than 80% affected farms. About $50m per annum economic losses were attributed to the root rot disease in Australia. Root-lesion nematode, *Pratylenchus zeae* was reported on a higher proportion in Australia and all parasitic nematodes are estimated to cause an economic loss of ~$80m annually (Blair and Stirling 2007).

9.1.2.5 Insect Pests

Worldwide the yield loss in sugarcane due to insect damage accounts for more than 10% (Ricaud et al. 1989). The crop protection cost in sugarcane amounted to AUD 111 million in 1996 in Australia of which AUD 14 million and 97.4 million were accounted towards the production loss and management costs for the pests and diseases respectively (McLeod et al. 1999). In Brazil, losses due to *Diatraea saccharalis* differed between seasons. For each per cent of bored internode the sugar yield losses were estimated to be 8.83 and 19.8% in the first and second season respectively with significant differences in the quality of sugar (Rossato et al. 2013). In Louisiana, losses and management costs due to *D. saccharalis* is more than USD 8 million (Wilson 2021). The major borer pests of sugarcane cause yield losses of nearly 25–30% (Kalunke et al. 2009).

In South Africa, the stalk borer *Eldana saccharina* and thrips *Fulmekiola serrata* seriously affect the sugarcane yields (Keeping et al. 2014). The major borer pest in Mauritius is *Chilo sacchariphagus* with 40–60% infestation (Soma and Ganeshan 1998) and the top borer *Scirpophaga excerptalis* in India and Indonesia (Mukunthan 1989; Koerniati et al. 2020) cause enormous losses to farmers and sugar industry.

A loss of 0.25% sugar yield was observed for every one percent increase in the infestation levels of *D. saccharalis* (Gallo et al. 2002). In Panama, infestation of the stalk borer *Diatraeata bernelle* led to losses in fiber, cane weight and sugar recovery. In comparison with canes with no damage (level 0), canes with damage (level 3) yielded 2.56t lesser sugar per hectare. There was a positive correlation between internodes bored and loss of sugar ranged from 12.9 to 26.47% (Valdespino et al. 2016). Significant financial losses in major sugarcane areas of China had been incurred due to a host of factors such as continued increase in the borer population, stalk damage as well as dead hearts in maturity phase of crop and resultant reduction in sugar and cane yields (Xie et al. 2012; Li et al. 2013a, b, c). In China, 45% in cane yield and 6% sucrose were observed due to combined infestation of *Chilo infuscatellus* and *Tetramoera schistaceana* (Li et al. 2017a, b).

In Indonesia, cane height and other cane traits were negatively affected by moth borers among which *S. excerptalis* and stem borer caused a loss of 40.8 and 15% in stalk mass (Goebel et al. 2014). In India, *C. infuscatellus* causes 55–60% reduction in mother shoots by killing of meristems and 43–76% reduction in tillers and eventually 16–43% cane yield is reduced (Thirumurugan et al. 2006; Geetha et al. 2018).

In Ethiopia, combined infestation of stalk borer pests, *Scirpophaga calamistis, Eldana saccharina* and *Chilo partellus* resulted in significant losses on stalk length (10.24%), cane yield (24.86%), and sugar recovery (34.34%). The overall potential loss in yield was 27.3% and the damage was the highest in the grand growth phase of the sugarcane (Michael et al. 2018). Since its introduction in Reunion, Mauritius and Madagascar during the nineteenth century, *C. sacchariphagus* is a serious pest on sugarcane. Yield loss during heavy infestations was found to be 30% in many commercial varieties in comparison to the resistant variety (R570). Several field trials over multiple crop seasons established that the variety R579 was relatively more susceptible to *C. sacchariphagus* than R570 (Rochat et al. 2001).

In the Belize, heavy incidence of the frog hopper (*Aneolamia varia*) resulted in 10% loss of cane yield during 2006–2007 in the northern region (Thomas and Bautista 2020). White grubs are a serious constraint in sugarcane production in all countries cultivating sugarcane (Allsopp et al. 1991; Goble 2012) causing 25–80% loss in cane yield in India (Prasad and Thakur 1959; Tippannavar 2013; Lamani et al. 2017), 39% of yield reduction in Australia (Sosa 1984) and a yield loss ranging from 23 to 55 tonnes per hectare (McArthur and Leslie 2004) in South Africa.

9.1.3 Growing Importance in the Face of Climate Change and Increasing Population

Impact of climate change is witnessed across the continents and most of the crops under cultivation and animals face this threat. Across the countries climate change is expected to significantly affect sugarcane agriculture, specifically in the developing nations probably due to low capacity to adaptive strategies, highly prone to natural calamities and inadequate research infrastructure and management strategies (Zhao and Li 2015a, b). Climate change induced frequency and intensity of extreme environments may negatively affect sugarcane production and probably continue to be affected. Further, geographic location and mitigation strategies will decide the degree of impact caused by climate change on sugarcane. The key factors such as weather and CO_2 in the atmosphere, temperature, rainfall etc. influence the crop production, especially in developing countries. Cane and sugar production have fluctuated with climate extremities in different countries, especially drought and precipitation.

Plant response to drought, heat, cold, salinity, high CO_2 concentrations, weeds, disease and pests in the changing climate are the best studied abiotic and biotic stresses (Pandey et al. 2017; Suzuki et al. 2014). Severe weather conditions have caused more incidences of diseases and overwintering pests with the corresponding input cost for control them. Changes in the precipitation and high diurnal temperature majorly influence the prevalence of insect pests (Hussain et al. 2018). Deviations from the regular patterns of temperatures may probably lead to changes in pest and disease incidences and this can impact crop production (Rosenzweig et al. 2014). Baez-Gonzalez et al. (2018) have suggested such associations with the infestations of sugarcane pests.

Since sugarcane crop is in the field for over 10 months, day and night temperature, rainfall pattern, and distribution and duration of light may have a key influence on growth of the crop. Further, they influence distribution of different pest and diseases in the crop during various growth phases and seasons. Deressa et al. (2005) observed a temperature increase by 2 °C and rainfall by 7% (doubling of CO_2) has negative impacts on sugarcane production in all sugarcane-growing regions of South Africa. Nevertheless, there are reports on positive side on raised CO_2 in controlled conditions enhanced water use efficiency, photosynthesis and biomass resulting high yield and productivity in sugarcane (de Souza et al. 2008). The enhanced temperature may change the incubation period of the pathogen in the host, may shorten the life cycle of the pathogen, may increase the spore numbers and more number of generations in crop cycle. Warm winters with high night temperatures enhances the survival of pathogens, life cycle of insect vectors, higher sporulating capacity and secondary aerial infection (Harvell et al. 2002).

Many pathogens spread their spore with help of wind and rain for a long distance. The wind direction may introduce the pathogen to the new areas where the crop is being grown and if the environment is favourable for infection and disease development, there is a chance for introduction of new diseases. Brown rust severity in

sugarcane has occurred in different countries or disease was introduced to new territories. Also rust resistant varieties quickly became susceptible due to faster gain of virulence by the new pathogenic races. Occurrence of orange rust was confirmed in Florida, Costa Rica, Guatemala, Nicaragua and Panama in 2007. Concerns were expressed over the sudden appearance of the disease in the American continent, probably due to climate changes (Viswanathan and Selvakumar 2021).

Smut outbreak was noticed on the east coast of Australia for the first time during 2006. Although it is due to climate change or not, it became a serious challenge to Australian sugar industry by initiating smut resistance programme (Croft et al. 2008a, b). Usually dry weather favoured the shedding the spread of smut spores in the field whereas a wet weather and rain negatively affects the spread. Since smut is distributed throughout sugarcane growing countries, it may emerge as a major constraint to cane cultivation in warmer environments. Pokkah boeng was earlier regarded as a minor constraint in India, however, its serious epidemics across the country in India is suspected due to favourable climatic factors for the disease development (Viswanathan 2018, 2020).

It is well established that abiotic and biotic factors influence disease development in sugarcane. Hence it is speculated that any impact to crop growth due to climate change would aggravate the crop to YLD seriously. In addition, climate changes on the vector i.e. sugarcane aphid *Melanaphis sacchari* in sugarcane ecosystem will also cause changes in disease epidemiology and disease build-up. Under field conditions in Guadeloupe, aphid population and YL disease progress had shown a correlation between them. In this study, precipitation during the first weeks of sugarcane growth showed a negative correlation to *M. sacchari* dispersal in the field and suggested that lack of rain or poor rain in initial crop phases favors severe YLD in a susceptible sugarcane variety (Daugrois et al. 2011). Similarly, late spring and early summer had the first *M. sacchari* incursion and aphid flow in Louisiana and this coincided with a high sequential increase of YLD (McAllister et al. 2008). Studies conducted at Coimbatore for four seasons revealed that precipitation pattern has a temporal fluctuation in aphid population (Viswanathan et al. 2022b).

Pest dynamics is synchronous with the vagaries of climate whether the changes are transient weather changes or seasonal or long term. As drought stress increases sugarcane vulnerability to pests (Showler 2012) and thus, developing multi-stress resistant varieties are vital (Dlamini 2021). For instance, in sugarcane, the borer *E. loftini* infestation increased during drought conditions. Crops irrigated adequately with well water had 82.8–90.2% lesser *E. loftini* eggs than those raised under drought situations (Showler and Castro 2010), as the leaves of drought stressed plants released oviposition cues. Similarly, during drought overproduction of reactive oxidative species (ROS) occurs, which escalates different pests including nematodes infections (Tsaniklidis et al. 2021).

Some of cultural practices as stalk burning before harvest or trash burning after harvest, mainly following during manual operations, impacts the climate severely, causing enormous heat and pollution disrupting the environmental balance. Self-detrashing varieties to minimize the drudgery of manual harvest and using the trash for mulching could be the options to refrain from trash or stalk-burning. Change

in pest status due to the variation in climate has been reported. Of the borers *D. saccharalis* and *D. flavipennella*, the dominant species changed from the former to the latter within a decade and the main reason suggested was intensive irregular rains favouring the latter (de Freitas et al. 2007).

9.1.4 Limitations of Traditional Breeding and Rational of Genome Designing

The major aim of any crop improvement activity would be to introgress one or a few favorable genes from donor into highly adopted variety, and to recover most of the recipient parental genome as rapidly as possible. Breeding for biotic and abiotic stress requires identification of stress tolerant genotypes mostly from the germplasm and accumulating their genes in current commercial cultivars. During the last 50 years, a remarkable accomplishment was made in plant breeding program by developing new improved sugarcane cultivars. Major emphasis was laid on sourcing genes contributing to better productivity and adaptability from related species and wild relatives through genetic manipulation at cultivar, interspecific or intergeneric level. Breeding for stress resistance through conventional means is challenging due to lack of knowledge on inheritance of disease resistance, transfer of undesirable genes from the wild accessions along with desirable traits and the presence of reproductive barriers especially in interspecific and intergeneric crosses.

Plant breeding has seen a major transition in the past decade as advances in biological sciences helped in evolving tools that can be applied to commonly accepted field techniques. Molecular markers have become a handy tool to accelerate plant breeding process by selecting desirable genotypes by following the genes or chromosomal segments in the crosses using markers that are closely linked to them. This is particularly important in the case of genes governing biotic and abiotic stresses where traditional methods of screening for the trait are laborious and time consuming. Sugarcane suffers from damages caused by various insect pests either by direct feeding of plant parts or by transmitting important viral diseases. Insecticides are used as a major control strategy to combat different insect pests. However, it was established that continuous use of insecticides results in development of resistance to the chemicals among the target insects and unintended harmful effects occur to beneficial insect population of pollinators, parasitoids and predators in the ecosystem. Hence, the best approach is to evolve plant varieties that are resistant to insects. For several years, breeding varieties for disease and pest resistance has been taken up. The inherent difficulties in the conventional screening and the misleading results in screening efforts, probably due to the polygenic control of resistance makes marker assisted selection (MAS) for pest and disease resistance a viable alternative. In marker-assisted selection, the selection is not on the elusive trait of interest but on the reliable molecular markers closely associated with the trait. Being environmentally independent and scorable even at very early stage of development; molecular markers ensure quicker

and clear-cut analysis at lower cost than phenotypic testing. Screening with molecular markers would be helpful especially when the trait is under polygenic control, most commonly seen in the case of pest and disease resistance. Biotechnological interventions play an important role to assist and improve classical plant breeding by integrating genomic tools that renders plant breeding programs more focussed, precision and less time consuming.

9.2 Description on Different Biotic Stresses

Throughout the world, negative impact of pests and diseases in sugarcane is reported and every sugarcane growing country suffers from insects and pathogens, although the type of causative organism varies. Nearly 125 diseases of fungal, bacterial, viral, phytoplasmal and nematode pathogens were reported from different continents (Rott et al. 2000). Although efforts are being made for the last 100 years to develop resistant varieties to various biotic constraints, the crop succumbs to many pests and diseases. The disease incidences and spread to new areas increased in different countries during the past decades. As per the report of International Society of Sugar Cane Technologists (ISSCT), each year several millions of dollars are lost due to diseases in sugarcane. Due to different diseases, each nation lose about 10–15% of their sugar production. Amongst, red rot, smut, and wilt are the major stalk diseases caused by fungal pathogens occur widespread across the sugarcane growing countries. Among the bacterial diseases, leaf scald (LSD) and ratoon stunting (RSD) caused by *Xanthomonas albilineans* and *Leifsonia xyli* subsp *xyli*, respectively occur in almost all the countries. Gumming disease and red stripe, the other bacterial diseases are known to inflict crop losses in some countries. Mosaic and yellow leaf (YLD) are the major viral diseases occur in all the sugarcane growing countries and affect sugarcane production considerably (ElSayed et al. 2015; Holkar et al. 2020; Lu et al. 2021). Besides these, phytoplasma diseases such as sugarcane grassy shoot (SCGS) and sugarcane white leaf (SCWL) seriously affect cane production in several countries in Asia. Foliar diseases such as rusts, eye spot, pokkah boeng, yellow spot, brown spot, ring spot, brown stripe, etc. occur throughout the world and their severity depends on the prevailing environmental conditions. Apart from these diseases, Sugarcane bacilliform virus causing leaf fleck has emerged as a serious constraint in different countries (Viswanathan et al. 2019a). Besides, Fiji disease confined to Australia and neighbouring countries and Pachymetra root rot limited to Australia are of regional importance.

9.2.1 Fungal Diseases

9.2.1.1 Red Rot (*Colletotrichum Falcatum* Went)

It seriously affects crop production in the countries like Bangladesh, India, Indonesia, Myanmar, Nepal, Pakistan, Thailand, Vietnam, and other Asian countries and is considered as a major stalk disease in USA, Brazil, Austrasia, Cuba, South Africa etc. Overall, the disease was reported from 77 countries in almost all the continents (Singh and Lal 2000). The fungal pathogen *C. falcatum* with perfect stage *Glomerella tucumanensis* [Speg.] Arx & Muller is associated with red rot. In Louisiana, the pathogen deteriorates the planted stalks or stubbles of sugarcane and this leads to failures in crop establishment (Hossain et al. 2020; Viswanathan 2021a). Sudden discolouration and drying of foliage, lesions of the rind and death of the affected stools are the field symptoms of red rot in a standing crop (Fig. 9.1a). The disease has been a serious menace from 1900 onwards in almost all the sugarcane growing countries when the disease was carried through seed canes from South East Asia. Over the decades, the disease menace has been reduced in many countries except South and South East Asia, where still epidemic occurrences of the disease destroy several thousands of ha. The pathogen causes extensive rotting of internal tissues and affected tissue turns red, hence it is called as 'red rot'. Typically, affected canes exhibit rotting of internal tissues with varying shades of red with characteristic white spots, perpendicular to the long axis of the cane (Fig. 9.1d).

The historic failures of elite sugarcane varieties in the past due to *C. falcatum* epiphytotics have started from the cv Co 205, the first man made hybrid sugarcane variety to the recent epiphytotics on Co 0238 were attributed to origin of new *C. falcatum* pathotypes. The new variants have gradually adapted to the new varieties which were hitherto resistant to the pathogen (Viswanathan et al. 2003a, 2022a; Viswanathan 2017, 2021a, b). Earlier, the new variants caused varietal breakdown or failures were designated as dark and light isolates based on the cultural morphology and usually light types were reported as virulent. In 1990s, a systematic study was conducted with a set of *Saccharum* spp and sugarcane hybrid varieties as differentials to characterize and designate the pathogenic variants in India (Padmanaban et al. 1996) and so far 13 pathotypes of *C. falcatum* were designated from different states (Table 9.1). This system of characterizing *C. falcatum* pathotypes ensures uniformity of using same pathotype to screen a common set of sugarcane varieties in advanced varietal trials in a region by different research centres (Viswanathan 2018).

C. falcatum exhibits enormous variation for pathogenicity, and also dynamic changes in virulence (Viswanathan et al. 2017a). Earlier studies of Malathi et al. (2006) revealed adaptation of *C. falcatum* to host varieties. In this, a resistant interaction becomes susceptible after repeated inoculations of the less virulent isolate on a resistant variety. Subsequent biochemical and molecular studies revealed pathogenicity factors that aid in pathogenicity of *C. falcatum* (Malathi and Viswanathan 2012a, b). Recently, detailed studies on red rot development from soil borne inoculum and plug method of inoculation on a set of varieties were conducted

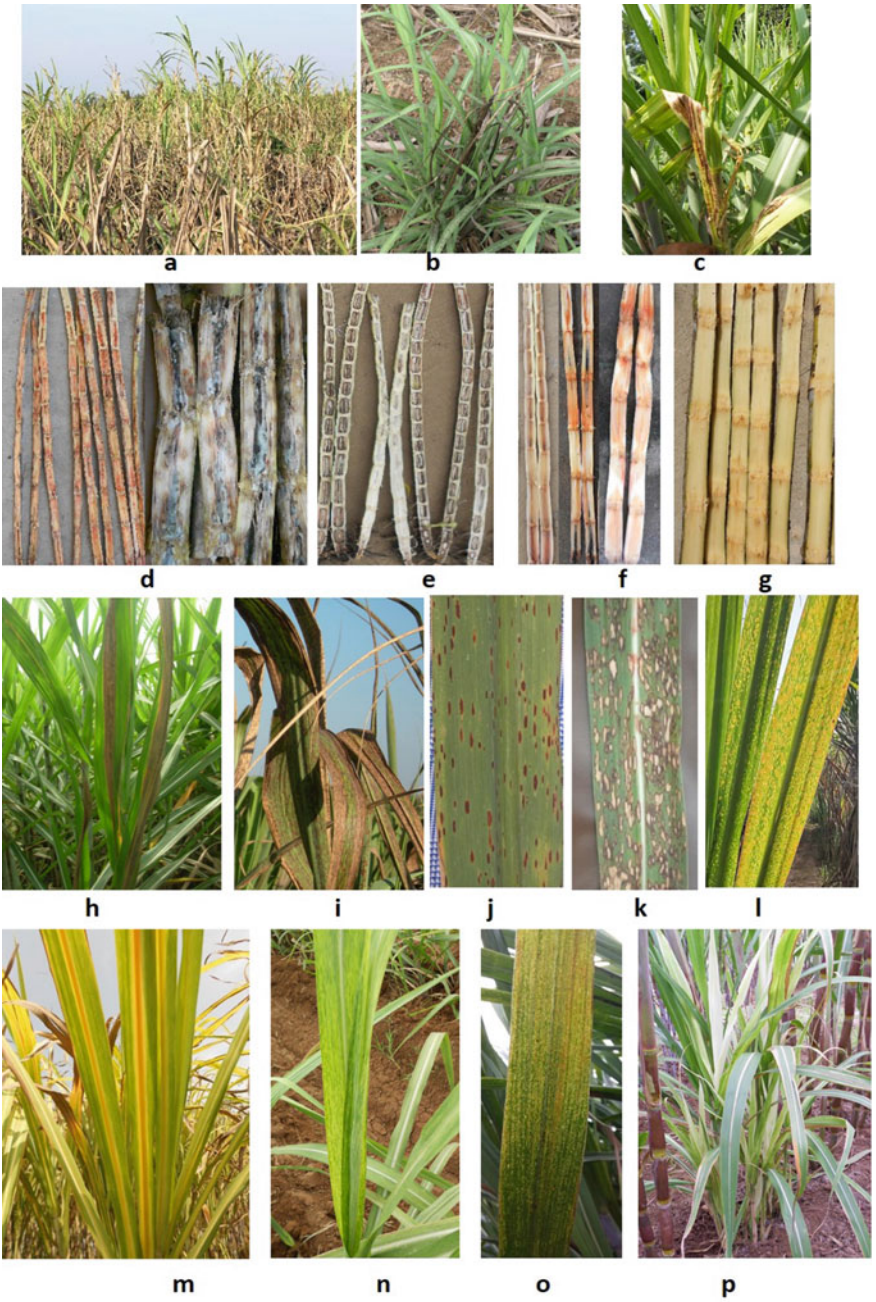

Fig. 9.1 Characteristic symptoms of major diseases of sugarcane. **a** Red rot-field symptoms, **b** smut, **c** pokkah boeng, **d** red rot-internal symptoms, **e** wilt internal symptoms, **f** pineapple disease, **g** ratoon stunting disease-internal symptoms, **h** leaf scald, **i** brown rust, **j** brown spot, **k** ring spot, **l** yellow spot, **m** yellow leaf disease, **n** mosaic, **o** leaf fleck, **p** grassy shoot disease

Table 9.1 Designated pathotypes of *Colletotrichum falcatum* in India

Pathotype	Host variety	Year of collection	Region
CF01	Co 1148	1997	Subtropical
CF02	Co 7717	1997	Subtropical
CF03	CoJ 64	1997	Subtropical
CF04	Co 419	1997	Tropical
CF05	Co 997	1997	Tropical
CF06	CoC 671	1997	Tropical
CF07	CoJ 64	2006	Subtropical
CF08	CoJ 84/CoJ 64	2006	Subtropical
CF09	CoS 767	2006	Subtropical
CF10	85A261	2006	Tropical
CF11	CoJ 64	2006	Subtropical
CF12	Co 94012	2009	Tropical
Cf13	Co 0238	2018	Subtropical

under field conditions. Pathogenicity of *C. falcatum* pathotypes from these assays clearly revealed that a pathotype exhibits a host adaptation to cause the disease in sugarcane (Viswanathan et al. 2020a, b). Further, the inoculum surviving in the soil makes repeated attempts to infect the host, finally succeeds to cause the disease in the field. By this, host resistance in a variety is compromised and 'resistance breakdown' or 'varietal breakdown' occurs (Viswanathan and Selvakumar 2020).

9.2.1.2 Smut

The whip smut caused by *Sporisorium scitamineum* (Phylum: Basidiomycota, Order: Ustilaginales) is a widespread disease of sugarcane across the continents, affecting both cane yield and sucrose content, hence substantial economic losses occur during severe cases (Bhuiyan et al. 2021; Rajput et al. 2021; Sundar et al. 2012). Emergence of long culmicolous smut whip (sorus) in growing point is the characteristic symptom of the disease or such whips on the axial buds and secondary tillers. The smut whips may be up to 1.5 m in length and contain millions of black teliospores (Fig. 9.1b). Severity of the disease is influenced by prevailing pathogenic races, environmental conditions, number of ratoons and varieties grown. Globally, efforts were made to identify race profile of *S. scitamineum* by assessing whip development in 14 locations across 10 countries on a set of standard host differentials. Although this study revealed existence of variability among *S. scitamineum* populations, a high level of pathogen diversity was found only in Taiwan (Grisham 2001).

Molecular studies with *S. scitamineum* isolates from 15 cane growing countries against 17 microsatellites revealed existence of a very low level diversity among African and American population as compared to the Asian population, which seemed as the major source of diversity in smut pathogen (Raboin et al.

2007). Although, molecular variation in smut pathogen has an association with their geographic origin, evidence for co-evolution between the host and the pathogen is lacking in China (Que et al. 2012). However, the studies from India suggested that *S. scitamineum* isolates originated from main sugarcane producing states exhibited a significant genetic and pathogenic variation. Further, prevailing environmental conditions and the varieties grown in the region are found to govern such pathogenic variation (Barnabas et al. 2018).

9.2.1.3 Wilt

Fusarium sacchari (E.J. Butler & H. Khan) W. Gams, (1971) (Nectriaceae, Hypocreales, Sordariomycetes, Ascomycota) is associated with the disease. Wilt is an important stalk disease, seriously affect production and productivity of sugarcane in different countries. Currently, it occurs in Bangladesh, India, Iran, Malaysia, Myanmar, Nepal, Pakistan and Thailand (Hossain et al. 2017; Rao and Agnihotri 2000; Viswanathan 2013a, 2018). Characteristic symptoms of wilt include stunted growth, drying of canes and internally, pith cavities and discolouration of the stalk tissues (Fig. 9.1e). In India, the disease occurs throughout the cane growing areas however; Indo-Gangetic plains of subtropical region, Gujarat and East Coastal deltaic regions witness disease severity to very high levels (Viswanathan 2018; Viswanathan et al. 2006). Only in the recent years, cause of wilt by *F. sacchari* has been established based on detailed pathogenicity and molecular analyses in India (Viswanathan et al. 2011a). The pathogen exhibits enormous variability for cultural and morphological characters (Poongothai et al. 2014a, b) and among the molecular markers, ISSR is more efficient followed by RAPD and rDNA IGS-RFLP to group the isolates (Poongothai et al. 2015).

9.2.1.4 Pokkah Boeng

Pokkah boeng (PB) is Javanese term meaning distorted or malformed spindle in sugarcane (Fig. 9.1c), earlier considered as a minor disease but now it occurs in many countries, devastating sugarcane productivity. Several *Fusarium* spp cause the disease and the following species *F. sacchari, F. andiyazi, F. verticillioides, F. proliferatum,* and *F. subglutinans* are reported from various continents/regions (Martin et al. 1989; McFarlane and Rutherford 2005; Govender et al. 2010; Mohammadi et al. 2012; Khani et al. 2013; Nordahliawate et al. 2008; Viswanathan 2020). *F. verticillioides, F. sacchari, F. proliferatum,* and *F. oxysporum* were reported as the casual organism in China, however, *F. verticillioides* is the dominant species associated with PB (Lin et al. 2014a; Bao et al. 2016; Meng et al. 2020). In the country, the disease occurs throughout year during both wet and dry seasons (Lin et al. 2014a). Further, *F. verticillioides* and *F. proliferatum* are reported as the cause of the disease in sugarcane and among the two, the former accounts for more than 90% of the records in the country. To confirm identity of the two species infecting sugarcane, a

species-specific PCR assay was developed (Lin et al. 2014a). In India, *F. sacchari* and *F. proliferatum* were isolated from the affected sugarcane; however, the former is frequently isolated from the infected samples (Viswanathan et al. 2017b). Majority of 55% *Fusarium* spp associated PB with knife cut symptoms in Iran was found to be as *F. verticillioides* and *F. subglutinans, F. proliferatum*, and *F. semitectum* are the other species associated with the disease. Almost all the isolates were pathogenic except *F. semitectum* isolates and among pathogenic species, *F. verticillioides* and *F. subglutinans* isolates were more pathogenic than isolates of *F. proliferatum* (Taher Khani et al. 2013). *F. verticillioides* and *F. proliferatum* were reported as the PB-associated pathogen in Mexico (Rosas-Guevara et al. 2014). Morphological features and molecular phylogenetic analyses grouped PB associated Fusaria and this broadly grouped them into two species *F. verticillioides* and *F. proliferatum* closely related to *F. sacchari* and *F. fujikuroi,* respectively (Leslie and Summerell 2006).

9.2.1.5 Pineapple Disease (Sett Rot)

In sugarcane, pineapple disease is a serious constraint and it causes rotting of the seed cane setts and rotting of standing canes. *Ceratocystis paradoxa* is the causative organism (anamorph: *Thielaviopsis paradoxa*). The disease is referred as pineapple disease because of sweet smell coming out of the diseased sugarcane was similar to ripened pineapple fruit. The disease is reported in more than 50 countries of both tropical and temperate regions. The disease causes 15–20% losses in sett germination, post germination death of seed cane sprouts and 10–15 tonne losses per hectare in cane yield (Girard and Rott 2000; Viswanathan 2012a, b). The pathogen affects standing canes particularly after damages caused by animal bites, lodging, water logging and red rot or wilt (Fig. 9.1f).

9.2.1.6 Rusts

Worldwide, two rusts, brown and orange rusts are regularly recorded on sugarcane (Rott et al. 2000). The former is caused by *Puccinia melanocephala* (Syd. & P. Syd) and the latter is caused by *P. kuehnii* (W. Kruger) E.J. Butler. During 2008, tawny rust, a new sugarcane rust, also referred as African sugarcane rust, was recorded in South Africa for the first time (Martin et al. 2017). Brown rust, also referred as common rust was recorded in ~29 sugarcane growing countries during 1980s, whereas currently it is reported from more than 40 countries (Egan 1980; EPPO 2019a). Severe outbreaks of brown rusts in Southern Karnataka on the cvs CoVc 03165, Co 0323 and other varieties like Co 94008, Co 98005, CoC 671, Co 94012 and VSI 434 with severe losses to crop production were recorded in the past (Fig. 9.1i) (Viswanathan 2012a; Selvakumar and Viswanathan 2019). Although orange rust was reported from ~18 sugarcane growing countries before 1980, the recent reports suggest that the rust occurs in nearly 45 countries, in the continents of Asia, Oceania, Africa and America (Egan 1980; Martin et al. 2017; Saumtally et al. 2011; EPPO 2019b). It was first

recorded during 2007 on the variety CP 80-1743 in Florida and subsequently it was recorded in other countries in America (Chavarría et al. 2009; Flores et al. 2009; Ovalle et al. 2008; Barbasso et al. 2010; Comstock et al. 2010).

Aerial spread of rust spores is of great concern since it will spread rapidly to the new areas in sugarcane growing countries. New variants of rust pathogens cause breakdown of resistance hence, many outstanding varieties under cultivation turn to be susceptible or resistant varieties have a tendency to pick up the disease slowly in the field (Braithwaite et al. 2009).

9.2.1.7 Other Foliar Diseases

Brown spot (*Cercospora longipes* E. J. Butler [1906]) is economically important in countries and regions where high relative humidity and mild temperatures of ~20–22 °C prevail (Saumtally and Sullivan 2000) (Fig. 9.1j). The disease is severely affecting productivity in the susceptible varieties like CoM 0265 in the tropical India and brown spot epidemics curtailed the spread of the variety in North Karnataka and South Maharashtra (Viswanathan and Ashwin 2020). Brown stripe caused by fungal pathogen *Bipolaris stenospilus* (teleomorph: *Cochliobolus stenospila*) is reported from various countries with severe damages to sugarcane cultivation in Cuba, Louisiana, Australia, Caribbean islands, Taiwan, India etc. The disease is favoured by factors such as drought or nutrient deficiencies resulting in huge losses to the crop (Martin and Egan 1989). Downy mildew caused by *Peronosclerospora sacchari* is characterized by leaf stripes of creamy white that become red upon aging with stunting of affected clumps. The disease is reported from Pacific, South Asia and South East Asian regions (Suma and Magarey 2000). Serious outbreaks have occurred in Australia, Fiji, Philippines and Taiwan and heavy yield loss is reported on susceptible varieties from 38 to 58% in Philippines. The yield losses can range from 20 to 90% with severe losses. Eye spot is another foliar disease caused by *Biploaris sacchari* is recognized by small "eye shape like" spots on laminar tissues and long streaks, several feet in length and sometimes 1/3 of an inch in width on susceptible varieties. The disease is considered as a minor disease and reported worldwide in the tropics and sub-tropics covering Africa, Asia, Americas, the Caribbean, Europe and Oceania (Comstock 2000). In India a severe disease outbreak occurred on the popular variety Co 419 in Karnataka (Kumaraswamy and Rabindra 1978). Cool moist weather favors the disease development. Ring spot, another minor disease caused by *Leptosphaeria sacchari*, generally infects the senescing leaves in tropical, high rainfall areas with humid conditions and is reported in more than 80 countries. Except the terminal leaves, the disease affects the entire foliage, hence it shows a burnt appearance from a distance. The disease may become a serious one in susceptible varieties if the preventive measures are not taken under disease favourable conditions (Fig. 9.1k) (Croft 2000). Similarly, yellow spot (*Mycovellosiella koepkei*) is prevalent in high relative humidity and heavy rainfall areas of sugarcane growing countries (Fig. 9.1l). The disease is of seasonal importance in sugarcane, reported worldwide from mild to severe form in India, East Asia, Central and South Pacific Islands, and also occurs

in Australia and Africa, Guyana, Trinidad, Barbados, Jamaica, Central and South America and North America. High yield loss is reported when 35–50% areas of the top 8–10 young leaves are affected due to the damage to the photosynthetic tissues. Sucrose content was affected in early maturing varieties and yield loss was reported in late maturing varieties under epidemic conditions (Ricaud and Autrey 1989).

9.2.1.8 Other Fungal/Oomycete Diseases

The other fungal diseases affecting sugarcane, reported worldwide in different countries with or without economic damages (Rott et al. 2000) are listed below:

Australian basal stem, root and sheath rot—unidentified basidiomycete fungus,

Banded sclerotial disease—*Thantephorous sasakii/T. cucumeris*,

Black leaf spot (tar spot)—*Phyllachora sacchari*,

Covered smut—*Sporisorium cruentum, Spacelotheca erianthi* and *Sporisoium schweinfurthiana*,

Dry top rot—*Ligniera vasculorum* (a plasmodiophoromycete fungus),

Ergot—*Claviceps purpurea*,

Leaf blight—*Leptosphaeria taiwanensis*,

Leaf scorch—*Stagnospora sacchari* and *Leptosphaeria bicolor*,

Marasmius basal stem, root and sheath rot—*Marasmius sacchari*,

Pachymetra root rot—*Pachymetra chaunorhiza*,

Pythium root rot—*Pythium arrhenomanes*,

Ramu orange leaf—unidentified Exobasidiales,

Red leaf spot (purple spot)—*Dimeriella sacchari*,

Red rot of leaf sheath—*Corticium rolfsii*,

Red spot of leaf sheath—*Mycovellosiella vaginae*,

Rind disease and sour rot—*Phaeocytostroma sacchari*,

Root and basal stem rot—*Xylaria* cf. *warburgii/X. arbuscular*,

Sclerophthora disease—*Sclerophthora macrospora*,

Sheath rot—*Cytospora sacchari*,

Veneer blotch—*Deightoniella papuana*,

White speck—*Elsinoe sacchari*,

Zonate leaf spot—*Gloeocercospora sorghi.*

9.2.2 Bacterial Diseases

9.2.2.1 Ratoon Stunting Disease (RSD)

Leifsonia xyli subsp. *xyli* (Lxx), the xylem-limiting bacterium is an unique bacterium causing RSD recorded almost in all the countries and is considered as a major disease constraint among the various sugarcane diseases (Viswanathan 2001a, 2016; Putra and Damayanti 2012; Taher-Khani et al. 2013; Li et al. 2014; Magarey et al. 2021). The disease is characterized by a stunted cane growth, which indicates reduced cane thickness, internode numbers and tillers. Also the internodes exhibit irregular shapes and ratoon crops express more pronounced disease symptoms. Usually RSD affected crops show a pale canopy due to loss of vigour and when it occurs with viral diseases of mosaic and YLD, a severe degeneration in the crop is noticed. Other than growth reduction, the disease is not recognized except orange-red nodal discolouration (Fig. 9.1g); hence the disease presence is largely ignored in many countries. It primarily affects cane yield, whereas other key economic parameter like sugar content show marginal impact. The disease expresses more severity in ratoons as well as in rainfed crops. RSD incidences varied from 48.9 to 100% depending on the sugarcane variety in China and it is the most significant disease constraint in the country, found widespread among the principal sugarcane diseases (Wei et al. 2019). In India, Lxx along with other viruses causing mosaic and YLD seriously affect cane productivity by means of varietal degeneration (Viswanathan 2004, 2016). Genome of Lxx is 2.6 Mb in length with 2,044 predicted open reading frames and genome analysis identified putative pathogenicity genes such as pectinase, lysozyme, wilt-inducing protein, desaturase and cellulase (Monteiro-Vitorello et al. 2004).

9.2.2.2 Leaf Scald

Xanthomonas albilineans, the gram −ve bacterium causing leaf scald disease (LSD) occurs in about 60 countries growing sugarcane, including Argentina, Australia, China, Brazil, India, Mauritius, Cuba, Reunion islands, Thailand, USA, etc. Like RSD, it is also a major disease of sugarcane and occurs worldwide (Rott and Davis 2000a; Lin et al. 2018). Typical manifestation of LSD vary from a narrow, single, white, sharp stripes or longitudinal blights to total wilting and necrosis of affected lamina, resulting in death of entire clumps (Fig. 9.1h) (Ricaud and Ryan 1989; Rott and Davis 2000a). Genetic relatedness of 218 *X. albilineans* isolates representing 31 regions worldwide revealed divergent populations of the bacterial pathogen. Worldwide, a narrow dispersal of the pathogenic variants was found (Davis et al. 1997). Recently *X. albilineans* complete genome was sequenced. The genome comprises a 3724 kb circular chromosome with a 31,536 bp plasmid. Whole genome analysis revealed an intra-species variability of *X. albilineans* and it further provided resources to explore its pathogenic potential and virulence (Zhang et al. 2020).

9.2.2.3 Red Stripe

Red stripe caused by the bacterial pathogen *Acidovorax avenae* subsp *avenae* (Aaa) in sugarcane occurs throughout the sugarcane growing countries, however its severity varies depending on the varieties under cultivation and prevailing environment. The disease manifests its symptoms in two phases viz. leaf stripe and top rot. Of the two, the latter is deleterious and causes severe crop losses since top rot phase causes death of the growing meristem or stunted cane growth (Martin and Wismer 1989; Rott and Davis 2000b). In molecular analyses, strains of Aaa in Argentina and other countries exhibited high degree of genetic variation (Fontana et al. 2013, 2019; Li et al. 2017a, b). The draft genome of Aaa is sequenced to ~5646 kb and it has a GC content of 68.6% (Fontana et al. 2016).

9.2.2.4 Other Bacterial Diseases

Other than these bacterial diseases, occurrences of the following bacterial diseases were reported in different countries, mostly as minor or seasonal diseases from different countries (Rott et al. 2000).

Bacterial mottle (*Pectobacterium chrysanthemi*),

False red stripe (*Xanthomonas* sp.),

Gumming (*Xanthomonas axonopodis* pv. *vasculorum*),

Mottled stripe (*Herbaspirillum rubrisubalbicans*),

Red streak (*Pseudomonas syringae* pv. *syringae*),

Spindle rot (*Acidovorax avenae* subsp. *avenae*).

9.2.3 Viruses

9.2.3.1 Yellow Leaf (YL) Disease

It was first reported during 1989 in Hawaii and later from other countries. Currently it occurs throughout cane growing countries and attained status of a major production constraint in India (El-Sayed et al. 2015; Holkar et al. 2020; Viswanathan 2021c). Sugarcane yellow leaf virus (ScYLV), a *Polerovirus*, is associated with YLD worldwide and the virus systemically infects phloem cells. The disease is characterized by mid rib yellowing, bunching of leaves in the spindle, drying of discoloured midrib and leaf tissues (Fig. 9.1m). Variation in the virus genome has been studied in detail based on complete genomes. Currently, 10 ScYLV genotypes occurring worldwide viz. from Brazil (BRA), China (CHN1-3), Colombia (COL), Cuba (CUB), Hawaii (HAW), India (IND), Peru (PER) and Reunion Island (REU) were characterized

(Moonan and Mirkov 2002; Abu Ahmad et al. 2006a, b; Chinnaraja et al. 2013; ElSayed et al. 2011; Gao et al. 2012; Lin et al. 2014a, b; Viswanathan et al. 2008a; Wang and Zhou 2010; Wang et al. 2012). The BRA genotype occurs in most of the countries but others are confined to few nations (ElSayed et al. 2015). Khalil et al. (2018) grouped 498 ScYLV isolates reported all over the world into 10 genotypes according to geographic origins.

9.2.3.2 Sugarcane Mosaic

Sugarcane mosaic virus (SCMV) subgroup of *Potyviridae* and Sugarcane streak mosaic virus (SCSMV) are associated with mosaic in sugarcane and the disease prevails worldwide (Viswanathan et al. 2018b). The viruses infect sugarcane, maize, sorghum, and many other grasses and cause yield losses. The disease symptoms are characterized by moderate to prominent forms of mosaic on leaves and in severe cases, entire leaf turn pale or yellow and causing yield decline (Fig. 9.1n). SCMV and SCSMV together or separately cause the disease in Asian countries, whereas Americas have infections of SCMV and/or Sorghum mosaic virus (SrMV). SCMV is predominantly reported from Africa and Australia however, recently infections of SCSMV have been reported from Ivory Coast (Koike and Gillaspie 1989; Grisham 2000; Gemechu et al. 2004; Chatenet et al. 2005; Xu et al. 2008; Viswanathan and Karuppaiah 2010; Gonçalves et al. 2012; Wu et al. 2012; Putra et al. 2014; Viswanathan et al. 2018b; Daugrois et al. 2020). Complete genomes SCMV were characterized from many countries with reports on prevalence of new strains, variation in genomes and recombinant isolates (Viswanathan et al. 2009, 2018b; Moradi et al. 2016; Xie et al. 2016; Bagyalakshmi et al. 2019b; Lu et al. 2021). SCSMV reported earlier as an unassigned member of *Potyviridae* (Hema et al. 1999); later it was characterized to a new genus 'Susmovirus' of *Potyviridae* (Viswanathan et al. 2008b) and it was subsequently rechristened as '*Poacevirus*'. The genome of SCSMV is characterized based on whole genome basis. Many whole genomes of SCSMV from Myanmar, China, Pakistan, India, Indonesia, Japan, and Thailand were reported (Fellers et al. 2009; Xu et al. 2010; Parameswari et al. 2013; Liang et al. 2016).

9.2.3.3 Leaf Fleck

Leaf fleck caused by Sugarcane bacilliform virus (SCBV) is a *Badnavirus* (*Caulimoviridae*). It was initially detected from Cuba in 1985 and subsequently from Morocco in 1986 (Lockhart and Autrey 1988). In India, the virus was initially reported from *Saccharum officinarum* and other germplasm clones (Viswanathan et al. 1996, 1999; Viswanathan and Premachandran 1998). However, recently prevalence of leaf fleck in severe form was recorded under field conditions on most of the popular cultivars under cultivation (Viswanathan et al. 2019a). Besides its occurrence worldwide, the virus is regularly detected in quarantine (Viswanathan et al. 2018b). Typically the disease symptom start as minute chlorotic specks, expand in size,

turn to yellowish and red and gradually the symptoms spread to entire leaf lamina. The severe expression of the disease on the older leaves and in severe cases entire foliage dries (Fig. 9.1o). SCBV exhibits enormous genomic variation (Rao et al. 2014). Initially two SCBV species Sugarcane bacilliform Morocco virus (SCBMV) and Sugarcane bacilliform Ireng Maleng virus (SCBIMV) were designated from Morocco and Australia, respectively (Bouhida et al. 1993; Geijskes et al. 2002). Later, complete genomes of SCBV from China, Guadeloupe and India were reported (Muller et al. 2011; Karuppaiah et al. 2013; Sun et al. 2016).

9.2.3.4 Other Viruses

Among the 23 virus species reported to infect sugarcane, SCMV, SCMV, ScYLV and SCBV are common in most of sugarcane growing countries (Boukari et al. 2020). In Australia, Fiji leaf gall caused by Fiji disease virus (FDV) was a major constraint to cane production (Smith 2000). Sugarcane mild mosaic virus (SCMMV), a *Closterovirus* has been reported as mixed infections with SCBV in germplasm from few countries (Lockhart and Autrey 2000). Peanut clump virus (PCV), a *Pecluvirus*, associated with red leaf mottle has been reported mostly reported from African countries (Rott and Chatenet 2000). Sugarcane striate mosaic associated virus (SCSMaV) associated with sugarcane striate mosaic disease, taxonomically intermediate between the genera *Carlavirus* and *Foveavirus* was reported from central Queensland, Australia (Thompson and Randles 2001). Ramu stunt virus with sequence homology to *Tenuivirus* genus causes Ramu stunt a serious constraint of sugarcane and is confined to Papua New Guinea (Braithwaite et al. 2019). There are reports of eight *Geminiviridae* members of the genus *Mastrevirus* species viz. Maize streak virus, Saccharum streak virus, Sugarcane streak virus, Sugarcane chlorotic streak virus, Sugarcane streak Egypt virus, Sugarcane white streak virus, Sugarcane streak Reunion virus, and Sugarcane striate virus (Bock et al. 1974; Peterschmitt et al. 1991; Hughes et al. 1993; Bigarre et al. 1999; van Antwerpen et al. 2008; Lawry et al. 2009; Candresse et al. 2014; Boukari et al. 2017, 2020; Yahaya et al. 2017). Two probable new viruses in *Umbravirus* and *Chrysovirus* genera were also reported after metagenomics studies (Filloux et al. 2018).

9.2.4 Phytoplasma Diseases

Sugarcane grassy shoot (SCGS) and sugarcane white leaf (SCWL) are the major diseases caused by phytoplasma and are confined to Asian countries and were not reported outside the continent. The countries, Bangladesh, China, Malaysia, Myanmar, Nepal, India, Thailand, Pakistan, Sri Lanka, Sudan, and Vietnam, and reported varying intensities of these diseases (Rishi and Chen 1989; Chen and Kusalwong 2000; Viswanathan 2000; Nithya et al. 2020). Both the diseases are characterized by excessive tillering, sprouting of axillary buds, leaves become short,

leathery and chlorotic and affected stools fail to produce millable (harvestable) canes (Fig. 9.1p). In the field, the diseases mainly spread through disease affected seed canes. The leafhoppers *Matsumuratettix hiroglyphicus* and *Yamatotettix flavovittatus* are the two known reported vectors for secondary spread SCWL disease in the field (Wongkaew and Fletcher 2004; Hanboonsong et al. 2006). However, role of insect vectors in spreading SCGS disease is not reported under field conditions. Usually the ratoon crops suffer severely and low cane productivity especially in the ratoons in these countries is attributed to severe outbreak of SCGS and SCWL diseases. SCWL phytoplasma and SCGS phytoplasma have close relations and come under the 16SrXI group (Wongkaew et al. 1997; Sdoodee et al. 1999). Detailed studies to characterize the phytoplasma associated with SCGS revealed occurrence of 16SrXI-B and 16SrXI-F strains in India however, there was no relation between phenotypic symptoms on sugarcane and the associated strains of phytoplasma (Nasare et al. 2007; Viswanathan et al. 2011b; Rao et al. 2017; Yadav et al. 2017). Recently, 0.505 Mb draft sequence of SCGS-phytoplasma genome from India was revealed with GC content of 19.86%, along with a putative plasmid of 2.9 kb (Kirdat et al. 2020).

9.2.5 Nematodes

Plant-parasitic nematodes occur worldwide in sugarcane. Species the following genera *Circonemella, Criconemella, Helicotylenchus*, *Hemicycliophora*, *Hemicriconemoides*, *Hoplolaimus*, *Pratylenchus*, *Rotylenchulus*, *Scutellonema*, *Meloidogyne, Ogma* and *Tylenchorhynchus* infecting sugarcane in 24 countries including Australia, Brazil, India, Kenya, Mauritius, Pakistan and South Africa (Stirling and Blair 2000; Ramouthar and Bhuiyan 2018). Symptoms of swellings and galls or lesions of varying dimensions are observed due to nematode infections. Such damages to root by the nematodes reduce plant growth which causes reduced tiller production and poor canopy coverage. Australian reports show 5–20% losses caused by nematodes every year and this loss is estimated to be more than $80 million in cane productivity. In the country, *Pratylenchus zeae* (lesion nematode) and *Meloidogyne* spp., especially *M. javanica* (root-knot nematode) are the major nematodes reported to impact sugarcane cultivation (Blair and Stirling 2007). For root-lesion nematodes, none of the commercial varieties evaluated in Australia are resistant. Recently, *Saccharum spontaneum*, was identified as a source of resistance to *Pratylenchus zeae* and *M. javanica* in Australia (Bhuiyan et al. 2019).

9.2.6 Occurrence and Distribution of Important Insect Pests

9.2.6.1 Major Borer Pests in Sugarcane

Most of the major pests of sugarcane are crambids *Chilo* spp. and *Diatraea* spp. of Lepidoptera, the former distributed throughout Africa and Asia and the latter confined to the new world (Bleszynski 1969). In tropics, *Sesamia* spp. and *Scirpophaga* spp. occur in large scale. The common sugarcane borers with the alternate crop hosts, occurring across the continents (Table 9.2) and in Asia are listed (Table 9.3).

Among the sugarcane pests, lepidopteran stalk borers are important pests (OECD 2016) causing enormous damage (Li et al. 2017a, b) leading to loss of quality (Sallam et al. 2010; Souza et al. 2013) and yield (Mengistu and Selvaraj 2013; Sattar et al. 2016) worldwide. They include the sugarcane giant borer *Telchin licus* (Drury) (Triana et al. 2020; Dinardo-Miranda and Fracasso 2013) in the Central and South America; the sugarcane stem borer *Diatraea saccharalis* (F.) in the Americas and the Caribbean region (Francischini et al. 2019), the *Eoreuma loftini* (Dyar), the Mexican borer in South Texas (Showler and Reagan 2017), in Mexico (Rodríguez-del-Bosque and Reyes-Méndez 2013), *Eldana saccharina* Walker, the African stem borer in South Africa (Keeping et al. 2014), the spotted borer *Chilo sacchariphagus* (Bojer) in China, South Africa, Swaziland and Mauritius, Réunion, Madagascar and Mozambique (Bezuidenhout et al. 2008), *Chilo sacchariphagus indicus* (Kapur), the internode borer in India (Geetha et al. 2010), *Proceras venosatus* Wlk (Weng et al. 2006), *Chilo infuscatellus* (Snellen), *C. sacchariphagus, Tetramoera schistaceana, S. inferens* and *Scirpophaga intacta* (Snellen) in China (Zhang et al. 2019).

Across the world atleast fifty crambid and noctuid borers of *Chilo, Sesamia* and *Diatraea* genera infest sugarcane (Wijayanti et al. 2021) while 36 species of them were recorded by Sallam (2006) in Asia and islands in the Indian ocean. In the old world, *Chilo* and *Sesamia* occur but *Diatraea* is a pest in the new world. The constantly occurring important stalk borers belong to the *Chilo* genus that are extensive and intensively distributed in Indian Ocean Islands (Williams 1983) and Mozambique, Africa (Youdeowi 1989; Kfir et al. 2002), China (Rossato et al. 2013) causing severe loss and easily spread by vegetative propagation (Rossato et al. 2013). In Indonesia, *C. sacchariphagus, C. auricilius* and *Scirpophaga excerptalis* are the most important sugarcane borers (Goebel et al. 2014) and *C. infuscatellus, T. schistaceana*, *S. inferens* and *Phragmataecia castenea* are minor pests (Achadian et al. 2011).

The noctuid sugarcane pink borer *S. inferens* has extended distribution in the East Asia (China, Japan) and many of the Asian countries including Philippines, Bhutan, Malaysia, Bangladesh, Brunei, Taiwan, Korea, Nepal, and Srilanka, (Jeevanandham et al. 2020) and infests various graminaceous hosts like finger millet, maize, sorghum wheat, rice, and citronella grass besides sugarcane (Fletcher 1920). This polyphagy enables persistent occurrence throughout the year in the ecosystem allowing the pest to multiply rapidly in the most favorable host before transferring on to sugarcane. Vast distribution of the stem borers *Sesamia calamistis* Hampson and *Chilo partellus*

Table 9.2 Major borer pests of sugarcane in the world

Pest species	Local name[a]	Alternative crop hosts
Argyroploce (*Tetramoera schistaceana* [Sn.])	Grey borer, sugarcane shoot borer	Nil
Chilo sacchariphagus (Bojr.)	Spotted borer	Rice, sorghum, maize
Chilo auricilius Dudgeon	Stalk borer, gold-fringed rice borer	Sugarcane, rice, maize and sorghum
Chilo infuscatellus (Snellen)	Striped stem borer, early shoot borer, yellow top borer	Rice, oat, maize, barley, sorghum, *Andropogon* sorghum
Chilo agamemnon Bleszynski	Purple lined borer, lesser sugar cane borer	Maize, rice, sugarcane, sorghum
Diatraea saccharalis (Fabricius)	Sugarcane stalk borer	Maize, rice, sorghum
Chilo terrenellus (Pagenstecher)	Sugarcane borer, sugarcane internode borer	Nil
Diatraea flavipennella (Box)	*Broca pequena da cana-de-acucar*	Nil
Diatraea indigenella (D. & H.)	Stem borer	Maize, sorghum
Diatraea considerate (Heinrich)	Stalk borer	Maize, sorghum
Diatraea rosa (Heinrich)	Stem borer	Nil
Diatraea grandiosella (Dyar)	South western corn borer	Maize
Diatraea tabernella Dyar	Stalk borer	Nil
Eldana saccharina (Walker)	Sugarcane stalk borer, African sugarcane stem borer, Eldana borer	Maize, sorghum, cassava
Eoreuma loftini (Dyar)	Mexican rice borer	Rice, maize, sorghum
Scirpohaga excerptalis (Walker)	The white top borer or sugarcane top borer	Rice, wheat
Sesamia inferens (Walker)	Purple borer	Wheat, maize, sorghum, ragi, rice
Sesamia grisescens (Walker)	Ramu shoot borer, the pink sugarcane borer, shoot borer, sugarcane borer, pink stalk borer,	Napier grass *Pennisetum purpureum*
Sesamia cretica (Lederer)	Sorghum borer, durra stem borer, sorghum stem borer, purple stem borer, the corn stem borer, sugarcane pink borer, pink corn borer, maize borer, large corn borer, greater sugarcane borer	Maize, sorghum

(continued)

Table 9.2 (continued)

Pest species	Local name[a]	Alternative crop hosts
Tryporyzanivella intacta (Sn)	Sugarcane top moth borer	Nil
Telchin licus licus	Sugarcane giant borer, banana stem borer	Banana
Coleoptera *Metamasius hemipterus* (L.)	West Indian cane weevil	Banana, pineapple, palms, maize
Rhabdoscelus obscurus (Boisd)	New Guinea cane weevil borer, beetle borer, cane weevil borer, New Guinea sugarcane weevil, Hawaiian sugarcane borer, sugarcane weevil borer	Palms, banana, maize

[a]Local names are those by which the pest is addressed in literature or by farmers

(Swinhoe) in main land Africa, and spread of *Sesamia cretica* Ledere upto Southern Europe has been reported by Sallam (2006). In South East Asia, *Chilo auricilius* is a major sugarcane borer and a serious stalk borer in northern India (Neupane 1990).

In India, of the more than 200 pests recorded on sugarcane only a few borers and sucking pests severely affect the cane yield and quality of the produce (Figs. 9.2 and 9.3). The loss due to insect pests in sugarcane production is 20–25% (Kumar et al. 2019).

9.2.6.2 Genetic Divergence of Insect Populations

Very few records of biotypes of insects on sugarcane are available. Genetic divergence of the borer species *D. saccharalis* between the populations of southern United States, Mexico and Brazil was observed (Pashley et al. 1990). Different biotypes of the sugarcane moth borers that belong to the genera of *Diatraea, Chilo, Eoreuma, Sesamia* and *Bathytricha* within collection localities and across their distribution could be identified through molecular characterization of COII and 16S sequences (Lange et al. 2004). Though (Joyce et al. 2016), the widely distributed *D. saccharalis* is still assumed to be a single species Joyce et al. (2014) demonstrated the existence of two different genotypes in United States.

Divergence analyses often has the ability envisage the expansion and invasion of a pest. Francischini et al. (2019) analyzed the genetic diversity of *D. saccharalis* in America through molecular markers targeting entire genome and comparing the mitochondrial gene sequences. The clustering analyses indicated three distinct groups, which showed the distribution pattern of genetic diversity in the Americas suggested possible extensive spread through human-mediated movement. In India, the host based genetic divergence in the populations of *S. inferens* was established through SSRs (simple sequence repeats) analysis (Reetha and Mohan 2018).

Table 9.3 Major stemborers in Asia with their host plants and distribution (Adapted from Hittori 1971)

Species	Host plant					Distribution												
	Rice	Sugarcane	Maize	Wheat	Others	China	Japan	Formosa	Philippines	Indonesia	Malaysia	Vietnam	Thailand	Burma	Pakistan	India	Srilanka	Others
C. infuscatellus		✓	✓		✓			✓	✓	✓				✓		✓	✓	Afghanistan, Central Asia, Timor, Vulcan
C. sacchariphagus		✓			✓				✓	✓	✓							Madagascar, Mauritius, Reunion
C. s. indicus	✓	✓			✓													
Chilo tumidicostalis		✓														✓		Nepal
Chilo auricilius	✓	✓	✓		✓			✓	✓	✓			✓	✓		✓	✓	Bhutan, Nepal, Sikkim, Sangir, Molucca
Chilo partellus	✓	✓	✓		✓										✓	✓	✓	Afghanistan, Africa
Chilo polychrysus	✓	✓	✓		✓	✓			✓	✓	✓		✓			✓		Assam
C.s. straminellus		✓			✓	✓	✓	✓										
Acigona steniellus		✓										✓				✓		
Tryporyza nivella		✓				✓	✓	✓	✓	✓				✓		✓	✓	New Zealand, Africa
T.n. intacta		✓			✓					✓								
Sesamia inferens	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Korea

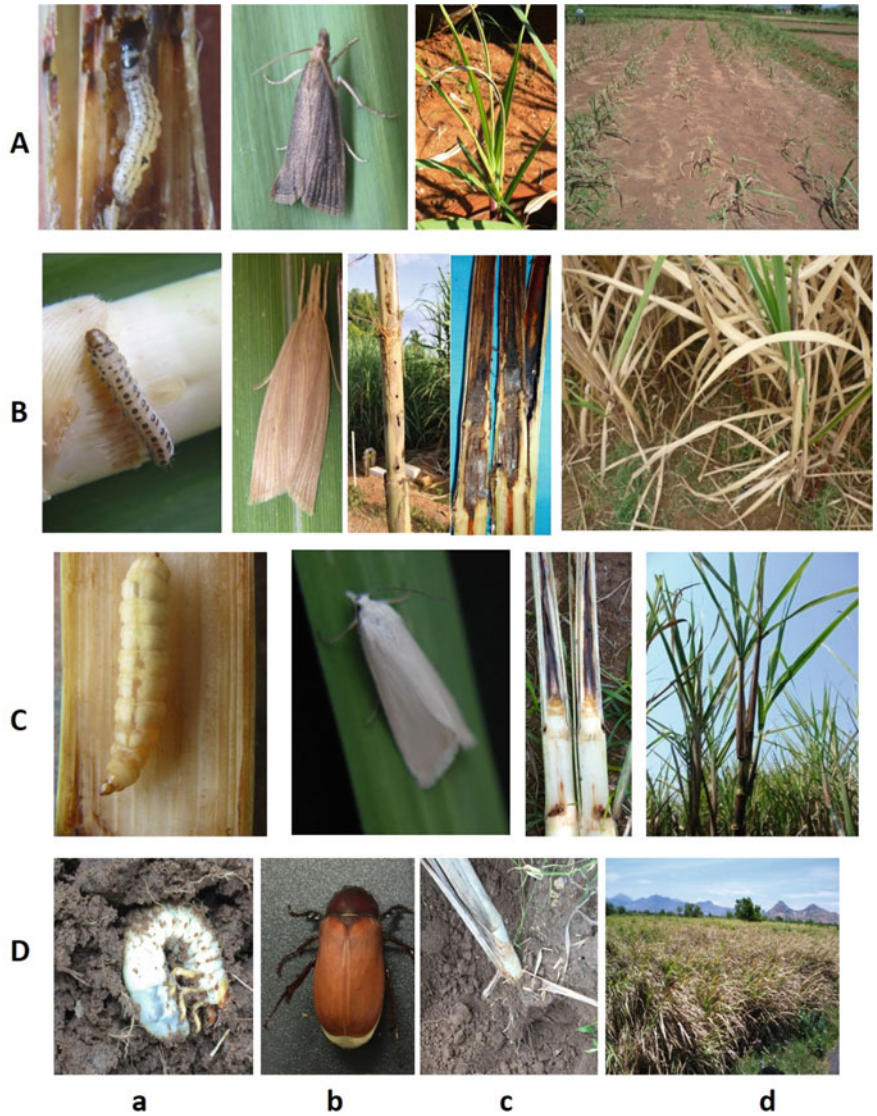

Fig. 9.2 Major insect pests of sugarcane; **A** shoot borer: *Chilo infuscatellus*, **B** internode borer: *Chilo sacchariphagus indicus*, **C** top borer: *Scirphophaga excerptalis*, **D** white grub: *Holotrichia serrata*; **a** larva (grub), **b** adult, **c** damaged plant, **d** affected field

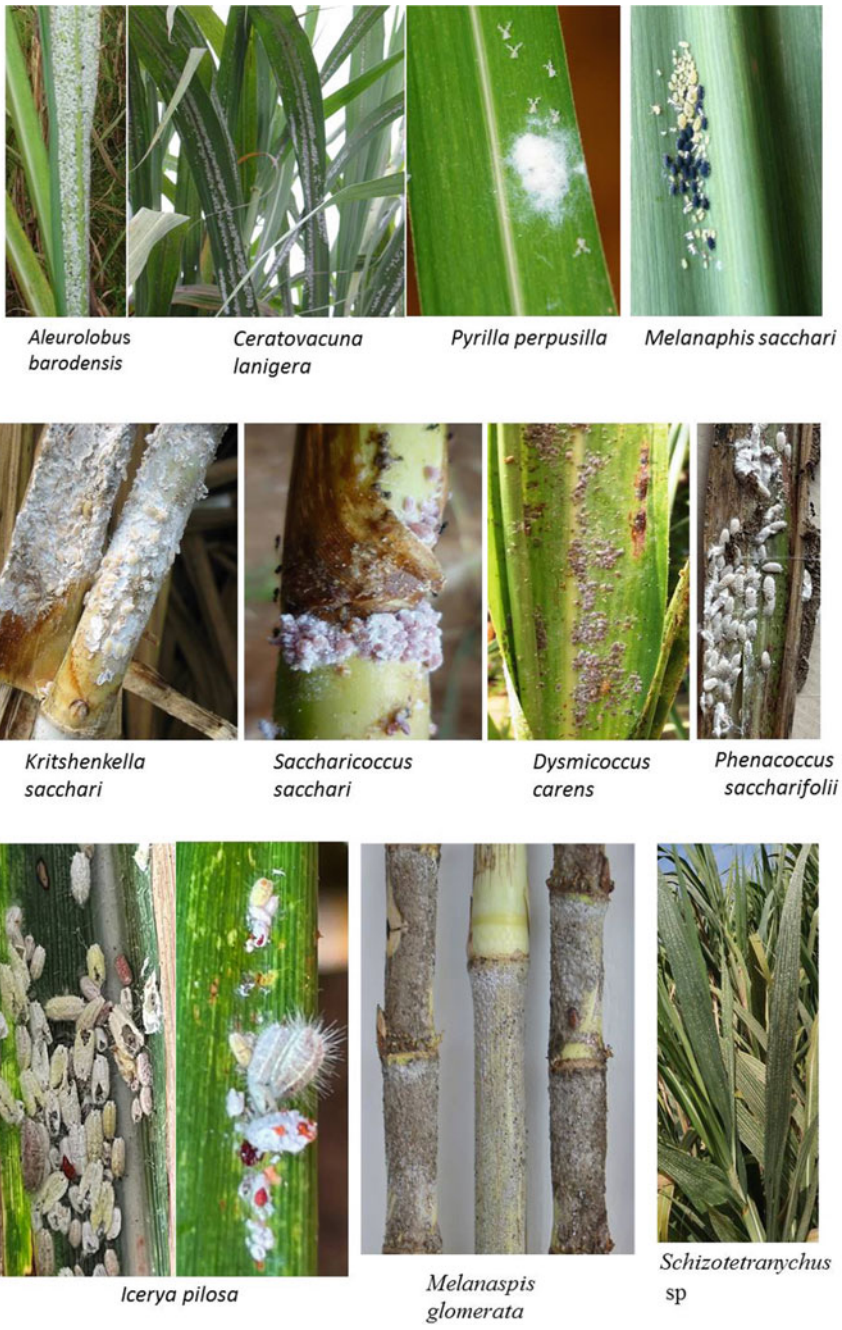

Fig. 9.3 Major sucking pests of sugarcane

9.2.7 Stages and Extent of Damage

9.2.7.1 Diseases

In sugarcane, vegetative propagation through seed canes (setts) is commonly practiced across the countries. The crop is harvested during 12–18 months after planting. Rarely, the crop is harvested 24 months after planting in places like Hawaii. After harvest of the plant crop, the second crop referred as ratoon is raised from the stubbles for many seasons. Number of ratoons again varies from country to country. In India, two ratoons are common, of course there are also 20–25 ratoons successfully grown in certain isolated pockets. Overall, sugarcane crop is grown as a plantation crop in large estates in different countries especially in the continents Americas, Australia and Africa, however, the farmers grow sugarcane in small holdings, in most of the Asian countries. Hence type of cultivation has a major influence on pest and disease outbreaks and management strategies to be adopted. Major fungal, bacterial, viral and phytoplasmal diseases are transmitted mainly through infected seed canes (setts). Hence the infected setts introduce diseases in the field and its manifestation may occur during 0–60 days in germination phase, active tillering during 60–150 days, grand growth during 150–270 days and maturity phase from 270 days to till harvest, depending on the initial pathogen load, additional inoculum in the soil, inoculum carried through secondary sources (air, water, vectors etc.) and prevailing environment. Similarly, inoculum left in the stubbles of plant or ratoon crops serves as primary inoculum for the succeeding ratoon crops.

Since many stalk diseases like red rot and wilt cause death of entire stalks, such canes become unfit for sugar extraction and left in the field. Farmers bear the loss in cane yield due to death of canes (Fig. 9.1a, b, d, e). During the milling process, infected canes either partial or full crushed with healthy canes, spoil juice quality thereby reduces sugar yield. When canes are purchased based on tonnage as practiced in many Asian countries, the millers bear the loss in sugar yield. Practically, diseased canes are also taken together with healthy canes to the mills. Wherever sorting system followed in cane yards of the mills, all the dead canes due to red rot, wilt or sometimes pineapple disease are removed and only healthy canes are taken for milling. The first author recorded heaps of rejected wilt affected canes in the yards and this scenario portrays supply of healthy canes to milling and also prevalence of severe wilt in the east costal region in India (Viswanathan 2012a, b, 2020). Such sorting processes are now discontinued in many mills due to labour scarcity and industries suffer due to poor juice quality in the milling process, especially in deltaic regions and areas prone for waterlogging. Scale insect infestation is a serious issue in sugarcane, because the insects cannot be removed from the canes (Fig. 9.3). Crushing of the insect affected canes affect the juice quality due to direct loss in sugar by the insect feeding and chemical compounds from crushed insects also impair juice quality. Apart from red rot and wilt, smut causes severe yield losses in terms of number canes produced in a stool or area. Foliar diseases affect cane growth and yield and ultimately sugar yield, depending on the severity of infection during tillering and grand growth

phase. Infection of these diseases during maturity phase does not affect cane yield significantly since the crop does not grow much during this phase or flowering takes place. However, severe infection during this phase affects sucrose accumulation in the canes.

Overall, severity of the stalk diseases and varietal degeneration caused by non-fungal diseases is more in ratoon crops. Poor ratoon productivity in canes in many countries is attributed to degeneration of canes and also due to increased availability of fungal inoculum to cause severe diseases in cases of red rot, wilt and smut. Crop losses to a tune of 100% are recorded on several occasions in India after severe outbreaks of these three major fungal diseases, mostly in ratoons (Viswanathan 2018, 2020, 2021a). Severe disease outbreaks or varietal degeneration restricts number of ratoon crops and this affects profitability to farmers and sugar mills.

9.2.7.2 Factors Affecting Insect Pest Infestation

Climate, cultivars, cultivation practices and disturbance in the ecosystem determines a pest status in any given location or in a crop. Climate, cultivar and crop type majorly impacted the infestation and damage of sugarcane stalk borers in different locations (White et al. 2001; Mengistu and Selvaraj 2013). For example, population and thus the intensity of *D. saccharalis* infestation is affected by the adopted cultivars, varying temperature and moisture conditions throughout the year and population of natural enemies among others. Multiple overlapping generations throughout the year and the occluded larval and pupal stages of the pest inside the plant make it difficult to estimate populations and control the pest. The response of a variety to borers varied according to the different climatic conditions. Humidity was the most important factors limiting the borer infestations. The unirrigated fields were found to be more infested than irrigated ones. Plant canes tend to be more susceptible than ratoons (Williams 1983). Rajabalee et al. (1990) showed a positive correlation between the percent internodes damage and sugar loss.

When new areas are brought under cultivation of existing crop or in case of introduction of new crop into a location, the pest status gets altered. In Brazil, extended area under sugarcane and application of vinasse had been suspected to boost the population buildup of the root pest *Sphenophorus levis* that often kills the host plants (Martins et al. 2020). Geographic distribution of the insect pests on sugarcane is extremely narrow except for a few that are cosmopolitan (Mengistu and Selvaraj 2013). In Indonesia, species predomination among different borer species is impacted by several ecological components such as climate, cropping system varieties and edaphic factors resulting in changes in the individual pest behavior biology and population dynamics in borer composition and distribution (Wijanaythi et al. 2021). Soil moisture and soil clumping might interfere with underground movement of borer and thus suppress the population. Changes in species predominance have been observed due to the change in weather patterns and varieties. In China, increasing temperature during winter and introduction of newer varieties has altered the species

dominance in the borer complex. Reversal in the status, damage potential and distribution of the important borers *Tetramoera schistaceana* and *Chilo sacchariphagus* (Leul and Thangavel 2013; Li et al. 2013a) was observed.

Biochemical composition of the plant, i.e., the internal plant environment of the cane influences the build-up of the pest. Higher infestation of root knot nematodes in sugarcane, *Meloidogyne* spp. was linked to lower concentrations of free amino acids in plants (Heppner et al. 2008). Sugarcane plants stressed by drought had higher levels of dry leaf tissue and elevated concentrations of amino acids in the cane stalks, such plants were preferred for oviposition by *E. loftini* (Showler and Castro 2010). In Bihar, India, *C. auricilus* became a major pest due to the excessive usage of nitrogen fertilizers, extension of area under soft and high sugar varieties (Kumar et al. 1987).

9.2.8 Conventional Methods of Control

9.2.8.1 Diseases Management

Disease-Free Planting Materials

Multiplication of sugarcane through setts favors carry over of most of the pathogens except foliar pathogens through seed canes (setts) and hence planting of disease-free seed canes is emphasized to prevent disease introduction in the field. Disease-free canes are raised in designated nursery plots. In case of non-fungal diseases, the disease can be managed effectively only through healthy seed nursery programme. Nowadays, tissue (meristem) culture is recommended to produce viral and phytoplasma-free planting materials; however, molecular assays for the designated pathogens are done to ensure a total freedom of the pathogens in the seedlings. After YLD assumed a major menace to sugarcane production in India, sugarcane varieties were multiplied in large-scale through meristem culture. To support this venture, molecular diagnosis was made compulsory to produce ScYLV-free plantlets (Viswanathan 2012a, b). Additionally, the plantlets were also indexed for SCMV, SCSMV and SCGS phytoplasma to address varietal degeneration in India (Viswanathan 2016, 2021c; Viswanathan et al. 2018c). Efficacy of meristem culture in virus elimination has been validated in RT-qPCR assays by comparing virus titre in virus-free seedlings and asymptomatic plants in the field (Chinnaraja et al. 2014).

Elimination of SCGS phytoplasma through tissue (meristem) culture combined with PCR assays in healthy seed programme has been found as a major approach to control the disease under field conditions. Further, multiplication of disease-free plants through single bud settling nurseries ensures very rapid multiplication of healthy seed for the popular varieties in India (Viswanathan 2016, 2018; Viswanathan et al. 2018c). Although, heat treatment of seed canes either by aerated steam or moist hot air practiced in India, it is partially effective in inactivating the phytoplasma (Viswanathan 2016, 2018). By tissue (meristem tip) culture SCWL-plants are produced extensively in Thailand (Wongkaew and Fletcher 2004). Change of

cane planting time from autumn to spring season reduces the SCWL disease incidence due to reduced population of *M. hiroglyphicus* during low temperature during winter.

Cultural

Combining agronomical measures along with the use of disease free seed canes have completely controlled WLD in Taiwan (Leu and Kusalwong 2000). However, in tropical country like Thailand, sanitation, planting WLD-free planting materials and use of green manure plants in crop rotation are the most appropriate strategies to contain the disease (Wongkaew 2012). Since the vector insects movement is low in the field, the insecticides can be effectively used against them. Hence an integrated WLD-free seed cane production methodology is recommended in Thailand involving tissue culture, multiplying seed canes in large, isolated areas, and applying insecticides to control vectors (Hanboonsong et al. 2021).

Fiji leaf gall was effectively managed through an integrated approach of involving virus-free or certified seed, resistant cultivars and effective quarantine in Australia. In addition, to manage Fiji leaf gall, sugarcane varieties with certain level resistance is desired since during the favourable conditions for plant hopper vectors, susceptible varieties succumb to the disease and severe outbreaks occur. Further, to reduce inoculum availability in the field, virus-free seed is necessary to manage this disease (Smith 2000).

Disease Resistance

Disease resistance in the varieties is an important component in red rot management strategy in sugarcane and the new varieties with high sugar and cane yield are recommended for cultivation in India only if they possess red rot resistance. By this strategy, the disease epidemics were overcome inspite of the ravages caused by each of the red rot epidemics occurred during the past 120 years (Viswanathan 2021b). Much progress has been achieved in identifying resistant parents, inheritance of disease resistance and resistant clones in the germplasm, parents and inter-specific and -generic hybrids (Ram et al. 2005; Babu et al. 2010; Nair et al. 2017; Viswanathan et al. 2017c, 2018d, 2021b). Further, new screening methods, rapid screening of large number of clones under controlled conditions, a method to assess field tolerance and a new method to assess nodal resistance to *C. falcatum* by cotton swab inoculation were developed and being used (Mohanraj et al. 2012; Viswanathan et al. 2018a). Comparison of red rot ratings in the controlled condition testing method with the standard plug method revealed that this rapid screening method possesses adequate precision and matching disease reactions with plug method (Viswanathan et al. 2021c). Recent studies conducted at ICAR-SBI demonstrated *C. falcatum* soil borne inoculum as a source to induce disease development in sugarcane varieties and established field tolerance potential of the varieties to different pathotypes (Viswanathan et al. 2020a).

C. falcatum gradually adapts to the newly released varieties and comes out with new variants capable of breaking host resistance; hence the pathotypes are characterized regularly and resistance to the new pathotypes in the varieties, parental clones and germplasm is updated regularly (Malathi et al. 2006; Viswanathan and Selvakumar 2020; Viswanathan et al. 2022a).

Before 1998, ~70% of the varieties under cultivation in Australia were susceptible to smut (Sundar et al. 2012) hence a systematic breeding for sugarcane smut resistance has been initiated. This has brought a significant increase from 0.4 to 52% in smut-resistant crosses in Australian breeding programs from 2000 to 2007 (Croft et al. 2008a, b) and by the end of 2011, smut-resistant clones nearly doubled (Bhuiyan et al. 2013a, b). Similarly, many elite smut-resistant sugarcane cultivars were developed in different countries through their breeding programs.

As like red rot and smut, managing wilt through host resistance in sugarcane is a viable strategy to reduce crop losses in epidemic regions. Hence the pre-release varieties are artificially inoculated on the standing canes of the varieties following the standard plug method to assess wilt resistance in India. Alternatively, the pathogen is applied to the root zone to allow the pathogen to infect the canes from root and stalk. Recently a 0–9 scale was developed to rate sugarcane varieties for wilt resistance from R to HS (Viswanathan et al. 2022c). Further resistance to wilt and PB in sugarcane was identified among the parental clones numbering 700 maintained at National Hybridization Garden (NHG), the Indian national facility being used by ~24 sugarcane research centres to develop new sugarcane varieties. Sudden outbreak of these Fusarium diseases benefitted to identify sources of resistance among the parents that are frequently used in sugarcane breeding (Viswanathan et al. 2014a, 2019b). Overall it was found that parental clones of subtropical states exhibited relatively more resistance to these diseases as compared to tropical clones.

Although, YLD can be managed by healthy seed nursery programme, it is costly and seed replacements need recurring cost under field conditions. Hence host resistance to ScYLV has been studied in detail and resistant sources in the germplasm, varieties and parental clones in different countries. In Florida, *S. spontaneum* was identified as the most resistant group for ScYLV with 7% incidence whereas, *S. officinarum* was the most susceptible group with 76% in world collections of sugarcane and related grasses (Comstock et al. 2001). ScYLV infection showed a wide variation in the range of 0–100% in Colombia. In a cross of resistant male parent and a susceptible female parent resulted in progenies of YLD resistance (Victoria et al. 2005). Artificial virus inoculation procedures using aphids were developed in Louisiana, Hawaii, Brazil and other countries to develop YLD resistance in sugarcane varieties (Viswanathan 2021c). Studies of Fartek et al. (2014) found a positive phenotypic and genetic correlation among *Melanaphis sacchari* resistance in sugarcane varieties and disease incidence. They found a two-fold lower mean virus incidence in 22 resistant varieties than the 159 susceptible varieties. Previously, Zhu et al. (2010a, b) revealed that YLD-tolerant cultivars have limited ScYLV population than the disease susceptible ones. Recent studies of Burbano et al. (2021) in Brazil found a greater broad-sense heritability of 68% in qRT-PCR assays for ScYLV whereas it was 52.62% in

YLD-phenotypic expression to identify possible immune clones to the virus. ICAR-SBI, Coimbatore, India conserves the largest collections of sugarcane germplasm of nearly 4066 different *Saccharum* spp and hybrids at Kannur, Kerala, India. Detailed surveys were conducted for YLD incidence and severity in the germplasm clones, using the new 0–5 resistant grading system, identified about 463 and 773 among the hybrid genotypes and *Saccharum* spp, respectively as resistant sources (Viswanathan et al. 2016a).

By developing effective mechanical inoculation assays for SCMV, screening for virus resistance in sugarcane clones was successfully done, under greenhouse conditions (Chaves-Bedoya et al. 2011; Gemechu et al. 2004; Pinto et al. 2013; Srisink et al. 1994). Recently, da Silva et al. (2015) reported a combination of phenotypic evaluation of mosaic and SCMV diagnosis by ELISA assays to efficiently select mosaic-resistance sources. This has helped to detect the virus in asymptomatic genotypes and to identify 22 genotypes as resistant to SCMV Rib-1 strain. In China, mosaic phenotyping in sugarcane germplasm was done and identified resistant sources to mosaic from intergeneric hybrids of *S. officinarum* and *E. rockii* and *Erianthus arundinaceus* and *S. spontaneum* clones to SCSMV, SCMV and SrMV using severity grades of mosaic and RT-PCR (Li et al. 2013b, 2018, 2019). At ICAR-SBI, Coimbatore a 0–6 scale was developed to screen the *Saccharum* spp germplasm for mosaic resistance. This study clearly indicated that mosaic is widely prevalent in the germplasm maintained at Agali and about 97% of the genotypes/varieties have infections of SCMV and SCSMV, either alone or together. Indexing through RT-PCR assays revealed infections of SCSMV, SCMV and both in 77, 51 and 46 of the 88 *Saccharum* spp clones. Three *S. robustum* cones were identified as mosaic resistant from the study and probably these clones would be the donors for mosaic resistance in India. Further, the 0–6 scale adopted will be highly useful to phenotype mosaic resistance in other *Saccharum* spp clones and parental clones (Bagyalakshmi and Viswanathan 2021).

Work done at Australian researchers clearly demonstrated that Fiji leaf gall disease can be controlled though resistant varieties. The breeding strategy involves screening all the clones for resistance, avoiding crosses between susceptible parents and developing better methods of rating for resistance like breeding plant hoppers on virus-infected sugarcane in the glasshouse and subjecting new clones to a defined hopper numbers, rating clones based on percent of virus-infected plants and disease symptoms severity (Smith 2000).

Different methods and scales were developed for rust screening for resistance in various countries. Leaf whorl inoculation was optimized in Florida, where in uredospores @10^5 per ml were placed in whorls of spindles and rust development was observed after 30 days. Alternatively, wrapping of rust affected leaves with young shoots was found to be superior as compared to dusting or spraying uredospores. A 0–4 rust rating scale was adopted and graded them as resistant to susceptible; this criterion was followed to identify orange rust resistance in sugarcane clones under field conditions by adopting leaf whorl method of inoculation (Sood et al. 2009, 2013). In China, sugarcane germplasm was sprayed with uredospores suspension and screened for rust resistance using 0–9 scale (Wang et al. 2013). Under Indian conditions, leaf whorl inoculation method was found ideal to artificially induce severe

rust on susceptible clones and the method was recommended to screen sugarcane varieties for brown rust resistance (Viswanathan, unpublished). At Coimbatore in tropical India, 275 parental clones of sugarcane were screened under field conditions for rust resistance and among them ~60% of clones were rust-free and 20% were moderately resistant. In the remaining 20% susceptible group, about 13 and 7% behaved as moderately susceptible and susceptible, respectively (Selvakumar et al. 2018).

RSD resistance is assessed based on the pathogen population densities in sugarcane (Roach 1992; Davis et al. 1994; Miller et al. 1995). Limited colonization by the pathogen is found associated with LSD resistance in sugarcane varieties and wild relatives of *Saccharum* spp (Rott et al. 1997). Host resistance is the major strategy followed to manage all the leaf spot diseases, red stripe and pokkah boeng in different countries. At times, resistance breeding is focused on red rot, wilt, smut, mosaic or other diseases specific to their region hence foliar diseases are not given due weightage. In many countries susceptible varieties are rejected during varietal selection process; only disease resistant types are advanced and recommended for cultivation. Although this method does not give true resistant types, it is followed in places where resources and man power are limited.

Biocontrol Approaches

In sugarcane, effectiveness of native plant growth promoting rhizobacteria (PGPR) and fungal antagonist *Trichoderma* against *C. falcatum* was established under laboratory conditions and field situations (Viswanathan and Samiyappan 2002a, 2008; Malathi et al. 2008; Singh et al. 2008; Hassan et al. 2010, 2012; Joshi et al. 2019; Viswanathan and Malathi 2019). PGPR mediated mechanism of induced systemic resistance was governed by specific induction of oxidative enzymes and PR-proteins. Further, antagonistic activities and biocontrol potential of endophytic PGPR strains were established against *C. falcatum* (Viswanathan and Samiyappan 2002b; Viswanathan et al. 2003b, c; Jayakumar et al. 2021).

Chemical Control

As mentioned earlier, pathogenic inoculum from sett- and soil serve as primary sources for spread of different diseases in sugarcane, hence sett treatment with fungicides or bioagents will reduce fungal disease development from seed canes and soil. Practically no diagnosable disease symptoms exist on the setts, hence sett treatment with fungicides is recommended in disease prone areas or susceptible varieties are grown. Thiophanate methyl, a systemic fungicide was found effective against *C. falcatum* (Malathi et al. 2004). The same fungicide was found to be compatible with *Pseudomonas fluorescens* strains and the combination exhibited more efficacy against the pathogen inoculum survives in the soil (Malathi et al. 2002). Conventional immersion of setts in fungicide solution for a short duration is ineffective

against the deep-seated fungal propagule; to overcome this concern, mechanized fungicide treatment of setts with fungicide was developed and improved efficacy of fungicides and disease control was demonstrated under field conditions against smut and red rot diseases (Viswanathan et al. 2016b; Malathi et al. 2017). Recently Shailbala (2016) reported efficacy of the fungicide combination Azoxystrobin 18.2% + Difenoconazole 11.4% w/w SC against red rot, smut and rust diseases in sugarcane.

Currently both the sugarcane rusts are common in Florida. The farmers prefer to grow high yielding cultivars though they are brown rust susceptible by adopting fungicide sprays. A high yielding cv CP96-1252, but moderately susceptible to brown rust is preferred by the growers in Florida hence it occupied 29% of the sugarcane area during 2016 (Raid et al. 2018; Rott 2018; Van Weelden et al. 2017). Recently Chaulagain et al. (2019a) reported 40–42% less rust severity in the brown rust affected field after spraying fluxapyroxad + pyraclostrobin on the popular cv CP 96–1252 during three seasons and obtained an increase of 27–35% in mean stalk weight. For effective control of brown rust, they suggested a minimum of two fungicide sprays. They also found beneficial effects of fungicide sprays in orange rust susceptible varieties in Florida and recommended fungicide sprays with different modes of action such as two successive sprays of fluxapyroxad + pyraclostrobin and later one spray of pyraclostrobin, fluxapyroxad, metconazole or fluxapyroxad + pyraclostrobin during three succeeding months, to prevent emergence of fungicide resistant pathotypes (Chaulagain et al. 2019b).

Spraying of 60 g streptomycin + tetracycline in 500 l water per ha in the two month crops at 15-days intervals has been found effective to manage LSD severity in Tamil Nadu, India. Further, spraying of this antibiotic combination immediately after noticing the disease symptoms reduced LSD severity significantly (Viswanathan 2012a).

Heat Treatment

Across the countries, hot-water treatments are used to inactivate the pathogens in seed cane. Immersing setts in running water (ambient-temperature) for 40 h and then by hot water treatment for 3–4 h at 50 °C before planting is used to inactivate LSD bacterium. This treatment is reported to provide control efficacy of 95% (Rott and Davis 2000a). Treating RSD affected seed canes with hot water at 50 ± 0.5 °C improved bud germination, crop growth, higher cane yield in the range of 9.5–54.7% and sugar content in the range of 0.68–1.7% (Wei et al. 2019). Moist hot air treatment or aerated stream therapy is followed in different sugar mills to inactivate pathogens causing RSD and grassy shoot with partial success. There were claims on their efficacy against sett borne fungal inocula of red rot or smut, however only incomplete success was achieved. To inactivate grassy shoot phytoplasmas in the setts, information on varied thermal tolerance in the varieties and varying regimes of temperature and timing are required (Viswanathan 2001b).

9.2.8.2 Insect Pest Management

Host Plant Resistance—Borer Pests

Unlike other crops, efforts of resistance breeding in sugarcane are rather lagging probably because of its genetic complexity and polygenic inheritance (White et al. 2010). Mostly, field observations on damage help to identify the resistant varieties among the already popular varieties in a locale and conventional breeding for insect resistance is not in vogue in sugarcane. Between two major varieties cultivated in Reunion, R570 was more resistant than R579 (Nibouche and Tibére 2010). Differences in the varietal response to *D. saccharalis* were observed by White et al. (2008) in Louisiana.

Though voluminous work has been published on screening or evaluation of resistant varieties to borers of sugarcane globally, quantification of the benefits accrued due to prevention of pest or increase in yield has not been accomplished. For example, with regard to *C. sacchariphagus* in sugarcane, no information is available on the resistance status or the gain accrued by improved varieties in Reunion (Nibouche and Tibere 2009). Further, a constantly changing varietal scenario undermines the efforts of identifying resistant varieties, as the popular varieties gain area under cultivation only based on their performance with reference to major diseases, cane yield and quality.

Morphological characteristics are primary indicators of borer resistance in sugarcane. Several traits in sugarcane correlated to borer resistance were leaf width, color of the stalk, fiber composition, waxy rind, self-de-trashing leaves, elongated spindles, slim stalks, thin erect leaves, elevated fiber composition, content, wax, increased crop vigor, juice percentage, and attraction to ovipositing females (Reagan et al. 2008). However, through the years, the rind hardness and fiber content of the cane have been suggested, tested and found to be correlated with borer resistance (Reagan and Martin 1982; Bessinet al. 1990). Despite their importance in breeding for resistance against *D. saccharalis*, constant search for varieties with lower borer infestation would also lead to accretion of unfavorable characters like increased fiber content and lower sucrose thus reducing sugar recovery (White et al. 2006). Borer resistant varieties are currently cultivated on more than 60% of sugarcane area in Louisiana (Wilson 2021).

In an effort to identify sources of multiple pest resistance among the genotypes from *S. barberi, S. robustum, S. spontaneum* and *Erianthus* sp., a few genotypes had exhibited resistance to two to five major sugarcane pests (Mukunthan 1994) and it could be seen that *S. robustum* and *Erianthus* had more genotypes with multiple pest resistance compared to *S. barberi* while *S. sinense* and *S. spontaneum* did not possess any such genotype. Cane length as well as the length and girth of the vulnerable portion had a positive correlation with *C. sacchariphagus indicus* infestation on 20 genotypes tested. The sugar content, phenol, cellulose and tannin played a role in INB resistance in sugarcane (Asha et al. 2019). Antibiosis resulting in lower weight gain and prolonged larval duration was exhibited by the *D. saccharalis* resistant variety, HO 08-9003 (White et al. 2011; Salgado et al. 2021). Poor neonate establishment

due to rind hardness conferred resistance to *D. saccharalis* and nine fold differences of such response were observed among the cultivars (Salgado et al. 2021). Tomaz et al. (2018) Identified sugarcane varieties with resistance to leaf feeding as well as stalk entry and tunnelling, for use in breeding programs to achieve increased level of borer resistance.

In Louisiana, decades of sustained efforts have been made since 2001 to breed resistant cultivars for management of the stalk borers, *E. loftini* and *D. saccharalis* (Wilson 2011; Wilson et al. 2015; Reay-Jones et al. 2003; Reagan et al. 2008) through continuous rigorous screening and monitoring protocols and programs (Reagan and Martin 1982; Schexnayder et al. 2001; Posey et al. 2006; Reagan et al. 2008). Three resistant varieties, L 99-226, HOCP 04-838 and L 01-299 are currently recommended for resistance to stem borers in Louisiana. Similarly, five among the recommended commercial varieties have been declared to be susceptible to *D. saccharalis*. Among the currently popular sugarcane cultivars 5–10 fold differing levels of borer resistance has been observed (Wilson et al. 2015, 2021).

Among the South African sugarcane varieties, N21 is highly tolerant to drought (Kvedaraset al. 2009). The survival and developmental rates of insects increases during drought due to increased plant nutrient levels, lowered plant defense, and availability of improved temperature niches (Atkinson and Nuss 1989). Hence Reagan and Mulcahy (2019) suggested that drought tolerant varieties can be successfully used in managing the borer populations during the adverse climatic conditions. Borer resistance in sugarcane is polygenic, governing any resistant trait and several genotypes showing antixenosis and antibiosis against borers have been identified in Brazil (Pimentel et al. 2017). Complex genetic control of sugarcane borer resistance with high genetic variation among Brazilian sugarcane genotypes was demonstrated by Tomaz et al. (2018, 2019). Notable work on single clone and family selection for breeding resistance to *E. saccharina* had been done by several workers (Farrag et al. 2018; Zhou 2015, 2016; Zhou and Mokwele 2016).

Cultural Control

Routine or specific field operations, planting time, irrigation/fertilizer schedule, spacing, harvesting are the common aspects of crop husbandry which can incidentally or intentionally favor or suppress build-up of pests is understood as cultural control. This is an eco-friendly non-chemical method but can be expensive, time consuming and execution may be dependent on several factors in crop cultivation. Increased incidence of stalk borers in ratoon crops is a consequence of elimination of the natural enemies (Macedo and Araujo 2000), due to the stalk burning of the previous crop as compared to non-burned sugarcane (Pholan et al. 2005).

In Brazil, movement and utilization of infested sugarcane seedlings has been the major factor for incursion and increased infestation of billbug *S. levis*. Also, application of vinasse (a byproduct during the production of ethanol from molasses) through fertigation increases the pest population, as the volatiles from vanasse are

attractive to the beetles. The increased soil moisture levels due to vinasse applications enhance the survival of the billbug populations (Martins et al. 2020).

In South Africa, Keeping et al. (2014) observed increased survival of *E. saccharina* due to higher rates of nitrogen application while silica application reduced the infestation and this protection was greater on susceptible varieties. A loss of 30% sugar yield due to *E. saccharina* in susceptible varieties could be prevented through foliar application of silicon (Keeping and Meyer 2002). Further, susceptible varieties grown in soils under water-stress but rich in silicone reinforced the barrier effect of the stalk to the borer (Pene et al. 2018). Flooding has been suggested for the management of white grub such as *Tomarus subtropicus* (Buss 2003) but this method is inoperable in areas of poor water supply where the infestations with white grub are intense. De-trashing combined with spraying of imidacloprid reduced *A. barodensis* resulting in higher yield and sucrose (Rao et al. 2011). Repeated ploughing during May–June exposes the hibernating population of white grubs to natural enemies like birds, pigs and dogs for predation.

Chemical Control

The insecticides applications for borer management have not been adopted as they had not been economically viable (Showler and Reagan 2017) and hugely impractical due to the large canopy. It is further difficult to manage the internal feeders, which were inaccessible for the sprays. As the larvae of *E. saccharina* bored inside the sugarcane within 24 h after hatching, insecticidal sprays were rendered useless (Heathcote 1984). Similar is the case with many other borers such as *E. loftini* in South Africa, *C. sacchariphagus* in many Asian countries and *C. sacchariphagus indicus* in India.

Selection of chemicals and application strategies determine the efficacy against borers. In Louisiana, if systemic chemicals were applied as high volume spray, early in the season, sugarcane could be protected from *D. saccharalis* injury, as the concentrations remained effective even after eight weeks post application (Wilson et al. 2021). Synthetic neonicotinoids, imidacloprid and thiamethoxam are highly effective against the sugarcane whitefly species specifically *Aleurolobus barodensis* (Bhavani and Rao 2013; Chaudhary and Jaipal 2006; Vijayaraghavan and Regupathy 2006). Among the newer chemicals, thiamethoxam (Vijayaraghavan and Regupathy 2006) and dinotefuran (Koohzad-Mohammadi et al. 2017) brought down the populations of *A. barodensi* and *N. andropogonis*, respectively.

The most effective insecticide combination may be detrimental to conservation of biocontrol agents. A combination of thiacloprid with deltamethrin was effective against *N. andropogonii* but harmful to the two parasitoids. Hence use of pyriproxyfen (a juvenile hormone) was the safest insecticide with >50% parasitoids emergence could be more prudent (Behnam-Oskuyee et al. 2020). Integrated pest management (IPM) program had effectively used for whitefly management. In this regard, IPM involving stripping the affected leaves, spraying imidacloprid, azadirachtin, 2% urea reduced *A. barodensis* population resulting in higher yield of sugar and cane

(Bhavani and Rao 2013). Similarly, a combination of de-trashing, release of *Chrysoperla carnea*, yellow sticky traps, pesticide application reduced the population of *A. barodensis* to 0.48 per cm^2 of leaf (Bhatti et al. 2019).

Biocontrol

Since the scope of chemical control is restricted due to the dense crop canopy specifically during the grand growth phase, natural control of pests thrives in sugarcane ecosystem. However, the ecological differences among ecosystems may influence the success of biocontrol agents. Sugarcane cultivated yearlong or as a semiperennial offers a relatively stable habitat (Kfir et al. 2002). For establishment and success of natural enemies, habitat stability is crucial (Hall and Ehler 1979; Cameron et al. 1993). However, climate change can restrict the adaptability and success or may often result in the undesirable impact on non-target insects (Lu et al. 2015). Temperature essentially impacts both establishment and efficacy of a natural enemy (Lu et al. 2013). Biopesticides are eco-friendly. Since they aid in the the reduced application of toxic chemical insecticides, safer food and cleaner environment can be ascertained (Hall and Menn 1999).

The most prominent parasitoids that had been frequently used in classical biological control programs in sugarcane are *Cotesia flavipes* and *Xanthopimpla stemmator* (Hymenoptera: Ichneumonidae). Their successful establishment has been proven on several occasions. In Asia and Indian Ocean islands, *C. flavipes* has a broad host range occurring on $4/5^{th}$ of recorded species of the borers (Sallam 2006). Conlong and Goebel (2002) found that the introduced parasitoid *X. stemmator* remarkably brought down the INB infestation at all the released sites in Mozambique. Soil application of the fungus *Metarhizium anisopliae* at the time of earthing up would significantly effective in reducing white grub population and recorded higher yield than untreated control (Purwar 2013; Lamani et al. 2017; Thirumurugan et al. 2020).

9.3 Genetic Resources of Resistance Genes

9.3.1 Available Germplasm

The genus *Saccharum* consists of *S. officinarum*, *S. sinense, S. barberi*, *S. edule* (cultivated species) *S. spontaneum* and *S. robustum* (wild species) (D'Hont et al. 1998; Irvine 1999). Two duplicated world collections of sugarcane germplasm 'World collections of sugarcane germplasm' are preserved by ICAR-SBI, Coimbatore, Tamil Nadu at Kannur in Kerala, India and the 'National Germplasm Repository' in USA at Subtropical Horticulture Research Station, USDA-ARS, Miami, Florida. Constituents of Miami collections predominantly include *S. officinarum*, *S. spontaneum* and sugarcane hybrids. It also has other *Saccharum* spp. and species of

other genera such as *Imperata* spp., *Coix gigantea*, *Miscanthus floridulus*, *M. sinensis*, *Miscanthus* spp., *Miscanthus* hybrids, *Narenga porphyrocoma*, *Sorghum plumosum*, and *S. arundinaceum*. There were 1002 accessions maintained at Miami mostly the survivors of Andrew Hurricane occurred in 1992 and other fresh accessions (Spurthi et al. 2014). ICAR-SBI, Coimbatore, India regularly undertakes the collection, characterization, conservation in the respective repositories and documentation of genetic resources including *Saccharum* spp and allied genera. Besides, the institute maintains the basic species of clones and related genera of *Sachharum* complex. Further, ICAR-SBI also maintains Indian hybrids, Indo-American clones, improved clones of *S. barberi* (Population improved barberi-PIB), *S. robustum* (Population improved robustum-PIR), *S. officinarum* (Population improved officinarum-PIR), *S. spontaneum* (Population improved spontaneum-PIS), interspecific hybrids (ISH) and intergeneric hybrids (IGH). About 2680 'Co' selections (hybrids) developed by ICAR-SBI, since its inception in 1912 are maintained at Coimbatore, India and these are part of the longest sugarcane improvement activities across the globe (Amalraj and Balasundaram 2009) and that is active now also. The germplasm availability of predominantly used species clones of *Saccharum* complex and related genera at major breeding centres of the world are presented below.

S.No.	Species	India	USA	Brazil[a]	China#	Thailand[b]
1.	*S. officinarum*	764	238	103	32	4785[c]
2.	*S. barberi*	43	38	29	3	–
3.	*S. sinense*	29	22	17	25	–
4.	*S. edule*	16	–	3	–	–
5.	*S. robustum*	145	45	39	6	–
6.	*S. spontaneum*	978	319	211	690	991
7.	*Erianthus* spp.	202	–	40	404	957

[a]Sum of the collections maintained at Serra do Oura, Biovertis, Gran Bio and Devaneio (*Source* Cursi et al. 2022)
[b]Sum of collection maintained at Department of Agriculture (DOA), Khon Kaen University (KKU), Kasetsart University (KU), Office of the Cane and Sugar Board (OCSB) and MitrPhol (MitrPhol Innocation and Research Centre (MitrPhol) (*Source* Khumla et al. 2022)
[c]including *Saccharum* spp. hybrids
#*Source* Zhang and Govindaraju (2018a, b)

Besides above breeding materials, the Thailand breeding population has accessions of *Miscanthus* and *Sclerostachya*; *Narenga*, *Imperata* and *Pennisetum* in China. Brazil has *Miscanthus*, unknown species and hybrids in their germplasm collections. These genetic materials have substantial diversity for various traits and many useful genes for many phenotypic characters, cane and sugar yield, biomass and resistance to different stresses etc and serve as excellent breeding materials for sugarcane advancement.

9.3.2 Primary Gene Pool

This sugarcane gene pool comprises five different species of *Saccharum* and their hybrids, which hybridize easily among them. Major part of the species germplasm had been screened for red rot resistance. Very few clones of *Saccharum* spp. showed resistance to red rot (Alexander 1987; Malathi and Viswanathan 2012b), whereas, the number of red rot resistant clones in *S. barberi* was relatively more as compared to other cultivated species (Alexander and Rao 1976). Among the *S. officinarum* germplasm, seven clones were reported to be resistant to red rot and 15 clones were moderately resistant (Sreenivasan and Nair 1991). Baragaua, Seleri and Saipan G showed resistant consistently. About 237 clones were found to resistance or moderately resistance to smut. Alexander (1987) reported ~95 *S. officinarum* clones from the world germplasm collections as resistant to smut. Naidu and Sreenivasan (1987) reported that *S. officinarum* (97 out of 428 clones) and *S. spontaneum* (137 out of 324 clones) had the highest resistant sources among five species clones of *Saccharum* whereas *S. sinense* (15), *S. barberi* (9) and *S. robustum* (3) showed a lower level of resistance against smut pathogen. Unlike resistance to red rot, more number of *S. officinarum*, many *S. spontaneum* and *S. robustum* clones showed resistance to smut (Srinivasan and Alexander 1971; Alexander et al. 1983).

Viswanathan et al. (2016a) reported that in case of *Saccharum* spp, 86% of *S. robustum* were resistant to YLD, followed by *S. sinense* (80%), *S. officinarum* (78%), and *S. barberi* (76%) in the germplasm collections maintained at Kannur, India. Similarly, occurrence of YLD in the germplasm collections of sugarcane at Miami, USA was highest in *S. officinarum* (75.8%) followed by *S. robustum* (62.5%), *S. sinense* (46.2%), *S. barberi* (13.6%), and *S. spontaneum* (7.0%). A cross involving a YLD-susceptible noble cane and a resistant wild relative gave a higher percentage of clones, which were free from the virus for more than 10 seasons. Similarly, the wild parent (IND-81-146) remained free from virus infections during the period indicating that *S. spontaneum* is highly tolerant to ScYLV whereas, *S. officinarum*, is highly susceptible. In sugarcane, resistance to YLD was found as a dominant trait (Comstock et al. 2001).

Chu et al. (1982) presumed that modern commercial varieties possess rust susceptible genes transmitted from some *S. officinarum* clones. Later, inheritance of rust resistance was studied with self-progeny of the sugarcane cv R570. Reaction of the progenies to rust was assessed under controlled greenhouse and field trials and rust phenotyping revealed a definite 3:1 segregation for resistant and susceptible reactions in the progenies. This study indicated that the brown rust resistance is probably governed by a dominant resistant gene and identified 'Bru1' gene in the cv R570. The Bru1 gene was found to check infections caused by different brown rust isolates from various geographic locations (Asnaghi et al. 2001; Daugrois et al. 1996). Later, the second major brown rust resistant gene 'Bru2' was identified and it prevented rust fungal sporulation (Raboin et al. 2006; Costet et al. 2012). Heritability for rust resistance was reported to be intermediate (Tai et al. 1981; Gonzales et al. 1987). Further, high narrow-sense and broad-sense heritability values of 0.84 and 0.73 respectively,

for rust resistance were reported (Comstock et al. 1992). In another study, Hogarth et al. (1993) reported 0.84 and 0.78 heritability values for rust. Costet et al. (2012) analysed 380 recent varieties and breeding clones from different breeding centers of more than 30 across the globe with 22 molecular markers. They identified 17 genotypes viz., B 41227, Co 214, MEX 73 523, MQ 76 53, N 53 216, NCo 334, R 84 693, Q 127, Q 136, R 570, R 572, R 573, R 575, R 577, H 72-8597, R 579, R 83 1592 genetically linked to Bru1 as the stable resistant source for rust.

9.3.3 Secondary Gene Pool

S. spontaneum and *S. robustum,* the wild species of sugarcane constitute the secondary gene pool of sugarcane. It is very interesting to note that sugarcane improvement started with utilisation of secondary gene pool sugarcane rather than primary gene pool and the source for red rot resistance is mainly contributed by *S. spontaneum* (Srinivasan and Alexander 1971; Natarajan et al. 2001). A large number of clones with red rot resistance are available in *S. spontaneum* germplasm collection maintained at ICAR-SBI (Alexander et al. 1983). Of the 170 *S. spontaenum* clones, 69 were resistant and 59 moderately resistant to red rot (Kandasami et al. 1983) and 91 of the *S. spontaneum* clones were resistant to smut. Five *S. spontaneum* clones were found to be resistant to rust and 91 clones resistant to ratoon stunting disease. Among the 30 Japanese wild sugarcane (*S. spontaneum*) accessions and five cultivars, JW 90, Iriomote 8, Iriomote 15, Iriomote 28, and T16 were found resistant and the cultivar Ni F8 was moderately resistant to the only one race of smut pathogen prevalent in Japan (Sakaigaichia et al. 2018). Interspecific hybrids (ISH) developed from the tall, thick and broad leaved *S. spontaneum* from Arunachal Pradesh produced progenies, of which 35% were resistant to red rot and only 7% were highly susceptible (Mohanraj and Nair 2012). Inter-specific crosses involving PIO, PIS and PIR clones and commercial varieties exhibited higher scope for developing red rot resistant progenies and gave about 35% red rot resistant progenies with heterotic vigour for other economic traits. Three F_1 progenies of improved *S. officinarum* x *S. spontaneum* cross combination viz., 96-38, 96-195, 95-108 (Alarmelu et al. 2010) produced more resistant progenies.

9.3.4 Tertiary Gene Pool

Genetic diversity tertiary gene pool of sugarcane which include the allied genera such *Eriatnus, Miscanthus*, *Narenga*, *Sclerostachya*, *Imperata* and *Pennisetum* are a treasure house of much value for the sugarcane breeding programme in future. Among them, *Erianthus* offers greater scope by being an important source for higher biomass production, pest and diseases resistance and tolerance to abiotic stresses. However, success of *Saccharum* x *Erianthus* hybridisation is low because of high rate of selfs

and resemblance of hybrids with *Saccharum* parent. After developing *Erianthus* specific markers, intergeneric hybrids (IGH) involving *S. officinarum*, *S. robustum*, *S. spontaneum* and commercials with *Erianthus* were developed. These IGHs showed superiority over the ISHs involving *S. spontaneum* for sucrose content (Mohanraj and Nair 2010). Sorghum x *S. officinarum* hybrids have been developed that are unique in having sorghum cytoplasm (Nair 1999). Amongst the six taxonomic groups of *Saccharum* spp. comprising five different species of *Saccharum* and *Erianthus* spp, the clones belonging to the *Erianthus* spp section *Ripidium* were found to be the most resistant clones whereas clones of *S. officinarum* and *S. robustum* are highly susceptible (HS) (Burner et al. 1993). A total of 79 backcross progenies (BC1 and BC2) of *E. arundinaceus* were assessed for smut resistance. In this study, seven BC1 and three BC2 derivatives of *E. arundinaceus* exhibited greater resistance against smut pathogen and these lines may serve as potential donors for smut resistance in sugarcane (Shen et al. 2014). Among the *Erianthus* germplasm, 10 clones were stated to be resistant to red rot (Sreenivasan et al. 2001). Mukunthan and Nirmala (2002) screened 285 accessions of *S. barberi*, *S. robustum*, *S. sinense*, *S. sponataneum* and *Erianthus* species for their response to white grubs and reported that 61 clones are tolerant. The majority of the tolerant clones were accessions of *Erianthus* collections. Pest reaction of 20 *Erianthus* clones had been reported with respect to 7 key pest of sugarcane (Sreenivasan et al. 2001).

9.4 Glimpses on Classical Genetics and Traditional Breeding

9.4.1 Classical Mapping Efforts

9.4.1.1 Morphological Characterization

S. officinarum (noble canes) is characterized by having showy colours with juicy stalks and broad leaves. These are known for their thick stalks and high sucrose content. Though single cane weight is high, its tillering ability is poor. Hence, noble canes were replaced by the improved inter-specific hybrid varieties involving *S. officinarum* and *S. spontaneum*; however, some noble canes are cultivated for festivals and for chewing purposes in many Asian countries. The clones of *S. barberi* are thin, hardy with narrow or medium leaves. In spite of having very high tillering ability, the yield is poor because of very low single cane weight. *S. sinense* known as Chinses canes have medium thick canes with good tillering ability and satisfactory level of yield and sucrose. *S. robustum,* a wild relative of sugarcane has medium thick canes and broad leaves. The other wild relative of sugarcane, *S. spontaneum* shows wide variation from grassy type to thin cane types (Ramana Rao et al. 1979). *S. barberi* clones were classified into four groups, viz., Mungo, Saretha, Sunnabile and Nargori

based on the morphology and *S. sinense* was placed under Pansahi group (Barber 1916, 1918).

9.4.1.2 Cytological Studies in *Saccharum* Complex

Jagathesan et al. (1970) observed the chromosome number in 585 *S. officinarum* clones from the World collections of sugarcane and identified typical $2n = 80$ and atypical $2n \neq 80$. Chromosome analyses of about 442 *S. spontaneum* clones from various locations in India established polyploid aneuploid nature of the species. The Indian accessions had $2n = 40$–80, 80–112 and 112–118 from central, eastern and western regions, respectively (Panje and Babu 1960). Among the number of cytotypes in *S. spontaneum* reported, $2n = 64$ is the most common cytotype and distributed in most parts of India (Nair and Praneetha 2006). Among the 30 *S. officinarum* and 20 *S. spontaneum* clones studied, *S. officinarum* clones had $2n = 80$ except NG 77-26 which had $2n = 70$ whereas in *S. spontaneum,* the number of chromosomes was in the range of $2n = 64$–72 (Sobhakumari 2009). Sobhakumari (2020) based on the chromosome analysis of 524 *S. spontaneum* accessions inferred that North-eastern region of India was found to have a higher evolutionary activity in *S. spontaneum* due to multiple cytotypes occurrence and sympatric growth with other species and genera. Crosses between Vellai an *S officinarum* with $2n = 80$ and Coimbatore local, an *S. spontaneum* with $2n = 64$ resulted in hybrids having $2n = 112$ having $2n + n$ chromosome transmission (Dutt and Rao 1933). This mechanism favoured the transmission of whole nuclear genome of noble canes to hybrids resulting in superiority of hybrids for most of economic traits. However when atypical *S. officinarum* clones were crossed with *S. spontaneum*, $n + n$ transmission was observed. Parthasarathy and Rao (1947) reported somatic chromosome number of five forms of *Sclerostachya fusca* collected from different locations as $2n = 30$. Chromosome number in *Erianthus munja*, *E. ravennae* and *E. arundinaceous* clones were determined (Rao and Raghavan 1951). *E. ravennae* had only one cytotype of $2n = 20$ while the other two species had $2n = 30$, 40, and 60 chromosomes. Later, a detailed survey on the chromosome number of the *S. spontaneum*, *S. officinarum* and *Erianthus* spp., *Narenga*, *Sclerostachya* and *Imperata* collected from North east India was made (Sreenivasan and Sreenivasan 1994). Modern sugarcane cultivars originate from hybrid derivatives obtained from the cross combinations involving noble canes with $x = 10$; $2n = 8x = 80$ (*S. officinarum*) and wild canes with $x = 8$; $2n = 5x\text{-}16x = 40$–128 (*S. spontaneum*). The progeny clones have 130–140 chromosomes, of which, *S officinarum* contribute 70–80% and *S. spontaneum* 10–20%. The residual 10% are of recombinants between these two *Saccharum* spp (D'Hont et al. 1996). Differential contribution of the male and female predecessor was revealed by a genomic study involving in situ- and fluorescent in situ-hybridization assays in the hybrid R 570 genome. Earlier, isozyme variation was used as potential biochemical markers in sugarcane genetics and breeding (Glaszmann et al. 1989). This work has given the way for the use of markers as an effective means of finding linkage groups in genome of sugarcane.

9.4.2 Breeding Objectives

Improving the sugarcane yield and sucrose content in the varieties are the most important breeding objectives. Under the worst scenario of climate change, where the minor diseases becoming major, the fast development of biotypes of insect pests and of pathotypes of pathogens and increased virulence of pathogens, development of pests and diseases resistant varieties become foremost important. Red rot was considered once as diseases of subtropical India and smut as diseases of tropical region. In subtropical breeding programme, red rot resistance was given major emphasis while smut resistance in the tropical India. Nowadays red rot prevails in most of the states in India and new varieties succumb to the disease before their potential realized (Viswanathan 2021a, b). Hence, without red rot resistance, no sugarcane variety gets released or notified for commercial cultivation in India. Ratoon stunting causing yield loss up-to 15–50% in South Africa (Bailey and Bechet 1986) and 29% in Fiji (Johnson and Tyagi 2010) is a major constraint in sugarcane across the nations. This made the sugarcane breeders to consider this disease during their selection process in these countries. Before 1998, majority of the sugarcane varieties in Australia were smut susceptible which caused yield loss upto 26%, hence, resistance breeding to smut has become one of the primary objectives in Australian sugarcane varietal development program (Sundar et al. 2012). The climate changes also necessitate developing varieties tolerant to water logging, drought, salinity, cold, frost and other climatic extremities. Winter ratooning ability is important breeding objective of developing varieties for sub-tropical region of India. Some of the other objectives in sugarcane breeding like short duration varieties, special varieties for jaggery production, high fibre, high biomass etc. are driven by the demand of sugar industries. However, recently sugarcane has also become a bio energy crop (Souza et al. 2014) for producing ethanol. Nowadays, technological advancement in other fields made possible to find more utility for the by-products of sugarcane viz., filter muds, molasses (for cane ethanol, other alcohols, acetic acid, citric acid, cattle feed and cooking fuel, baggase (fuel, fibreboard, paper, bioplastics, power generation, biogas, fertilizer) etc. (Moore et al. 2013). Accordingly, the breeding objectives in sugarcane improvement are dynamic according to the priorities and future requirement.

9.4.2.1 Positive and Negative Selection in Sugarcane

In sugarcane, most of yield and sucrose quality traits are selected in positive side. The objective of breeding varieties for the purpose of bio-ethanol production can be met by developing varieties with very high sucrose content. In case of fibre content, a negative selection is effected. However, this trait is selected positively when we breed for energy canes. In case of tropical regions of India like Karnataka and southern parts of Maharashtra, flowering is a negative character and hence this trait is selected negatively. Other trait where negative selection is practiced in sugarcane is crop maturity duration. Recently developed variety at ICAR-SBI, Coimbatore, Co 11015,

the high sucrose % at 8th month of crop maturity on par with that of ruling variety Co 86032 at 12th month was achieved (Durai et al. 2020). This variety was developed applying the negative selection for days to crop maturity.

9.4.3 Achievements in Classical Breeding

The idea of utilizing a wild species for improvement of cultivated crop was thought out and initiated in sugarcane breeding at Coimbatore during 1912 and now it is being practiced in most of the crop improvement programmes across the world. The first hybrid Co 205 developed from the cross between *S. officinarum* and its wild relative *S. spontaneum* recorded 50% more yield than the indigenous varieties in Punjab, India and well adopted to the climatic and soil conditions of the subtropical region because of the ancestry of *S. spontaneum*. There had been steady improvement in sucrose content in the varieties bred at Coimbatore from mean of 15.89% prior to 1960s to mean of 19.54% in the 2000s (Hemaprabha et al. 2012). During the last two decades, substantial improvement in cane yield was observed in the major sugarcane growing countries. A significant improvement in sugarcane yield of Colombia was achieved from 5 t of sugar/ha/year during the year 1950 to 12 t/ha/year in 2000 (Cock 2001). During the same period, sugarcane production in Brazil has increased from 64 to 70 t ha. In Florida, from 1968 to 2000, sucrose, cane yield and sugar yield of the commercial cultivars progressively increased by 26.0, 15.5 and 47.0%, respectively (Edmé et al. 2005). In China, during the past 60 years (1961–2013), a rapid increase in sugarcane production from 2.643 MT to 126.13 MT was observed. Tremendous improvement in cane productivity (24.0–67.4 t/ha) and the mean sucrose % (less than 13% to >14.5%), with some varieties now record an average over 16% sucrose was observed during October to April (Zhang and Govindaraju 2018a, b). Increase in sugarcane productivity of Australian sugarcane varieties attained at the end of 1999 (95 t/ha) could be credited chiefly to the genetic improvement of varieties (Ming et al. 2010). Apart from yield and quality improvement, all other traits for stress tolerance are expected from the sugarcane varieties. Red rot problem in India is majorly managed through deployment of resistance varieties. Sugarcane cultivation in problematic areas was possible because of development of varieties with tolerance mechanism in sugarcane for drought, salinity, water logging etc. Among the diseases that affect the sugarcane production in Louisiana, smut, brown rust, orange rust, pokkah boeng, leaf scald, red stripe and top rot, mosaic (both sorghum mosaic virus (SrMV) and SCMV), are primarily managed through host plant resistance (https://www.lsuagcenter.com/).

9.4.4 Limitations of Traditional Breeding and Rationale for Molecular Breeding

The main objective of any plant breeding programme is to introgress one or a few favourable genes from donor into highly adopted variety and to recover most of the recipient parental genome as rapidly as possible. Breeding for biotic and abiotic stress requires identification of stress tolerant genotypes mostly from the germplasm and accumulating them in current commercial cultivars. Remarkable achievement was achieved during the past five decades in evolving new improved crop cultivars through traditional breeding in sugarcane. Major emphasis was laid on sourcing genes contributing to better productivity and adaptability from related species and wild relatives through genetic manipulation at cultivar, interspecific or intergeneric level. Breeding for stress resistance through conventional means is challenged by a poor understanding on inheritance of disease resistance, transfer of undesirable genes from the wild accessions along with desirable traits and the presence of reproductive barriers especially in interspecific and intergeneric crosses.

Most of modern varieties of sugarcane are products of only few inter-specific crosses involving around 15–20 genotypes developed at Java and India (Roach 1989). Even now in classical breeding programme, old genetic materials are widely used in crosses thus restricting the few recombinations from the original founder parents leading to narrow genetic base. Among the plant species available on earth, genetics of sugarcane is found as one of the most complex. Nevertheless, gene mining of *Saccharum* spp complex by genomic research helps the breeders (Abberton et al. 2016) to incorporate the variety of alleles in the breeding materials. Sucrose and cane are primary products from sugarcane. However, there was no significant improvement in top sugarcane producing countries in the last two decades for cane yield (Yadav et al. 2020). Further, the increase in sugar yield in most of the varieties of Florida was because of increase in cane yield rather than sugar (Zhao and Li 2015a, b). There was not a marked difference with respect commercial cane sugar (CCS) % between the older and new varieties of Australia (Jackson 2005). These facts clearly indicate that further improvement in sugarcane is possible by understanding molecular mechanism of sugar accumulation and metabolism. It was demonstrated that knocking down the pyrophosphate: fructose 6-phosphate 1-phosphotransferase (PFP) activity enhances sucrose accumulation in immature internodes of canes (Groenewald and Botha 2008).

Plant breeding has seen a major transition in the past decade as advances in biological sciences helped in evolving tools that can be applied to commonly accepted field techniques. In the context of plant breeding, molecular markers became a handy tool in selecting desirable genotypes by following the genes or chromosomal segments in the crosses using markers that are closely linked to them. This is particularly important in the case of genes governing biotic and abiotic stresses where traditional methods of screening for the trait are laborious and time consuming. Sugarcane suffers from insect damage, either by directly damaging the crop tissues or by its role as vectors of plant viruses. Continuous use of chemicals to protect the crop plants against insects harms the environment seriously. Hence it is essential to evolve plant

varieties that are resistant to insects. For several years, breeding varieties for disease and pest resistance has been taken up. The inherent difficulties in the conventional screening and the misleading results in screening efforts, probably due to the polygenic control of resistance makes marker assisted selection (MAS) for resistant to biotic constraints a viable alternative. In MAS, selection is not on the elusive trait of interest but on the reliable molecular markers closely associated with the trait. Being environmentally independent and scorable even at very early stage of development, molecular markers ensure quicker and clear cut analysis at lower cost than phenotypic testing. Screening with molecular markers would be helpful especially when the trait is under polygenic control, most commonly seen in the case of pest and disease resistance.

In the age of climate change, transgenic technology in sugarcane is boon by developing transgenic events tolerant to various biotic and abiotic constraints. The very first transgenic variety having tolerant to drought was commercially released in Indonesia. This transgenic genotype has plant cells stabilizing compound called betaine a bacterial gene (http://www.thejakartapost.com/). Progress to develop varieties resistant to stalk borer, ScYLV and herbicide resistance was possible through transgenic approach (Arencibia et al. 1997; Gilbert et al. 2009; Enríquez-Obregón 1998). Further, to tap the potential of cellulose in the leaves of sugarcane and bagasse, lignin is to be modified into simpler form which can be easily degraded by modifying its chemical structure through genetic engineering and studies were initiated in Brazil (http://agencia.fapesp.br/en/167560) and Australia (Harrison et al. 2011). Presently sugarcane is considered as an ideal plant for producing medicinal and industrial values like therapeutic protein and natural precursors of biopolymers (Wang et al. 2005; Petrasovits et al. 2007). In order to fulfil these objectives of utilising the sugarcane in present and future, molecular approaches are essentially required along with conventional breeding.

9.5 Marker-Assisted Breeding for Resistance Traits

9.5.1 Germplasm Characterization and DUS

The concept of plant variety protection received the emphasis by Agreement on Trade Related Aspects of Intellectual Property Rights (TRIPs). Being a member to TRIPs, it is mandatory to protect varieties of crop plant by patents, by an efficient *sui generis* system, or by both in India. The choice of *sui generis* method of plant crop varieties protection was selected by Indian Government and 'Protection of Plant Varieties and Farmers' Right Act in 2001 was enacted to encourage research, variety development, protection to varieties, ensures farmers rights and for the growth of seed industry. To claim the protection under this act, the variety must satisfy four criteria of novelty (variety should not be commercialized for more than one year before the grant of protection and in case of tress of vines earlier than six years),

distinctness (variety must be distinguishable from already available old varieties by one or more identifiable morphological, physiological and other characters), uniformity (in appearance), stability (expression of the essential traits remains unchanged over the successive generations of propagation) (DUS) (Anonymous 2006).

DUS testing is done to know the whether the new variety bred in particular species is distinct from already available old varieties and the character(s) of distinctness is expressed stably over the period. ICAR-SBI, Coimbatore is designated as a National DUS center by the Protection of Plant Varieties & Farmers' Rights Authority (PPVFRA) for sugarcane. The sugarcane varieties are subjected to the DUS testing at four centers viz., Coimbatore and Agali for tropical varieties and at Lucknow and Karnal for sub-tropical ones.

In case of sugarcane, seed quantity of 400 single buds taken from top portion of the 8–10 month old mother cane are taken as planting materials for DUS testing. Care is taken to select the setts for planting from healthy and vigorous material without any incidence of pest and diseases. Further, the seed material for DUS testing is expected to have high level genetic purity, uniformity and phyto-sanitary standards and it should not be taken from in vitro propagation and not subjected to any chemical or bio-physical treatment. The DUS test is conducted on payment of testing fee by the nominating centers.

In case sugarcane varieties developed from a sub-tropical region of the country, the DUS testing is done at two locations viz., ICAR-SBI, Regional Centre, Karnal, Haryana and ICAR-Indian Institute for Sugarcanes Research, Lucknow, Uttar Pradesh. ICAR-IISR maintains reference varieties of sugarcane numbering 153 that includes released and notified varieties from Central Varietal Release Committee (CVRC) and state governments and clones from advanced varietal trials (AVT) of All India coordinated research project on sugarcane (AICRP[S]) from different sugarcane research centers (Anonymous 2019). Similarly, a total of 167 reference varieties are maintained at ICAR-SBI Regional Centre, Karnal. A total 233 reference varieties are maintained clonally at ICAR-SBI at two of its test locations viz., ICAR-SBI, Coimbatore and its Research Centre in Agali clonally and separately for DUS testing of the varieties developed from tropical region of the country (Anonymous 2020). DUS testing guidelines are used to record DUS characters on the reference collections and the reference varieties were characterized and the database of the all the reference varieties are developed along with photographs of the major morphological features (Anonymous 2020). Three characters viz., growth habit, leaf blade, leaf sheath adherence are taken to group the reference varieties. A group of reference varieties to which candidate variety shows most similarity is selected to plant along with candidate variety for DUS testing. The DUS trial is conducted in plot size of four rows of 6 m length with row to spacing of 0.90 m. Observations specific to different stage of crop maturity stages as per the guidelines of PPV and FRA are recorded to see the distinctness and similarity of candidate varieties to the reference varieties.

Morphological characters on growth habit, leaf sheath hairiness, shape of ligule, shape of inner auricle, colour of dew lap, leaf blade curvature and leaf blade width are seen during the end of grand growth phase of the crop (240 days). During the

maturity phase (300 days), those adhering on leaf sheath, colour of inter-node both exposed and not exposed to sun, characteristic features of inter-node like, diameter, shape, alignment, growth crack (split), rind surface appearance and waxiness, bud characters like shape of bud, size of bud, bud groove, bud cushion, bud tip in relation to growth ring, prominence of growth ring and width of root band are observed. Cane characters like cane height, number of millable canes, pithiness and internode cross section are recorded during harvest (360 days) of the crop.

9.5.2 Molecular Markers for Biotic Stresses

Conventional plant breeding has contributed immensely on improving yield in major crops and with the advancement of modern genomic tools a paradigm shift is being made in evolving better varieties. Breeding programs aim at introgressing one or a few favorable genes from donor into highly adopted variety while at the same time recovering most of the recipient parental genome. Breeding for biotic and abiotic stresses requires identification of tolerant genotypes mostly from the germplasm, and hence the knowledge on the genetics of resistance/tolerance is very valuable. Remarkable accomplishments were achieved during the last five decades in evolving new improved crop cultivars resistant to plant diseases and pests through plant breeding. Genomic tools are beginning to support plant breeding programs in a way as never before in evolving crop varieties, resilient to various biotic and abiotic stresses with improved productivity. Major emphasis was laid on sourcing genes contributing to better productivity and adaptability from related species and wild relatives through genetic manipulation at interspecific or intergeneric level. Breeding for stress tolerance through conventional means is challenging due to poor understanding on inheritance of resistance to diseases, transfer of undesirable genes from the wild accessions along with desirable traits and the presence of reproductive barriers especially in interspecific and intergeneric crosses and needless to say that the time taken in evolving such varieties is very long.

9.5.2.1 Red Rot

In sugarcane breeding programs especially in India, red rot resistance is a prerequisite in identification and commercial release of new varieties. Also, prevalence of many *C. falcatum* pathotypes complicates breeding for *C. falcatum* resistance (Viswanathan 2021b). Further, inheritance patterns to red rot resistance/susceptibility are not clear since the studies revealed existence of both race-specific and non-specific (vertical and horizontal) resistance (Babu et al. 2010). Hence, sugarcane genome complexity does not permit any genetic manipulation for *C. falcatum* resistance by conventional breeding gene introgression methods. The only way of getting resistant varieties is screening the highly variable F_1 progeny population and the subsequent clonal propagation for *C. falcatum* resistance. However, there is need to categorize

genomic regions imparting resistance against constantly evolving new variants of *C. falcatum*. In sugarcane, almost all the traits of interest are quantitative and multi-allelic (Selvi and Nair 2010) and mapping them even with the currently available genomic and bioinformatics resources and tools is a tedious process.

9.5.2.2 Rust

MAS has been highly successful in sugarcane breeding for rust resistance. Genetic basis of rust resistance among the selfed progenies of the resistant cv R570 established a 3:1 segregation ratio for resistant and susceptible. Resistant allele 'Bru1' was identified in the cv R570, which is dominant, single copy and monogenic (Daugrois et al. 1996). To locate the major gene on the cv R570, genetic map based on restriction fragment length polymorphism (RFLP) was used (Grivet et al. 1996). Resistance to brown rust was found to be very much transmissible and additive; hence breeding for disease resistance became fast and effective. Simple inheritance to brown rust in a complex polyploidy crop like sugarcane favored bacterial artificial chromosome (BAC) library construction and map based cloning of the cv R570. In the context of genomic complex of sugarcane Bru1 became the finely categorized Mendelian trait and Bru1 provided resistance against many rust races of the pathogen (Asnaghi et al. 2001). Even though the cv R570 has been cultivated intensively in Réunion Island, resistance breakdown to the gene has not been observed for nearly two decades.

9.5.2.3 Yellow Leaf Disease

Only a very few genetic studies were made to describe YLD resistance in sugarcane. Using a quantitative triat loci (QTL) strategy of involving progenies between a susceptible (S) variety and a resistant (R) clone, the first key quantitative trait allele (QTA) was tagged for ScLYV resistance and named as *Ryl*1 (Costet et al. 2012). Here, resistance in the 196 progenies of R570 (S) x MQ76-53 (R) was evaluated using tissue-blot immunoassay (TBIA) for 10 years. Genotyping was accomplished with different molecular markers (1299 amplified fragment length polymorphism (AFLP), 247 simple-sequence repeats (SSR), 115 RFLP) resulting in 2822 polymorphic markers. The major QTA *Ryl*1 contributed 32% for YLD resistance in the resistant cv MQ76-53.

9.5.2.4 Other Diseases

Another association mapping study by McIntyre et al. (2005) with 192 progenies made from a cross between the cv Q117 and a clone 74C42. In fact, this was the first association mapping study attempted in sugarcane. The Q1 progeny were evaluated for their disease resistance to pachymetra root rot (PRR), brown rust and genotyped using RFLP (7 RFLP and 31 resistance gene analogues), 31 AFLP and 30 SSR

markers. An elite clone set consisting of 154 clones representing diverse Australian breeding material was used to validate the identified markers. Linkage map and association analysis were carried out and 30 markers were identified for brown rust and PRR. The total phenotypic variations described by the specific markers were in the range of 4–16% for PRR and 4–18% for brown rust. QTL's identified from biparental cross were validated in the elite clone set. Three markers were found highly associated for PRR and one marker was significantly associated to brown rust. This study provided a foundation that association mapping can be successfully employed in sugarcane crop.

Gouy et al. (2015) screened 183 sugarcane accessions representing worldwide sugarcane germplasm with SSR, DArT, and AFLP (1406 AFLP, 1892 DArT and 29 SSR) markers, and the population was characterized for agro-morphological and disease resistance characters across five locations. R12H16_PCR marker located in the Bru1 gene was used as a diagnostic marker. Diagnostic markers are derived from the polymorphism that directly contributes to the trait or in strong linkage disequilibrium (LD) with allele.

A mapping panel consisting of 154 sugarcane clones representing parental materials and cultivated varieties were studied for markers associated to disease resistance PRR, Fiji leaf gall, leaf scald and smut. Genetic analysis with AFLP (1068) and SSR (141) markers indicated that the number of markers identified almost halved when population structure was considered for all the diseases except for leaf gall. The numbers of markers significantly associated at $P \leq 0.001$ within groups were 12 for smut, 5 for leaf scald, 4 for Fiji leaf gall and 5 for Pachymetra (Wei et al. 2006).

These genes which are often identified as candidate genes with several other gene sets in other biotic as well as abiotic studies not only in sugarcane but also in other crops, are being studied further and probably these would support directly to red rot resistance, and potentially apply MAS in sugarcane breeding. Although association between identified markers and phenotype is not well established in sugarcane for routine selection process, it could be a valuable means to understand the resistance potential of the genotypes used in the breeding programs.

9.5.2.5 Insect Pests

The genome of sugarcane, a complex polyploid with QTL for borer pests remains relatively unexplored. In sugarcane, differentially expressed cDNA fragments for sugarcane stalk borer Eldana were identified by Butterfield et al. (2004). Using an RFLP approach, genes involved in resistance mechanisms such as peroxidase, catalases and several receptor kinases were probed on a set of population of 78 sugarcane clones. They identified 69 polymorphisms exhibiting correlation with Eldana resistance followed by 59 to smut, and 35 to SCMV. Distinct markers with the largest effects accounted for 20.2% of the variation in case of Eldana and 15.9% of the phenotypic variation in smut score.

Randomly amplified polymorphic DNA (RAPD) and SSR markers were applied to assess genomic diversity amongst cane cultivars varying in resistance for top

borer and to identify their association with borer resistance and susceptibility. DNA from R, moderately resistant (MR) and HS clones were bulked and screened with polymorphic primers. Sixty-two of the 125 primers generated polymorphic profiles. Among them OPC201020, NKS7186, NKS8334, NKS61221 and NKS9615 showed a relation with top borer resistance/susceptibility in resistant varieties whereas two markers NKS5684 and OPV17917 showed such relation in the susceptible varieties. Finally, these markers were validated with a set of foreign hybrids showing resistance and identified three NKS7186, NKS61221 and OPV17917 that are useful for screening top borer resistance in sugarcane (Selvi et al. 2008).

Inter specific and intra specific breeding for resistance in sugarcane is viable due to the genetic compatibility and availability of resistance sources. A wild relative of sugarcane, *Erianthus arundinaceus* has tolerance against abiotic stresses (Shabbir et al. 2021) which may also have tolerance to different pests therefore they can be extensively used in current sugarcane improvement programs to develop varieties with insect pest resistance and high sucrose (Cai et al. 2012).

9.6 Genomics-Aided Breeding for Resistance Traits

9.6.1 Structural and Functional Genomic Resources Developed

With the recent advances in genome sequencing technologies, several relevant genes were identified/characterized and novel information on process and pathways are continually emerging. The major developments include the development of molecular markers, generation of expressed sequence tag (EST) databases, gene expression methodologies, development of microarray technologies, development of computational abilities and algorithms etc., sequencing of the several plant genomes and transcriptomes along with the advances in automated sequencing. These developments enabled the structural and functional characterization of several genes governing economically important traits and their further use in enhancing the breeding efficiency. The largest transcriptome resource for sugarcane (http://sucest.lbi.ic.unicamp.br/en/), containing about 238,000 ESTs sourced from 26 cDNA libraries of 12 cane varieties. The cDNA libraries represented different stages of crop development and environment and subjected to different biotic treatments (Arruda 2001). Brazilian modern cultivar's (SP80-3280) gene space assembly was created and that comprises 373,869 genes of the whole sequence with their upstream regions to identify regulatory promoter elements, BACs of R570 and SP80-3280 and CRISPOR, a CRISPR/Cas9 assisting tool. The SUCEST-FUN database also hosts functional genomics resources for insect and pathogen interaction with sugarcane.

Nuclear genomes of modern cultivars have two sub-genomes; the one from *S. officinarum* with basic monoploid genome size of about 1 Gb and the other from *S. spontaneum* with size of 750–843 mb (Zhang et al. 2012). Linkage mapping

in autopolyploid is difficult because of arbitrary combination of many homologous chromosomes, detection of many spots/bands by nucleic acid probe/primers and segregation of alleles with different dosage level (Ming et al. 2010). Recently, French sugarcane variety R 570 (2n = 115) was selected by the sugarcane genome sequencing initiative (SUGEST) and this variety is characterized intensively (Aitken et al. 2016). Other cultivars, used for gene sequencing are SP 80-3280, Q 165, LA 9 Purple and IJ 76-514 (*S. officinarum*), SES 208 and Mandalay (*S. spontaneum*). First linkage map was created from the progenies of a cross combination of *S. spontaneum* and its doubled haploid having 64 linkage groups from 276 RFLPs with 208 single dose randomly primed PCR loci of *Saccharum* complex (da Silva et al. 1995). Linkage groups in all the nine available linkages have partially represented less than 50% of the genome of the genotypes taken for study (Ming et al. 2010). IND 81-146 with about 58% of genome coverage had the fewest chromosome number (26–28), which is the best criteria for selecting the genotypes for a saturated genetic map.

The differential chromosome number (100–130), plenty of transposans/retrotransposons present through the genome, repetitive elements and differential ploidy levels for genes account for about 50% of genome in the crop making sugarcane monoploid genome 10 times bigger than the model plant species like *Arabidopsis*. The large and complex polyploidy nuclear genome and organeller genome of sugarcane are responsible for less advancement in sugarcane genomics.

A monoploid reference sequence of sugarcane hybrid cv R570, an allele defined genome of *S. spontaneum* and a long read reference transcriptome are some of the sugarcane genomics resources developed recently (Hoang et al. 2017, Garsmeur et al. 2018; Zhang et al. 2018). Further, advances in proteomic research resulted in expansion of a huge reference proteomes of around 20,382 as on January 2022, in the Uniprot database, consisting more than 8714 bacterial, 10,069 viral, and 1805 eukaryote proteomes. For structural genomics, the protein database PDB, hosts various information on crystal structure, electron microscopy, x-ray diffraction studies, and nuclear magnetic resonance (NMR) studies of proteins from plants, viruses, bacteria and fungi. For sugarcane to be specific, crystal structures of defensin (de Paula et al. 2011), canecystatin (Valadares et al. 2013), sugarcane serine/threonine protein kinase SAPK10 (Righetto et al. 2019; PDB Accession 5WAX), UDP-glucose pyrophosphorylase (Cotrim et al. 2018), an antifungal protein Sugarwin (Maia et al. 2021) are available in PDB. In addition structural information from model plants like Arabidopsis (1720) entries, maize (23), Tobacco (10), rice (28), sorghum (18, consisting of important genes like peroxidase, caffeoyl-CoA O-methyltransferase, Phenylalanine ammonia-lyase), structure of effector protein, chitin deacytylase, fungal alcohol oxidase etc. from *Colletotrichum* species (10), structure of proteins for fungal toxin, replication protein, kinesin etc. from *Ustilago* species are available for references.

The large volume of sequence data generated by next-generation sequencing (NGS) are simultaneously characterized functionally using high-throughput assays, DNA microarray, gene chips, serial analysis of gene expression (SAGE), oligoarrays, and single cell RNA sequencing etc. to identify candidate genes on a large scale. A huge DNA sequence information were generated from these projects and

the online databases such as http://www.ncbi.nlm.nih.go (NCBI-National Centre for Biotechnology Information), http://www.tigr.org (TIGR-The Institute of Genome Research), http://www.ebi.ac.uk (EBI-European Bioinformatics Institute) have all the sequence deposits. The availability of genome sequences for several crops and microbes CCBI Database as on January 2022, (Eukaryotes [20672 out of which 1754 are plant genomes https://www.ncbi.nlm.nih.gov/genome/browse/#!/eukaryotes/Plants]), Prokaryotes (372,288), Viruses (46,556), Plasmids (34,863) and Organelles (21,232). Recent progress in genome editing (GE) methods has made advances in breed for practically any given desired character. Improvements in GE tools like transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs) have made it possible to manipulate accurately any gene of interest by the molecular biologists. By applying gene-editing approach, non-genetically modified (Non-GMO) crop plants with the desired gene of interest or trait will be achieved and this will contribute to enhanced yield by effectively managing various biotic and abiotic stresses.

The new genomic science allowed the scientists to investigate the plant genome by many approaches and on a dimension, which was earlier unthinkable. Shortly, plant breeders will have the choice of using many genes, to develop a desired plant of required genetic makeup with more efficiency than the past. Moreover, in the coming days interaction between different genes that work together in plants to give a desired crop features will be achieved through genomics. By this, a combination of desired genes could be assembled into cultivars by means of very accurate plant breeding procedures. Thus a combination of biotechnology and genomics programs is greatly aiding to confront the many challenges facing production, management, and sustainability in agriculture.

9.6.2 Genome-Wide Association Study (GWAS) and Genomic Selection (GS)

Genomic-selection method initiated by Meuwissen et al. (2001) is a new approach for selection of individuals in breeding experiments and is appropriate for the augmentation of complex-traits requiring long-term field experiments. Genomic-selection utilize the whole-marker information by concurrently calculating the outcome of each marker covering the whole genome to anticipate the genetic-value of individuals. Because of its complex genome (aneu-polyploidy), genetics and genomics research has not been very successful in sugarcane, unlike other crops. However, with the advances in genomics and decreasing costs of NGS tools, development of high-density markers enabled genetic maps is currently possible. During last two decades, GWAS were developed to find QTLs related to biotic resistance in sugarcane (Debibakas et al. 2014; Gouy et al. 2015; Singh et al. 2016; Gutierrez et al. 2018; Islam et al. 2018; Yang et al. 2018, 2019; You et al. 2019, 2020; Aono et al. 2020; Pimenta et al. 2021).

9.6.2.1 Red Rot

An association mapping approach used on a panel of 119 sugarcane genotypes fingerprinted for 945 single sequence repeats alleles was carried out to find markers associated with resistance to three pathotypes *C. falcatum* of viz., CF01, CF08 and CF09 (Singh et al. 2016). Mixed-linear models identified four markers that were able to describe ten to sixteen percentage of individual trait variation. General linear model (GLM) analysis identified three (IISR_90_360; IISR_298a_140; IISR_256_240), one (IISR_198_170) and five (IISR_148_200; IISR-137-240; IISR_46b_170; SCB10_410; ESTA69_400) markers linked with resistance to the pathotypes CF01, CF08 and CF09, respectively. MLM identified only four markers viz., IISR_256_240 & IISR_298a_140 for the pathotype CF01 and IISR_137_240, IISR_46b_170 for the pathotype CF09, which were able to elucidate 16.6, 10.7, 14.5, and 11.7%, respectively of the total phenotypic variation. Many genes involved in host-defense like Serine/threonine protein kinase, MAP Kinase-4, Transporter-1, Cytochrome-P450, Ring finger-domain protein, Glycerol-3-Phosphate, and others were confined to the region of these four markers. Similarly, from ICAR-SBI, Coimbatore, the populations of BO 91 × Co 775, Co canes, Co 86002 × BO 91 and CoM 0265 × Co 775 were scored for red rot resistance and identified more number of clones under MR category. Clonal selection was done in all the populations for sucrose, red rot reaction and other criteria and broad sense heritability was calculated for all the traits. The heritability % for the red rot trait from the populations ranged from 94 to 97%. All the genotyped clones subjected to develop genomic selection/prediction models. BayesA, BayesB, BL, genomic best linear unbiased prediction (GBLUP), Reproducing Kernel Hilbert space (RKHS) Single models showed significant Single nucleotide polymorphisms (SNPs) for the sucrose and red rot traits. The correlation between prediction models for the sucrose trait with training and testing population was high (>0.9) and the prediction accuracy was high for 100 testing population (>0.65 for the for the sucrose trait). The prediction models for red rot resistance showed the accuracy of 0.56 (Manimekalai et al. unpublished).

9.6.2.2 Smut

Recently hybrid-transcript based mapping assembly method was followed to decode genome-wide expression conversion at iso-form level and alternative splicing (AS) land-scapes regulation in a moderately resistant genotype following *Sporisorium scitamineum* inoculation (Bedre et al. 2019). Approximately 5000 (14%) sugarcane genes were identified using de novo and comparative genome-wide transcript mapping that undergo alternative splicing in reaction to *S. scitamineum* infection. A total of eight hundred and ninety-six events have been established that expressed differentially at various stages of infection with *S. scitamineum.* The open reading

frames (ORFs) of these genes deciphered changed proteins, which change and regulate cell wall, reactive oxygen species (ROS) homeostasis, transcription and defense-hormone signaling. Although AS approach has cleared the path up to some extent but still there are many folds that have to come out. In addition to the abovementioned methods, there are new opportunities to develop emerging GWAS method for sugarcane and *S. scitamineum,* which allows for the concurrent detection of genes interconnect between sugarcane and its pathogen.

9.6.2.3 Rust

Several works have been executed by different workers in sugarcane to know the resistance to *Puccinia kuehnii*. Yang et al. (2018) based on genome-wide association method detected 3 quantitative-trait loci (qORR4, qORR109 and qORR102) for sugarcane rust in a population of 173 progenies attained from a bi-parental crosses (CP88-1762 and CP95-1039). Resistance gene encoded maker-G1 was identified through PCR method. Probably the putative QTL and G1-marker identified in this work can be successfully used in breeding programmes to ease the selection process for orange rust disease management. Later, Yang et al. (2019) evaluated 308 accessions from the World germplasm collections maintained at Miami for resistance to *P. kuehnii* by genotyping and phenotyping studies. They also characterized many DNA sequence variants by NGS through target-enrichment sequencing. They detected 91 putative DNA-markers and eighty two candidate genes remarkably related to resistance to orange rust. These results throw lights on the genetic bases of the rust resistance in sugarcane. Although MAS is successful for rust resistance, all the related alleles are yet to be discovered and the accompanying regions vary between genotypes, thus regulating methodology generalization. Aono et al. (2020) used GBS method to find out the genomic regions resistant to brown rust in full sib progenies. They detected about 14,540 SNPs that guided to achieve 50% Mean Prediction Accuracy. By this method, they attained up to 95% accuracy with 131 SNPs dataset related to brown rust resistance.

9.6.2.4 Leaf Scald

Molecular markers resistant to leaf scald disease (Causal organism: *Xanthomonas albilineans*) through MAS were developed by Gutierrez et al. (2018) with progenies from a cross of resistant (LCP 85-384) and susceptible (L 99-226) parental cultivars. QTL analysis detected 8 genomic regions on 7 linkage groups controlling leaf scald response. QTLs qLSR77; qLSR37; qLSR262 and qLSR104 were accumulative for 30 percentage of the resistance response. They were able to locate one representative gene around three QTLs using syntenic information of sorghum reference genome and comparative genome analysis. Upon *X. albilineans* infection of meristematic or lamina tissues, a clear upregulation in their expression of the genes linked to major QTLs was found. c5_1527, the codominant marker tightly allied to the QTL can

be used along with other linked SNP markers as diagnostic markers in MAS for *X. albilienans* resistance in sugarcane.

9.6.2.5 Ratoon Stunting Disease

You et al. (2020) evaluated 146 individuals of selfed progenies of the cv CP80-1827 resistant to ratoon stunting disease through GWAS. Eighty-two Lxx resistant genes were recognized by exploring the twenty-three quantitative trait loci regions on their tune in with forty-four genes on Sorghum genome, twenty genes from the genome of sugarcane cv R570, and 18 genes from *Saccharum spontaneum* genome. They recognized quantitative trait loci administering ratoon stunting resistance along with the associated single nucleotide polymorphism markers will help in MAS to reduce the depletion of sugarcane yield due to this disease.

9.6.2.6 Yellow Leaf Disease

A GWAS was performed to identify QTL governing YLD resistance on a sugarcane varieties panel numbering 189 (Debibakas et al. 2014). About 3949 DArT and AFLP polymorphic markers were used to fingerprint the panel. During the study, the varieties were phenotyped for the virus infections in 2 trials for 2 crop seasons at Guadeloupe, which has conducive conditions for natural infections of ScYLV. For ScYLV resistance, 6 independent markers were identified in relation with phenotype, but 2 markers were identified frequently during the GWAS analysis. After detailed bioinformatic analyses it was found that many genes involved in interaction of the plant with the aphid vector or the virus are found in the marker regions. Later, two QTLs administering yellow leaf virus resistance were detected and cumulatively they reduced disease incidence by 31% (Islam et al. 2018). These earlier reports improved understanding of the molecular resistance mechanisms to ScYLV. But this study were carried out either on bi-parental populations or in cultivar panels and this hinders the chance of identifying the potential ScYLV resistant loci owing to contracted genetic basis in elite sugarcane cultivars. Hence, to avoid these limitations, high density markers were used and found ninety one putative-markers and eighty two significantly associated candidate genes for YLD resistance (Yang et al. 2019). Recently, an Axiom Sugarcane100K SNP array was constructed using more than three hundred *Saccharum* spp lines (TES method) from the sugarcane germplasm and this generated about 4 million single nucleotide polymorphisms. QTL analysis identified eighteen QTLs controlling ScYLV resistance segregating in the 2 mapping populations, harboring twenty-seven disease resistant genes (You et al. 2019). Progress made in identifying QTLs for ScYLV resistance has been very positive to identify YLD resistant parental lines or germplasm. We are hopeful that GWAS application will ably support breeding for YLD resistance in sugarcane.

9.7 Recent Concepts and Strategies Developed

9.7.1 CRISPR/Cas System Mediated Resistance to Sugarcane Diseases

Despite the tremendous advances made through traditional sugarcane initiatives, long breeding cycle of 12–14 years, slow breeding improvement, narrow genetic variability, complex polyploidy of the genome and poor fertility obligated to produce novel varieties make further genetic gain of superior sugarcane varieties difficult (Babu et al. 2021; Ram et al. 2021). Achievements made using genetic engineering technique for incorporating tolerance/resistance to biotic stress in sugarcane is given in Table 9.4. Due to complex nature of biotic stress resistance in sugarcane and the lack of genetic information posed a serious challenge. Advances in genomics, such as NGS strategies and the availability of the mosaic monoploid genome of sugarcane, facilitated identifying new genes linked to both biotic and abiotic traits, expanded understanding of the response of the crops to stress, and these developments are likely to speed up the development of sugarcane-based products (Babu et al. 2021).

New genomic modification tool using the cleavage mechanism of RNA-guided Cas9 with the target specificity allows precise control of the gene editing (Doudna and Charpentier 2014). Gene editing was successfully demonstrated in plant systems for various traits (Feng et al. 2013; Li et al. 2013c; Nekrasov et al. 2013; Shan et al. 2013; Xie and Yinong 2013). Genome-editing technologies include clustered regularly interspaced short palindromic repeats/CRISPR-associated systems (CRISPR/Cas9), zinc finger nucleases (ZFNs), sequence specific nucleases (SSNs), meganucleases (MNs), transcription activator like effector nucleases (TALENs), CRISPR/Cas12a (Cpf1, CRISPR from Prevotella and Francisella 1), and Cas9-derived DNA base editors. Though application of genome editing was successfully demonstrated in sugarcane for improvement of agronomically significant characters (Jung et al. 2012; Kannan et al. 2018; Zhao et al. 2021; Eid et al. 2021; Oz et al. 2021) (Table 9.4), gene editing for inducing resistance to biotic stresses is not carried out till date.

Table 9.4 Application of genome editing in sugarcane for improvement of agronomic traits

Targeted genes	Technique	Improved traits	Repair pathway	References
COMT	TALEN	Reduction in lignin content for bioethanol production	NHEJ	Jung and Altpeter (2016), Kannan et al. (2018)
ALS	CRISPR	Herbicide tolerance	HDR	Oz et al. (2021)

9.7.2 Nanotechnology

Nanotechnology is the term related to resources and processes involving particles of 100 nm. Nanotechnology has the ability to create massive changes with ecological earnings in agricultural systems offering a chance to exercise a more proficient, safe and precise control of time and location of pesticide release (Kuzma and VerHage 2006). The use of nano particles in the new formulations of pesticides and insect repellants has been reviewed by El-Wakeil et al. (2017). Application of nanotechnology has led to better pest management among other benefits with least impact on environment (Hofmann et al. 2020). Thus subsequently mitigating consequences of climate change Nanoparticles could be used in the new formulations of pesticides preparation and insect repellants. Nanoparticle-mediated gene delivery in many plant species is more efficient than the traditional technologies due to the higher efficiency of genetic transformation (Ahmar et al. 2021).

Nano pesticides can replace the conventional pesticides as they deliver higher efficay at lower doses minimizing negative effects (Kah et al. 2018, 2019; Ahmar et al. 2021). Prolonged sustainable efficacy is ensured due to the accurate delivery and slow gradation of active components (Chhipa 2017). The pesticide nanoemulsions have certain advantages over other methods such as broad range of applicability, superior adherence on target sites with better perviousness (Feng et al. 2018). Globally, in the nano pesticide market, insecticides formed the highest share of 41% revenue in 2019 (Research Corridor 2020). Several reports and reviews have adequately emphasized the merits and risks associated with nanopesticides, and their fate in the environment (Sharma et al. 2017; Peixoto et al. 2021; Rajiv et al. 2020; Nguyen et al. 2012; Adak et al. 2012; Shukla et al. 2020; Kah et al. 2013; Mukherjee et al. 2016). Agricultural products may retain the nanopesticide residues (García et al. 2010) and thus enhanced persistence of pesticide molecules in the target organism or plant achieved through nanocapsules or nanoemulsions may pose greater risk (de Francisco and García-Estepa 2018).

9.8 Genetic Engineering for Resistance Traits

9.8.1 Establishment of Genetic Transformation in Sugarcane

The lack of resistant sources in *Saccharum* germplasm to many diseases or absence of viable management practices has opened new avenues especially genetic engineering and gene editing to circumvent the constraints and to improve production of sugarcane. Due to inaccessible crop canopy, insect pest management in sugarcane by chemical application is difficult. Moreover, borer larvae are impervious to chemical control as larvae are cryptic internal feeders. Although traditional insect host-plant resistance involves quantitative attributes at numerous loci, progress in developing a resistant cultivar has been limited. The availability of resistance gene

sources in the breeding pool as well as onerous screening procedures make traditional breeding for resistant types difficult. Chawdhary and Vasil (1992) successfully used particle bombardment and electroporation methods to transfer pBarGUS genes into sugarcane suspension cell cultures. Following that, tremendous progress was made in sugarcane genetic transformation and transgenic sugarcane development for a variety of traits. Among the several approaches used to introduce the desired gene in sugarcane protoplasts, cells or calli, *Agrobacterium* mediated transformation (Arencibia et al. 1998; Joyce et al. 2010; Kalunke et al. 2009; Manickavasagam et al. 2004; Mayavan et al. 2013) is popular. The other methods of gene transfers involved either chemicals (Chen et al. 1987) or devices (Franks and Birch 1991; Snyman et al. 2006; Babu and Nerkar 2012) or electrical perforations in the target tissue (Rathus and Birch 1992).

Genetic transformation in sugarcane has been extremely successful and transgenics for various biotic stresses developed (Babu et al. 2021) are listed in Table 9.5. These include resistance to diseases such as mosaic, yellow leaf, leaf scald, red rot etc. (Jain et al. 2007; Gilbert et al. 2009; Zhang et al. 1999; Kanchana 2007) and to pests like sugarcane borers (Kalunke et al. 2009; Gao et al. 2016), Sugarcane has also been modified genetically for the better economically important traits namely yield of sugar, quality of juice (Botha and Groenewald 2001; Vickers et al. 2005) and value-added unique sugar that is more beneficial to consumers (Wang et al. 2005).

9.8.2 Disease Resistance in Sugarcane

9.8.2.1 Viral Diseases

In order to induce resistance to viral diseases, SCMV-coat protein (SCMV-CP) gene of was transferred by genetic transformation in sugarcane. The transgenic lines of sugarcane plants carrying the coat protein gene was tested and found to be superior to that of non-transformed plants. Sugarcane hybrid CC84-75 was transformed through particle bombardment using ScYLV coat protein DNA fragment. Most of the PCR positive for ScYLV coat protein exhibited negative for ScYLV even after 10 months after infection (Rangel et al. 2005). Microprojectile transformation of sugarcane cv Q124 with FDV segment 9 ORF1 resulted in resistance to Fiji disease. Of the 47 transgenic lines investigated, some of the resistant lines showed no Fiji disease symptoms (McQualter et al. 2001).

Transgenic sugarcane lines conferring with mosaic resistance in high yielding and high sucrose varieties were developed in many countries through particle gun bombardment methods (Ingelbrecht et al. 1999; Yao et al. 2004; Gilbert et al. 2005; Guo et al. 2008). However, the transgenic lines had high copy numbers of target gene inserts (Arencibia et al. 1998) and transgenic lines obtained through gun bombardment methods had difficulties to prove the sites of insertion, and border sequences. Post-transcriptional gene silencing (PTGS) mediated transgene development was reported as the most widely adapted method to confer mosaic resistance in high

Table 9.5 Sugarcane transgenics developed for different biotic stress resistance/tolerance

Disease	Gene used	References
SCMV	Sugarcane mosaic virus coat protein (SCMV-CP)	Joyce et al. (1998) Apriasti et al. (2018)
Sugarcane leaf scald	albicidin detoxifying (albD)	Zhang et al. (1999)
SrMV	Sorghum mosaic virus coat protein (SrMV-CP)	Ingelbrecht et al. (1999)
ScYLV	Sugarcane yellow leaf virus coat protein (ScYLV-CP)	Rangel et al. (2005)
SCMV	Sugarcane mosaic virus coat protein (SCMV-CP)	Gilbert et al. 2005
Sugarcane rust (*Puccinia melanocephala*)	Glucanase, chitinase & aprotinin 24	Enriquez et al. (2000)
Fiji leaf gall	Fiji disease virus segment 9 ORF 1 (FDVS9 ORF 1)	McQualter et al. (2001)
Red rot (*Colletotrichum falcatum*)	Dm-Anti microbial peptide 1 (amp1) and chitinase	Kanchana (2007)
Red rot (*C. falcatum*)	Chitinase and 1,3-β-glucanse	Kanchana (2007)
Red rot (*C. falcatum*)	*Trichoderma* β-1,3-glucanase gene	Nayyar et al. (2017)
Red rot (*C. falcatum*)	barley chitinase class-II genes and *HarChit*and *HarCho*	Ijaz et al. (2018), Tariq et al. (2018)
Insect pests	Gene used	References
D. saccharalis	Crystal toxin gene (cry1Ab)	Braga et al. (2003)
C. infuscatellus	Crystal toxin gene (cry1Ab)	Arvinth et al. (2010)
Sugarcane borers	Crystal toxin gene (cry1Aa3)	Kalunke et al. (2009)
P. venosatus	*Bt*Crystal toxin gene (cry1Ac)-modified	Weng et al. (2006, 2011)
Cane grub	*Galanthus nivalis* L. (snowdrop) agglutinin (gna)	Legaspi and Mirkov (2000)
Cane grub	*G. nivalis* (snowdrop) agglutinin (gna)	Nutt et al. (1999)
E. loftini	*G. nivalis* (snowdrop) agglutinin (gna)	Setamou et al. (2002a, b)
E. loftini	*G. nivalis* (snowdrop) agglutinin (gna)	Tomov and Bernal. (2003)
Sugarcane stalk borer	*G. nivalis* (snowdrop) agglutinin (gna)	Irvine and Mirkov (1997)
E. loftini	*Galanthus nivalis* L. (snowdrop) agglutinin (gna)	Nutt et al. (1999)
Ceratoacuna lanigera	Snow drop lectin	Zhangsun et al. (2007), Romeis et al. (2003)

(continued)

Table 9.5 (continued)

Insect pests	Gene used	References
Top borer *S. excerptalis*	Aprotinin	Christy et al. (2009)
Early shoot borer (*Chilo infuscatellus* Snell)	Crystal toxin gene (cryIF)	Thorat et al. (2017)
Sugarcane weevil	Sugarcane cysteine peptidase inhibitor 1 (CaneCPI1)	Schneider et al. (2017)
D. saccharalis, Ceratovacuna lanigera	AVAc-SKTI	Deng et al. (2008)
Sphenophorus levis	*HIS Cane CPI-1*	Ribeiro et al. (2008)
E. loftini	*gna*	Setamou et al. (2002a)

yielding and high sucrose mosaic susceptible varieties. During 2005, the first SCMV resistant transgenic lines developed with untranslatable SCMV strain E-CP gene by following biolistic transformation methods in USA and these lines were evaluated for agronomic performance and field disease resistance. Around 100 transgenic lines derived from the cvs CP 84-1198 and CP 80-1827 when evaluated for resistance against the disease and agronomic traits in one plant crop and two ratoons, the transgenics developed from CP 84-1198 had recorded a significant improvement in cane yield and sucrose with reduced mosaic disease incidence (Gilbert et al. 2005).

In China, transgenic sugarcane lines resistant to mosaic were developed using Sorghum mosaic virus (SrMV) (SrMV) CP gene by following RNA interference (RNAi) approach. The RNAi vector pGII00-HACP contained hairpin interference sequence and herbicide-tolerant gene, *cp4-epsps* and the expression cassette was transferred to sugarcane cv ROC22 by following *Agrobacterium*-mediated transformation. The SrMV transgenic lines were confirmed by challenge inoculation and herbicide screening. The genetically modified cv ROC22 were reported with 87.5% SrMV resistance rate (Guo et al. 2015). Similarly, RNA silencing approach was followed to develop transgenic sugarcane against SCMV with the expression of a short hairpin RNAs (shRNA) directing SCMV-CP gene, in Punjab province in Pakistan. Based on SCMV conserved CP region, two independent shRNA transgenic lines expressing stem and loop sequences derivative of microRNA, sof-MIR168a—an active regulatory miRNA in sugarcane, siRNA 2 and siRNA4 were engineered as RNAi constructs with the polyubiquitin promoter control. Particle bombardment method was used to deliver the constructs into sugarcane cvs SPF-234 and NSG-311 as separate experimentations. Challenging the transgenic lines with SCMV by mechanical inoculation revealed that the degree of mosaic resistance is more in shRNA4 transgenic lines in both cultivars with 80–90% reduction of SCMV-CP gene expression (Aslam et al. 2018).

In India, efforts were made to develop mosaic resistant transgenic sugarcane plants with RNAi technology using the SCSMV suppressor protein genes SCSMV-P1 and HC-Pro. Both the gene constructs were evaluated in model plant *Nicotiana tabacum* under GFP tagged transient expression assay in that the P1 gene was identified as

playing a major role of RNA silencing suppressor (Bagyalakshmi and Viswanathan 2020). Recently, Hidayati et al. (2021) made a comparison of RNAi and pathogen-derived resistance (PDR) approaches toassess effectiveness of transgenic sugarcane plants with resistance to SCMV in Indonesia. Transgenic plants harbouring RNAi mediated resistance were reported with high level of SCMV resistance based on delayed symptom expression at 26 dpi with mosaic symptoms only 50% of the inoculated plants as compared to 77.8% in PDR transgenic plants and with less number of plants with 36.7 kDa SCMV-coat protein. With this RNAi mechanism generated SiRNA mediated control was reported as effective against the SCMV.

Agronomic evaluation of five independent sugarcane transgenic clones with SCMV resistance was done based on field performance, resistance to the virus, and stability of transgene in comparisons with Badila, the wild-type parental clone in China. All the transgenic lines were reported with higher tonnes of cane/ha, higher sucrose % along with low mosaic incidence than Badila. Among the five independent sugarcane transgenic lines, the line B48 showed very high resistant to the virus with only 3% or less incidence. Further, the resistant line recorded an average of yield of 102.72 t/ha, whereas the parental clone Badila recorded 67.2% lesser cane yield and the transgene expressed stably over many vegetative generations. With this study, the China has developed a transgenic Badila as a valuable SCMV resistant germplasm source for future development of mosaic resistant genotypes (Yao et al. 2017).

In Florida, USA Gilbert et al. (2009) developed two transgenic clones (6–1 and 6–2) resistant to ScYLV from the CP92-1666 cultivar by particle bombardment methods using two different transformation vectors under the same maize ubiquitin promoter with untranslatable ScYLV-CP gene construct in antisense orientation; and the other construct with modified antibacterial Cecropin B gene along with *npt*II selectable marker gene. The transgenic lines as well as tissue culture material had shown low yield potential compared to the parental cultivar in plant crop followed by two subsequent ratoons. But, transgenic lines had a high level of ScYLV resistance with very low infection rates of 0–5% compared to 98% in parent cultivar. This study revealed that transgenic lines cannot be acceptable for commercial cultivation as such due to poor yield potential but serve as donor parents to develop ScYLV resistance. Later, Glynn et al. (2010) reported that it could be overcome or reduced by transgene transfer to sexual progeny of sugarcane true seeds. In the same way, Zhu et al. (2010a, b) developed transgenic lines resistant to ScYLV from a susceptible cv H62-4671 in Hawaii using the particle bombardment method. Two different transformation constructs were used, one with untranslatable CP gene of ScYLV in a sense orientation driven by a maize ubiquitin promoter while the other with *npt*II selectable marker under a sugarcane ubiquitin promoter. Based on viral titer and symptom phenotype, the transgenic lines were evaluated. Of nine transgenic lines, six exhibited ScYLV resistance with atleast 10^3 fold lower virus titer than the wild susceptible parent.

9.8.2.2 Other Diseases

Nine putative transgenics harboring the chitinase gene was tested against *Colletotrichum falcatum* and eight plants showed susceptible reaction whereas a single transgenic G11-1 showed partial resistance. Increased resistance was observed in transgenics GM-8 and GM-9, which co-expressed with Dm-antimicrobial peptide (Amp1) and chitinase. On the other hand two sugarcane transgenics P-2 and P-4 harbouring the genes chitinase and β-1,3-glucanase, respectively showed moderate tolerance to red rot (Kanchana 2007). The transgenic sugarcane lines of the cv CoJ 83 expressing *Trichoderma* spp β-1,3-glucanase gene exhibited tolerance to *C. falcatum* CF08 and CF09 pathotypes in glass house environment. The expressed gene in parenchymatous tissues in stalks inhibited fungal growth by lysis. Further, the expressed protein of β-1,3-glucanase gene sliced β-1,3-glycosidic bonds that causes damage to mycelia of *C. falcatum* (Nayyar et al. 2017). Further, transgenic lines with expression of *HarChit* encoding Chitinase and *HarCho* encoding Chitosanase are found to show strong inhibition against *C. falcatum* (Ijaz et al. 2018; Tariq et al. 2018). These studies clearly showed potential of the barley chitinase class-II genes to inhibit red rot pathogen in sugarcane stalk tissues.

9.8.3 Insect Pests Resistance in Sugarcane

Genetic transformation in sugarcane has helped to fortify a superior variety that already excels in most agronomic features but is susceptible to pests. Introduction of insecticidal genes through transformation enhances pest resistance in sugarcane thus maximizing and sustaining the crop yields even though IPM approaches complement the previously existing tolerance (Allsopp and Manner 1997; Allsopp and Suasaard 2000). Several genes that confer insect resistance have been found from various sources and effectively used in commercial sugarcane genetic transformation for pest management (Table 9.5). The widely exploited insect resistance genes include protease inhibitors, crystal toxins, lectins, secondary plant metabolites, proteins that inactivate ribosomal activity, and viruses. These genes are used either singly or in combination to generate commercially valuable insect resistant transgenic plants. Sugarcane transformed with proteinase inhibitor genes were resistant to grubs (Atkinson et al. 1993; Falco and Silva-Filho 2003; Nutt et al. 2001). Significant growth inhibition was observed in stalk borers reared on sugarcane transformed with lectin genes (Legaspi and Mirkov 2000). *Bt*-transformed sugarcane was found to be resistant to *D. saccharalis* (Arencibia et al. 1997; Wu et al. 2009). ELISA studies of the integrated Cry 1Aa 3 gene showed ten-fold increase in the level of expression (Kalunke et al. 2009). Borer larvae fed with transformed sugarcane possessing a gene coding for aprotinin suffered significant weight loss (upto 99.8%) which could be due to the cumulative antibiosis effect (Christy et al. 2009). Transgenic sugarcane lines over expressing Cry1F showed resistance to *C. infuscatellus* (Thorat et al.

2017). Arvinth et al. (2009, 2010) developed transgenic sugarcane expressing cry 1 Ab gene for management of shoot borer.

Transgenic sugarcane plants expressing both Cry1Ab and EPSPS were resistant to *D. saccharalis* and tolerated the herbicide Glyphosate but were agronomically poorer than the native sugarcane plants. Also, variations in the copy number of the target fragment in the transformants and expression of both of the target genes in less than 70% of the resistant plantlets (Wang et al. 2017a, b), probably due to exogenous gene silencing were the other issues.

Success of transformation and its inheritance in progeny plants are determined by the integration and expression of the desired gene in the genomic DNA of the plant. *Agrobacterium* mediated gene transfers in sugarcane by Dessoky et al. (2020) resulted in less than 25% of transgenics with varying levels of integration and the expression of cry1Ac gene. Only two transgenic sugarcane lines showed highest toxicity against the borer *S. cretica* at lower concentration of toxin, which may be due to single copy of the gene integrated. Different concentrations of endotoxin produced by each sugarcane transgenic line possessing *Cry1Ab- Cry1Ac* (Koerniati et al. 2020) could affect the efficacy against the target pest *Scripophaga excerptalis.* No differences in the morphological traits in the transformant sugarcane plants with resistant gene targeting *D. saccharalis* and the aphid *C. lanigera* were observed though the growth was slower compared to the non-transformed plants (Deng et al. 2008).

Work on transgenics for cane grub resistance is scarce. In Australia, the transgenics caused severe antibiosis in cane grub *Antitrogus consanguineus.* Grubs reared on sugarcane incorporated with a proteinase inhibitor gene attained <5% weight of those raised on non-transformed sugarcane. In yet another instance, *Dermolepida albohirtum* larvae on sugarcane transgenics with lectin gene attained less than 21%larval weight in controls (Nutt et al. 2001).

Despite the proven successes in transgenic sugarcane development, it remains a strenuous process involving rigorous and complicated procedures of tissue culture and regeneration that have to be standardized for each sugarcane genotype. Therefore, it is a tedious procedural challenge to standardize and evolve effective transformation protocols for every new sugarcane variety. Besides these, molecular protocols required for commercial release. Even after the transgenic event is achieved, due to genetic complexity and absence of a completelyanalysed reference genome for sugarcane, it is extremely difficult to execute the molecular studies to ascertain the number of copies, expression levels, insertion site and create other data for commercial release by regulatory authorities (Budeguer et al. 2021).

9.8.4 Safety of Transgenic Sugarcane

In Brazil, the earliest transgenic sugarcane variety (Bt sugarcane CTC175) expressing the Cry1Ab protein to manage *D. saccharalis* has been approved in 2018 for commercial production and distribution (ISAAA 2018a; Gianotto et al. 2018, 2019). US FDA

issued approval for Bt sugarcane from Brazil by declaring the sugar from such canes was not different that obtained from non-transformed varieties (ISAAA 2018b). Two more sugarcane events expressing cry1Ac gene (CTC91087-6 and CTC93209-4) have also been released in Brazil, recently (ISAAA 2021).

Sugar from Bt sugarcane has been proven to be safe for consumption (Gianotto et al. 2018). Sugar derived from genetically modified sugarcane was not found the products of introduced genes and was not any different from that from non-transgenic canes thus cultivating genetically modified varieties should be continue to maintain the way sugar is used as food source (Joyce et al. 2013; Cullis et al. 2014; Gianotto et al. 2018, 2019; Lajoloi et al. 2021). In South Africa positive consumer acceptance of sugar from genetically modified cane has been reported (Vermeulen et al. 2020).

Safety and impact of transgenic, specifically Bt-crops on non-target organisms have been studied in many crops (Abbas 2018; Marques et al. 2018) though such studies are scare in sugarcane. The Bt-transformed sugarcane did not have any negative impact on the structure or diversity of microbes or enzymes in the rhizosphere (Zhou et al. 2016). However, the proteins of the transformants may be toxic to parasitoids and may interfere with their ability to locate their hosts (Schuler et al. 1999).

9.9 Future Perspectives

9.9.1 Potential for Expansion of Productivity

As discussed in Sect. 9.1, increased cane productivity and sugar/ethanol production in the past 50 years was attributed to expansion of sugarcane area. However, we cannot ignore the genetic gains achieved through conventional breeding. Although outbreaks of different diseases or insect pests occurred in different continents, they have been contained by varietal replacements. Further, as discussed in the introduction, many countries experience yield plateaus attributed by pests and diseases, declining soil fertility and climatic conditions (Yadav et al. 2020). In India, varietal degeneration as the cause for decline in sugarcane productivity in many popular varieties was established. By this, yield potential of a variety comes down after few years in the field due to systemic accumulation of non-fungal pathogens causing RSD, YLD and mosaic (Viswanathan 2001a, 2016; Viswanathan and Balamuralikrishnan 2005; Viswanathan et al. 2014b). In addition, phytoplasmas causing SCWL and SCGS diseases affect productivity in the ratoons in almost all the countries in South and South East Asia (Rishi and Chen 1989; Nithya et al. 2020). The major fungal diseases like smut, red rot and wilt are tackled by host resistance, whereas, we could not manage the non-fungal diseases owing to various reasons. Lack of resistant sources, ignorance of impact caused by these pathogens, complication of disease management by disease spreading insect vectors and non-adoption of healthy seed programmes contribute to perpetuation of non-fungal pathogens hence poor cane

yield under field conditions. Recently, many success stories have emerged from different countries to manage YLD, WLD, RSD and other non-fungal diseases by adopting an integrated approach of obtaining healthy seed after meristem culture, molecular indexing of the mother plants or seed canes and heat treatment of seed canes and disease surveillance and monitoring under field conditions (Hanboonsong et al. 2021; Viswanathan et al. 2018d; Wongkaew and Fletcher 2004; Wei et al. 2019). By adopting ScYLV-free nursery programme, potential yield of 250 tonnes/ha was achieved under tropical India in the popular cv Co 86032 and the disease epidemic was managed (Viswanathan et al. 2018d). Such successful disease management programmes will address varietal degeneration due to non-fungal diseases and there is a scope of getting additional cane production of 60–80 million tonnes of canes in India without increasing cane area (Viswanathan 2018). Hence, vertical expansion of cane growth is the only way to meet increased demand for sugarcane in most of the countries. However, regular deployment of resistant varieties to different biotic constraints with improved cane and sugar yield potential is needed to take advantage of genetic gain in them.

9.9.2 Potential for Expansion into Non-traditional Areas

Raising demand for sugar and ethanol accelerated expansion of sugar industry across the continents. Hence, global sugarcane production witnessed three-fold increase during the last five decades however, it is contributed by the drastic increase in area of crop cultivation in the major cane growing countries like Brazil, India, China, and Thailand (Zhao and Li 2015a, b). Sugarcane needs copious water for its growth that too throughout the year. Hence, irrigation water availability throughout the growing season is a key for cane cultivation in many of the Asian countries. At this situation, expanding sugarcane in nontraditional areas will be difficult in these countries. Whereas cane cultivation is expanding in many African countries during the last 20 years by clearing forest land or reserving land for sugarcane from other land categories. Scope of rainfed sugarcane cultivation or availability of irrigation water from perennial water sources like Nile or other rivers favored cane area expansion. Many sugar estates in the continent realized good yield from virgin soil with high organic matter. In contrast, scope of expanding cane acreage is limited in many Asian regions due to land requirement for other crops, especially food crops. Further, the present cane area is also impacted by rainfall in counties like India, China and Thailand.

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Chapter 10
Designing Tobacco Genomes for Resistance to Biotic Stresses

K. Sarala, C. Nanda, K. Baghyalakshmi, Reza Darvishzadeh, K. Prabhakara Rao, U. Sreedhar, and K. Gangadhara

Abstract Tobacco is one of the important commercial crops in the world and is cultivated in more than 120 countries. Various biotic stresses viz. pests, diseases and parasitic weeds infect tobacco from seedling stage to leaf harvest and during post-harvest leaf management there-by severely affecting its leaf yield and quality. Development and deployment of host plant resistance is a sustainable option to minimize these losses. A number of varieties resistant to major biotic stresses viz. TMV, back shank, brown spot, blue mold, powdery mildew, root-knot nematodes, cater pillar, aphid etc. infecting tobacco were developed through conventional breeding. However, lack of reliable sources of resistance, narrow genetic variability, natural barriers of crossing among existing species, longer period required for developing stable homogeneous lines, undesirable associations between the resistant gene and yield and quality contributing characters either due to pleiotropic effects of the resistance gene per se, or due to linkage drag effects caused by the presence of deleterious genes linked to resistant gene, laborious process of screening/phenotyping segregating generations, etc. are slowing down the progress in developing tobacco varieties resistant to biotic stresses through traditional breeding. These limitations can be successfully overcome through molecular breeding and genome designing

K. Sarala (✉) · K. Prabhakara Rao · U. Sreedhar
ICAR-Central Tobacco Research Institute, Rajahmundry, Andhra Pradesh, India
e-mail: sarala.ctri@gmail.com; K.Sarala@icar.gov.in

C. Nanda
ICAR-Central Tobacco Research Institute, Research Station, Hunsur, Karnataka, India
e-mail: C.Nanda@icar.gov.in

K. Baghyalakshmi
ICAR-Central Institute for Cotton Research, Research Station, Coimbatore, India
e-mail: kauverik@gmail.com

R. Darvishzadeh
Department of Plant Breeding and Biotechnology, Faculty of Agriculture, Urmia University, Urmia, Iran
e-mail: r.darvishzadeh2016@gmail.com

K. Gangadhara
ICAR-Central Tobacco Research Institute, Research Station, Kandukur, Andhra Pradesh, India
e-mail: gangadhara.k1@icar.gov.in

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strategies. In this chapter, the current knowledge about genetic resources, the status of utilization of molecular markers in germplasm characterization and development of molecular maps, identified linked markers and quantitative trait loci (QTLs) to various biotic stresses, omics resources characterized, resistant genes cloned, accessible genomic resource databases etc. were summarized for their effective utilization in designing tobacco genome for higher yields and biotic stress resistance. In addition, advances in Marker-assisted selection (MAS) strategies, gene editing technologies and other genome designing strategies, and their possible utilization in designing tobacco genotypes for biotic stress resistance were also discussed.

Keywords Tobacco · *Nicotiana* · Diseases · Pests · Stress · Resistance · Genetic maps · Diversity · Genome sequencing · Markers · Genomic resources · QTL · Genome designing · MAS · Gene editing · Cloning · Databases

10.1 Introduction

10.1.1 Economic Importance of the Crop

Tobacco (*Nicotiana* spp.), also termed as "golden leaf", is an important commercial crop in the world and is cultivated in more than 120 countries (FAO 2019). China, India, Brazil, United Republic of Tanzania, Indonesia, Zimbabwe, Malawi, USA, Zambia, Mozambique, Turkey, Democratic People's Republic of Korea, Bangladesh, Argentina and Pakistan, are some of the major countries growing tobacco. It is grown on less than one percent of the world's agricultural land, and on a wide variety of soils and climate. Currently, tobacco is being grown in an area of 3.62 million hectares with the global tobacco production of 6.69 million tons (FAO 2019). China is the largest tobacco producer in the world with 2.61 million tons/year and India comes second with 0.8 million tons of yearly production. China and India together produce more than 50% of World's total. Dried tobacco leaves are mainly used for smoking in the form of cigarettes, cigars, pipe tobacco, and flavored shisha tobacco. Leaf is also consumed as snuff, chewing tobacco, dipping tobacco and snus. It is generating huge revenue to the National governments in addition to providing employment to billions of people worldwide.

10.1.2 Reduction in Yield and Quality due to Biotic Stresses

Diseases and pests infect tobacco from seedling until leaf harvest (and even during the curing process) there-by affecting yield and quality of leaf which is the economic product. However, the extent of losses due to pests and diseases varies from year to year and location to location depending upon the weather and percentage of pest infection (Seebold et al. 2007).

According to a rough estimate, the world annual loss due to tobacco diseases is in excess of 5.7 hundred million dollars (Hossain 2020). Loss in yield due to leaf blight and black shank varies from 2 to 10% annually depending on weather conditions. Frog eye disease infection lowers nicotine (46.7%) and reducing sugars (24.3%) contents in tobacco leaves and affect leaf quality (Patel et al. 2001). In severe cases frog eye spots coalesce to become bigger spots leading to drying of leaves (Lucas 1975). Losses to the crop caused by hallow stack range from 5 to 30% (Roy et al. 2012). Tobacco brown spot caused by *Alternaria* fungal species is one of the most damaging diseases, and results in significant yield losses (Luo et al. 2009a; Mo et al. 2012). *Alternaria* produces a variety of toxic secondary metabolites that damage plant tissues resulting in necrotic lesions and premature leaf aging there-by affecting leaf quality (Melton and Shew 2000). Tobacco blue mold is one of the major foliar diseases in the United States and Canada that causes an annual loss of more than $200 million (Schiltz 1981; Heist et al. 2002). Annual losses exceeding $200 million recorded in North America due to blue mold epidemics (Nesmith 1984; Heist et al. 2002). Blue mold caused severe losses in Cuba between 1978 and 1980 (Pérez et al. 2003). *Fusarium* wilt adversely affects the tobacco crop in view of its seed transmissibility and its ability to survive in soil without a host plant for years (LaMondia 2015). Wilt in addition to causing severe disease, it may render heavily infested fields not suitable from tobacco production. The losses due to bacterial or granville wilt exceed up to 7% of the crop in South Carolina in 1998 (Fortnum and Martin 1998).

Tobacco Mosaic Virus (TMV) was estimated to cause a loss of one million dollars each year between 1960 and 1965 in North Carolina's flue-cured tobacco (Gooding 1969). Damage caused by TMV infection depends on the stage of infection and genetic structure of the variety and affects both yield and quality. TMV infection of younger plants results in a greater yield loss than infection of older plants. Early infection can result in up to 60% reduction in crop value (Valleau and Johnson 1927). When plants were inoculated with virus at transplanting, 20–31% (Wolf and Moss 1933) loss in yield and 24% in value was reported while late infections result in reduced yield losses of 13–17% at topping (Wolf and Moss 1933; Johnson et al. 1983; Hossain 2020). In severe leaf curl infection, yield losses vary from 60 to 70% as infected leaves do not cure well. While in mild infections, the yield loss is very marginal (Valand and Muniyappa 1992).

Plant parasitic nematodes cause severe damage to tobacco crop. Worldwide loss in tobacco yield due to nematodes was estimated to the tune of 14.7% (Sasser and Freckman 1987). Estimation of losses in production of transplants and cured leaf yield of bidi tobacco in India due to root-knot nematodes reported to be around 50% (Markose and Patel 1977; Shah et al. 1983; Patel et al. 1986). The symptoms may get aggravated under drought conditions as nematodes directly impact the plants' ability to uptake water and nutrients along with predisposition to secondary pathogens (i.e. *Fusarium* wilt).

Crop yield losses due to root parasite, *Orobanche* spp. reported to vary from 20 to 75% depending on the time of infection and the availability of soil moisture (CABI

2021a). Annual yield decline due to *Orobanche* is estimated about $1.3 to $2.6 billion in Middle East (Aly 2007).

Severe incidence of ground beetles reduces crop stand up to 50–60% (Sitaramaiah et al. 1999) necessitating replanting which not only adds to the cost of cultivation, but reduce the yield and quality of tobacco due to variation in crop growth. The tobacco caterpillar (*Spodoptera)* incidence causes up to 80–100% loss of transplantable seedlings in the nursery (ICAR-CTRI 2021a) and 33–71% yield loss in planted crop of tobacco (Sitaramaiah et al. 1994). The white fly infestation causes stunted plant growth leading to considerable yield reduction. Tobacco aphid causes not only loss of cured leaf and bright leaf yields (125 kg and 70 kg/ha, respectively) but also leads to deterioration of leaf quality due to development of sooty mold (Sitaramaiah et al. 1994). Further, aphid and white fly transmit viral diseases causing additional yield losses. In FCV tobacco, early incidence of capsule borer, recorded up to 2891 and 426 kg/ha loss in green leaf and cured leaf yields, respectively (Sreedhar et al. 2005). The average estimated seed loss due to capsule borer was around 89 kg/ha in chewing tobacco (Chari et al. 1983). In the years of severe incidence of capsule borer, seed production may be severely affected resulting in huge losses.

Thus, based on the percent infestation and measures taken for control in field condition, losses in tobacco due to biotic stresses may range from minimum to 100% in nursery and up 80% in planted crop. These stresses not only affect yield, but also cause reduction in leaf quality and hence, require suitable control measures to contain the losses.

10.1.3 Growing Importance in the Face of Climate Change and Increasing Population

Climate change is affecting every country on every continent. It is disrupting national economies and affecting lives. Weather patterns are altering, sea levels are rising, and weather events are becoming extreme. Year 2019 was the second warmest year and the end of the warmest decade (2010–2019) ever recorded. Carbon dioxide (CO_2) levels and other greenhouse gases in the atmosphere rose to new records in 2019 (United Nations 2019).

Climate change is likely to alter the balance between pests, their natural enemies and their hosts. Climate change is an important factor driving the spread of pests and diseases. It affect the population size, survival rate and geographical distribution of pests as well as the intensity, development and geographical distribution of diseases (Doody 2020). Temperature and rainfall are the big drivers of shifts in how and where pests and diseases spread. Since 1960, crop pests and diseases have been found to move at an average of 3 km a year in the direction of the earth's north and south poles as temperatures increase (Bebber et al. 2013). Higher temperatures and precipitation levels can slow the growth and reproduction of some pest species and destroy their

eggs and larvae by washing off from the host plant. Climate change also impacts the ecology and biology of insect pest. Increase in temperature would cause migration of insect species towards higher latitudes. Studies using computer prediction models on crop yield losses show that the crop yield loss worldwide is likely to increase by 10–25% due to global warming.

Insect pests of crop plants are highly affected by global climate change. The changing climate could result in insect outbreaks, migration, change in biodiversity, species extinction, change in host shift, and emergence of new pests or biotypes (Kumar and Singh 2016). Insect pest and disease problems in tobacco have shown a shift in the recent past due to climate, ecosystem, and technological changes. There has been an overall decline of budworm and a rise in incidence of tobacco caterpillar, aphid, white fly, stem borer, ground beetle, *Cucumber Mosaic Virus* (CMV) and *Orobanche* (Sreedhar 2016). The damage due to certain pests like ground beetles is usually noticed more in drought years and during prolonged hot spells immediately after planting of tobacco (Sitaramaiah et al. 1999). Climate change also impacts disease incidence and their severities. In case of higher latent infection, the entire tobacco crop can be wiped out due to hallow stack in the event of increased rainfall and water logging (ICAR-CTRI 2005, 2006). Tobacco plants aged 35–60 days were found to be highly susceptible to bacterial wilt infection in the field following high temperature (25–30 °C) and rainfall. Thus, climate change is likely to change the pests and disease composition and their severities in tobacco, requiring preparedness to contain the possible crop losses.

10.1.4 Limitations of Traditional Breeding and Rational of Genome Designing

One of the strategies for effectively managing the menace of biotic stresses in tobacco is through host plant resistance. Conventional breeding played a significant role in developing tobacco genotypes for higher yields and biotic stress resistance in the past and continues to be important for tobacco improvement. However, it has its limitations in terms of availability of sources of resistance and other desirable traits, narrow genetic variability, barriers for natural crossing among existing species, undesirable associations between the presence of a resistant gene and yield and quality contributing characters either due to pleiotropic effects of the resistance gene per se, or due to linkage drag effects caused by the presence of deleterious genes linked to gene of interest (Chaplin et al. 1966; Chaplin and Mann 1978; Legg et al. 1981; Zeven et al. 1983; Friebe et al. 1996; Brown 2002). Also, suppressed recombination within introgressed chromatin (Paterson et al. 1990; Liharska et al. 1996) can make it difficult to alleviate linkage drag effects through back crossing (Stam and Zeven 1981; Young and Tanksely 1989). This can even complicate the efforts to discriminate between pleiotropic and linkage drag effects (Purrington 2000; Brown 2002). Other limitations in conventional breeding are the time required (relatively longer)

to combine different genes and laborious process of screening/phenotyping segregating generations in developing a variety. Traditional breeding depends on phenotypic screening for biotic stress resistance. Phenotypic screening for biotic stress resistance under natural conditions is not reliable as the stress incidence depend on various environmental factors which are highly variable under field conditions. Screening under artificial conditions is dependent on crop stage and creation of favorable conditions for infection development. Therefore, screening at early crop stages may not be possible and hence, large undesirable plants have to be handled up to full crop stage before rejection. Such screening related issues are acting as limitations in achieving progress in resistance breeding.

The recent technological advancements makes it possible to design plant genomes with desirable phenotypes with high yield by accumulating favorable alleles and eliminating deleterious and undesirable alleles. Genome manipulation strategies alleviate the limitation faced in conventional breeding as they depend on precise modification or editing of genome regions responsible for a phenotypic trait. The complete knowledge about target trait in terms of genomic areas and genes responsible, gene expression patterns and regulation, linked markers/quantitative trait loci (QTLs) etc. aids in targeted manipulation of desirable traits. Such modifications overcome the linkage drag effects and undesirable gene associations and reduce the time required for resistance gene transfer. Molecular marker-assisted breeding for fore ground and background selection helps in saving time required in classical breeding and assists in selection of characters in precise manner in segregating populations from early stages itself without artificial inoculations and environmental influence. The current advancements in gene editing technologies are making it possible to modify and edit target genes to yield desirable phenotypes avoiding nontarget effects observed when mutation breeding employed. In genome-assisted breeding (GAB), selection of genotypes based on their breeding values that are estimated considering the all available information on markers will pave the way for the development of genotypes with all the favorable alleles for achieving maximum attainable yields along with desirable quality and resistance to biotic stresses. The time taken for developing genotypes with desirable genes can be reduced through precise manipulation of genomes without requiring generations of selfing to achieve homozygosity. Transgenic and cisgenic transfer of desirable genes from unrelated and distantly related species will enlarge the sources of resistance and other desirable traits. Genome edited crops may be considered as non-GMO (Genetically Modified Organisms) plants and hence, may avoid GMO regulations. Thus, genome designing offers greater scope for accelerated varietal development and overcomes the limitations of conventional breeding.

10.2 Description on Different Biotic Stresses

10.2.1 Taxonomy of Diseases and Insects Infecting Tobacco

Several species of insect pests, diseases and root parasite pose serious threat to the tobacco crop adversely affecting the leaf yield and quality. Some pests also transmit virus pathogens that cause viral diseases (Lucas 1975; Gopalachari 1984; Doroszewska et al. 2013).

10.2.1.1 Insect Pests Infesting Tobacco Crop

Number of insects attack tobacco and cause damage to various degrees. The taxonomic details of insect pests that infest tobacco crop in majority of its growing areas are given at Table 10.1. Insects belonging to various families of Lepidoptera, Homoptera, Coleoptera and Hemiptera orders found to attack tobacco crop. Among

Table 10.1 Taxonomic details of insect pests infesting tobacco crop

Insect pest	Scientific name	Order	Family
Tobacco caterpillar	*Spodoptera litura* (Fabricius)	Lepidoptera	Noctuidae
Beet armyworm	*Spodoptera exigua* (Boisduval)	Lepidoptera	Noctuidae
Tobacco horn worm	*Manduca Sexta (Linnaeus)*	Lepidoptera	Sphingidae
Stem borer	*Scrobipalpa heliopa* (Lower)	Lepidoptera	Gelechiidae
Whitefly	*Bemisia tabaci* (Gennadius)	Homoptera	Aleurodidae
Tobacco Flea Beetle	*Epitrix hirtipennis* (Meisheimer)	Coleoptera	Chrysomelidae
Ground beetles	*Mesomorphus villiger* (Blanchard), *Seleron latipes* Guer, *Opatroides frater* Farum	Coleoptera	Tenebrionidae
Wireworms	*Conoderus vespertinus* (Fabricius)	Coleoptera	Elateridae
Stink bug	*Nezara viridula* (Linnaeus), *Euschitus servus* (Say)	Hemiptera	Pentatomidae
Tobacco aphid	*Myzus persicae nicotianae* (Blackman)	Homoptera	Aphididae
Tobacco budworm	*Heliothis virescens* (Fabricius)	Lepidoptera	Noctuidae
Budworm/Capsule borer	*Helicoverpa armigera* (Hubner)	Lepidoptera	Noctuidae
Cotton Mealybug	*Phenacoccus solenopsis* (Tinsley)	Hemiptera	Pseudococcidae

them, tobacco caterpillar, *Spodoptera litura* (nursery and main field), stem borer, *Scrobipalpa heliopa* (nursery and main field), whitefly, *Bemisia tabaci* (nursery and main field) vector of *Leaf Curl Virus* disease, tobacco aphid, *Myzus persicae nicotianae*, bud worm and seed capsule borer, *Helicoverpa armigera* (main field) are the major pests, whereas ground beetles, *Mesomorphus villiger* and *Spodoptera exigua* are minor and sporadic in nature. Others pets cause insignificant damage to the crop.

10.2.1.2 Microorganisms that Cause Diseases in Tobacco

Number of fungal, bacterial and viral pathogens infect tobacco causing economic losses (Lucas 1975; Gopalachari 1984; Shew and Lucas 1991)). The taxonomic details of various organisms causing disease in tobacco areas detailed at Table 10.2. Major fungal diseases are damping off, leaf blight and black shank, brown spot and blue mold; Bacterial diseases are leaf spots, wilt and hallow stack; viral diseases are TMV, leaf curl, CMV etc. Mixed infections of viruses are quite frequently observed in tobacco (Blancard et al. 1999).

10.2.1.3 Nematodes Infecting Tobacco

Two types of nematodes mostly affect tobacco causing malformation on tobacco root. They are root-knot nematodes (*Meloidogyne* spp.) and cyst or gall nematodes (*Globodera* spp.). Both the nematodes belongs to Kingdom Animalia, Phylum Nematoda, Class Secernentea, Subclasses Tylenchia and family Heteroderidae. Out of several *Meloidogyne* spp., *M. incognita* (Kofoid and White) and *M. javanica* (Treub) are the widespread and the most damaging ones on tobacco Two other species, *M. arenaria* and *M. hapla* (Chitwood 1949) have limited distribution and cause less damage. Several subspecies of *Globodera* viz. *G. tabacum*; *G. tabacum solanacearum*, *G. solanacearum*; *G. tabacum virginiae*, *G. virginianae* etc. have been reported on tobacco in the world (CABI 2021b). Other nematodes that cause damage in few areas are reniform (*Rotylenchulus reniformis*) and stunt nematodes (*Tylenchorhynchus vulgaris*) (Bairwa and Patel 2016).

10.2.1.4 Broomrape (*Orobanche* spp.)

Orobanche generally known as broomrape is a root parasites on tobacco and belongs to Orobanchaceae family (Gevezova et al. 2012). This genus is divided into four sections: Gymnocaulis Nutt., Myzorrhiza (Phil.) Beck, Trionychon Wallr., and Orobanche (syn. Osproleon Wallr.) (Greuter et al. 2000). The most important species (from an agronomic perspective) are found in the sections Trionychon and Orobanche. Section Trionychon includes *O. ramosa* L. and *O. aegyptiaca* (Paran et al. 1997). *O. cernua*, *O. ramosa* and *O. aegyptiaca* found to cause damage in tobacco.

Table 10.2 Taxonomic details of microorganisms that cause diseases in tobacco

Disease	Causal organism	Phylum	Order	Family
Fungal diseases				
Damping off	*Pythium aphanidarmatum* (Edson) Fitzp	Oomycota	Peronosporales	Pythiaceae
Leaf blight and Black shank	*Phytophthora parasitica* f. spp. *nicotianae* (van Breda de Hann)	Oomycota	Peronosporales	Peronsporaceae
Brown spot	*Alternaria alternate* (Fries) Keissler	Ascomycota	Pleosporales	Plepsporaceae
Blue mold	*Peronospora hyoscyami* f. spp. *tabacina*	Oomycota	Peronosporales	Peronsporaceae
Fusarium wilt	*Fusarium oxysporum* Schlechtend.: Fr. f. spp. *nicotianae* (J. Johnson) W.C. Snyder & H.N. Hans	Ascomycota	Hypocreales	Nectriaceae
Frog eye leaf spot	*Cercospora nicotianae* EII. and Ev	Ascomycota	Capnodiales	Mycosphaerellaceae
Curvularia leaf spot	*Curvularia verrucosa* Sivan	Ascomycota	Pleosporales	Plepsporaceae
Powdery mildew	*Erysiphe cichoracearum* var. *nicotianae* (D.C.) V.P. Heluta, *Golovinomyces cichoracearum* var. *cichoracearum*	Ascomycota	Erysiphales	Erysiphaceae
Black root rot	*Thielaviopsis basicola* Syn. *Chalara elegans*	Ascomycota	Microascales	Ceratocystidaceae
Bacterial diseases				
Angular leaf spot	*Pseudomonas syringae* pv. *angulate* Ellis and Everh	Proteobacteria	Pseudomonadales	Pseudomonadaceae
Bacterial leaf spot	*Dickeya dadantii*	Proteobacteria	Enterobactereales	Pectobacteriaceae
Bacterial wilt	*Ralstonia solanacearum*	Proteobacteria	Burkholderiales	Urkholderiaceae
Hollow stalk	*Erwinia carotovora* sub spp. *carotovora*	Proteobacteria	Enterobactereales	Pectobacteriaceae
Viral diseases				
Tobacco Mosaic Virus	*Tobacco Mosaic Virus* (TMV)	Kitrinoviricota	Martellivirales	Virgaviridae
Potato Virus Y	*Potato Virus Y* (PVY)	Pisuviricta	Patatavirales	Potyviridae

(continued)

Table 10.2 (continued)

Disease	Causal organism	Phylum	Order	Family
Tobacco Leaf Curl Virus	*Tobacco Leaf Curl Virus* (TLCV)	Cressdnaviricota	Geplafuvirales	Geminiviridae
Cucumber Mosaic Virus	*Cucumber Mosaic Virus* (CMV)	Kitrinoviricota	Martellivirales	Bromoviridae
Tobacco Distorting Virus	*Tobacco Distorting Virus*	Pisuviricota	Sobelivirales	Luteoviridae
Tobacco Etch Virus	*Tobacco Etch Virus*	Pisuviricota	Patatavirales	Potyviridae

10.2.2 Races, Isolates, Biotypes

Race/isolates/biotypes that are distinguishable based on host differentials exist in causal organisms of few biotic stresses of tobacco. *B. tabaci* is documented as a complex of cryptic species with two most important biotypes, MEAM1 (Middle East-Asia Minor 1; biotype B) and MED (Mediterranean; biotype Q) (Yao et al. 2017). Two races, race 0 and race 1 found to exist in *Phytophthora parasitica f.* sp. *Nicotianae* (Woodend and Mudzengerere 1992). Likewise, two races viz. race 0 and race 1 are reported in *Pseudomonas syringae* pv. *tabaci* causing wildfire. Two isolates, PVY^{NTN} and $PVY^{N}W$ in the PVY^{N} strain group that produce necrotic symptoms on "VaVa" plants (BURLEY 21, K 326, NC 95) found to infect tobacco (Verrier and Doroszewska 2018). CMV constitutes two subgroups, I and II based on severity of symptoms and virulence (Blume et al. 2017). TMV has mutated into many strains and strains such as TMV-O, TMV-C and TMV-N can infect most members of Solanaceae (Holmes 1946).

10.2.3 Stages and Extent of Damage

Biotic stresses infest and cause damage to tobacco at different stages of crop growth from seedling stage in the nursery to seed collection stage in the main field. The damage caused by some major insect pests, diseases, nematodes and *Orobanche* in tobacco are discussed here.

10.2.3.1 Insect Pests

Tobacco caterpillar (*S. litura*): The tobacco caterpillar is one of the most destructive polyphagous pests worldwide (Xue et al. 2010). The young caterpillars feed on the leaf tissues in both nursery and main field. Larvae feed voraciously leaving only veins and petioles and also cut the stems of small and tender seedlings, hence, known as cut worms. In severe cases, larva feed the entire lamina leaving only veins and petioles leading to leaf skeletonisation and heavy defoliation.

Tobacco hornworm (*M. sexta*): The tobacco hornworm is a common pest of plants in the family Solanaceae, that includes tobacco, eggplant, tomato, pepper, various ornamentals and weeds (del Campo and Renwick 1999). They are voracious feeders and may completely defoliate plants if not controlled. *M. sexta* has a large distribution throughout the New World, occurring as far south as Chile.

Stem borer (*S. heliopa*): The larva bore inside the stem and midribs in nursery as well as in the transplanted crop and feed on internal tissues. As a result, swelling appears where the borer stays. Borer infested seedlings when planted in the field remain stunted and sometimes unusual branching of the plant is seen.

Whitefly (*B. tabaci*): Whitefly is a complex cryptic species and destructive insect pest, reported to attack and damage about 600 plant species (Nombela and Muniz 2010). Whiteflies are small fly like insects seen on the underside of leaves. Both adults and nymphs suck the sap from the leaves and transmit the tobacco leaf curl virus disease to the healthy seedlings/plants. The virus infected plants are stunted and twisted; leaves are puckered and thickened with abnormally prominent veins. "B" biotype was found to infest tobacco and transmit TLCV.

Ground beetles (*M. villiger)*: These beetles damage newly transplanted tobacco plants by gnawing/cutting the tender stem, resulting in death of the seedlings causing up to 50–60% gaps in the field (Sitaramaiah et al. 1999).

Tobacco aphid (*M. persicae nicotianae*): In case of heavy infestation, hundreds of aphid can be seen on the underside of the leaf. By constantly sucking the sap from the leaf they debilitate the plant and there-by retard the growth. They secrete sugary juice known as 'honey dew' on the leaf due to which sooty mold develops rendering the leaf unfit for curing. In addition, they also transmit virus diseases eg. CMV, *Rosette* or *Bushy Top Virus* etc.

Tobacco budworm (*H. virescens*): It is principally a field crop pest, attacking such crops as tobacco, alfalfa, clover, cotton and soybean. The budworm larvae make holes in shoots and flower buds. Sometimes larve can be found on the growing tips, the leaf petioles and the stems. In the absence of reproductive tissue, the larvae would feed on leaf material.

Budworm/Capsule borer (*H. armigera*): It is a polyphagous pest. During the vegetative phase (30–50 days), it feeds on the terminal bud and surrounding young leaves causing loss. Generally, one larva is seen on terminal buds in earlier stages and during flowering phase more than one borer per plant is seen. During flowering and capsule formation stage, larvae feed on flower buds and bore the capsules to feed on the developing seeds.

10.2.3.2 Fungal Diseases

Damping off (*Pythium* spp.): Damping off is one of the most important diseases of tobacco nurseries and is responsible for poor stand of seedlings or complete loss of nursery beds. It is caused by several soil inhabiting fungi predominant being *P. aphanidermatum* (Edson) Fitz; *P. debatyanum* Hesse, *Phytophthora* spp. and sometimes *Rhizoctonia solani* are also involved. The disease attacks the root or stem region near the soil surface. The disease may appear at any stage of the seedlings but maximum damage is observed 5–6 weeks after sowing causing rotting of the tiny seedlings. Older seedlings show shriveling and dark brown discoloration of stem at the base and ultimately collapse and topple over. The wet rotting and collapse of seedlings start in circular patches and may extend to the entire bed, if unchecked.

Leaf blight and Black shank (*P. parasitica* f. sp. *nicotianae*): Disease occur both in nursery and field crop. Young tiny seedlings in the nursery rot and die suddenly. Seedlings show blackening of roots and stem at ground level. Under continuous wet weather conditions, large circular to irregular water-soaked patches appear on the leaf

surface causing leaf blight. Symptoms of black-shank on the transplanted tobacco are seen in the form of blackening of roots and stalk. Blackening of the stalk starts at the base near the soil gradually extending upwards up to 30 cm or more above the ground level. The leaves turn yellow and the whole plants wilt and die.

Brown spot (*A. alternate*): It is a disease of senescence. The symptoms on older leaves appear as small water- soaked lesions which enlarge quickly. Once the spots enlarge, the center of the spots die and become brown, leaving a clear demarcation between diseased and healthy tissue. Circular brown spot lesions with concentric rings appear on lower leaves. In severe infection spots enlarge, coalesce and damage large areas making leaf dark brown, aged and worthless.

Blue mold of tobacco (*P. hyoscyami*f. sp. *tabacina*): Blue mold is one of the most important foliar diseases of tobacco that causes significant losses in the America, south-eastern Europe and the Middle East. It is highly destructive to tobacco seed beds, transplants and production fields. Single or groups of yellow lesions appear on the older and shaded leaves. The spots, often, grow together to form light brown necrotic areas. Leaves become puckered, distorted, large portions disintegrated and may lead to fall apart of the entire leaf. Blue mold can destroy all leaves at any growth stage in the event of continuous favorable weather conditions. Lesions may also appear on buds, flowers, and capsules.

Fusarium wilt (*F. oxysporum* f. sp. *nicotianae*): *Fusarium* infection causes chlorosis, wilting and necrosis of tobacco leaves that leads to stunted growth and death. The symptoms often appear vertically on one side of the plant or even one side of the leaf midvein. Diagnostic chocolate-brown discoloration of the vascular tissue develops up to top of the plant. In due course of time, the discoloration on the exterior of the green stalk becomes visible.

Frog eye leaf spot (*C. nicotianae*): Generally, this disease is seen 4–5 weeks after germination and 30 days after transplanting and on the harvested crop. Round shaped brown spots akin to frog-eye form appear on the lower leaves of the seedlings. Spots appear initially on lower leaves and spread gradually to upper leaves. Under hot, dry weather frog eye lesions may be pin point in size and would not be recognized.

Powdery mildew (*E. cichoracearum* var. *nicotianae*): Powdery mildew, also known as white mold or ash disease, occurs in all types of tobacco in several Asian countries, Oceania, the Mediterranean, Africa and Canada. The disease causes severe damage in flue-cured tobacco compared to other types of tobacco. Under favorable conditions of low temperatures (16–23 °C) and high humidity, white patches spread to upper leaves, enlarge and cover the entire surface of the leaf. Mildew affected leaves get scorched on curing and show brown patches or blemishes rendering them unfit for marketing or reduce their commercial value.

Black root rot (*T. basicola*): Infected roots appear dark brown or black due to the presence of large numbers of black spores and rotting of root tissues greatly reduces the number of roots. Large scars may be seen on the main tap root. The above-ground symptoms show temporary nutrient deficiency symptoms, stunting and irregular growth of the tobacco.

10.2.3.3 Bacterial Diseases

Angular leaf spot (*P. syringae* pv. *angulate*): *Pseudomonas syringae* pv. *angulate* causes angular dark brown to black colored spots surrounded by yellow halo. Lesions are restricted between veins and leaves appear puckered and tears easily. It was regarded as a mutant of *Pseudomonas syringae* pv. *tabaci*, which does not produce tobtoxinine (Braun 1955). *P. syringae* pv. *tabaci* causes a severe type of angular leaf spot well known as wild fire in Tamil Nadu, India (Gnanamanickam et al. 1977).

Bacterial leaf spot (*D. dadantii*): It affect *bidi* and late planted FCV tobacco in Karnataka, India. Plant develops circular water soaked yellow lesions with a minute brown centre, which expand with a translucent border and wide chlorotic halo (Wolf and Foster 1917). Causes vascular discoloration of the stems, wilting and stunting (Johnson 1923; Komatsu et al. 2002).

Bacterial wilt (*R. solanacearum*): Also known as 'Granville wilt', the disease affects both nursery and field crop. In the field, the first symptom of the disease is drooping of 1–2 leaves during day and their recover during evening. One half of the affected leaves become flaccid, a characteristic symptom of bacterial wilt of tobacco. On slow progression of the disease, the affected leaves turn light green and may gradually turn yellow, midribs and veins get flaccid and large leaves may droop in an umbrella like fashion.

Hollow stalk (*E. carotovora* sub sp. *carotovora*): Hollow stalk has been reported from U.S.A, Canada, India and China (Roy et al. 2008). The disease may appear at any time due to stem injury but it is commonly observed 35–40 days after topping operations. Pith undergoes rapid browning and hollowing due to soft rot and tissue collapse eventually. Initially, the top leaves wilt and the infection slowly spreads downward. Black leg phase of the disease is characterized by the formation of black stripes or bands girdling the stalk and cured leaves.

10.2.3.4 Viral Diseases

Tobacco Mosaic Virus (TMV): TMV is worldwide in distribution and reduces cured leaf yield, quality and price. It is sap transmissible and spreads mainly through mechanical means. Characteristic symptoms include irregular mosaic pattern of dark and light green areas on leaf, leaf malformation and stunted plant growth. Infected young leaves are often malformed and show puckering or wrinkling. "Mosaic burn" with large, irregular, burned or necrotic areas appear on affected mature leaves causing extensive loss to the crop.

Potato Virus Y (PVY): This virus is aphid transmitted and affects tobacco crop worldwide. The virus is transmitted by aphids. This virus causes tobacco veinal necrosis or vein banding *i.e.* appearance of dark green bands along the brown necrotic veins. Necrosis extends to the vascular region and plants die out of pith necrosis.

Tobacco Leaf Curl Virus (TLCV): Caused by a virus of the genus begomovirus belonging to Geminiviridae. TLCV is transmitted by the insect vector, whitefly, *B. tabaci*. Diseased leaves become brittle, puckered, exhibit downward curling of

margins with enations or leafy outgrowths on the under surface of leaves. Leaves show vein clearing, abnormal vein thickening and twisting of petioles. Internodes get shortened resulting in dwarfing of the plant. Late infected plants show mild symptoms with small sized inwardly curled top leaves.

Cucumber Mosaic Virus (CMV): CMV enters into the leaf through wounds, principally those made by aphid. Infected plants show typical mottling and mosaic patterns, narrowing and distortion of the leaves, sometimes accompanied by plant stunting. Under severe infection, vein banding, inter veinal yellowing are observed accompanied with leaf blisters, shriveled, chlorotic and necrotic lines causing filiform leaves. Mild CMV strains would cause a faint mottling of the leaves.

Tobacco Distorting Virus: This virus causes abnormal suckering, stunting of the plant. Leaves get mottled, puckered, distorted with rat-tails. Only long midrib is seen without lamina at times.

Tobacco Etch Virus: It is transmitted by sap and also by aphids in a non-persistent manner. It infests solanaceous plants, and causes rat-tailing. On lower leaves vein clearing is seen along with some necrotic lines or etching. Mottling is seen with chlorotic and necrotic spots.

10.2.3.5 Nematode Diseases and Broomrape

Meloidogyne spp.: In most countries *Meloidogyne* spp. (root-knot nematodes) are considered as major factors limiting the production of tobacco crops. The nematode infection causes root galls and plant stunting. As nematodes damage plants at root level, above ground symptoms may not be prominent. At moderate to high infection, plants may appear wilted, yellowed and stunted.

Globodera spp.: *Globodera* presence on the roots is evident by the numerous cysts scattered over young or old roots with resultant reduced growth. The cysts of the species vary in sizes. Cyst nematodes cause lesser damage on tobacco compared to *Meloidogyne* spp.

Broomrape (*Orobanche* spp.): Broomrape affects the growth, development and morphogenesis of tobacco in the main field (Krishnamurthy 1994).

10.2.4 Control Methods

10.2.4.1 Cultural Methods of Control

Selection of disease free nursery site, deep summer ploughing, raising the seed beds, rabbing seed beds, use of optimum seed rate and regulation of watering to avoid dampness etc. controls fungal diseases in the nursery.

Deep summer ploughing, Soil fumigation, crop rotation, intercropping, companion/boarder crops (maize, sorghum, Tagetes etc.), trap cropping (castor as ovipositional trap for insects; sesame, jowar, black gram, green gram etc. for

Orobanche), application of fermented farm yard manure (avoid *Orobanche* seed) etc. have been highly effective in the management of insect, nematode, viral diseases and *Orobanche* weed (Chari et al.1999; Sreedhar et al. 2007).

The phyto-sanitary measures that are adopted for controlling insect and diseases in tobacco include adopting right time of planting, transplanting of healthy seedlings, removal and destruction of diseased/virus infected plants and alternate hosts from the field, use of recommended fertilizer doses (including potash and avoiding excess nitrogen), collecting and destroying the crop debris after harvest etc.

Planning inter-culture operations in infected fields at the end, disinfection of implements before entering healthy fields, washing hands with soap water before and after entering infected fields, prevention of smoking and use of other tobacco products, etc. effectively control viral diseases and *Orobanche* (Lucas 1975). Physical removal of *Orobanche* before its flowering and burning reduce *Orobanche* incidence in the next season (Krishnamurthy 1994).

10.2.4.2 Chemical Methods of Control

Chemicals recommended and their doses for the control of insects and diseases vary from country to country. Guidance Residue Levels (GRLs) have been developed by the CORESTA (Cooperation Centre for Scientific Research Relative to Tobacco) Agro-Chemical Advisory Committee (ACAC) for guiding tobacco growers and those in the tobacco industry interested in Crop Protection Agents (CPAs) application and implementation of Good Agricultural Practice (GAP) in tobacco production (CORESTA 2020). The importing countries buy tobaccos when the pesticide residues in the cured leaf are below established GRLs and Maximum residue level (MRL) fixed by them. In view of the increasing stringency of regulations controlling the registration and use of CPAs resulted in an ever decreasing list of insecticides registered and recommended for use on tobacco (CORESTA 2020). Hence, new selective and low active ingredient (ai) insecticides, bio-pesticides and novel molecules are been evaluated and recommended to cope up with pesticide residue problem (Sreedhar 2020).

10.2.4.3 Biocontrol Methods with Natural Products and Biotic Agents

In view of the increasing concern about pesticide residues in tobacco, biopesticides viz. *Bacillus thuringiensis* var. kurstaki based biopesticides, Neem seed kernel suspension (NSKS), *Nuclear Polyhedrosis Virus* (NPV), Fungal pathogens viz. *Nomuraea rileyi*, *Beauveria bassiana,Verticillium lecani, Trichoderma harzianum, T. viride* etc. and bacterium, *Pseudomonas aeruginosa,* VA *mycorrhiza Glomus fasciculatum, Azotobacter* etc. have emerged as a strong component of integrated pest management strategies in tobacco (Chari et al. 1996; Sreeramulu et al. 1998; Ramaprasad et al. 2000; Dam et al. 2010; Sreedhar et al. 2014).

10.2.4.4 Integrated Management of Pests and Disease (IPM/IDM)

Integration of host plant resistance, cultural, chemical and biological management are always necessary for effectively controlling various diseases, nematodes and *Orobanche* without environmental pollution and for minimum residues of Crop Protection Agents (CPA), which is an issue of concern in exported tobacco. Bio-intensive IPM modules with genetic, cultural, biological and habitat management techniques as major components along with need based use of selective insecticides were highly effective in reducing pest damage, enhancement of natural enemy activity and helps in increasing the production of residue free tobaccos with favorable economics (Rao et al. 1994; Sitaramaiah et al. 2002; Sreedhar and Subbarao 2014).

10.2.5 Traditional Breeding Methods

Tobacco is a self-pollinated crop with very low natural out-crossing and all the breeding methods that are applicable to self-pollinated crops, such as introduction, mass selection, pure line breeding, pedigree method, back cross breeding, hybridization etc., are being utilized in tobacco (Bowman and Sisson 2000; Sarala et al. 2012). Introduction has played an important role in the initial years till 1960s in introducing tobacco cultivation into European, Australian and Asian countries. Pure line selection, hybridization of selected parents followed by selection in the segregating populations through pedigree, back cross and recurrent selection methods was the most pre-dominant methods adopted in breeding high yielding tobacco varieties worldwide. Back-cross breeding was used for the efficient transfer of a number of resistant factors viz. TMV (fromVamorr-50), Powdery mildew (Kofun), Black shank (Beinhart 1000-1), Caterpillar (DWFC), *Fusarium* wilt (Speight G33) etc. into tobacco varieties, from other tobacco types and wild species (Sarala et al. 2012). Backcrossing to the common tobacco variety was practiced to eliminate the genetic drag due to undesirable alleles and to recover the plant type and quality characteristics of the adapted variety. Distant hybridization/Interspecific hybridization was used to introgress useful genes like resistance to TMV, wild fire, black shank, brown spot, black root rot, blue mold, aphid, tobacco caterpillar, root knot nematode, powdery mildew, *Tomato Spotted Wilt Virus* (TSWV), PVY, cyst nematode etc. (Milla et al. 2005; Sarala et al. 2012). Incompatibility barriers in interspecific hybridization were overcome through utilization of hormones, bridge cross technique and in vitro rescue methods (Ramavarma et al. 1980). Mutation breeding has also played an important role in creating variability and developing high yielding improved tobacco varieties and parental materials for use in breeding programs (Sarala et al. 2012).

10.2.6 Use of Morphological Markers

The characters which can be readily detected by phenotype and useful to identify and characterize plants is referred to as morphological marker. In tobacco, these markers are mostly related to variation in plant (shape, habit, height, intermodal length etc.), leaf (number of economic leaves, size, color, shape, margin, tip shape, maturity interval period, etc.) floral characters (time of 50% flowering, size, color, development of stamens, height of pistil relative to stamens, etc.), capsule (shape and size), and seed (shape, color and surface characters) (Sarala et al. 2018). Morphological markers generally require neither sophisticated equipment nor preparatory procedures for scoring. Monogenic or oligogenic morphological traits are generally simple, rapid, and inexpensive to score, even from preserved specimens (Bretting and Widrlechner 1995).

Morphological, karyotypical and physiological characters have been used to study the genetic background of tobacco (Goodspeed 1954; Zhang 1994; Lu 1997). Morphological markers played an important role in breeding improved tobacco varieties till date and continue to be effective in future also. As leaf is the economical product, improvement in number of harvestable leaves and leaf weight is important in realizing higher yields in tobacco along with desirable chemical quality characters. Sarala et al. (2005) observed continuous improvements for plant height, total leaves, harvestable leaves, days to flowering, leaf area, leaf growth rate, specific leaf weight and carotene from old varieties to recently released tobacco varieties. They further suggested that improvement in leaf number, leaf area and specific leaf weight in future cultivars can results in obtaining higher yields.

Conventional plant-breeding approaches in tobacco, as in other crops, rely on morphological markers representing desirable agronomic and product characteristics (that is, phenotypes including resistance to biotic stresses) for the selection of the parents, for creation of variation through their crossing or mutagenesis from the tobacco germplasm and for identification of targeted genotypes in segregating generations. Resistance related morphological characters are only expressed when there is an incidence of pests/diseases and such characterization may not be possible under natural conditions. These morphological traits are limited in number, influenced by the plant growth stages and various environmental factors (Eagles et al. 2001) because of which phenotypic identification could be misleading due to complex genotype and environment interaction that governs the trait of interest. Consequently, development of resistant varieties requires more than twelve years in case of recessive traits. As these markers are generally expressed late into the development of tobacco plant and are highly influenced by the environmental factors or growing conditions, their detection is dependent on the development stage of the crop. Further, they are less polymorphic, and exhibit dominance, pleiotropy and epistasis. In view of these limitations, genotypic markers that can be identified in early stages without environmental influence are highly useful in accelerating the tobacco varietal improvement.

10.2.7 Limitations and Prospect of Genomic Designing

Conventional biotic resistance breeding approaches in tobacco are phenotype-based, time-taking and resource-intensive. Hence, the progress thus obtained through traditional tobacco breeding is slow and is hampered by linkage drag and lack of easily transferrable sources of resistance. The genome designing strategies overcome this as they depend on the knowledge of the genome composition of the plants and gene sequence information. Targeted modification or designing of plant genome including addition of alien genes will accelerate the tobacco varietal developmental process through precise manipulation of gene functions for higher yields and stress resistance. Transfer of resistance from distance wild relatives and other unrelated sources to cultivated tobacco can also be successfully made using genome designing through trans- and cis-genesis approaches involving various processes viz. gene mapping, identification, gene transfer, gene editing etc. Marker-assisted and genome assisted breeding are going to be the order of the day with rapidly evolving technological advancements.

Availability of draft genome of *N. tabacum* and few wild species and various data sharing and analysis platforms (databases) in recent times, making it possible to understand genes, their sequences and linked molecular markers for target traits with the involvement of innovative bioinformatics tools and comparative genomic studies. The information thus available can effectively utilized to edit the genome sequences with rapidly evolving relatively precise gene editing technologies viz. meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), homing endonucleases, clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) 9 etc. However, gene editing technologies yet times do suffer from lower specificity due to their off-targets side effects (Khan 2019). Availability of high density of molecular maps and genome information further helpful in providing the knowledge of linked molecular markers and QTLs that are either closely linked or with in the target gene (s) and also allow map-based cloning of target traits. Linked markers and QTLs identified in tobacco for various biotic stresses is going to pave the way for marker-assisted breeding for resistant traits. The available information can be effectively utilized for estimating the breeding value of individuals in genome assisted breeding and accordingly selections can be made.

However, genetic engineering tools have certain limitations, including time-consuming and complicated protocols, potential tissue damage, DNA incorporation in the host genome, and low transformation efficiency. Unlike tradition breeding strategies, genome designing technology is resource intensive and require technology expertise for handling the processes.

10.3 Genetic Resources of Resistance/Tolerance Genes

Availability of stable and heritable sources of resistance to biotic stresses is essential in any crop while breeding resistant varieties. The gene pool consists of various easily crossable tobacco lines and *Nicotiana* species are to be explored for available variability and resistance factors in developing tobacco cultivars resistant. In case of non-availability in any of these gene pools it needs to be created through mutations or incorporated through genome designing.

Currently, large number of *Nicotiana* species and cultivated tobacco varieties are available (Lewis 2011). Around 83 species are available within the genus *Nicotiana* (Berbec and Doroszewska 2020). Currently there are 92 *Nicotiana* species and varieties listed at the Taxonomy Browser of National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi) and 307 records of *Nicotiana* species and varieties available in The Plant List database (http://www.theplantlist.org/tpl1.1/search?q=Nicotiana). International Plant Name Index (http://www.ipni.org/ipni/plantnamesearchpage.do) contains 450 records on the keyword '*Nicotiana*'. Large number of these species are reliable sources of resistance to biotic stresses that infest tobacco crop (Lewis 2011). Another important advantage of wild *Nicotiana* species is their cytoplasmic genomes, which have provided the source of cytoplasmic male sterility (CMS) for developing male-sterile isolines of inbred lines and cultivars. CMS is a prerequisite for technically feasible and economically viable seed production of hybrid cultivars. Various available sources of resistance in tobacco genetic resources are discussed below.

10.3.1 Primary Gene Pool

The primary gene pool consists of tobacco genotypes that can easily be crossed to produce fertile offspring with the cultivated tobacco. They may be cultivated ones and in wild gene pools. The cultivated gene pool covers commercial varieties of the crop, as well as landraces. While the wild gene pool comprises putative ancestors and closely related species that show a fair degree of fertile relationships with the domesticated tobacco. Large number of varieties are developed by breeders in different countries and fairly large collections of germplasm are available in *N. tabacum* and *N. rustica* that are resistant to various pests and diseases (Table 10.3). As gene transfers from such sources is easy, the first priority of the breeders is to explore them for the availability of resistance to target biotic stresses and transfer them to cultivated varieties through either traditional method of breeding or genetic engineering.

Table 10.3 Few genetic resources of resistance/tolerance available in the primary gene pool of tobacco

S. No	Disease	Resistance sources	Reference
1	Leaf blight and Black shank	Beinhart 1000–1, Coker 411, F 180, F 210, MC 1610, Reams 64 and Va 770 resistant to all the races of *P. parasitica* var. *nicotianae*	Abdul Wajid et al. (1987)
		Coker 371, KT 200, Beinhart	Martinez et al. (2011)
		Coker 371-Gold, Oxford 1, NC 95, Speight NF3	Bowman and Sisson (2000)
2	Brown spot	16 Cigar wrapper tobacco lines	Monga and Dobhal (1987)
		Jati tobacco: Vaishali Special, PT-76 and Gandak Bahar	Shenoi et al. (2002)
		Banket A1	Lapham (1976)
3	Black root rot	KY 15, KT 17	Martinez et al. (2011)
		PB19	Bai et al. (1995)
4	*Tobacco Mosaic Virus*	Colombian variety 'Ambalema'	Nolla and Roque (1933)
		8 FCV, 10 Burley, 10 Air cured, 3 rustica, 2 each of hookah and cigar and 1 each of *natu* and snuff types	Reddy and Nagarajan (1981)
		Banket A1	Lapham (1976)
		TN 90, Burley 21, Kentucky 56	Sierro et al. (2014)
		TMVRR-1, TMVRR-2 and TMVRR-3	Sastri et al. (1981)
		Bidi cultivars: MRGTH 1, GT 9 2001 Cheroot variety: S 5 FCV cultivars: VT-1158, Jayasri-MR, Godavari special, CTRI special –MR, CTRI Sulakshana and FCR-15	https://ctri.icar.gov.in/files/varieties.pdf
		Coker 176, SC 71 and SC 72, NC 567	Bowman and Sisson (2000)
5	*Tobacco Distorting Virus*	DG 3	Gopalachari (1984)

(continued)

Table 10.3 (continued)

S. No	Disease	Resistance sources	Reference
6	*Poty Virus*	TN 86	Miller (1987)
		TI 1406 (Virgin A Mutant)	Noguchi et al. (1999)
7	*Tobacco Vein Mottling Virus* (TVMV) *and Tobacco Etch Virus* (TEV)	TN 86	Miller (1987)
8	Bacterial wilt	Oxford 26	Bowman and Sisson (2000)
9	Granville wilt	NC 95, Speight NF3	Bowman and Sisson (2000)
10	Wild fire	NC 567	Bowman and Sisson (2000)
		Banket A1	Lapham (1976)
11	Hollow stalk	*N. rustica* germplasm accessions, White Pathar and Bengthuli	ICAR-CTRI (2010)
12	Budworm	CU 263	Bowman and Sisson (2000)
		I 514, TI 1687, TI 536, TI 675, TI 1068, TI 1656, CU 1097	Sreedhar (2011)
13	Aphid	TI 1687, TI 675, TI 1068, TI 1656, CU 1097, KDH 959	Sreedhar (2011)
14	Whitefly/Leaf Curl	I 514, CU 1097 TI 1687, TI 675, TI 1068, TI 536, KDH 959	Sreedhar (2011)

(continued)

Table 10.3 (continued)

S. No	Disease	Resistance sources	Reference
15	Tobacco caterpillar	DWFC, Abhirami (CR)	Joshi et al. (1978)
		I 514, CU 263, TI 536, TI 1687, CU 1097, KDH 959 and TN 86	Sreedhar (2011)
16	Root-knot nematodes	Florida 22, Speight NF3	Bowman and Sisson (2000)
		Bhavya	https://ctri.icar.gov.in/files/varieties.pdf
17	Cyst nematodes	NC 567	Bowman and Sisson (2000)

10.3.2 Secondary Gene Pool

The secondary gene pool referred to wild relatives of tobacco that are discrete from the cultivated species and still closely related in such a way that are crossable with cultivated species to at least certain extent to produce few fertile offspring. Genetic resources collected from gene centers as closely related species, primitive cultivars, old land races evolved and adopted to different environments are valuable source for resistance to biotic stresses. Majority of *Nicotiana* species (58 No.) would hybridize either with *N. tabacum* or at least with one other sister *Nicotiana* species. Some (*N. mutabilis, N. petunioides, N. attenuata, N. corymbosa, N. linearis, N. burbidgeae, N. thyrsiflora*, and *N. wigandioides*) hybridize with at least one other *Nicotiana* but not with *N. tabacum* (Berbec and Doroszewska 2020). *N. tabacum* found to produce inviable hybrids *with N. africana* ($2n = 46$), *N. excelsior* ($2n = 38$), *N. goodspeedii* ($2n = 40$), *N. gossei* ($2n = 36$), *N. maritima* ($2n = 32$), *N. megalosiphon* ($2n = 40$) and *N. velutina* ($2n = 32$) after crossing at 28 °C (Tezuka et al. 2010). However, Type II hybrid lethality with the characteristic symptoms of browning of hypocotyl and roots observed in these crosses was suppressed at elevated temperatures (34 or 36 °C). Utilization of genes from these materials in breeding program is tedious due to incompatibility and undesirable linkages. Modern molecular and biotechnological tools can be an aid in overcoming such difficulties.

10.3.3 Tertiary Gene Pool

This pool made up of even more distantly related crop wild relative species. Fourteen species (*N. azambujae, N. acaulis, N. ameghinoi, N. paa, N. cutleri, N. longibracteata, N.* spegazzini*, N. faucicola, N. fatuhivensis, N. heterantha, N. stenocarpa, N. truncata, N. monoschizocarpa,* and *N. symonii*) had no hybridization records (Berbec and Doroszewska 2020) with *N. tabacum.* Genes present in both the S and T subgenomes of *N. tabacum* appear to be responsible for hybrid lethality in crosses with incompatible *Nicotiana* species (Tezuka and Marubashi 2012). Use of specific breeding techniques, such as bridge crosses, various sorts of treatments of male and female flower parts, and in vitro techniques like ovary/ovule culture and embryo rescue, chromosome and genome manipulation, such as increasing (polyploidization) or decreasing (haploidization) the number of genomes, different methods of partial genome transfer that include chromosome addition and substitution lines, mutagenesis and cell fusion, translocation breeding, exchange of nuclear and cytoplasmic genomes (mitochondrial and/or chloroplastic), grafting, marker-assisted breeding (MAB), in vitro tissue culture etc. and genetic engineering are needed to transfer genes from such pools (Weil et al. 2010).

10.3.4 Artificially Induced/Incorporated Traits/Genes

When source of resistance is not available in any of the above pools, creation of resistance through mutations (physical and chemical mutagens), genetic engineering for transfer of alien genes, gene manipulation and genome editing technologies are to be adopted in developing resistant cultivars.

Some source of resistance for biotic stresses available in primary gene pool of cultivated tobacco are provided at Table 10.3. Various resistant factors introgressed to *N. tabacum* from *Nicotiana* Species (secondary and tertiary pools) are listed at Table 10.4.

10.4 Glimpses of Classical Genetics and Traditional Breeding

10.4.1 Classical Mapping Efforts

In tobacco, initial gene mapping efforts were made by Anderson and De Winton (1931), East (1932), and Brieger (1935) with the identification of linkage between a pollen color factor and the sterility factors. Brieger estimated that S and br are about 20 crossover units apart in the first linkage group. Brieger (1935) has summarized the data on linkages in *N. langsdorfii* and *N. sanderae*, and established with some degree of certainty the first two linkage groups (1) Self-sterility allele (S) and lethality (I), (2) C is the basic gene for anthocyanin color (C) and a recessive gene causing a peculiar type of growth cr (crassa). Smith (1937) confirmed the existence of linkage between self-sterility and pollen anthocyanin color. The first accounts of monosomics in *N. tabacum* was reported by Clausen and Goodspeed (1926) and they established two types of monosomics in which haplo-C (called "corrugated)") found to be associated with the chromosome where the basic color factor, Wh, is positioned. Later, Clausen and Cameron (1944) developed complete set of 24 monosomics and studied the association in transmission between monosomics and mendelian characters which led to location of 18 genes on nine chromosomes. Though inheritance of number of traits studied and the linkage of few of them established with other traits, genetic linkage maps are not fully developed in tobacco due to its allopolyploid nature (Suen et al. 1997; Narayanan et al. 2003).

Table 10.4 Biotic stress resistance traits introgressions to *N. tabacum* from wild *Nicotiana* species

Resistance trait	Source	Reference
Tobacco Mosaic Virus	*N. glutinosa*	Holmes (1938), Tbbnovsky (1941), Valleau (1952), Apple et al. (1963)
Wild fire and angular leaf spot	*N. longiflora* (against *P. syringae* pv *tabaci* race 0)	Clayton (1947)
	N. rustica (against *P. syringae* pv *tabaci*race 0 and 1)	Stavely and Skoog (1976), Woodend and Mudzengerere (1992)
Black shank (*P. parasitica* var *nicotianae*) (race 0)	*N. longijflora*	Valleau et al. (1960)
	N. plumbaginifolia	Chaplin (1962)
	N. rustica	Woodend and Mudzengerer (1992)
Brown spot	Immune- N. *debneyi; R*esistant- *N. exiqua, N. glutinosa, N. longifolia, N. nesophila* and *N. plumibaginifolia*	Reddy et al. (1976)
Black root rot	*N. debneyi*	Clayton (1969)
Blue mold	*N. velutina*	Clayton (1967), Clayton et al. (1967), Lea (1963)
	N. debneyi	Clayton et al. (1967)
	N. goodspeedii	Wark (1970)
	N. goodspeedii and *N exigua*	Gillham et al. (1977)
Aphid	*N. gossei*, *N. repanda, N. trignophylla, N. umbratica* and *N. excelsior*	Thurston (1961)
	N. gossei	Sarala et al. (2012)
Tobacco caterpillar	*N. gossei*, *N. benthamiana*, *N. repanda*, *N. nesophila* and *N. gossei*	Joshi and Sitaramaiah (1975), Sarala et al. (2012)
Root knot nematode (*M. javanica*)	*N. longiflora*	Ternouth et al. (1986), Sarala et al. (2012)
	N. amplexicaulis	Sarala et al. (2012)
	N. repanda	Ternouth et al. (1986)
	N. tomentosa	Clayton et al. (1958), Yi et al. (1998b)
Powdery mildew	*N. debneyi*, *N. glutinosa*, *N. tomentisoformis*	Smeeton and Ternouth (1992)
Tomato Spotted Wilt Virus	*N. alata*	Gajos (1988)
Potato Virus Y	*N. africana*	Lewis (2005)
	N. tomentosoformis	Legg and Smeeton (1999)

(continued)

Table 10.4 (continued)

Resistance trait	Source	Reference
Tobacco cyst nematode (*G. tabacum*)	*N. plumbaginifolia*	Chaplin (1962), Johnson et al. (2002)

10.4.2 Limitations of Classical Endeavors and Utility of Molecular Mapping

Though genes are very useful markers but they are by no means ideal for mapping studies. Mapping based on morphological markers is tedious and time taking and genes governing quantitative traits cannot be mapped (Worland et al. 1987). Limited number of visual phenotypes whose inheritance could be studied and complications arising in analyzing them due to pleiotropic effect of genes is one of the major limitations for gene (trait) mapping through classical approach. Allopolyploid nature of tobacco making it difficult to score individual gene effects and recombination events at phenotype level due to epistatic interactions between homeo alleles present on constituent genomes and their functional redundancy posing a problem in classical mapping.

For making the gene maps more comprehensive, identification of characteristics that were clearly distinctive and less complex than visual ones is necessary. But, only a fraction of the total number of genes in tobacco exist in allelic forms that can be distinguished conveniently making it difficult to construct classical maps. One of the reasons why our knowledge of the details of inheritance in tobacco was so meager, is because of the prevailingly quantitative or semi-quantitative nature of majority of character including flower color in tobacco (Clausen and Cameron 1944). Gene maps are, therefore, not very comprehensive in tobacco and a detailed map based entirely on genes is not available in tobacco.

As the plant breeding progressed, biochemical markers such as protein and isozyme markers were developed (Markert and Moller 1959). Protein and isozyme markers have been successfully applied in the detection of genetic diversity, population structure, gene flow and population subdivision in tobacco (Mateu-Andres and De Paco 2005). The isozymes and other proteins mostly have neutral effect on plant phenotype and are often expressed co-dominantly making them discriminate easily between homozygote and heterozygote. However, due to their limited availability and the requirement of a different protocol for each isozyme system, utilization of protein and isozyme markers in plant breeding programs and mapping endeavors is very limited.

Hence, there is a need for other types of marker which are abundantly available, stable over environments and can be easily classified into distinct categories. DNA based molecular markers developed in 1990 could satisfy these requirements. Because molecular markers are so abundant in a genome and can easily be detected, when mapped by linkage analysis, they fill the voids between genes of known phenotype. In mapping, DNA marker is not important in itself but, the heterozygous

site is merely a convenient reference point for marking the chromosomal locations. However, the molecular marker development and genetic map construction in tobacco have lagged behind other Solanaceae crops such as the tomato, potato, and pepper plants (Tanksley et al. 1992; Barchi et al. 2007). The molecular marker based maps can be effective anchoring points for identification of linked traits for their isolation, cloning and also for use in marker-assisted breeding.

10.4.3 Breeding Objectives

The tobacco breeding mainly aims at enhancing leaf yield potential of the cultivar in addition to maintaining leaf quality, and resistance to biotic and abiotic stresses. Tobacco leaf yield is a dependent variable and is the result of the associated yield attributing independent traits such as plant height, number of leaves, leaves length and width, days to maturity, resistance to biotic and abiotic stresses (Sarala et al. 2005). Some of these traits, in breeding improved high tobacco varieties, are to be selected in positive direction such as number of leaves, days to maturity, resistance, leaf length and width etc. while some of the traits such as plant height, leaf intermodal length, reducing sugars and alkaloids etc. in negative direction. Breeding for improved (higher level) resistance to various biotic stresses as well as finding novel or improved sources of resistance remains a central part of majority of the present flue-cured breeding programs.

Bowman and Sisson (2000) analyzed the relative change for several traits of flue-cured tobacco cultivars grown in the early 1960s compared to the late 1990s and they concluded that yield, leaves per plant and days to flower were selected in positive direction while plant height, intermodal length, reducing sugars and total alkaloids were selected in negative direction. Breeders were successful in increasing the yield despite maintaining leaf quality (both are negatively correlated). The newer cultivars were modified to have longer growing period, more leaves prior to flower bud formation, relatively lower plant height, internodal length, reducing sugars and total alkaloids. Sarala et al. (2005) observed positive selection for plant height, total leaves, harvestable leaves, days to flowering, leaf area, leaf growth rate, specific leaf weight and carotene in tobacco varieties.

10.4.4 Classical Breeding Achievements

Traditional tobacco breeding aimed at developing improved tobacco varieties with higher yield, better leaf quality, resistance to pests and diseases. Significant progress has been made over the years in enhancing the tobacco leaf yield through both varietal and hybrid development, in addition to improving disease and insect resistance without significantly sacrificing in ease of curing. The classical breeding achievements in tobacco are enumerated here.

10.4.4.1 Yield and Quality

Breeders have made progress in improving tobacco leaf yield and quality over years (Bowman and Sisson 2000; Sarala et al. 2005). A number of high yielding varieties have been released so far using conventional breeding methods (https://crosscreekseed.com; https://content.ces.ncsu.edu/flue-cured-tobacco-information; ICAR-CTRI 2021b). Release of two high yielding flue-cured tobacco cultivars viz. Coker 139 (in 1955) and K 326 (in 1981) have provided important germplasm for yield enhancement and have been used widely by breeders as parental lines since release. K 326 is the most successful tobacco cultivar in the 20th century with its cultivation in majority of the tobacco growing countries (Bowman and Sisson 2000). With the development of high yielding disease resistant lines and CMS lines, F_1 hybrids were developed in tobacco. Hybrid burley cultivars have been available since 1960s, but few flue-cured hybrids were released before the late 1990s (Bowman and Sisson 2000; Sarala et al. 2012). Key advantage of hybrids found to be the speed and ease of obtaning multiple disease resistant ones based on the selection of desirable parental lines having target traits. Several hybrids of Burley and FCV are released and are under cultivation worldwide (https://crosscreekseed.com; https://content.ces.ncsu.edu/flue-cured-tobacco-information; ICAR-CTRI 2021b). One of the main reasons for the popularity of hybrids is the repeal of the 1944 Federal Tobacco Seed Law that prohibited the sale of domestic tobacco seed in abroad. Hybrid cultivars ensure the proprietary status of a new cultivar and seed sales, and distribution can be controlled (Bowman and Sisson 2000).

10.4.4.2 Disease Resistance

Much of the early tobacco breeding carried out was concentrated in incorporation of resistance to diseases that were the primary factors limiting production (Bowman and Sisson 2000). The varieties developed in recent years found to combine disease resistance with high yield potential. Conventional breeding and interspecific hybridization were used to transfer disease resistance traits viz. TMV, wild fire, black shank, brown spot, black root rot, blue mold, root knot nematode, powdery mildew, TSWV, PVY, cyst nematode etc. (Bowman and Sisson 2000; Sarala et al. 2012). Most commercial varieties are highly susceptible to blue mold disease (Rufty and Main 1989) and functional/partial resistance reported in *N. debneyi*, *N. velutina*, *N. goodspeedii* and *N. exigua* (Clayton 1968; Gillham et al. 1977; Wark 1970) have been transferred through interspecific hybridization (Milla et al. 2005). N gene conferring TMV resistance from *N. glutinosa* was successfully transferred to *N. tabacum* by bridge cross technique (Clausen and Goodspeed 1925; Holmes 1938). Majority of the present flue-cured tobacco cultivars possess resistance to important diseases such as TMV, black shank, Granville wilt and root knot nematodes. However, resistance to black shank and Granville wilt are variable among cultivars (Bowman and Sisson 2000). The five CMV genes from wild *Nicotiana* Species were pyramided into the tobacco

line Holmes and resistance derived from Holmes for multiple virus resistance has been incorporated into many cultivars (Holmes 1960, 1961; Wan et al. 1983).

Once resistance to a single disease had been achieved, pyramiding resistance to several diseases into a single cultivar became a top priority (Bowman and Sisson 2000). This objective is being continued into the twenty-first century. Majority of the current flue-cured tobacco cultivars have resistance to Granville wilt, black shank, and root knot nematodes, even though levels of resistance is highly variable among cultivars. Many of the modern hybrids possess resistance to two or more biotic stress (https://crosscreekseed.com; https://content.ces.ncsu.edu/flue-cured-tobacco-information; ICAR-CTRI 2021b). The popular FCV cultivar, K 326 is resistant to the common root-knot nematodes and have low level of resistance to black shank and bacterial wilt (https://crosscreekseed.com/usa-varieties/usa-flue-cured-tobacco-seed/k-326/). While, popular burley tobacco cultivar, Banket A1 is resistant to brown spot, wildfire, and TMV (Lapham 1976).

10.4.4.3 Insect Resistance

Unlike in the case of disease resistance, incorporation of insect resistance is not met much success in tobacco. A tobacco variety, CU 263 having moderate level of resistance to tobacco budworms was released in 1995. Tobacco varieties with resistance to tobacco aphids (*Myzus nicotianae*) and tobacco caterpillar have been developed for commercial cultivation in India (Joshi and Sitaramaiah 1975; Joshi et al. 1978; Murthy et al. 2014). FCV variety, CTRI Sulakshana is tolerant to aphids and resistant to TMV. Meenakshi (CR) and Abirami (CR) are caterpillar resistance chewing tobacco varieties developed through back cross breeding (ICAR-CTRI 2021b).

10.4.5 Limitations of Traditional Breeding and Rationale for Molecular Breeding

Major limitation in traditional breeding in tobacco are undesirable gene associations, pleiotropic gene effects and linkage drag effects caused by the presence of deleterious genes linked to gene of interest (Legg et al. 1981; Zeven et al. 1983; Friebe et al. 1996; Brown 2002). Chaplin et al. (1966), Chaplin and Mann (1978) and suppressed recombination within introgressed chromatin (Paterson et al. 1990; Liharska et al. 1996) making it difficult to alleviate linkage drag effects through back crossing (Stam and Zeven 1981; Young and Tanksely 1989). Sources of resistance from easily crossable or that can be transferred through special techniques such as bridge crossing, embryo rescue, chemical treatments are required for developing resistance species.

With the advent of advanced molecular breeding techniques like, marker-assisted selection, plant transformation, CRISPER/CAS9 gene editing etc. only gene of interest can be modified or incorporated into the selected cultivar in short span of

time without these limitations. Molecular marker-assisted plant breeding for fore ground and background selection with speed breeding strategies will help in saving time.

10.5 Brief on Diversity Analysis

The major interest to study the genetic diversity in tobacco is for the conservation of genetic resources, broadening of the genetic base and cultivar development in breeding programs. Narrow genetic diversity can lead to crop losses due to reduced flexibility of varieties to combat infestations of new strains of pests or pathogens or to adapt populations to changing environmental conditions such as increasing temperatures or salinity (Moon et al. 2009a). The study of the genetic diversity is important for identifying sources for economically important traits and diverse parental combinations to create segregating progenies with maximum genetic variability for further selection in breeding (Barrett and Kidwell 1998). Utilization of diverse parents helps in developing tobacco varieties having diverse genetic backgrounds with improvement in yield and quality along with stress resistance traits. Deploying varieties with diverse sources of resistance will reduce genetic vulnerability of cultivars to evolving races and biotype of insects and pathogens. Thus, genetic diversity is essential for continued progress in breeding varieties for adaptation to future environmental challenges.

10.5.1 Phenotype-Based Diversity Analysis

Phenotypic viz. morphological, karyotypical and physiological characters have been used to study the genetic diversity of tobacco germplasm (Goodspeed 1954; Zhang 1994; Lu 1997; Zhang et al. 2005). Till date several agro-morphological traits (Zhang 1994; Wenping et al. 2009; Zeba and Isbat 2011; Sarala et al. 2018; Baghyalakshmi et al. 2018), chemical and cytological traits (Tso et al. 1983; Okumus and Gulumser 2001; El-Morsy et al. 2009; Darvishzadeh et al. 2011) have been used to study the genetic variation of tobacco germplasm. Agro-morphological traits usually vary with environment and affect diversity estimates under different environments (Lu 1997). Germplasm are screened for identifying sources of resistance to various biotic stresses under artificial conditions based on the extent and type of damage caused to the crop through phenotyping.

The tolerance/resistance mechanism to various stresses is now being extensively studied through newer techniques of phenotyping namely high throughput phenotyping where the system quantifies a number of traits within a described set of plant population with automated image collection and analysis, thus effectively streamlining the plant phenomics. This technology is gaining importance due to its non-destructive sampling methods, rapid screening of larger population and automated

data analysis. To measure tolerance, visual cameras are used to capture the plant growth, architecture, chlorosis and necrosis all of which can be negatively affected by insect infestation. Photosynthetic activity of crop plants can be measured in terms of chlorophyll fluorescence through fluorescence cameras as an indication of tolerance mechanisms occurring in response to insect attack (Buschmann and Lichtenthaler 1998). The technology can have numerous applications in the measure of insect damage and plant resistance to insects (Goggin et al. 2015). Ultimately the use of high throughput phenotyping systems could possibly reduce the amount of labour and screening time to identify plants tolerant to insects.

10.5.2 Genotype-Based Diversity Analysis

Before the advent of DNA markers, morphological, karyotypical, physiological and isozyme markers are mainly used in diversity analysis of tobacco. However, morphological characters usually differ with environments. The karyotypical characters are limited in number and the study of genotypic diversity based on isozyme variation is restricted to a small number of loci that control few polymorphic enzyme systems (Lu 1997). Until now, only limited information has been available on the relationship between morphological variability and genome diversity in cultured tobacco. Later, attempts were made to examine the degree of relatedness among tobacco cultivars and diversity of germplasm based on variability at DNA level. About 77% of the total genomic DNA content is composed of repetitive sequences in tobacco and therefore, the remaining non-repetitive sequences are responsible for morphological and quality trait variability (Narayan 1987).

With the advantages such as highly polymorphic nature, codominant inheritance, easy access, fast assay, high reproducibility and easy exchange of data between laboratories, different types of molecular markers have been introduced over the last two decades, that has revolutionized the entire scenario of biological sciences including tobacco (Liu and Zhang 2008). Molecular markers provide a relatively unbiased estimation of genetic diversity in plants and are abundantly available throughout the genome. DNA-based molecular markers found to be versatile tools having applications in various fields such as characterization of genetic variability, genome fingerprinting, genome mapping, gene localization, analysis of genome evolution, population genetics, taxonomy, genome comparisons, gene mapping, quantitative trait loci analysis, marker-assisted breeding diagnostics, etc.

Restricted fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites or simple sequence repeat (SSR), single-nucleotide polymorphism (SNP), inter simple sequence repeats (ISSR) etc. have been employed in studying genetic diversity, gene mapping and marker-assisted breeding of tobacco.

RFLPs were the most popular molecular markers in late eighties due to their reproducibility, locus-specificity, mendilian inheritance and codominant nature (Nadeem et al. 2018). RFLP methodology is simple and require no special equipment. Another

advantage of RFLP is that the sequence used as a probe need not be known RFLPs, being co dominant markers, can detect coupling phase of DNA molecules as DNA fragments from all homologous chromosomes are detected. RFLPs are highly reliable markers in linkage analysis and breeding as they can easily differentiate whether the linked trait is present in a homozygous or heterozygous state in an individual and such information highly desirable for recessive traits (Winter and Kahl 1995). However, the large amount of DNA required for restriction digestion and laborious Southern blotting technique hampered the utility of the assay. Further, it is time-taking and labor-intensive and only few out of numerous markers may be polymorphic making it highly inconvenient especially for crosses between closely-related species. RFLP markers were the first molecular markers used in tobacco research specially to study the function of few cloned genes (Bretting and Widrlechner 1995).

Invention of polymerase chain reaction (PCR) technology and PCR-based markers such as RAPD and AFLP emerged in the beginning of nineties and later microsatellite markers were used by different workers to study genetic diversity in tobacco. Compared to RFLP, these PCR based markers are preferred because of the relative ease with which PCR assays can be carried out. RAPD technology utilizes short synthetic arbitrary oligonucleotides primers to amplify fragments. It methodology is simple, time-saving, and requires only small amount of genomic DNA. Due to these advantages, RAPD technology has been widely used in studying genetic diversity, localization of target genes, genetic mapping and evolution genetics. However, RAPD are anonymous and the level of their reproducibility is very low due to the non-specific binding of short, random primers. AFLP technology combines the power of RFLP with the flexibility of PCR-based markers. It offers a universal, multi-locus marker tool that can be applied to complex genomes from any organism. Though the AFLPs markers are advantageous in terms of their reproducibility and sensitivity, their detection method is lengthy, laborious and not amenable to automation. Both RAPD (Xu et al. 1998; Del Piano et al. 2000; Evanno et al. 2005; Zhang et al. 2005, 2008a; Arslan and Okumus 2006; Sarala and Rao 2008; Sivaraju et al. 2008; Denduangboripant et al. 2010; D'hoop et al. 2010) and AFLP (Huang et al. 2008; Zhang et al. 2008a; Chuanyin et al. 2009; Liu et al. 2009a) were used to analyze the genetic diversity and varietal identification in tobacco. In tobacco, the RAPD technique has also been used to identify markers linked to genes for resistance to pathogens (Bai et al. 1995; Yi et al. 1998a, b; Johnson et al. 2002; Collard et al. 2005; Julio et al. 2006; Gong et al. 2020). Based on AFLP markers, five TMV-resistant tobacco accessions were found to carry a TMV resistance gene (N gene derived from *N. glutinosa* L.) on chromosome H and seven accessions were found to carry a resistance factor on an alternative chromosome (Lewis et al. 2005).

Soon after the discovery of SSR markers in late 90 s and the beginning of twenty-first century, they became markers of choice as they could able to eliminate all drawbacks of earlier DNA marker technologies (Jafar et al. 2012). SSRs were highly reproducible, polymorphic, and amenable to automation. In spite of higher working costs, SSR markers are extensively employed in plant molecular genetics and breeding. Development of PCR-based molecular markers is, in general, inefficient in tobacco in view of the very limited genetic diversity between tobacco cultivars, particularly

between those of the same type (Del Piano et al. 2000; Julio et al. 2006; Rossi et al. 2001) and that cultivated tobacco is a tetraploid species with a very large genome (Livingstone et al. 1999; Ren and Timko 2001; Doganlar et al. 2002). However, Bindler et al. (2007) for the first time reported the use of microsatellite markers in the identification of tobacco varieties. They used around 637 functional SSR markers out of which 282 were highly polymorphic and were used for variety identification. Since then, SSR markers are being used in estimating diversity in tobacco. Later, an additional set of 5119 new and functional SSR markers were developed for mapping and diversity studies (Bindler et al. 2011). Tong et al. (2012b) published a set of 1365 genomic SSRs and 3521 expressed sequence tag (EST)-SSRs, which only slightly overlapped with the set published by Bindler et al. (2007, 2011). Madhav et al. (2015) developed a new set of microsatellite markers using 70 motifs (which includes perfect and imperfect repeat) and validated their applicability in differentiating different types of tobacco and diverse cultivars of Flue Cured Virginia (FCV) tobacco, and the transferability of these markers in a wide range of *Nicotiana* species. Cai et al. (2015) used EST databases of tobacco for developing EST-SSR markers and validated them in studying the genetic differentiation between *N. rustica* and *N. tabacum*, and between oriental tobacco and other accessions of *N. tabacum*. Later, Wang et al. (2018) identified a huge number of SSRs through comparative genome wide characterization of ~20 Gb sequences from seven species viz. *N. benthamiana, N. sylvestris, N. tomentosiformis, and N. otophora,* and *from N. tabacum* cultivars TN90, K326, and BX representing a ~74%, ~80%, ~78%, ~81%, ~84%, and ~73% of each of the genomes, respectively. Their study resulted in the development of a total of 1,224,048 non-redundant NIX (*Nicotiana* multiple (X) genome) markers (SSRs), of which 99.98% are novel (Wang et al. 2018). SSR markers development in tobacco has led to the analysis of molecular diversity (Moon et al. 2009b; Davalieva et al. 2010; Fricano et al. 2012; Gholizadeh et al. 2012; Prabhakararao et al. 2012; Xiang et al. 2017) of genetic resources, DUS testing (Binbin et al. 2020), genetic relatedness of cultivated varieties (Moon et al. 2008), estimating the changes in diversity due to breeding interventions (Moon et al. 2009a) and also for the identification of linked markers and QTLs to various biotic stresses that were discussed later in this chapter.

Genetic diversity in tobacco varieties is also been assessed using Inter simple sequence repeats (ISSR) markers (Yang et al. 2005, 2007; Qi et al. 2006) and inter-retrotransposon amplification polymorphism (IRAP) markers (Yang et al. 2007).

In recent years, SNP markers, first discovered in human genome, proved to be universal, abundant, ubiquitous, and amenable to high- and ultra-high-throughput automation are being identified and used (Jafar et al. 2012). Although SNPs are less polymorphic than SSR markers, their biallelic nature compensate this drawback (Ghosh et al. 2002). The application of SNPs in tobacco is complicated and challenging due to its tetraploid nature and complex genetic architecture (Ganal et al. 2009). However, recent studies identified number of SNPs in tobacco (Xiao et al. 2015; Thimmegowda et al. 2018; Tong et al. 2020) that are being utilized in the identification of markers linked to important traits in tobacco entries including biotic stresses in addition to development of molecular maps, and studying genome structure and organization.

Wang et al. (2021) used genotyping-by-sequencing (GBS) technique on 113 cigar tobacco accessions for the identification of 47 core Kompetitive allele specific PCR (KASP) and 24 candidate core markers utilizing SNP data. KASP markers are able to discriminate between two alleles of a SNP using a common reverse primer paired with two forward primers, one specific to each allele. Core markers were used for varietal identification and fingerprinting in 216 cigar germplasm accessions.

10.5.3 Relationship with Other Cultivated Species and Wild Relatives

Cultivated tobacco belongs to the genus *Nicotiana,* one of the five major genera of the family Solanaceae. Comparative morphological, cytological and biochemical studies, and examinations of organellar (plastid and mitochondrial) genome organization and analysis of molecular features, such as repetitive DNA sequences and the structure of various nuclear gene family members, has been employed to study the evolution and the genetic diversity in genus *Nicotiana* (Kostoff 1943; Goodspeed 1954; Komarnitsky et al. 1998; Lim et al. 2000; Liu and Zhang 2008).

The genus, *Nicotiana* resembles the two genera, *Cestrum* (8 pairs) and *Petunia* (7 pairs of chromosomes) (Darlington and Janaki Ammal 1945) in habit and habitat. The origin, evolution and relationships among various species of the genus have been summarized in three phylogenic arcs. In the first two arcs, the genus is envisaged as derived from a pre-generic reservoir of two related genera and evolving into three complexes, at the 12-paired level, that are hypothetical precursors of the three modern sub-genera. The third arc contains the present day species, at 12- and 24-paired chromosome level. Although 6-paired species of *Nicotiana* is not known, the predominance of 12- paired species and their compound morphological character, along with a frequency of 4–8 pairing with a mode of 6 pairs in large number of F_1 hybrids combining 12-paired species, indicates that 6 is the basic chromosome number for *Nicotiana* and both 12 and 24 are derived numbers. Additional evidence for this has been provided by secondary association and pairing in haploids of 12-paired species (Kostoff 1943; Goodspeed 1954). It is assumed that with a higher survival value, the allopolyploids have eliminated the older 6-paired types. Hybridization between the ancestral 6-paired and the present day 12-paired member was considered to be responsible for the evaluation of 9- and 10-paired species. The *N. tabacum* and *N. rustica* and other 24-paired species are modern descendants of the 12-paired progenitors entered into amphiploid origin. The aneuploid species of the section Suaveolentes with a chromosome number between 16 and 24 pairs are presumed to have originated as products of hybridization and segregation. Thus, among various evolutionary mechanisms, amphiploidy superimposed by amphiploidy seems to be the basic evolutionary process responsible for the 6- 12- 24-paired sequence in the genus (Goodspeed 1954). Further enlargement of the genus

took place through aneuploidy resulting from hybridization, genetic recombination and mutation.

The systematic classification of the genus was presented in detail by Goodspeed (1954) and Goodspeed and Thompson (1959) mainly based on cytogenetic studies involving chromosome morphology, behaviour in interspecific hybrids, aneuploids and amphiploids. Subsequently, additions to the classification were made by Burbridge (1960). In the revised systematics based on molecular research (Chase et al. 2003; Clarkson et al. 2004; Knapp et al. 2004), subgenera were dropped retaining the division into sections. As per the recent classification, *N. trigonophylla* Dun. was renamed *N. obtusifolia* Martens et Galeotti, *N. affinis* Hort is synonymous with *N. alata* Link et Otto, and *N. bigelovii* (Torrey) Watson with *N. quadrivalvis* Pursh. *N. sanderae* Hort. is considered to be a hybrid between *N. alata* and *N. forgetiana* Hemsl. (*Nicotiana x sanderae*) whereas *N. eastii* Kostoff is a variant of autotetraploid version of *N. suaveolens* Lehm. (Chase et al. 2003; Knapp et al. 2004).

N. tabacum and *N. rustica* are the cultivated species among the 83 wild species of *Nicotiana* genera (Lewis 2011; Berbec and Doroszewska 2020). *N. tabacum* is highly polymorphic with wide range of morphological types and diversified utilities viz. smoking, snuff, chewing, etc. Other species cultivated in smaller scale are *N. repanda* Willd ex Lehm., *N. attenuata* Torrey ex S. Watson and *N. quadrivalvis* Pursh are for smoking, *N. sylvestris* Spegazzini & Comes, *N. alata* Link and Otto, *N. langsdorffii* Weinmannm, *N. forgetiana* Hemsley, and *N. sanderae* (Hort) for ornamental and *N. glauca* Graham for industrial purpose (Lester and Hawkes 2001).

It is known that *N. tabacum* is natural amphidiploid i.e., allopolyploid ($2n = 4x = 48$) ascended by hybridization of wild progenitor species (*N. sylvestris* (S-genome) × *N. tomentosiformis* (T-genome) and *N. rustica* L. from a cross between *N. paniculata*/*N. knightiana* (P/K-genome) × *N. undulata* Ruiz & Pav. (U-genome) (Goodspeed 1954; Clarkson et al. 2005; Lim et al. 2005; Leitch et al. 2008; Edwards et al. 2017; Sierro et al. 2018). Comparison of whole-genomic sequences indicated that the genome of *N. sylvestris* and *N. tomentosiformis* contributed 53 and 47%, respectively, to the genome of *N. tabacum* (Sierro et al. 2014). The comparative mapping studies of the diploid ancestor of the T-genome (*N. tomentosiformis*) and a species related to the S-genome (*N. acuminata*) using conserved ortholog sequences (COS) and SSR markers revealed that the tetraploid tobacco genome has undergone a number of chromosomal rearrangements compared to these diploid genomes (Wu et al. 2009). Furthermore, in the same study, it was observed that a number of reciprocal translocations and inversions (>10) differentiate the ancestral tobacco genomes from the tomato genome. Gong et al. (2016) identified a large number of genome rearrangements occurring after the polyploidization event through mapping of ancestral genomes of *N. tomentosiformis* and *N. sylvestris* with SNP markers. Based on studying the cpSSRs and MtSSRs, S genome in *N. tobacum* was identified to originate from *N. Sylvestris* ancestor (Murad et al. 2002). The chloroplast genome of *N. otophora* revealed that *N. otophora* is a sister species to *N. tomentosiformis* within *Nicotiana* genus and *Atropha belladonna* and *Daturastramonium* are the closest relatives (Asaf et al. 2016).

Sierro et al. (2018) reported that 41% of *N. rustica* genome is originated from the paternal donor (*N. undulata*) and 59% from the maternal donor (*N. paniculata/N. knightiana*). Comparison of families of repetitive sequences, two from *N. paniculata* and one from *N. undulata* indicated that P- and U-genomes of *N. rustica* was similar to the putative parents, *N. paniculata* and *N. undulata*, respectively (Lim et al. 2005). Genomic in situ hybridization confirmed that *N. rustica* is originated as an allotetraploid from *N. paniculata* (maternal P-genome donor) and *N. undulata* (paternal U-genome donor) and interlocus sequence homogenization has caused the replacement of many *N. paniculata*-type intergenic spacer (IGS) of the 18-5.8-26S rDNA in *N. rustica* with an *N. undulata*-type of sequence (Matyasek et al. 2003). However, the study of nuclear and chloroplast genomes, and gene analyses showed that *N. knightiana* is closer to *N. rustica* compared to *N. paniculata.* Gene clustering revealed 14,623 ortholog groups common to other *Nicotiana* species and 207 unique to *N. rustica.* (Sierro et al. 2018).

Both *N. tabacum* and *N. rustica* shares its basic chromosome number of $n = 12$ with many other solanaceous species such as tomato, potato, pepper and eggplant (Lim et al. 2004; Clarkson et al. 2005). Microsynteny observed between the genomes of *N. tabacum* cv. TN90, K326 and BX and those of tomato and potato at the protein level (Sierro et al. 2014).

10.5.4 Relationship with Geographical Distribution

The genus, *Nicotiana*, considered to be recent origin, is presumed to have had its original habitat in and around the Andes region in South America and Central America, probably from the mild to low altitude forest margin (Goodspeed 1954). Although, occurring naturally as a perennial plant, tobacco is farmed as an annual crop. In the genus *Nicotiana*, 20 are native to Australia, one to Africa and 54 are indigenous to North /South America (Goodspeed 1954; Burbridge 1960; Clarkson et al. 2004). *N. benthamiana*, a species native to Australia, is used extensively as a model system to study various biological processes. *N. tabacum L.* (common tobacco) and *N. rustica L,* two species native to America, are the major cultivated species cultivated throughout the world.

Some 40% of *Nicotiana* species are allopolyploids, which have been generated independently in six polyploidy events several million years ago (Clarkson et al. 2004; Leitch et al. 2008). The taxonomically, some of the diploid genome donors that make up various allopolyploids are closely related and others belonging to distantly related taxonomic sections (Clarkson et al. 2004; Leitch et al. 2008).

Darvishzadeh et al. (2011) reported clustering of oriental-type tobacco genotypes based on morphological traits was in agreement with their geographical distribution and growth characteristics. However, the genetic diversity studies conducted using SSR markers in oriental (Darvishzadeh et al. 2011), and RAPD and AFLP markers in flue-cured tobaccos (Zhang et al. 2008a) could not indicate any clear pattern of their geographical origins. However, the clustering of tobacco genotypes based on

molecular diversity found to correspond to commercial classes (Flue-Cured, Burley, etc.), manufacturing trait and parentage (Sivaraju et al. 2008; Fricano et al. 2012).

10.5.5 Extent of Genetic Diversity

Existence of morphological diversity confirmed in various germplasm collections viz. FCV, burley, mutant lines, bidi, chewing, cheroot and cigar filler germplasm etc. (Baghyalakshmi et al. 2018; Sarala et al. 2018) maintained at tobacco genebank in India. Moon et al. (2009b) observed quite large average gene diversity among *N. tabacum* accessions from the U.S. *Nicotiana* Germplasm Collection compared FCV tobacco accessions. While, Fricano et al. (2012) reported lower SSR diversity per locus than in similar investigations carried out on TI accessions of tobacco. The genetic diversity assessment in tobacco cultivars indicated low degree of polymorphism (Xu et al. 1998; Del Piano et al. 2000; Rossi et al. 2001; Yang et al. 2005; Zhang et al. 2005; Julio et al. 2006). Richest genetic diversity reported for local group of tobacco varieties and lower diversity for introductions, and higher genetic similarity values between introductions and breeding group (Xiang et al. 2017). While, the variation among the tobacco lines were found to be higher concerning with the chemical nature of the plants (Tso et al. 1983; Darvishzadeh et al. 2011) as well as susceptibility to disease such as stem rot and powdery mildew (Darvishzadeh et al. 2010). The relatively low levels of diversity revealed by molecular markers in tobacco cultivars (Ren and Timko 2001) may be due to the utilization of only small proportion of the variability of the gene pools of the progenitor species in breeding programs (Lewis et al. 2007). The level of polymorphism among the varieties of *N. tabacum* observed to be higher compared to *N. rustica* (Sivaraju et al. 2008). However, wild tobacco accessions found to have higher genetic diversity (Chuanyin et al. 2009).

10.6 Association Mapping Studies

Association analysis, also known as linkage disequilibrium (LD) mapping or association mapping, uses the natural population as the target material. The analysis is based on linkage disequilibrium that detects the frequency of significant association between the genetic variation of markers with candidate genes and the target traits in a population (Bradbury et al. 2007; Pritchard et al. 2000). LD is defined as the non-random association of alleles at two or more loci (Fricano et al. 2012). Compared with QTL mapping, association analysis has two prominent advantages: (1) there is no need to construct population and genetic maps, and (2) uncovers (explores) elite genes from a certain number of germplasm resources in a single instance, providing

indication of genetic diversity. LD mapping has the potential to outperform traditional mapping and facilitate fine mapping in a random-mating population as only close linkage between markers and traits remains over several generations in such population.

10.6.1 Extent of Linkage Disequilibrium

In cultivated plants, extent of linkage disequilibrium is influenced by mating system, mutation rate, genetic drift, selection, recombination rate, gene conversion, and population size and structure (Flint-Garcia et al 2003). High-density genome fingerprinting could uncover long as well as short range LD. In the first case, in species with large genomes, a lower number of molecular markers can be tested (Waugh et al. 2009), although this will result in a lower mapping resolution. Conversely, short-range LD enables the fine mapping of causal polymorphisms, if large panels of markers are available (Myles et al. 2009).

The knowledge on the extent of LD is essential to determine the minimum distance between markers required for effective coverage when conducting genome-wide association studies (GWAS). Fricano et al. (2012) identified a set of 89 genotypes that captured the whole genetic diversity detected at the 49 SSR loci and evaluated LD using 422 SSR markers mapping on seven linkage groups. The pattern of intra-chromosomal LD revealed that LD in tobacco was clearly dependent on the population structure and was extended up to distances as great as 75 cM with $r2 > 0.05$ or up to 1 cM with $r2 > 0.2$.

10.6.2 Target Gene Based LD Studies

Thornsberry et al. (2001), for the first applied the association analysis in plants. Currently, LD has been used in association mapping to locate QTLs or major genes, based on the co-segregation of specific marker alleles and traits in tobacco (Zhu et al. 2008; Rafalski 2010). Zhang et al. (2012a, b) conducted association analysis on 13 agronomic traits in 258 flue-cured tobaccos and detected significant association of six agronomic traits with 18 sequence-related amplified polymorphism markers. Based on association analysis, Yu et al. (2014) found that the polymorphic loci of an SSR marker and six microsatellite-anchored fragment length polymorphism markers were significantly associated with the levels of tobacco-specific nitrosamines. Ren et al. (2014) found 24 SSR loci associated with aroma substances in tobacco and Basirnia et al. (2014) identified only one SSR locus from linkage group 13 that was significantly associated with low chloride accumulation rate in 70 oriental-type tobaccos. Fan et al. (2015) performed a marker–trait association analysis and obtained 11 SSR markers associated with potassium content in tobacco; five of the SSR markers were used to validate the stability of the associated markers in

130 tobacco germplasms. Through association mapping, Darvishzadeh (2016) identified the linkage of five SSR loci from linkage groups 2, 10, 11 and 18 of a tobacco reference map (Bindler et al. 2007, 2011) with gene(s) controlling *Orobanche* resistance in tobacco. Tahmasbali et al. (2021) by using mixed linear method identified a total of 16 loci to be significantly ($P < 0.05$) associated with the agronomic traits under normal (without *Orobanche*) and stress (with *Orobanche*) conditions with some common markers across normal and *Orobanche* conditions for few traits. Based on association mapping using 219 accessions for their responses to BS at two sites, Sun et al. (2018) detected six significant marker-trait associations and identified two probable candidate genes for resistance to BS (Nitab 4.5_0000264g0050.1 and Nitab 4.5_0000264g0130.1) among 31 predicted genes at locus Indel53.

SNPs, due to their abundance at genome-wide level, biallelic and reproducible nature, are considered to be the most desirable, precise and efficient tools for assessing the genetic characteristics of populations or germplasm, QTL mapping and facilitating the selection of breeding materials that bear desired genes/alleles or haplotypes in both plant genetics and breeding programs (Pace et al. 2015; Mora et al. 2016). Tong et al. (2020) made simultaneous association analysis of leaf chemistry traits in natural populations with a large amount of tobacco germplasms based on genome-wide SNP markers.

10.6.3 Genome Wide LD Studies

Association mapping studies largely depend on the genetic structure of the population. Molecular diversity in germplasm collections can be utilized to reconstruct the population structure in tobacco for association studies (Moon et al. 2009b). Ganesh et al. (2014) analyzed genetic structure of 135 FCV (Flue Cured Virginia) tobacco genotypes and observed that 25 unlinked SSR markers delineated 135 FCV genotypes revealing a total of 85 alleles with an average of 3.4 alleles per locus. However, as on date, Genome wide LD studies are not reported in tobacco.

10.6.4 Future Potential for the Application of Association Studies for Germplasm Enhancement

The population-based association study explores the availability of broader genetic variations with wider background for marker-trait correlations (i.e., many alleles evaluated simultaneously). Such studies provide higher resolution maps because of the utilization of majority recombination events from a large number of meiosis throughout the germplasm development history and exploits historically measured trait data for association without the development of expensive and tedious biparental populations in a time saving and cost-effective way (Abdurakhmonov

and Abdukarimov 2008). Linkage disequilibrium (LD)-based association study, as a high-resolution, broader allele coverage, and cost-effective gene tagging approach provides an opportunity to dissect and exploit existing natural variations in tobacco germplasm resources for tobacco improvement. Owing to the availability of large collection of germplasm resources in tobacco over worldwide, association studies help to detect neutrally inherited markers in close proximity to the genetic causatives or genes controlling the complex quantitative target traits including resistance to biotic stresses for further germplasm enhancement and exploitation.

10.7 Brief Account of Molecular Mapping of Resistance Genes and QTLs

10.7.1 Brief History of Mapping Efforts

Gene mapping in tobacco found to initiate with the identification of linkage between a pollen color factor and the sterility factors (Anderson and De Winton 1931; East 1932; Brieger 1935; Smith 1937). Clausen and Goodspeed (1926) developed two types of monosomics and showed that both haplo-C (then called "corrugated") and basic color factor, *Wh* are associated with a chromosome. Later, Clausen and Cameron (1944) studied the association in transmission between 24 monosomics developed by them and mendelian characters which led to location of 18 genes in nine chromosomes. Though genes controlling various characters and their linkages with other genes are identified, detailed map based entirely on genes is not available in tobacco (Suen et al. 1997; Narayanan et al. 2003).

However, with the advent of DNA markers in early nineties, efforts were made to map tobacco genome using molecular markers. Initially, RFLP, RAPD and AFLPs markers were used to map and tag resistant genes linked to biotic stresses. In 2001, for the first time, RFLP and RAPD markers were used to map *Nicotiana* spp. (Lin et al. 2001). Later, RAPD, AFLPs and ISSR were used in construction of genetic maps (Lin et al. 2001; Nishi et al. 2003; Julio et al. 2006; Xiao et al. 2006). After the discovery of SSR markers in late 1990s, for the first time SSR based molecular map showing 24 linkage groups was developed in *N. tabacum* (Bindler et al. 2007). This SSR map was improved further with identification of more number of SSRs (Bindler et al. 2011; Tong et al. 2012b). Recently, with identification of SNPs, high density SNP based tobacco genetic map has been developed with 24 linkage groups (Tong et al. 2020). Currently, maps are available for FCV, burley tobacco and their intra type. Maps are also available few *Nicotiana* spp.

10.7.2 Evolution of Marker Types

Molecular markers allow detection of variations or polymorphisms that exist among individuals for specific regions of DNA, thus serves as useful tools in mapping of genetic material. Molecular genetic markers, such as RFLP, RAPD, AFLP, SSRs and SNPs have been used in genetic linkage mapping and QTL mapping in tobacco (Liu and Zhang 2008).

Initially, molecular genetic markers such as RFLP, RAPD and AFLP were used in mapping studies. In the beginning of nineties, PCR-based RAPD markers were used by different workers to map and tag resistant genes linked to biotic stresses due to their relative ease in spite of the reproducibility issues. Though, reproducibility and sensitivity of AFLPs markers is higher, they were used in a limited extent in mapping of resistance gene sowing to their lengthy and laborious detection method and non-suitability to automation.

After the discovery of SSR markers in late 90s, SSRs and EST-SSRs became markers of choice for mapping in tobacco (Bindler et al. 2007, 2011; Tong et al. 2012b). Currently, more than 10,000 SSR markers available in tobacco for their use in QTL/gene mapping studies (Bindler et al. 2007, 2011; Tong et al. 2012b; Cai et al. 2015; Madhav et al. 2015). In addition, Wang et al. (2018) identified a huge number of about 1,200,000 non-redundant and novel NIX (*Nicotiana* multiple (X) genome) markers (SSRs) for use in tobacco.

In recent years, SNPs have been identified and used in mapping of tobacco genome. Xiao et al. (2015) developed SNPs using two different methods (with and without a reference genome) based on restriction-set associated DNA sequencing (RAD-seq). Thimmegowda et al. (2018) identified SNPs by whole-genome resequencing of 18 flue-cured Virginia (FCV) tobacco genotypes and positioned SNPs in linkage groups. Using the genome of *N. tabacum* (K326 cultivar) as a reference, Tong et al. (2020) identified and mapped 45,081 SNPs to 24 linkage groups in the tobacco genetic map.

In addition to the above markers; sequence-specific amplification polymorphism (SSAP), Sequence-related amplified polymorphism (SRAP), cleaved amplified polymorphic sequence (CAPS) and Diversity arrays technology (DArT) markers were also used in generating molecular linkage maps in tobacco.

10.7.3 Mapping Populations Used

For molecular mapping in tobacco, diverse populations viz. F_2 populations, doubled haploid (DH) lines, recombinant inbred lines (RILs), BC_1 progenies, BC_1F_1, BC_4F_3 populations etc. have been used as the mapping population (Table 10.5). Majority of the maps developed based on F_2 and DH populations and three maps developed based on NGS technologies. Practically 99–381 individuals are selected in a mapping population for higher resolution and fine mapping.

Table 10.5 Genetic linkage maps constructed in *Nicotiana* (in chronological order)

Type of tobacco	Population	Markers used	Linkage groups identified	Total length covered (cM)	References
Nicotiana species	99 F_2 plants from the cross *N. plumbaginifolia* × *N. longiflora*	69 RFLP & 102 RAPD	9	1062	Lin et al. (2001)
Burley	125 DH lines, derived from F_1 hybrids between *W6* and *Michinoku 1*	117 AFLPs	10	383	Nishi et al. (2003)
Flue-cured	DH population from a cross between Speight G-28 and NC2326	11 ISSRs &158 RAPDs	27	2094.6	Xiao et al. (2006)
Flue-cured	114 RILs	138 ISSR, AFLP & SSAP markers	18	707.6	Julio et al. (2006)
Flue-cured	186 F_2 derived from Hicks Broad Leaf and Red Russian	293 SSRs	24	1920	Bindler et al. (2007)
Flue-cured/burley	187 F_2 derived from the cross Taiyan 7 (FCV) and Bailei 21 (burley)	112 (92 SRAP and 10 ISSR)	26	1560.2	Ma et al. (2008)
Burley	92 DH population derived from Burley 37 and Burley 21	112 AFLP loci & 6 SRAP loci	22	1953.6 cM	Cai et al. (2009)
Wild diploid *Nicotiana* species	*N. tomentosiformis*	489 SSRs/CAPS	12	~1000 cM	Wu et al. (2010)
Wild diploid *Nicotiana* species	*N. acuminata*	308 SSRs/CAPS	12	~1000 cM	Wu et al. (2010)

(continued)

Table 10.5 (continued)

Type of tobacco	Population	Markers used	Linkage groups identified	Total length covered (cM)	References
Flue-cured	186 F2 derived from cultivar, Hicks Broad Leaf and Red Russian	2318 SSRs	24	3270	Bindler et al. (2011)
Flue-cured	207 DH population derived from FCV cultivars, HHDJY & Hicks Broad Leaf	851 (238 DArT & 613 SSRs)	24	2291	Lu et al. (2012)
N. tabacum	Parents [HD (Hong hua Da jin yuan) and RBST (resistance to black shank tobacco)] and their F_1 (HD × RBST) and 193 BC_1 progenies	Map 1: 4138 SNPs & Map 2: 2162 SNPs	24	Map 1: 1944.74 (with reference genome); Map 2: 2000.9 (de novo identification SNPs by RAD-seq)	Xiao et al. (2015)
Flue-cured tobacco	Specific locus-amplified fragment sequencing	4215 SNPs and 194 SSRs	24	2662.43	Gong et al. (2016)
Flue-cured tobacco	213 BC1s derived from Y3 and K326	626 SSRs	24	1120.45	Tong et al. (2016)
Flue-cured tobacco	Parents (Y3 & K326), their F_1 & 271 RILs	45,081 SNPs	24	3486.78 cM	Tong et al. (2020)
Flue-cured tobacco	Parents (NC82 & TT8), their F_1 & 200 BC_1F_1s	13,273 SNPs	24	3421.80	Cheng et al. (2019)
Flue-cured tobacco	Parents (Y3 & K326), their F_1 & 381 BC4F3s	24,142 SNPs	24	2885.36	Tong et al. (2021)

Note *RFLP* Restriction fragment length polymorphisms; *RAPD* Random amplified polymorphic DNA; *AFLP* Amplified fragment length polymorphism; *ISSR* Inter simple sequence repeat; *SSAP* Sequence-specific amplification polymorphisms; *SSR* Simple sequence repeats; *SRAP* Sequence-related amplified polymorphism; *CAPS* Cleaved amplified polymorphic sequences; *DArT* Diversity arrays technology; *SNP* single nucleotide polymorphisms, *DH* Double haploid lines; *RILs* Recombinant inbred lines

10.7.4 Mapping Software Used

Mapmaker program (Lander et al. 1987; Lin et al. 2001; Wu et al. 2010), JoinMap® 3.0 program (Van Ooijen and Voorrips 2001; Bindler et al. 2007, 2011) Map Manager QTXb20 (Manly et al. 2001; Bindler et al. 2011; http://www.mapmanager.org) and JoinMap 4.0 (Van Ooijen 2006; Lu et al. 2012; Tong et al. 2016) and LepMap3 software (Rastas 2017; Tong et al. 2020, 2021) were used in developing molecular maps in tobacco. Among various mapping softwares, JoinMap 3.0/4.0 program is the widely used one in tobacco for construction of molecular maps based on markers. LepMap3 software was used in the construction of maps using NGS data.

10.7.5 Maps of Different Generations

Genetic linkage maps are essential for studies of genetics, genomic structure, genomic evolution and for mapping essential traits. Construction of molecular genetic maps in tobacco have lagged behind other *Solanaceae* crops such as the tomato, potato, and pepper plants (Tanksley et al. 1992; Jacobs et al. 2004; Barchi et al. 2007). Till the end of twentieth century, very little information was available on genetic mapping and molecular development in tobacco (Suen et al. 1997). Construction of genetic linkage maps in tobacco was started since the beginning of twenty-first century (Lin et al. 2001). Various maps constructed are briefly discussed here.

10.7.5.1 Mapping of *Nicotiana* Species

Lin et al. (2001) constructed a genetic linkage map based on the 99 individuals of the F_2 plants derived from tobacco wild species, *Nicotiana plumbaginifolia* × *N. longiflora*. This map covers 1062 cM with the distribution of 60 RFLP and 59 RAPD loci on nine major linkage groups. Owing to the shortage of markers, the map has not coalesced into ten linkage groups, corresponding to the haploid chromosome number of *N. plumbaginifolia*. Later, another two maps for wild diploid *Nicotiana* species, *N. tomentosiformis* and *N. acuminata* with 12 linkage groups spanning ~1000 cM are generated by Wu et al. (2010). The *N. tomentosiformis* map was created with the combination of 489 SSR and CAPS markers and *N. acuminata* (closely related to *N. sylvestris*) with 308 SSR and CAPS markers (Wu et al. 2010).

10.7.5.2 Mapping of Burley Tobacco

Nishi et al. (2003) constructed a genetic linkage map of the burley tobacco containing 10 linkage groups based on DH lines, derived from F_1 hybrids between burley entries, W6 and Michinoku 1 using AFLP markers. Cai et al. (2009) constructed

currently available high density burley linkage map spanning 1953.6 cM using a double haploid (DH) population derived from a cross between Burley 37 with high nicotine content and Burley 21 with low nicotine content assembling 112 AFLP loci and six SRAP loci into 22 linkage groups (A1-A22).

10.7.5.3 Mapping of Flue-Cured Tobacco

The first linkage map of flue-cured tobacco based on a DH population from a cross between Speight G-28 and NC2326 was constructed by Xiao et al. (2006) using 169 ISSR/RAPD molecular markers covering 27 linkage groups. While Julio et al. (2006) constructed a molecular linkage map of flue-cured tobacco with 18 linkage groups covering 138 ISSR, AFLP and SSAP markers based on 114 flue-cured tobacco recombinant inbred lines.

Bindler et al. (2007) for the first time constructed an SSR based linkage map using F_2 plants derived from a cross of Hicks Broadleaf × Red Russian. Later, this map was further improved (Bindler et al. 2011) with the identification of more number of SSR markers. This high density SSR map of flue-cured tobacco constructed with 2318 SSR markers mapping on 24 linkage groups covering a total length of 3270 cM is the most widely used map of tobacco. The length of individual linkage groups in this map varied from 86 to 199 cM, and the average genetic distance between adjacent markers was 1.4 cM. However, in spite of the high marker density, there exists some gaps spanning ~16 cM in the map. (Fig. 10.1; Bindler et al. 2011). Tong et al. (2012a, b) utilized a population of 207 DH lines derived from a cross between 'Honghua Dajinyuan' and 'Hicks Broad Leaf' and constructed a genetic map of flue-cured tobacco consisting of 611 SSR loci distributed on 24 tentative linkage groups and covering a total length of 1882.1 cM with a mean distance of 3.1 cM between adjacent markers. Utilizing a 213 backcross (BC1) individuals derived from an intra-type cross between two flue-cured tobacco varieties, Y3 and K326, Tong et al. (2016) further constructed a genetic map consisting of 626 SSR loci distributed across 24 linkage groups and covering a total length of about 1120 cM with an average distance of 1.79 cM between adjacent markers.

Xiao et al. (2015) constructed two SNP linkage maps of flue-cured tobacco using two different methods (with and without a reference genome) based on restriction-set associated DNA sequencing (RAD-seq) of back cross population. Overall, 4138 and 2162 SNP markers with a total length of 1944.74 and 2000.9 cM were mapped to 24 linkage groups in these genetic maps based on reference genome and without reference, respectively. An SNP-based high density genetic map, *N. tabacum* 30 k Infinium HD consensus map 2015 (Fig. 10.2) was released at SOL Genome Network to facilitate the fine mapping of different trait of interest (https://solgenomics.net/cview/map.pl?map_version_id=178). Cheng et al. (2019) constructed a high-density SNP genetic map of flue-cured tobacco using restriction site-associated Illumina DNA sequencing. In this map, a total 13,273 SNP markers were mapped on 24 high-density tobacco genetic linkage groups spanning around 3422 cM, with an average distance of 0.26 cM between adjacent markers. While, Tong et al. (2020) performed

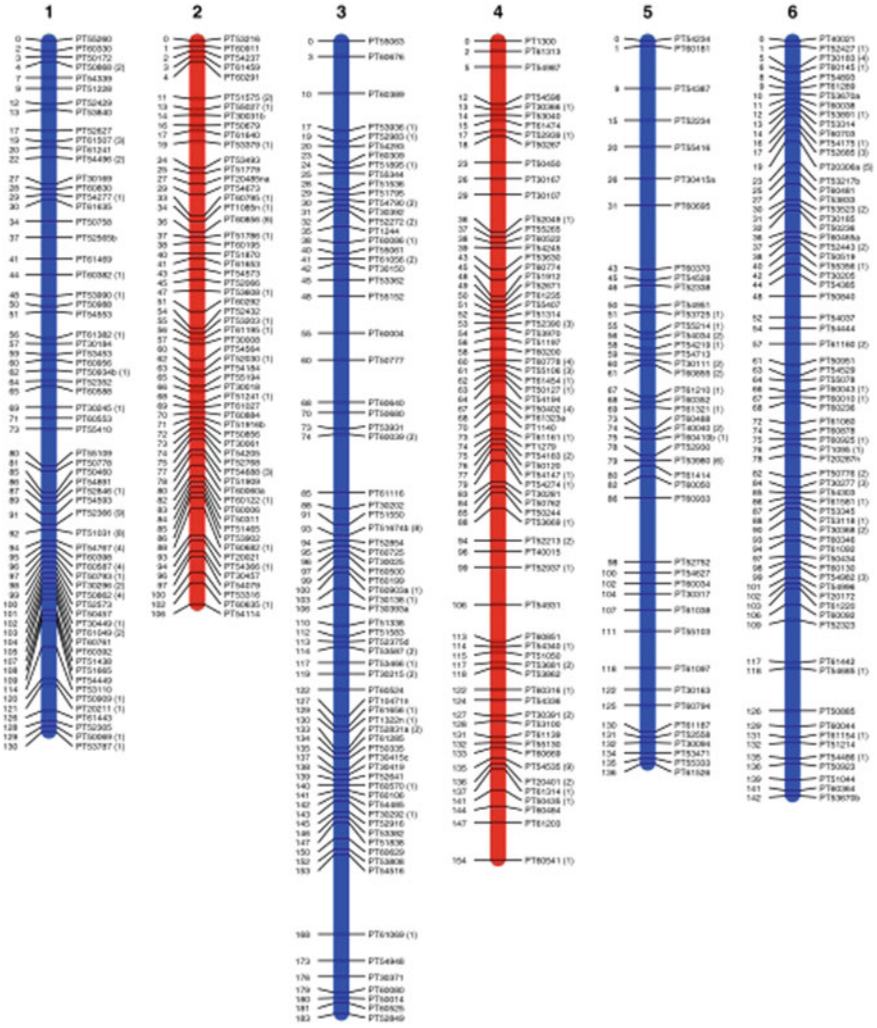

Fig. 10.1 Part SSR map (1–6 linkage groups) constructed by Bindler et al. (2011) with 2318 microsatellite markers covering a total length of 3270 cM

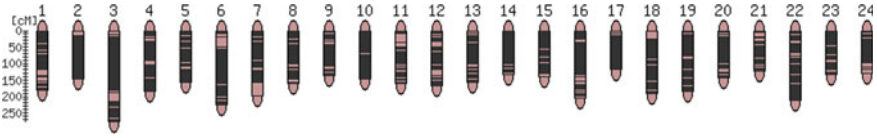

Fig. 10.2 *N. tabacum* 30 k Infinium HD consensus map 2015 (https://solgenomics.net/cview/map.pl?map_version_id=178)

whole-genome sequencing of an intraspecific RIL population, a F_1 generation and their parents and identified SNPs. Using the *N. tabacum* (K326 cultivar) genome as reference, a total of 45,081 identified SNP markers (with 7038 bin markers) were characterized to construct a high-density SNP genetic map of flue-cured tobacco spanning a genetic distance of around 3487 cM (Fig. 10.3). Tong et al. (2021) successfully constructed another high-density genetic map containing 24,142 SNP markers using a BC_4F_3 population derived from inbred of flue-cured tobacco lines Y3 (recurrent parent) and K326 (donor parent). This map included 4895 bin markers with a genetic distance of 2885.36 cM and an average genetic distance of 0.59 cM. Based on the genotype of these located markers, a binmap was also constructed using the chromosome as a unit.

Lu et al. (2012) developed a high-density integrated linkage map (2291 cM) of flue-cured tobacco that included 851 markers [238 DArT and 613 SSR] in 24 linkage groups. Gong et al. (2016) generated a high-density 2662.43 cM length integrated genetic map of flue-cured tobacco containing 4215 SNPs and 194 SSRs distributed

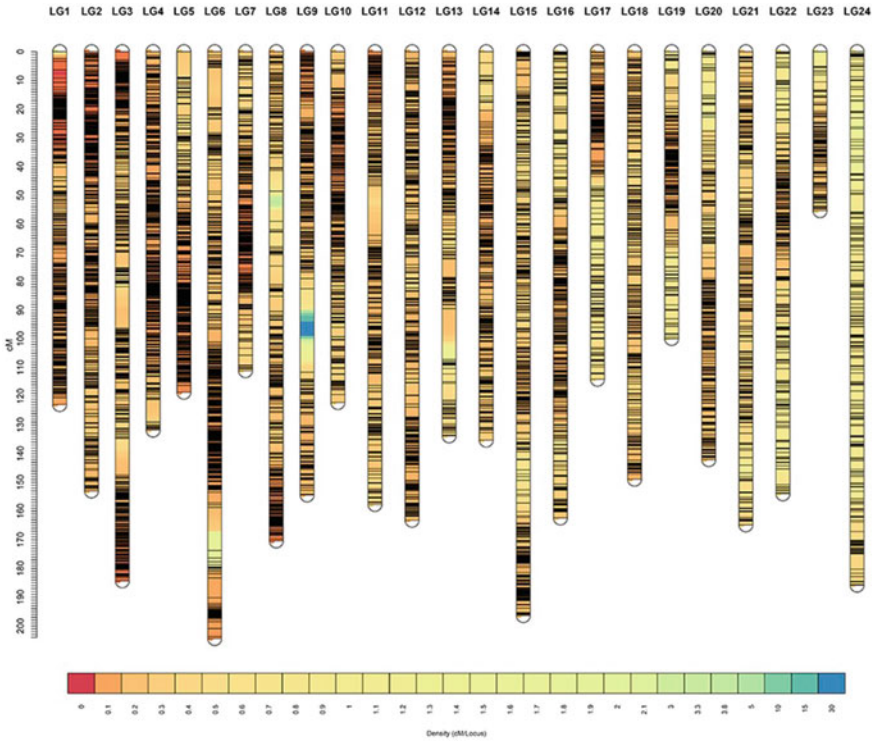

Fig. 10.3 Linkage map constructed based on the tobacco reference genome by Tong et al. (2020). These linkage maps were constructed with 45,081 SNP markers (with 7038 bin markers) covering a total length of 3486.78 cM

on 24 linkage groups (LGs) with an average distance of 0.60 cM between adjacent markers.

10.7.5.4 Intra Type Genetic Maps

Ma et al. (2008) constructed an intra type genetic map containing 26 linkage groups and 112 markers by using flue-cured and burley tobaccos based on SRAPs and ISSR markers.

Currently, the high-density maps in tobacco are constructed with SSR (Bindler et al. 2011) and SNP (Gong et al. 2016; Tong et al. 2020) markers. The widely referred SSR map of Bindler et al. (2011) was constructed with 2318 microsatellite markers covering a total length of 3270 cM while the SNP map of Tong et al. (2020) covers 3486.78 cM with 45,081 SNPs. The combination of SNPs and genetic maps, if developed, helps in designing precise breeding strategies and genomic selection in tobacco. Diverse genetic maps constructed can be effectively utilized in mapping QTLs, positional cloning, comparative genomics analysis, marker-assisted breeding and genomic selection etc. It would be necessary to further build the genetic linkage maps of tobacco in different cultivating types for their effective utilization in breeding of those types.

10.7.6 Enumeration of Mapping of Simply-Inherited Stress Related Traits

Availability of draft *Nicotiana* genome sequences (Sierro et al. 2014; Edwards et al. 2017)and high-density molecular maps in recent times are laying the foundation for trait discovery and fine mapping of trait of interest in tobacco (Yang et al. 2019). Linked molecular markers to various biotic stress namely black root rot, black shank, wildfire, blue mold, brown spot, powdery mildew, TMV, PVY, TSWV, root-knot etc. are identified in tobacco for their use in breeding programs are enumerated at Table 10.6. Majority of the markers found to be dominant ones.

10.7.7 Framework Maps and Markers for Mapping Resistance QTLs

Framework maps are being constructed mostly using SSR and SNP markers that are identified and mapped to linkage groups in tobacco (Tong et al. 2020; Lu et al. 2012). High density SSR map of Bindler et al. (2011) constructed with 2318 microsatellite markers covering a total length of 3270 cM and the SNP map of Tong et al. (2020) covering 3486.78 cM with 45,081 SNPs can be used in constructing framework maps

Table 10.6 Biotic stress resistance traits and linked markers in tobacco

Resistance trait	Gene	Marker	Linkage	Gene action	Reference
Black root rot, (ex *N. debneyi*)	–	SCAR	1 cM	Dominant	Julio et al. (2006)
Black root rot, (ex *N. debneyi*)	–	CAPS	Closely linked	Co-dominant	Qin et al. (2018)
Black shank (race 0)(ex *N. longiflora*)	*Phl*	SCAR	Closely linked	Dominant	Unpublished, personal communication
Black shank (race 0)(ex *N. plumbaginifolia*)	*Php*	RAPD	Closely linked	Dominant	Johnson et al. (2002)
Black Shank (Race 0) Coupling Phase	*Php*	SCAR	Closely linked	Dominant	Unpublished, personal communication
Black Shank (Race 0) Repulsion Phase	*Php*	SCAR	Closely linked	Dominant	Unpublished, personal communication
Wildfire (race 0)	–	RAPD	Closely linked	Dominant	Yi et al. (1998a)
Blue mold	–	SCAR	Closely linked	Dominant	Milla et al. (2005)
Blue mold	–	SCAR	Closely linked	Dominant	Julio et al. (2006)
Blue mold	–	SCAR	Closely linked	Dominant	Wu et al. (2015)
Brown spot	–	RAPD	Closely linked	Dominant	Zhang et al. (2008b)
Powdery mildew	*NtMLO1* *NtMLO2*	SSCP	Gene Specific	Co-dominant	Fujimura et al. (2015)
TMV	*N-gene*	SCAR	Genes specific	Dominant	Lewis (2005)
N-Gene (*N. Glutinosa* virus resistance)	*N-gene*	Gene specific	Gene specific	Dominant	Whitham et al. (1994)
Potyvirus susceptibility	*eiF4E1.S*	Gene specific	Gene specific	Dominant	Dluge et al. (2018)
PVY	*va*	SCAR	5.1 cM	Dominant	Julio et al. (2006)
PVY	*va*	RAPD	Closely linked	Dominant	Lewis (2005)
PVY	*va (eIF4E)*	Gene Specific	Gene Specific	Dominant	Julio et al. (2014)
PVY RBV	*eIF(iso)4E*	dCAPS	Mutation Specific	Co-dominant	Takakura et al. (2018)
TSWV	AFLP	SCAR	<5 cM	Dominant	Moon (2006)

(continued)

Table 10.6 (continued)

Resistance trait	Gene	Marker	Linkage	Gene action	Reference
Root-Knot, *M. incognita*	*Rk*	RAPD	Closely Linked	Dominant	Yi et al. (1998b)

Source CORESTA Guide No. 16—March 2019 (Yang et al. 2019)

while mapping various traits (Edwards et al. 2017).The release of the SNP-based high density genetic map, *N. tabacum* 30 k Infinium HD consensus map 2015 can be one of the finest resources for fine mapping any trait of interest (https://solgenomics.net/cview/map.pl?map_version_id=178).

RAPD and SSR markers are the most widely used markers in QTL mapping of biotic traits in tobacco followed by SNP and AFLP markers (Table 10.7). In few cases, RAPD markers are converted into SCAR markers for reproducibility and reliability. SCAR markers are developed after sequencing RAPD bands and designing 18–25 base PCR primers that can specifically amplify the sequenced DNA segment more reliably. CAPS, COS, Random amplified microsatellite polymorphism (RAMP), ISSRs and Target region amplification polymorphism (TRAP) are some of the other markers used. CAPS markers developed are the primers designed on the known sequence of a gene of Interest. COS primers used are universal primers based on sequence alignments of orthologs (genes that are conserved in sequence and copy number) from multiple solanaceous species. RAMP markers include SSR primers that amplify the genomic DNA in the presence or absence of RAPD primers (Liu et al. 2009b). TRAP are two PCR-based primers, one from target EST and the other is an arbitrary primer.

10.7.8 QTL Mapping Software Used

Mapmaker/Exp 3.0 is the most widely used QTL mapping software in QTL mapping various biotic traits in tobacco followed by various version of Join Map and Map Chart (Table 10.7). Mapmaker/QTL, QTL IciMapping 4.1, QTL Network 2.1, R/QTL, AYMY-SS, Stat Graphics Plus 5.0 and QTL Cartographer V 2.5 are some of the other softwares used.

10.7.9 Details on Trait Wise QTLs

QTLs have been identified for various biotic traits in tobacco for their use in gene introgression and genome selection (Table 10.7). Details of QTLs developed for resistance to Bacterial wilt, Brown spot, Black Shank and CMV are briefly discussed here.

Table 10.7 Details of few trait wise QTLs identified in tobacco

Trait	Cross	Population	Marker	Condition	QTL(s)	Linkage groups	Position (cM)	Variance (R^2 (%) c	LOD	Gene action		Software used	References
										Additive	Dominance		
Bacterial wilt	TI448AxEnshu	$F_{2:3}$ & $F_{2:4}$	SSR	Pooled	*qBWR-3a*	3	19.6	9	4.6	0.5	0.27	Mapmaker/Exp 3.0	Qian et al. (2013)
	TI448AxYanyam 97				*qBWR-3b*	3	21.1	19.7	6.17	0.03	0.3		
	TI448AxEnshu				*qBWR-5a*	5	68.8	17.3	4.12	0.08	0.31		
	TI448AxYanyam 97				*qBWR-5b*	5	43.6	17.4	3.18	0.03	0.28		
Brown spot	Jingyehuang × NC82	F_2	SSR	Field	*Jimo_2014*			18	7	−1.6	0.5	JoinMap 4.1, MapChart 2.2,	Sun et al. (2018)
		$F_{2:3}$			*Jimo_2014*			8.6	3.5	−5.1	0.4		
		$F_{2:3}$			*Jimo_2015*			10.1	4.2	−7.6	2.2		
		$F_{2:3}$			*Zhuzeng_2015*			9.4	3.9	−7.7	−2.0		
	Changbohuang × Jinyehuang	F_2	SSR	-	*qBS 2*	2a		8.1	4.9	8	−0.89	Mapmaker/ Exp 3.0b	Tong et al. (2012a)
					qBS 3	3a		14.3	6.3	10	12		
					qBS 5	5		8.7	5.11	8.3	−2.3		
Black Shank	Beinhart-1000 × Hicks	DH	Mircosatellite	Field	*1*		8	11.9	25	13		JoinMap 4	Ma et al. (2019)
					2		4	40.8	20	11			
					3		11	5.8	6.2	4.6			
					4		14	4	5.5	3.3			
	K326 x Beinhart 1000	Near isogenic lines (NILs), Sub NILs	Microsatellite, SNP	Field	*1*	7	76	35	15.9			R/QTL	Ma et al. (2020)
					2	15	108	25	12.4				
					3	17	88	8.5	4.8				

(continued)

Table 10.7 (continued)

Trait	Cross	Population	Marker	Condition	QTL(s)	Linkage groups	Position (cM)	Variance (R^2 (%) c	LOD	Gene action		Software used	References
										Additive	Dominance		
	Beinhart 1000–1 × Xiaohuangjin 1025	BC_1F_2	SSR	Field	*qBS7*	7	8.9	17.4	4.98	−6.9		Join Map 4.1	Zhang et al. (2018)
					qBS17	17	114.26	11.6	3.12	−1.62			
		$BC_1F_{2:3}$			*qBS7*	7	7.9	25.6	8.04	−16.82			
					qBS17	17	110.26	6.9	2.46	−9.37			
		$BC1F_{2:3}$			*qBS7*	7	7.9	17.48	5.55	−15.14			
					qBS17	17	111.26	10.30	3.48	−12.34			
	Yunyan 85 × Dabaijin 599	RILs	SNP	5 environments	*qBS7*	7	56	48.8	32	19.08	qBS7	QTL Ici Mapping 4.1	Gong et al. (2020)
				5 environments	*qBS14*	14	72.8	6.78	4.96	−7.3	qBS14		
				4 environments	*qBS16*	16	114	4.05	2.9	−5.7	qBS16		
				3 environments	*qBS24*	24	59.67	2.97	3.1	5.58	qBS24		
				3 environments	*qBS1*	1	46	2.63	2.47	−4.6	qBS1		
	Florida 301 × Hicks	RIL	Microsatellite	Field	*1*	7	62	16.9	8.7			Join-Map version 4.0, MapChart	Xiao et al. (2013)
				Green house				18.6	11.7				
				Field	*2*	24	9.5	9.2	3.5				

(continued)

Table 10.7 (continued)

Trait	Cross	Population	Marker	Condition	QTL(s)	Linkage groups	Position (cM)	Variance (R^2 (%) c	LOD	Gene action		Software used	References
										Additive	Dominance		
				Green house				15.3	7.04				
				Field	*3*	14	4.9	6	3				
				Green house				10	8.1				
				Field	*4*	3	23	–	–				
				Green house				5.6	4				
				Field	*5*	6	67	7.1	4.1				
				Green house				5.3	2.6				
				Field	*6*	4a	83	4.2	2.7				
				Green house				6.9	5				
				Field	*7*	11a	10	6.6	5.9				
				Green house				1.2	4.9				
CMV at seedling stage	TT8 × NC 82	F_1 and BC_1F_1	SNP	Seedling	*qID5*	5	40	7.70	3.39	−12.19	–	Join Map 4 Map Chart 2	Cheng et al. (2019)
					qD18	8	88	7.20	3.24	−5.00			

10.7.9.1 Resistance to Bacterial Wilt Disease

The resistance for bacterial wilt in tobacco is governed by either polygenes or in combination of polygenes and a major gene (Smith and Clayton 1948; Kelman 1953; Matsuda 1977; Hayward 1991). Furthermore, the influence of environment makes difficulty in screening/phenotyping, hence it is essential to identify closely linked molecular marker to developing resistant varieties through marker-assisted selection. Earlier, QTLs affecting the resistance to bacterial wilt have been reported on chromosome 6, chromosome 7, and chromosome 12 (Margin et al. 1999; Danesh et al. 1994). Nishi et al. (2003) detected a QTL for the bacterial wilt resistance of W6 explaining more than 30% of the variance. Qian et al. (2013) detected four QTLs viz. *qBWR-3a, qBWR-3b, qBWR-5a* and *qBWR-5b* in linkage group 3 and 5 strongly associated with resistance and explaining 9.00, 19.70, 17.30, and 17.40% of the variance in resistance, respectively. These loci had the close linkage with the markers PT20275 and PT3022. Lan et al. (2014) identified eight QTLs with significant main effects on chromosomes 2, 6, 12, 17 and 24 through genome wide QTL analysis. Also detected a major QTL (*qBWR17a*) on chromosome 17 which explained up to 30% of the phenotypic variation.

10.7.9.2 Resistance to Black Shank Disease

Clayton (1958) suggested that resistance to black shank disease in tobacco was simply inherited and controlled by recessive alleles. Moore and Powell (1959) reported resistance to be partially dominant and affected by modifying factors. Others have suggested that in the line Florida 301 resistance to be polygenic and additive in nature (Crews et al. 1964; Chaplin 1966). Incomplete penetrance and variable expressivity have probably complicated the interpretation of data generated to investigate the inheritance of resistance in this line. Vontimitta and Lewis (2012a, b) suggest that Beinhart 1000 and Florida 301 share a major gene affecting black shank resistance, but probably differ in allelic variability at a fair number of additional loci having smaller effects. The genetic control of black shank resistance in Florida 301 is of the classic polygenic type and that it is controlled by a combination of a few genes with large effects and a greater number of genes with small to intermediate effects. Zhang et al. (2018) identified stable QTLs (*qBS7 and qBS17*) for resistance to black shank disease using the F_2, $BC1F_2$ individuals and $BC1F_{2:3}$ lines derived from a cross between Beinhart 1000-1 and Xiaohuangjin 1025 (Fig. 10.4). QTL *qBS7* was mapped to the region between PT30174 and PT60621 and explained 17.40–25.60% of the phenotypic variance under various conditions. One major QTL, labeled as *Phn7.1*, was found to be the largest contributor to partial *P. nicotianae* resistance in the highly black shank resistant cigar tobacco cultivars, Beinhart 1000 and Florida 301 and highly resistant flue-cured tobacco cultivar, K346 (Vontimitta and Lewis 2012b; Xiao et al. 2013; Ma et al. 2019). A second QTL, *Phn15.1* with a large effect was identified on *N. tabacum* linkage group 15 in Beinhart 1000 (Ma et al. 2020). Gong et al. (2020) fine mapped the QTLs using the 177 $F_{7:8-9}$ recombinant

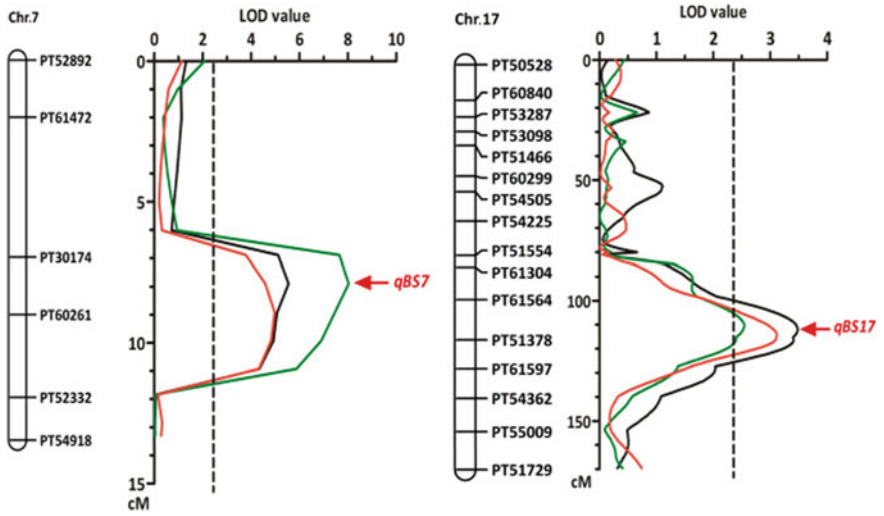

Fig. 10.4 Likelihood plots and position (cM) of the QTL associated with resistance to black shank in different conditions. Red line: LOD plots for the BC_1F_2 population; Green line: LOD plots for the $BC_1F_{2:3}$ population in the field; Black line: LOD plots for the $BC_1F_{2:3}$ population in the greenhouse; dashed lines represent the significant LOD threshold at the level of 2.35 (Zhang et al. 2018)

inbred lines generated from a cross between a resistant cultivar 'Yunyan 85' and a susceptible entry 'Dabaijin 599'. A total of 10 QTLs associated with resistance to *P. nicotianae* across multiple environments were detected and two major QTL *qBS7 and qBS14* were repeatedly identified under all five environments explaining 56 and 6.78% of the mean phenotypic variance with high logarithm of the odds (LOD) scores, respectively.

10.7.9.3 Resistance to Brown Spot

The cigar tobacco line Beinhart 1000 and flue-cured tobacco variety Jingyehuang are considered important sources of partial resistance in the USA and China, respectively. Beinhart 1000 is derived from a selection of tobacco Quin Diaz and presents a high level of partial resistance to brown spot. Using SSR markers, Tong et al. (2012a), detected three QTL for resistance to brown spot in tobacco on 2, 3, and 5 linkage groups using a F_2 population derived from a cross between a brown spot susceptible variety Changbohuang and the resistant source Jingyehuang (Fig. 10.5). The major QTL mapped on the genetic linkage group three found to explain 14.3% of the phenotypic variation. In a study, Sun et al. (2018) evaluated F_2, $F_{2:3}$ and $BC_3F_{2:3}$ populations developed from a cross between a source of brown spot resistance Jingyehuang and a brown spot susceptible flue-cured variety NC82 for field resistance under different environments and identified QTLs by linkage mapping.

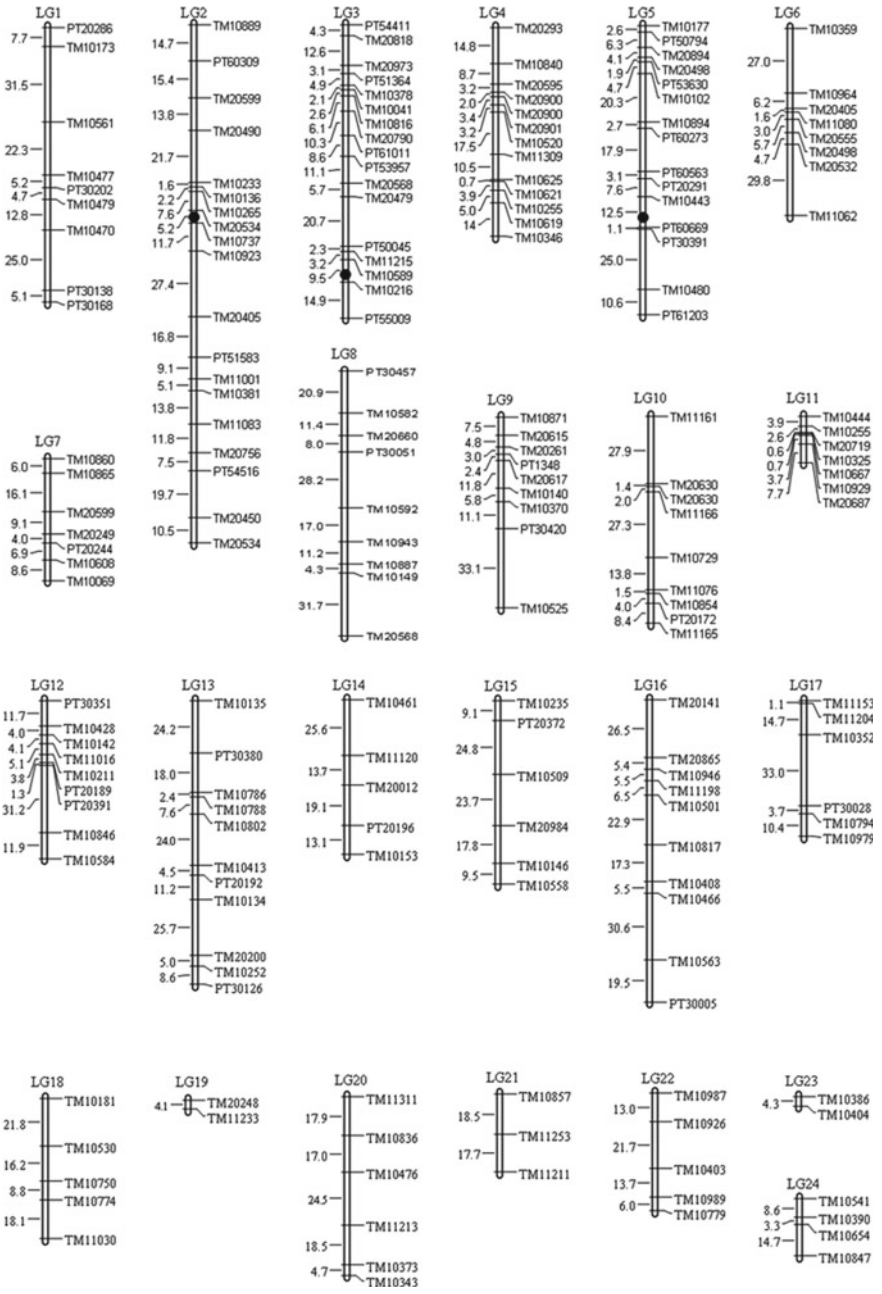

Fig. 10.5 Mapping of quantitative trait loci conferring resistance to brown spot in flue-cured tobacco (*N. tabacum*). Three QTLs for brown spot resistance were mapped on LG2a, LG3b and LG5, respectively (Tong et al. 2012a)

A major QTL mapped on chromosome 15 explained 8.6–18.0% of the phenotypic variation under diverse conditions. Furthermore, based on association mapping using 219 accessions for their responses to brown spot at two sites, six significant marker-trait associations were detected. Out of these markers, the marker Indel53 exhibited the most significant association with resistance to brown spot and explained around 21% of the phenotypic variation at the two sites. An approximately 2-Mb physical interval at the locus of marker Indel53 contained 31 predicted genes and two of these genes (*Nitab 4.5_0000264g0050.1 and Nitab 4.5_0000264g0130.1*) were identified as probable candidate genes for resistance to brown spot.

10.7.9.4 Resistance to *Cucumber Mosaic Virus*

Cheng et al. (2019) identified seven QTLs, including two for incidence of disease and four for disease index for CMV in BC_1F_1 population at seedling stage. qID5 for incidence of disease was mapped to the interval mk6533–mk646 on LGs 5 explaining 7.70% of the total phenotypic variance. For disease index, qDI8 mapped on LG 8 explained 7.20% of total phenotypic variance indicating stable genetic effects in diverse environments.

10.7.10 Mendelization of QTL

The QTL*, Phn7.1* was found to have an additive effect on resistance to black shank disease and the corresponding QTL was localized to within a genetic interval of approximately 3 cM (Ma et al. 2019).A second QTL*, Phn15.1* on *N. tabacum* linkage group 15 in Beinhart 1000 was localized to a genetic interval of approximately 2.7 centimorgans using subNILs containing varying amounts of Beinhart 1000-derived *Phn15.1*-associated genetic material (Ma et al. 2020). Incorporation of this allelic variability into breeding programs could increase the level, range, and durability of genetic resistance to *P. nicotianae* in to be released newly tobacco cultivars. However, *Phn15.1* is very closely linked to the gene *NtCPS2* associated with *Z*-abienol biosynthesis (Vontimitta et al. 2010). *Z*-abienol is a trichome exudate that contributes to flavor and aroma characteristics of Oriental and some cigar tobacco but is considered undesirable for flue-cured and burley tobacco. For the effective utilization of *Phn15.1* in flue-cured and burley tobacco cultivar development, Ma et al. (2020) disassociated the favorable Beinhart 1000 *Phn15.1* alleles from the Beinhart 1000 *NtCPS2* allele.

10.8 Marker-Assisted Breeding for Resistance Traits

Marker-assisted breeding (MAB) refers to a breeding program in which detection of DNA markers and selection of desirable genotypes are integrated. The status and prospects of MAB are discussed here under.

10.8.1 Germplasm Characterization and DUS

Germplasm are to be characterized for desirable traits and tightly linked molecular markers for the traits need to be identified. Identification of reliable linked markers is critically important to initiate a marker-assisted breeding program. Polymorphism for DNA markers are available throughout the genome and DNA markers can be detected at any stage of plant growth. The presence or absence of these markers is not affected by environments and usually do not directly affect the phenotype. Identification and selection of markers located in close proximity to the target gene or within the gene will ensure the success in selection of the target gene. Therefore, DNA markers are the predominant types of genetic markers for MAB (Xu 2010). These tightly linked markers can be utilization in MAB while screening parents, F_1 and other segregating materials for selecting plants with desirable traits. Molecular-assisted breeding of pest/disease resistant tobacco plants can help to identify the resistant plants in early stage, cut down on workload by way of avoiding inoculation procedure of pests/disease, increase selection efficiency, accelerate rapid utilization of the resistant sources, and shorten the breeding cycle (Liu and Zhang 2008).

The information on marker trait associations in germplasm lines and mapping populations can be obtained through gene mapping, QTL analysis, association mapping, classical mutant analysis, linkage or recombination analysis, bulked segregant analysis, etc. It is also essential to know the linkage state i.e. cis/trans (coupling or repulsion) linkage with the desired allele of the trait. Most commonly used molecular markers in tobacco include RAPD, AFLP, SSR, SCAR, CAPS, dCAPS (derived CAPS), and KASP (Yang et al. 2019). Each type of DNA markers has advantages and disadvantages for specific purposes. Relatively speaking, SSRs have most of the desirable features and availability of large number of SSRs make them markers of choice in tobacco. SNPs require a detailed understanding of single nucleotide DNA changes responsible for genetic variation among individuals. Fairly large number of SNPs have become available in tobacco making them important choice of markers for MAB in tobacco.

Genetic mapping, QTL analysis and association mapping (AM) have accelerated the dissection of genetic control of biotic stress resistant traits in tobacco. Large number of studies for screening germplasm and sources of resistance are made in tobacco to identify closely linked markers to various biotic stresses for their utilization introgression into cultivated varieties. Tightly linked markers are important tools for DUS characterization of varieties also. Tightly linked molecular markers/genic

markers/QTLs were identified for some of the traits conferring resistance to various biotic stresses particularly diseases in tobacco are listed at Tables 10.6 and 10.7. This information has the potential to make marker-assisted selection (MAS) a successful option for tobacco improvement.

10.8.2 Marker-Assisted Gene Introgression

Marker-assisted backcrossing (MABC) is the simplest form of marker-assisted gene introgression that is most widely and successfully used in transferring biotic stress resistant genes into elite cultivars. MABC aims to transfer one or a few genes/QTLs for resistance from one genetic source (donor parent) into a superior cultivar or elite breeding line (recurrent parent) to improve the stress resistance. In contrast to traditional backcrossing, MABC depends on the molecular markers linked to gene(s)/QTL(s) of interest in the place of phenotypic performance of target trait. MABC program with two types of selections viz. foreground selection for the marker allele(s) of donor parent at the target locus (ex. Resistance) and background selection for the marker alleles of recurrent parent in all genomic regions of desirable traits (agronomic traits) except the target locus may be made effective transfer of resistance into elite genotypes (Hospital 2003). Foreground selection ensures the transfer of target trait from donor parent and background selection takes care of the genome recovery of recurrent parent.

MAS can be used when other characters are to be combined from two parents along with resistance trait. However, MAS will be more effective for simply inherited character controlled by a few genes than for a highly complex character governed by large number of genes. In tobacco, SCAR markers were used in marker-assisted breeding of black shank, black root rot, PVY, blue mold, and TMV disease resistance breeding (Whitham et al. 1994; Johnson et al. 2002; Milla et al. 2005; Julio et al. 2006; Li and Miller 2010).

10.8.3 Gene Pyramiding

Pyramiding of multiple genes/QTLs may be achieved through multiple-parent crossing or complex crossing, multiple backcrossing, and recurrent selection. A suitable breeding scheme for marker-assisted gene pyramiding depends on the number of genes/QTLs required to be transferred, the number of parents that contain the desired genes/QTLs, the heritability of traits of interest, and other factors (e.g. cost of genotyping). Pyramiding of three or four desired genes/QTLs existing separately in three or four lines can be realized by three-way, four-way or double crossing, convergent backcrossing or stepwise backcrossing. More than four genes/QTLs can be pyramided by way of complex or multiple crossing and/or recurrent selection.

Gene pyramiding through MABC may be achieved through three different strategies or breeding schemes viz. stepwise, simultaneous/synchronized and convergent backcrossing or transfer. In the stepwise backcrossing, the target genes/QTLs are transferred from donor parents into the recurrent parent (RP) in order one after the other. In the first step of backcrossing, one gene/QTL is targeted and transferred, followed by next step of backcrossing for another gene/QTL, until all target genes/QTLs are introgressed into the RP. The advantage is that gene pyramiding through stepwise backcrossing is more precise and easier to adopt as it involves only one gene/QTL at a time requiring small population size and lower genotyping cost. The disadvantage of this method is it takes a longer time to complete. In the simultaneous or synchronized backcrossing, the recurrent parent is first crossed to each of the donor parents and the resultant single-cross F_1s are crossed with each other to produce two double-cross F_1sand then the two double-cross F_1s are crossed again to produce a hybrid integrating all target genes/QTLs in heterozygous state. The hybrid and/or progeny with heterozygous markers for all the target genes/QTLs are subsequently crossed back to the RP until the satisfactory recovery of the RP genome. Finally, homozygosity of the RP genome recovered line can be achieve through selfed. Simultaneous or synchronized backcrossing takes shorter time to transfer multiple genes, however, requires a large population and more number of genotyping as all target genes/QTLs are involved at the same time. Convergent backcrossing uses both stepwise and synchronized backcrossing strategies. First each of the target gene/QTLs from the donors are transferred separately into the recurrent parent through single crossing followed by backcrossing based on the linked markers to produce improved lines. The improved lines are crossed with each other and the resultant hybrids are then intercrossed to integrate all the genes/QTLs together to develop the final improved line with all the genes/QTLs pyramided. Convergent backcrossing not only reduce time (compared to stepwise transfer) but also easily fix and/or pyramid genes (compared to simultaneous transfer).

Marker-assisted complex or convergent crossing (MACC) can be undertaken to pyramid multiple genes/QTLs if all the parents are improved cultivars with complementary genes or favorable alleles for the traits of interest. In MACC, the hybrid of convergent crossing is self-pollinated for several consecutive generations along with MAS for target traits until genetically stable lines with desired marker alleles and traits have been developed. Detection and selection of most important genes/QTLs in early generations and less important markers in later generations can effectively reduce population size and avoid loss of important genes/QTLs.

Theoretically, application of MABC and MACC for pyramiding target genes/QTLs is possible in tobacco through various schemes discussed above. However, information is not currently available about the release of commercial cultivars resulted using these strategies.

10.8.4 Limitations and Prospects of MAS and MABC

Though MAS and MABC breeding has number of advantages, it may not be universally useful (Jiang 2013). Rapid DNA extraction technique and a high throughput system of marker detection are essential to handle a large number of samples and a large-scale screening of multiple markers in breeding programs. Development of suitable bioinformatics and statistical software packages are required for meeting the efficient and quick labeling, storing, retrieving, processing and analyzing large data set requirements, and even for integrating data sets available from other programs. Hence, the startup expenses and labor costs involved in MAS and MABC breeding are higher than conventional techniques making them not in the reach of all the researchers (Morris et al. 2003).

As the distance between the marker and the gene of interest increases, the chance of recombination between gene and marker increases there-by make the selection of resistant plants based on marker ineffective due to false positives. This may be avoided with the use of flanking markers on either side of the locus of interest in order to increase the probability that the desired gene is selected. Sometimes markers that were used to detect a locus may not be 'breeder-friendly'. Such markers viz. RFLP and RAPD may need to be converted into more reliable and easier to use markers. RFLP markers may be converted to STS (sequence tagged site) for detection via PCR protocols (Ribaut and Hoisington 1998) and RAPD markers into SCAR markers for reliable and repeatable amplifications (Milla et al. 2005; Lewis 2005). RAPD technique may be considered less reliable for MAS as RAPD results vary from lab to lab, largely due to low binding specificity of short (10-base) PCR primers. Hence, SCAR markers are developed by sequencing RAPD bands and designing more specific 18–25 base PCR primers to amplify the same DNA segment more reliably. Imprecise estimates of QTL locations and effects may result in slower progress than expected through MAS (Beavis 1998). Sometimes markers developed for MAS in one population may not be suitable for screening other populations due to absence of polymorphism for identified markers or lack of marker-trait association.

MAB is going to become a powerful and reliable tool in genetic manipulation of agronomically important traits in tobacco in view of the increasing utilization of molecular markers in various fields' viz. germplasm evaluation, genetic mapping, map-based gene discovery, characterization of traits etc. Currently available high density linkage maps in tobacco provide a framework for identifying marker-trait associations and selecting markers for MAB. Markers linked to resistant traits discussed in previous sections can effectively be utilized in MAB in tobacco. However, only the markers that are closely associated with the target traits or tightly linked to the genes can offer adequate promise for the success in practical breeding. Availability of new high-throughput marker genotyping platforms for the detection of SSR and SNP markers along with the sequencing information of cultivated and wild relatives of *Nicotiana* going to have a great impact on discovering marker trait associations that can be used for MAS in the future. Array-based methods such as DArT (Lu et al. 2012) and single feature polymorphism (SFP) detection

(Rostoks et al. 2005) offer prospects for lower-cost marker technology that can be used for whole-genome scans in tobacco. Rapid growth in genomics research and huge data generated from functional genomics in tobacco in the recent years is leading to the identification of many candidate genes for numerous traits including biotic stress resistance. SNPs within candidate genes could be extremely useful for 'association mapping' and circumvents the requirement for construction of linkage maps and QTL analysis for the genotypes that have not been mapped previously. The availability of large numbers of publicly available markers and the parallel development of user-friendly databases (Sol genome network, NCBI etc.) for the storage of marker and QTL data, increasing number of studies on genes and marker trait associations will undoubtedly encourage the more widespread use of MAS in tobacco.

Selection for all kinds of traits at seedling stagein MAB helps to minimize the costs as undesirable genotypes are eliminated at early stages. Closely linked markers allow the selection of disease/pest resistance traits even without the incidence of pests and diseases. MAS based on reliable markers tightly linked to the multiple genes/QTLs for traits of interest can be more effective in pyramiding desirable genes than conventional breeding. Use of co-dominance markers (e.g. SSR and SNP) in MAB allow effective selection of recessive alleles in the heterozygous state without selfing or test crossing, thus saves time and accelerate breeding progress. As more and more newer techniques are available genotypic assays based on molecular markers may be faster, cheaper and more accurate than conventional phenotypic assays and thus MAB may result in higher effectiveness and higher efficiency in terms of time, resources and efforts saved in future.

Conventional breeding methodologies have extensively proven successful in development of tobacco cultivars and germplasm. Subjective assessment and empirical selection plays a significant role in conventional breeding. As a new addition to the whole family of plant breeding, MAB has brought great challenges, opportunities and prospects for breeding crops including tobacco. However, as transgenic breeding or genetic manipulation does, MAB cannot replace conventional breeding but is a supplementary addition to conventional breeding. High genotyping costs and technical/equipment requirements of MAB will be major limiting factors for its large-scale deployment in the near future, especially in the developing countries (Collard and Mackill 2008). Therefore, integration of MAB into conventional breeding programs will be an optimistic strategy for tobacco improvement in the future. It can be expected that the drawbacks of MAB will be gradually overcome leading to its wide spread adoption in practical breeding programs as its theory, technology and application are further developed and improved.

10.9 Map-Based Cloning of Resistance Genes

10.9.1 Traits and Genes

High levels of redundancy between genes in the large and complex genome of tobacco with the absence of molecular markers and genomic resources till recent years made the identification and subsequent mapping of interesting mutants a very difficult prospect. However, having anchored 64% of the genome assembly to chromosomal locations in recent years, a possibility now exists to apply map-based biotic stress resistant gene discovery approaches in the species (Edwards et al. 2017). First reported instance of successful map based cloning in tobacco was done by Edwards et al. (2017) and cloned *NtEGY1* and *NtEGY2* homeologous candidate genes for *YB1* and *YB2* loci conferring white stem phenotype in recessive condition in burley tobacco. However, map-based cloning of pest and disease resistant genes based on genetic maps are not yet reported in tobacco.

10.9.2 Strategies: Chromosome Landing and Walking

Chromosome landing and walking strategies are used in identification of clones carrying gene of interest for map based cloning. Recently available high density genetic maps, genome sequences and Bacterial artificial chromosome (BAC) clones are paving the way for map based cloning of resistance genes in tobacco.

In the only reported case of map based cloning in tobacco, Edward et al. (2017) used a specific technique to clone genotyped pairs of NILs carrying dominant or recessive alleles of the *YB1* and *YB2* loci (cultivars SC58, NC95, and Coker 1) with a custom 30 K Infinium iSelect HD BeadChip SNP chip (Illumina Inc., San Diego, CA) used in developing a high density genetic map (*N. tabacum* 30 k Infinium HD consensus map 2015; https://solgenomics.net/cview/map.pl?map_version_id=178). Genomic regions comprising SNP polymorphisms that distinguished the nearly isogenic lines were identified and SNP markers closely linked to the loci were aligned to the genome assembly and predicted potential candidate genes. Coding regions of candidate genes were then amplified from first-strand cDNA from tobacco cultivars K326 and TN90 using the primers specifically designed. Amplified fragments were then cloned into a vector.

10.9.3 Genomic Libraries

Availability high-capacity genomic libraries are essential resources for physical mapping, comparative genome analysis, molecular cytogenetics etc. Such libraries

are also powerful tools for large-scale gene discovery, elucidation of gene function and regulation, and map-based cloning of target trait loci or genes associated with important agronomic and resistant traits for their further study and use in crop improvement programs. BAC libraries are the large DNA insert libraries (inserts of DNA up to 200,000 base pairs) of choice for genomics research. Cloning of larger DNA segments (more than 1000 kb) are possible with Yeast Artificial Chromosome (YAC) libraries and greatly facilitates chromosome walking and physical mapping around the target locus. While, transformation-competent artificial chromosome (TAC) libraries make it possible to clone and transfer genes efficiently into plants. In recent years, BAC libraries are constructed and utilized in tobacco for genome sequencing, mapping and comparative genome analysis. However, construction of YAC and TAC libraries are not currently reported in tobacco.

Tobacco Genome Initiative (TGI) generated BACs library (9.7-fold genome coverage) for assembling the partial genome of Hicks Broadleaf variety (Opperman et al. 2003; Rushton et al. 2008). Sierro et al. (2013b) used 425,088 BAC clone library for construction of physical map and ancestral annotation of tobacco cultivar, Hicks Broadleaf. Edwards et al. (2017) constructed two libraries containing 150,528 BACs from K 326 variety using *Hind*III or *Eco*RI, with average insert sizes of 115 kb and 135 kb, respectively (representing ~8 × coverage of the genome) and used for generating a whole-genome profile (WGP) map from sequence reads at *Eco*RI and *Hind*III restriction sites. Jingjing (2018) reported a tobacco genome sequence of the HongDa cultivar, which has been produced by the combination of BAC-to-BAC and whole-genome shotgun technologies. Dong et al. (2020) constructed a BAC library of 414,720 clones using blank shank resistant flue-cured tobacco line, 14–60 with an average insert size of 123 kb ranging from 97.0–145.5 kb covering 11 times of genome equivalents. Further confirmed the utility of this library by screening the library with gene specific primers. A BAC library of wild tobacco, *N. tomentosiformis*, one of the parent of *N. tabacum* was constructed with inserted DNA size ranging from 50 to 200 kb and an estimated average size of 110 kb (Yuhe 2012). These libraries are important resources for map based cloning of resistant traits through various strategies.

10.9.4 Test for Expression (Mutant Complementation)

Transformation of a cloned gene into mutant plant and looking for wild phenotype rescue will help to validate the function of the target gene. However, currently mutant complementation studies with cloned genes are not reported in tobacco due to the absence of map based cloning of functional genes in general and biotic stress resistant genes particular.

10.10 Genomics-Aided Breeding for Resistance Traits

10.10.1 Structural and Functional Genomic Resources Developed

The aim of structural genomics is to characterize the structure of the genome. Understanding the genome structure of an individual can be advantageous in manipulating genes and DNA fragments in that species. In tobacco, huge data on genomic resources have been generated from in-depth genomic studies by various researchers. These resources are available for furthering the research in the area of genomics, gene tagging, identification, isolation and cloning for genome assisted breeding in tobacco as well as other crop species.

With advances in next-generation sequencing (NGS) technologies, decoding the genome sequences of 12 *Nicotiana* species viz. *N. tabacum, N. rustica, N. attenuata, N. benthamiana, N. knightiana, N. obtusifolia, N. otophora, N. paniculata, N. undulata, N. tomentosiformis, N. sylvestris* and *N. glauca* has been completed. Sequencing details of *Nicotiana* Spp. are presented at NCBI website (https://www.ncbi.nlm.nih.gov/) and Sol Genome Network (SGN) (Asaf et al. 2016).

Plastid genomes of 10 tobacco species: *N. tabacum, N. attenuata, N. tomentosiformis, N. sylvestris, N. otophora, N. knightiana, N. rustica, N. paniculata, N. obtusifolia* and *N. glauca* are sequenced and data made available (Asaf et al. 2016; Mehmood et al. 2020).

The developments in Transcriptomics have resulted in the development of large data sets and tools for the progression of functional genomics in tobacco. A database of 2513 tobacco (*N. tabacum*) TFs representing all of the 64 well-characterized plant TF families created using a dataset of 1,159,022 gene-space sequence reads (GSRs) (Rushton et al. 2008). Further, the transcriptional activity for thousands of tobacco genes in different tissues throughout the lifecycle of the tobacco from seed to senescence based on tobacco expression microarray from a set of over 40 k unigenes (a set of transcripts that appear to stem from the same transcription locus) and gene expression in 19 different tobacco samples has been generated (Edwards et al. 2010). 772 of 2513 transcription factors earlier recognized in tobacco were mapped to the array, with 87% of them being expressed in at least one tissue in the generated Tobacco Expression Atlas (TobEA). Based on the co-expression of these transcription factors, putative transcriptional networks could be identified. SGN contains the collection of transcriptome sequences of *N. sylvestris* (32,276), *N. tomentosiformis* (31,961) and *N. tabacum* (26,284) from transcriptome projects and unigenes data sets of *N. sylvestris* (6300), *N. tabacum* (84,602) and *N. benthamiana* (16,024).

The data hosted at NCBI and SGN databases includes majority of the available genomic resources information pertaining to cultivated tobacco and wild *Nicotiana* spp. Hence, the available information in these databases was discussed here as an indicator of genome resource availability in *Nicotiana.* Large collection of data on nucleotides, genes and protein sequences submitted by various researchers on *Nicotiana* are available at NCBI site (Table 10.8). More than 33 lakh nucleotide

Table 10.8 Nucleotide, gene, SRA, GEO and protein sequences of *Nicotiana* spp. available at NCBI website (as on 30.09.2021)

Species	Nucleotide sequences	Gene sequences	Sequence read archive (SRA) data	Gene expression omnibus (GEO) data	Proteins
N. alata	238	–	–	–	265
N. amplexicaulis	–	129	–	–	174
N. attenuata	185,245	40,175	455	496	78,686
N. benthamiana	97,525	–	1570	585	1009
N. bonariensis	–	–	13	14	–
N. clevelandii	–	–	12	14	–
N. debneyi	61	129	–	–	195
N. glauca	107	129	30	14	335
N. glutinosa	196	–	–	–	184
N. knightiana	72,026	–	50	–	–
N. langsdorffii × *N. sanderae*	12,576	–	–	40	124
N. langsdorffii	59	–	–	–	–
N. linearis	–	–	6	–	–
N. megalosiphon	296	–	–	–	–
N. miersii	–	–	12	–	–
N. obtusifolia	–	–	17	–	116
N. occidentalis	–	–	7	–	–
N. otophora	–	155	8	–	240
N. paniculata	67,473	–	44	–	126
N. pauciflora	–	–	13	–	–
N. plumbaginifolia	710	–	22	23	404
N. repanda	133	129	–	–	260
N. rosulata	–	–	11	–	–
N. rustica	112,008	–	102	14	132
N. stocktonii	–	129	–	–	195
N. suaveolens	–	131	–	–	224
N. suaveolens × *N. tabacum*	140	–	–	–	–
N. sylvestris	385,277	40,544	41	22	49,040
N. tabacum	2,053,476	74,274	2589	1203	92,478
N. tomentosiformis	263,306	45,486	38	–	50,492
N. undulata	59,446	156	42		262
Nicotiana spp.	177	–	–	2438	–

(continued)

Table 10.8 (continued)

Species	Nucleotide sequences	Gene sequences	Sequence read archive (SRA) data	Gene expression omnibus (GEO) data	Proteins
Total	3,310,475	201,566	5082	4863	274,941

sequences of 20 *Nicotiana* spp. that includes genomic DNA/RNA, mRNA, cRNA, ncRNA, rRNA, tRNA and transcribed RNA is generated by various researchers and is available at NCBI website as on 30.09.2021 (Table 10.8). Among them, around 895,700 sequences are comprehensive, integrated, non-redundant, well-annotated set of reference sequences including genomic, transcript, and protein. These sequences also included 456,507 expressed sequence tags (ESTs) and 1,420,639 genomic survey sequences (GSS). Further, a total of over 201,560 records of gene sequences belonging to 12 *Nicotiana* spp. viz. *N. tabacum, N. tomentosiformis, N. sylvestris, N. attenuata, N. undulate, N. otophora, N. suaveolens, N. glauca, N. stocktonii, N. repanda, N. amplexicaulis and N. debneyi* are existing at NCBI (Table 10.8).

Sequence Read Archive (SRA) data is the largest publicly available repository of high throughput sequencing data and is accessible through several cloud providers and NCBI servers. The archive admits data from different branches of life including metagenomic and environmental surveys. SRA stores raw sequencing data and alignment information to enhance reproducibility and facilitate new discoveries through data analysis (https://www.ncbi.nlm.nih.gov/sra). Nearly, 5080 records of SRA data of 20 *Nicotiana* spp. are available at NCBI website (as on 30.09.2021) (Table 10.8). Around 4860 curated gene expression datasets as well as original series and platform records of 11 *Nicotiana* spp. are available at Gene Expression Omnibus (GEO) repository of NCBI as on 30.09.2021 (Table 10.8; https://www.ncbi.nlm.nih.gov/gds). Around 275,000 collection of protein sequences from several sources, including translations from annotated coding regions in GenBank, RefSeq and Third Party Annotation (TPA) Sequence, as well as records from other data bases are available for 20 *Nicotiana* spp. at NCBI (Table 10.8).

Genomic resource collection of SGN include information on genome sequences, transcriptome sequences, mRNAs, and predicted proteins of one or the other five wild *Nicotiana* spp. namely *N. attenuata, N. benthamiana, N. tomentosiformis, N. sylvestris* and *N. otophora* and four versions of *N. tabacum.* SGN also hosts 39 transcript libraries of *N. tabacum* and two of *N. sylvestris* are also present. Though both NCBI and SGN utilize the information from same bio-projects, the number of sequences, predicted proteins, CDS and mRNA for different species are not similar in these databases due the use of different bioinformatics softwares and gene prediction model in analyzing the sequences.

The proteomic data generated globally is stored and accessed through the Universal Protein Resource (UniProt). UniProt provides several sets of proteins (proteomes) thought to be expressed by organisms whose genomes have been

completely sequenced (https://www.uniprot.org/). There are 73,606 protein entries associated with *Nicotiana tabacum* proteome (UP000084051) in the UniProt database as on 31.03.2021.

10.10.2 Details of Genome Sequencing

10.10.2.1 Nuclear Genome Sequencing

Though, tobacco have the same basic chromosome number of $n = 12$ as of other solanaceous crops such as tomato, potato, chili and eggplant, its genome size (4.5 Gbp) is largest in the Solanaceae (Arumuganathan and Earle 1991) with a large proportion of repetitive sequences (Zimmerman and Goldberg 1977; Kenton et al. 1993; Leitch et al. 2008; Sierro et al. 2014). *N. tabacum* genome is 50% larger than the human genome. Analysis of the tobacco genome has been started in the last decade. Tobacco Genome Initiative (TGI) have initiated the first tobacco genome sequencing project in 2003 by the with the aim of sequencing the open reading frames of *N. tabacum* utilizing the methyl-filtration technology for reducing genome complexity (Opperman et al. 2003; Rushton et al. 2008). The Hicks Broadleaf variety, ancestor for some of the currently cultivated flue-cured tobacco cultivars, was chosen as the genotype for generation of bacterial artificial chromosome (BAC) libraries used for sequencing because of its low introgression content (Sierro et al. 2013b). The project was completed in 2007 and the sequences are available from the National Center for Biotechnology Information (NCBI) GenBank (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA29349). Around 689 Mb of genomic sequence generated through Sanger sequencing was assembled into 81,959 contigs with an average length of 1.2 kb and 871,255 singletons with an average length of 688 bp (Wang and Bennetzen 2015). However, the generated sequences comprised only a small portion of the tobacco genome, mainly because the sequencing technology (methylation filtration) was employed for only enriched genes, and for the portions of genes that are under methylated relative to TEs.

With the advances in next-generation sequencing (NGS) technologies, there is a rapid progress in sequencing of entire genomes of cultivated tobacco and important wild relatives in recent times (Table 10.9). Sequencing of three *N. tabacum* cultivars have been completed since 2014. Eleven complete genomes of wild *Nicotiana* species viz. *N. knightiana, N. paniculata, N. rustica, N. glauca, N. obtusifolia, N. otophora, N. attenuata, N. sylvestris, N. tomentosiformis, N. undulata* and *N. benthamiana* were sequenced and released since 2013. The scaffold level assemblies of *N. sylvestris* and *N. tomentosiformis* having 94.0× and 146.0× genome coverage and a length of 2222 and 1688 Mb, respectively being the first sequences released in 2013 (Sierro et al. 2013a). This was followed by the release of three genome assemblies of *N. tabacum,* one at Scaffold level (cv. TN90) and two at contig (cv. K326 and Basma Xanthi -BX) level by Philip Morris International in 2014 (Sierro et al. 2014). The length of these assemblies was around 3700 Mb with a GC content of 39% and genome coverages

Table 10.9 Genome sequencing details of *Nicotiana* species as (available at NCBI site)

Genome	Date published (dd-mm-yyyy)	Submitter	Assembly level	Assembly	Statistics	Genome coverage	Sequencing technology
N. sylvestris	08-05-2013	Philip Morris International R&D	Scaffold	Scaffolds: 253,918; Contigs: 392,998; N50: 26,744; L50: 21,230	Total length (Mb): 2221.99; GC%: 39.80	94.0×	Illumina HiSeq
N. tomentosiformis	10-05-2013	Philip Morris International R&D	Scaffold	Scaffolds: 159,548; Contigs: 215,609; N50: 34,051; L50: 13,932	Total length (Mb): 1688.47; GC%: 39.0999	146.0×	Illumina HiSeq
N. benthamiana	27-05-2014	National Academy of Agricultural Sciences	Contig	Scaffolds: 100,480; Contigs: 100,480; N50: 776; L50: 22,990	Total length (Mb): 61.9511; GC%: 39.6	79×	–
N. tabacum cv.TN90 (RefSeq Genome)	29-05-2014	Philip Morris International R&D	Scaffold	Scaffolds: 168,247; Contigs: 292,601; N50: 40,422; L50: 25,298	Total length (Mb): 3643.47; GC%: 39.2006	49.0×	Illumina HiSeq

(continued)

Table 10.9 (continued)

Genome	Date published (dd-mm-yyyy)	Submitter	Assembly level	Assembly	Statistics	Genome coverage	Sequencing technology
N. tabacum cv. K326 (sequencing & assembly)	29-05-2014	Philip Morris International R&D	Contig	Scaffolds: 582,565; Contigs: 711,416; N50: 24,673; L50: 40,137	Total length (Mb): 3732.64; GC%: 39	38.0×	Illumina HiSeq
N. tabacum cv.:BX (sequencing & assembly)	29-05-2014	Philip Morris International R&D	Contig	Scaffolds: 643,545; Contigs: 797,404; N50: 21,713; L50: 44,668	Total length (Mb): 3735.82; GC%: 39	29.0×	Illumina HiSeq
N. otophora	05-06-2014	Philip Morris International R&D	Contig	Scaffolds: 929,607; Contigs: 1,121,520; N50: 11,316; l50: 44,787	Total length (Mb): 2689.35; GC%: 39.1	66.0×	Illumina HiSeq
N. attenuata strain: UT (RefSeq, sequencing & assembly)	14-11-2016	Max Planck Institute for Chemical Ecology	Chromosome (12 haploid)	Scaffolds: 37,194; Contigs: 103,240; N50: 64,201; L50: 9648	Total length (Mb): 2,365.68; GC%: 41.333	30.0×	Illumina HiSeq

(continued)

Table 10.9 (continued)

Genome	Date published (dd-mm-yyyy)	Submitter	Assembly level	Assembly	Statistics	Genome coverage	Sequencing technology
N. obtusifolia	06-03-2017	Max Planck Institute for Chemical Ecology	Scaffold	Scaffolds: 53,128; Contigs: 93,877; N50: 39,219; L50: 9106	Total length (Mb): 1222.77; GC%: 38.4	50.0×	Illumina HiSeq
N. attenuata ecotype Arizona (sequencing & assembly	06-03-2017	Max Planck Institute for Chemical Ecology	Scaffold	Scaffolds: 951,503; Contigs: 2,556,970; N50: 1153; L50: 283,638	Total length (Mb): 1827.78; GC%: 29.1	18.0×	Illumina HiSeq
N. tabacum cv. K326 (sequencing and assembly)	28-06-2017	British American Tobacco	Scaffold	Scaffolds: 937,112; Contigs: 1,257,801; N50: 9081 L50: 108,397	Total length (Mb): 4646.65; GC%: 33.5	86×	Illumina HiSeq
N. glauca	16-02-2018	Saint Petersburg University	Scaffold	Scaffolds: 514,289 Contigs: 1,214,276; N50: 5814; L50: 143,017	Total length (Mb): 3222.83; GC%: 36.6	35.0×	Illumnina

(continued)

Table 10.9 (continued)

Genome	Date published (dd-mm-yyyy)	Submitter	Assembly level	Assembly	Statistics	Genome coverage	Sequencing technology
N. knightiana	02-05-2019	Philip Morris International R&D	Scaffold	Scaffolds: 160,415; Contigs: 368,149; N50: 28,120; L50: 23,129	Total length (Mb): 2298.94; GC%: 39.7	82.0×	Illumina HiSeq
N. paniculata	02-05-2019	Philip Morris International R&D	Scaffold	Scaffolds: 181,977 Contigs: 289,116; N50: 32,292; L50: 19,574	Total length (Mb): 2190.56; GC%: 39.4	100.0×	Illumina HiSeq
N. rustica	02-05-2019	Philip Morris International R&D	Scaffold	Scaffolds: 337,581 Contigs: 863,270; N50: 14,482; L50: 79,215	Total length (Mb): 4231.29; GC%: 40	90.0×	Illumina HiSeq
N. undulata	02-05-2019	Philip Morris International R&D	Scaffold	Scaffolds: 117,566 Contigs: 240,737; N50: 28,381; L50: 19,751	Total length (Mb): 1914.3; GC%: 40	112.0×	Illumina HiSeq

ranging from 29.0 to 49.0×, while the assembly of TN 90 is used as reference genome. Reference genome is a comprehensive, integrated, non-redundant and well-annotated set of sequences for a given genome. Later, an improved version of *N. tabacum* (cv. K 326) assembled at scaffold level covering the 86.0× of genome (4600 Mb) with a GC content of 33.5% was submitted by British American Tobacco in 2017 (Edward et al. 2017). This assembly covers over 4 Gb of non-N sequence (90% of predicted genome size), which is an increase from 3.6 Gb (81% of predicted genome size) in the previously published version (Sierro et al. 2014). This assembly achieved an N50 size of 2.17 Mb and anchored 64% of the genome to pseudomolecules; a significant increase from the previous value of 19% providing more complete coverage of the tobacco genome.

Currently, a total of 16 assemblies of 12 *Nicotiana* species viz. *N. sylvestris, N. tomentosiformis, N. benthamiana, N. tabacum, N. otophora, N. attenuata, N. obtusifolia, N. glauca, N. knightiana, N. paniculata, N. rustica* and *N. undulata* are obtainable at NCBI genbank with 18.0× to 146.0× genome coverages. Two assemblies are available for *N. attenuata* and four for *N. tabacum. N. attenuata* reference sequence (2366 Mb) is assembled at chromosomal level (12 haploid), while *N. tabacum* (K326, and Basma Xanthi) and *N. benthamiana* at contig level. All the other *Nicotiana* assemblies are available at scaffold level. Genome coverage of the sequences range from 18.0× in *N. attenuata* strain UT to 146.0× in *N. tomentosiformis*. GC content of the assemblies ranges from 29.1 to 41.3% and length ranges from 62 Mb in *N. benthamiana* (diploid genome) to 4647 Mb in *N. tabacum* cv. K326 (tetraploid). Most of the genomes were sequenced using high throughput Illumina Hiseq sequencing technology. The detail statistics including assembly level and their N50 and L50 values for each genome was also provided at Table 10.9.

Genomic resource collection of five wild *Nicotiana* spp. and four of *N. tabacum* are available at Sol Genome Network (SGN) (Fernandez-Pozo et al. 2015). They include contig level methylation filtered genome sequences generated under TGI project, and genomes, predicted proteins, and mRNAs *N. tabacum* cv. BX, *N. tabacum* cv. K326 and *N. tabacum* cv. TN90 and genome scaffolds, proteins and cDNA of improved K326 assembly (Sierro et al. 2014; Edwards et al. 2017). Further, scaffold level genome assemblies of five *Nicotiana* species namely *N. attenuata, N. tomentosiformis, N. benthamiana, N. sylvestris* and *N. otophora* also available at this database along with predicted proteins and mRNA for first four species. Chromosome level assembly is available for *N. attenuata.* Though the source of these assemblies is same as of NCBI, the sequence info is not similar due to differences in bioinformatics softwares employed in analysing the sequences.

10.10.2.2 Organelle Genome Sequencing

Plastid and mitochondrial genomes of tobacco are circular DNA molecules. Sequencing of chloroplast genome of tobacco, for the first time, was completed in 1986 (Shinozaki et al. 1986) and till then number of studies made to sequence chloroplast genomes of different entries of *N. tabacum* and its wild relatives. Currently,

Table 10.10 *Nicotiana* organelle genome resources available at NCBI (as on 31.03.2021)

Organelle reference genome	Sequence length (bp)	Archived complete genomes	PopSet	Genes	Proteins
Plastid					
N. tabacum (RefSeq)	155,943	124	10	144	98
N. attenuata	155,886	15	1	129	84
N. tomentosiformis	155,745	30	–	150	102
N. sylvestris	155,941	42	1	149	101
N. otophora	156,073	8	–	155	108
Mitochondrial					
N. tabacum	430,597	6	4	183	153
N. attenuata	394,341	1		68	40
N. sylvestris	430,597	1	1	64	37

sequencing of about 219 complete plastid genomes including 12 popset related to five *Nicotiana* spp. are completed (Table 10.10). The PopSet data is a collection of related DNA sequences derived from population, phylogenetic, mutation and ecosystem studies. The size of the plastid genome of *Nicotiana* species is around 0.156 Mb. In addition, recently, Mehmood et al. (2020) assembled the plastid genomes of five more tobacco species: *N. knightiana* (155,968 bp), *N. rustica* (155,849 bp), *N. paniculata* (155,689 bp), *N. obtusifolia* (156,022 bp) and *N. glauca* (155,917 bp) and made comparisons among themselves. Reference plastid genomes of five *Nicotiana* species namely *N. tabacum* (155,943 bp), *N. attenuata* (155,886 bp), *N. tomentosiformis* (155,745 bp), *N. sylvestris* (155,941 bp), *and N. otophora* (156,073 bp) are available at NCBI (Table 10.9).

Sequencing of mitochondrial complete genome of tobacco started in 2003 (Sugiyama et al. 2005)). Eight mitochondrial complete genome sequences including 5 popsets from three *Nicotiana* species namely *N. tabacum* (430,597 bp), *N. attenuata* (394,341 bp) and *N. sylvestris* (430,597 bp) are completed till date and the details are available at NCBI site (Table 10.10). Further, reference mitochondrial genomes are made available for *N. tabacum, N. attenuata* and *N. sylvestris*at the NCBI.

10.10.3 Gene Annotation

Genome annotation is the process of identifying functional elements along the sequence of a genome. Assigning function to genome sequence is necessary because the sequencing of DNA produces sequences of unknown function. Once a genome is sequenced, it needs to be annotated to understand its functions for its further successful utilization in genetic manipulation. In tobacco, both nuclear and organelle genomes are successfully annotated and annotation reports are available at various

data bases. Gene annotation records available at NCBI and SGN databases are discussed here they host majority data of the *Nicotiana* sp.

NCBI Eukaryotic Genome Annotation Pipeline is an automated annotation pipeline that annotates genes, transcripts and proteins on draft and finished genome assemblies. Currently, annotation reports are available for four *Nicotiana* species viz. *N. sylvestris*, *N. tabacum* cv. TN 90, *N. attenuata* strain UT and *N. tomentosiformis* at NCBI website (Table 10.11). The first annotation was released in 2014 for *N. sylvestris* followed by *N. tabacum* cv. TN 90 (in 2016), *N. attenuata* strain UT (2016) and *N. tomentosiformis* (2020). Before annotation the repeat sequences in the reference sequences were masked with RepeatMasker making use of curated libraries of

Table 10.11 Gene annotation reports of *Nicotiana* species (as per NCBI)

Species	*N. sylvestris*	*N. tabacum*	*N. attenuata*	*N. tomentosiformis*
Genome assembly name	Nsyl	Ntab-TN90	NIATTr2	Ntom_v01
Annotation release number	100	100	100	102
Release date	22-10-2014	04-05-2016	06-12-2016	21-04-2020
RefSeq scaffolds	253,917	168,245	37,135	159,547
% Masked with RepeatMasker	1.33%	3.00%	1.33%	1.49%
% Masked with WindowMasker	48.35%	51.43%	47.93%	53.07%
Genes and pseudogenes	40,317	73,946	39,977	45,485
• Protein-coding	33,678	61,526	34,094	31,842
• Non-coding	4667	9019	3886	11,549
• Pseudogenes	1972	3401	1997	2094
• Genes with variants	9033	14,549	6,946	10,545
mRNAs	48,059	84,001	44,491	50,010
• Known RefSeq (NM_)	0	0	0	18
• Model RefSeq (XM_)	48,059	84,001	44,491	49,992
Other RNAs	10,984	17,907	7156	17,677
• Known RefSeq (NR_)	0	0	0	0
• Model RefSeq (XR_)	10,212	16,461	6069	16,867
Coding sequences (CDS)	48,059	84,001	44,491	50,112
• Known RefSeq (NP_)	0	0	0	18
Model RefSeq (XP_)	48,059	84,001	44,491	50,094
Alignment of the annotated proteins to Ref Seq proteins				
A protein with an alignment covering 50% or more of the query		51,484	27,475	25,881
An alignment covering 95% or more of the query		23,115	12,029	11,730

Table 10.12 Gene annotation reports of *Nicotiana* species (as per SGN)

Nicotiana species	Reference scaffolds	Predicted proteins	mRNA
N. attenuata	37,182	33,449	33,449
N. benthamiana	56,094	57,140	–
N. tomentosiformis	159,548	53,753	85,853
N. sylvestris	253,917	54,497	87,234
N. tabacum cv. BX	420,216	86,009	146,748
N. tabacum cv. K326	382,373	85,994	145,503
N. tabacum cv. TN90	249,104	122,388	189,413
N. tabacum v4.5 (Edwards et al. 2017)	1,084,432	69,500	–

repeats and WindowMasker software (Morgulis et al. 2006). RepeatMasker masked 1.33–3.0% of genomes while WindowMasker masked 47.93–53.07%. Based on gene prediction tools, genes and pseudogenes, protein-coding and non-coding sequences etc. were estimated for all four genomes. While, alignments with RefSeq and other known transcripts and proteins were made to predict mRNAs, other RNAs and proteins. The final set of annotated proteins from the *Nicotiana* species was aligned against the available known RefSeq proteins from *Arabidopsis thaliana*, utilizing the high-quality proteins as the target and the annotated proteins as the query. Protein with an alignment covering 50% or more of the query and 95% or more of the query are identified in each of the annotated *Nicotiana* species. The details of the annotations are given at Table 10.11.

Annotated gene predictions are made available for published genomes of *N. attenuata*, *N. benthamiana, N. tomentosiformis, N. sylvestris* and 4 versions of *N. tabacum* at SGN site also (Table 10.12). For *N. tabacum,* predicted proteins were ranging from 69,500 to 122,388 and mRNA from 145,503 to 189,413. However, less number of proteins (33,449–54,497) and mRNA (33,449–87,234) were predicted for *Nicotiana* species compared to *N. tabacum.*

Nicotiana tabacum proteome (UP000084051) in the UniProt database contains 73,606 protein entries as on 31.03.2021 (https://www.uniprot.org/). Biotic stress response related proteins are also included in this list. Edward et al. (2017) identified predicted proteins showing good cross-over with the related *Solanaceae* species tomato and potato in addition to other flowering plants based on gene ontology analysis.

Annotations are also done for published organelle genome sequences predicting genes and proteins (Table 10.10). For plastid genomes, predicted genes in five *Nicotiana* spp. vary from 129 to 155 and proteins from 84 to 108. In mitochondria, number of genes and proteins predicted are more for cultivated species, *N. tabacum* (183; 153, respectively) than for wild species, *N. attenuata* (68; 40) and *N. sylvestris* (64; 37).

10.10.4 Impact on Germplasm Characterization and Gene Discovery

Sequencing of *Nicotiana* spp. made it possible to compare the genomes of *Nicotiana* spp. among themselves and with other solanaceous crops. These studies assisted in identifying the relationships between cultivated and wild species, and their progenitor species in terms of sequence similarities and genome rearrangements (Wu et al. 2009; Sierro et al. 2014; Asaf et al. 2016; Gong et al. 2016; Edwards et al. 2017). The synteny between the genomes of *N. tabacum* cv. TN90, K326 and BX and those of tomato and potato could be evaluated at the protein level which leads to the detection of homologous genes (Sierro et al. 2014).

Annotation of published genome sequences assisted in the identification of functional sequences, and predicted mRNAs and proteins that can be expressed in tobacco. Though comparison of genome assemblies, genomic regions responsible for virus tolerance was recognized in draft genomes (Sierro et al. 2014). Nearly perfect match of *N. glutinosa N* gene (source of TMV resistance in many tobacco cultivar) sequence was found on the draft genome sequence of a TMV resistant cultivar, TN 90 and weak identity in susceptible genomes (K326 and BX genomes). Genes that are differentially expressed between PVY resistant and susceptible RIL plants were identified based on comparison with a reference transcriptome (Julio et al. 2014). Further, through annotation of differentiation expressed genes, Julio and co-workers confirmed that functional *eIF4E* gene mapped on chromosome 21 of the tobacco genetic map is responsible for PVY susceptibility in majority of the tobacco lines carrying dominant *Va* locus. One copy each of *eIF4E1*, *eIF4E2* and *eIF (iso) 4E* gene were identified in the *N. sylvestris* genome, whereas two copies of *eIF4E1* and one copy of the other genes in *N. tomentosiformis* (Sierro et al. 2014). Except the *N. sylvestris eIF4E1* gene in TN90, all identified *N. sylvestris* and *N. tomentosiformis* genes were observed in TN90, K326 and BX genomes. With this observation, Sierro et al. (2014) confirmed that the genomic deletion of the S-form *eIF4E1* locus is responsible for TVMV, TEV and PVY resistance in TN90 and its presence for susceptibility in K326 and BX.

Based on sequencing of *Nicotiana* genomes and EST data, large number of SSRs and SNPs were identified (Bindler et al. 2011; Tong et al. 2012b, 2020; Cai et al. 2015; Xiao et al. 2015; Thimmegowda et al. 2018; Wang et al. 2018). The identified markers have been used for characterization of germplasm, diversity studies, DUS testing and genetic relatedness of cultivated varieties etc. (Moon et al. 2008, 2009a, b; Davalieva et al. 2010; Fricano et al. 2012; Gholizadeh et al. 2012; Prabhakararao et al. 2012; Xiang et al. 2017; Binbin et al. 2020). Wang et al. (2021) used core markers developed based on genotyping-by-sequencing for varietal identification and fingerprinting of cigar tobacco accessions. The high-density maps developed based on SSR and SNP markers will be useful in characterization of germplasm, and identification of target traits including biotic stresses. Genome-wide SNP markers were used for simultaneous association analysis of leaf chemistry traits in natural populations of tobacco germplasms (Tong et al. 2020). SSRs and SNPs were used

to identify QTLs linked to biotic stress resistant traits viz. Bacterial wilt (Qian et al. 2013), Brown spot (Tong et al. 2012a; Sun et al. 2018), black shank (Ma et al. 2019, 2020; Gong et al. 2020) and CMV (Cheng et al. 2019).

The release of *N. tabacum* 30 k Infinium HD consensus map 2015 also provides the tobacco genetic research community with resources to detect genome-wide DNA polymorphisms, fine map and clone their trait of interest. Genome-wide DNA polymorphisms could be detected using the custom 30 K Infinium iSelect HD BeadChip SNP chip (Edwards et al. 2017). Map based cloning of target traits is becoming a reality in tobacco with cloning of two homeologous candidate genes conferring white stem phenotype in recessive condition in burley tobacco (Edwards et al. 2017).

Advances in tobacco genomics provide further means to advance the understandings of diversity at species and gene levels, and allows DNA markers to hasten the pace of genetic improvement. Discovery of novel genes/alleles for any given trait could be obtained through genotyping-by-sequencing, whole-genome re-sequencing, Sequence-based mapping etc. Genomics tools also enable rapid identification and selection of novel beneficial genes and their controlled incorporation into germplasm. Similarly, genome-wide association studies (GWAS) could help to identify the genomic regions controlling traits of interest in diverse collection of germplasms that are genotyped and phenotyped for traits of interest through statistical associations between DNA polymorphisms and trait variations. Genomics possesses the potential to increase the diversity of alleles available to breeders through mining the gene pools of crop wild relatives (CWRs).

10.10.5 Application of Structural and Functional Genomics in Genomics-Assisted Breeding

Recent advances in high-throughput sequencing and phenotyping platforms are transforming molecular breeding to genomics-assisted breeding (GAB). GAB is going to be the key in designing future tobacco cultivars though optimizing the tobacco genomes with accumulation of beneficial alleles and eradicating deleterious alleles (Varshney et al. 2021). Availability of draft *Nicotiana* genomes, transcriptome and metabolome profiles is paving the way for understanding the genomic areas, their ancestral origins, genes, gene products, expression patterns, control elements and associated allelic variations responsible for resistance to biotic stresses. Availability of molecular markers (Sects. 10.5.2 and 10.7.2), high density genetic maps (Sect. 10.7), structural and functional resources (Sect. 10.10), identification of DNA markers linked to traits of interest, identification and mapping of trait specific QTLs in recent years is going to be the starting points for the utilization of GAB an important tool for tobacco improvement.

Currently, application of structural and functional genomics in GAB in tobacco is in initial stage. Application of DNA markers to facilitate marker-aided selection (MAS), a preliminary form of GAB, for tobacco improvement is in commencement

stage. As progress is made in genomics, genotypic and phenotypic datasets on training populations can be used to develop models to predict the breeding value of lines for genomic selection (GS). All the available marker data for a population can be used as predictors of breeding value of a line. Breeding value serves as a predictor of how well a plant will perform as a parent for crossing and generation advance in a breeding program, based on the resemblance of its genomic profile to other plants in the training populations that are known to have performed better in the target environment(s). The genotypic data obtained from a seed or seedling on all the favorable alleles can be used to predict the phenotypic performance of mature individuals without the need for extensive phenotypic evaluation over years and environments during GAB (Varshney et al. 2014). The time required for breeding will be drastically come down due to selection of desirable lines in early generations with accuracy without the influence of environment.

10.11 Recent Concepts and Strategies Developed

Genome designing involves all biotechnological interventions that results in accumulation of desirable alleles and elimination of undesirable alleles and gene combinations for realizing maximum possible genetic potential with tolerance to various stresses. In addition to MAS and genetic engineering, gene editing and nanotechnology are emerging as key concepts in genome designing of crop plants. Gene modification with these techniques are briefly discussed here.

10.11.1 Gene Editing

Gene editing (GE) techniques have revolutionized biological sciences via precise modifications in the genome of both plants and animals to yield desirable changes in the phenotypes. These technologies make structural changes to DNA of target genes or epigenetic changes to alter gene expression. The techniques used to edit or change the genomes are evolved from the earlier attempts like nuclease technologies, homing endonucleases, and certain chemical methods (Khan 2019). Molecular techniques like meganuclease (MegaN), TALENs, and ZFNs initially emerged as genome-editing technologies. Currently, there are several open engineering platforms available for construction of ZFNs and TALENs (Townsend et al. 2009). These initial technologies suffer from lower specificity due to their off-targets side effects. The latest discovery of the CRISPR/Cas9 nuclease system seems more encouraging in view of its higher efficiency and feasibility advancing the genome-engineering techniques to the level of molecular engineering. GE is broadly categorized into three generations: MegaN and ZFNs are first–generation tools, TALENs are second-generation tools, and CRISPR associated system is considered the third-generation tool. Third-generation GEs, e.g., CRISPR/Cas9, CRISPR–CRISPR from

Prevotella and *Francisella* 1 (Cpf1) were found to be powerful tools for the successful modification of genome sequence in a precise and straightforward manner (Ahmar et al. 2021). In terms of delivery to the plant cell, techniques such as ZFN technology, TALENs and CRISPR/Cas9 typically use either *Agrobacterium*-mediated or protoplast transformation.

Meganuclease, also termed molecular DNA scissors originate from microorganisms such as bacteria and yeasts and recognize relatively large DNA sequences (18 to 30 building blocks) in comparison with standard nucleases (Daboussi et al. 2015). Only very specific fragments that seldom occur in plant DNA are cut by meganucleases. Their potential to excise large pieces of DNA sequences was recognized as a genetic tool to modify DNA (Khan 2019). Double-strand breaks could be obtained at the target region in the genome under consideration through a sequence-specific nuclease. However, Meganuclease technology is costly and time-consuming (Townsend et al. 2009). The advantage with meganucleases is less toxicity in view of their natural occurrence and site specific cleavage ability. Modifying the target genes with specifically engineered meganucleases was first demonstrated in tobacco (Puchta 1999; Honig et al. 2015).

ZFNs were demonstrated as site-specific nucleases for cutting DNA at strictly defined sites, for the first time, in 1996. ZFNs are proteins composed of a zinc finger part and a nuclease part. By coupling the nuclease to a zinc finger, a protein that binds with great accuracy on a specific DNA fragment, the nuclease will only be able cut DNA at that location. Repair of ZFN-induced double-strand breaks (DSBs) with error-prone non-homologous end-joining (NHEJ) will result in introduction of insertion or deletion mutations (indels) at the site of the DSB. Alternatively, homology-directed repair of a DSB with an exogenously introduced donor template can promote introduction of alterations or insertions at or near the break site (Sander et al. 2011). ZFNs have been used to introduce specific mutations and transgene insertions that confer herbicide resistance in tobacco (Townsend et al. 2009).

TALEN effectors for DNA targeting were discovered in 2009. TALENS almost resemble ZFNs in terms of manufacturing and mode of action (Khan 2019). They are made by a similar principle where a restriction nuclease (*Fok*I) is bound to a DNA-binding protein domain called TAL effector that guides the nuclease to a specific DNA sequence. Fusing a nuclease and a TAL effector allow the nuclease to cut only at one specific place in the plant DNA. The construction of TALENs is quite easier and popular compared to ZFNs; however, repetitive sequences present in TALENs can enhance the rate of homologous recombination. TALENs can target 3 nt at a time thus making it slightly more site specific with fewer off-target effects compared to ZFNs that address only 1 nt. Methods were optimized for targeted modification of plant genomes using TALENs in tobacco (Zhang et al. 2013). Though, the mitochondrial genomes of higher plants, in general, are not transformable, TALENs could effectively be used to achieve targeted modification of the mitochondrial genomes of rice and Arabidopsis through mitochondrial localization signals (mitoTALENs; Arimura et al. 2020). Therefore, it is theoretically possible to engineer changes into any part of a plant's genome including tobacco using TALENs.

CRISPR Cas9/sgRNA system is a novel targeted genome-editing technique derived from bacterial immune system. It is an easy, less expensive, user friendly and quickly adopted genome editing tool and enables precise genomic modifications in many different organisms and tissues (Surender et al. 2016). Cas9 protein is an RNA guided endonuclease employed for creating targeted double-stranded breaks with a short RNA sequence to facilitate the recognition of the target site in animals and plants. Hence, CRISPR/Cas9 gene targeting requires a custom single guide RNA (sgRNA) that contains a targeting sequence (crRNA sequence) and a Cas9 nuclease-recruiting sequence (tracrRNA). The crRNA region is a 20-nucleotide sequence that is homologous to a region in target gene and will direct Cas9 nuclease activity. Development of genetically edited crops similar to those developed by conventional or mutation breeding using this technique makes it a promising and extremely versatile tool for genotype improvement. Inducible CRISPR/Cas9 system was developed to avoid constitutive expression of the Cas9 protein (Ren et al. 2020). Multiplexing could be performed using CRISPR/Cas9 simply through co-infiltration of multiple tobacco rattle viruses encoding different sgRNAs in tobacco (Ali et al. 2015). Through CRISPR/Cas9-mediated homologous recombination Hirohata et al. (2019) created three nucleotide substitutions (ATG to GCT) leading to herbicide chlorsulfuron (Cs) resistance. This technology has been used for improving seed oil in tobacco (Tian et al. 2020, 2021).

Engineered nuclease systems viz. ZFNs, TALENs, and CRISPR-Cas have emerged as innovative genome editing tools with their high genetic engineering efficiency and specificity. Successful demonstration of these techniques in tobacco promises its potential utilization in targeted editing of economically important traits in future.

10.11.2 Nanotechnology

The genetic engineering technique modifies plant cell genomes, involving the efficient delivery of modifier biomolecules as genetic cargo to targeted plant (Ahmar et al. 2021). Out of number of conventional tools for bimolecular deliveries, *Agrobacterium*-mediated transformation (AMT) and biolistic delivery of DNA are most widely used ones. AMT is being employed for the transformation of target DNA to the nuclear genome of a limited number of plant species. The AMT method results in random DNA integration, disruption of endogenous plant genes and alteration in gene expression arising from the inserted sites (Niazian et al. 2017). Biolistic delivery of DNA uses a high-pressure gene gun for directly targeting the plant tissues and randomly integrate the DNA into the chromosomal region across cell walls and membranes. This yet times results in destruction of tissues and multiple insertions in random portions of the plant genome (Toda et al. 2019). Because of the unavoidable high velocity of genetic cargo during biolistic delivery, the bombarded particles damage the cell wall through penetration and disrupt the homeostasis of target cell. Other less commonly used systems include electroporation, viral vectors

and chemical delivery. Polymer-based chemical transformation leads to cytotoxicity in plant cells because of the accumulation of high-density charged polymer-based genetic cargo. A decrease in charge results in the impairment of bioconjugated complex. The disadvantages with viral vectors are high host specificity and limited cargo size. In addition to biolistic methods, PEG-mediated transformation is one of the widely used methods for inserting genetic cargo into chloroplasts (Yu et al. 2020). This method allows to carry various genetic cargo types, such as DNA and RNAs (small interfering RNA [siRNA] and miRNA into target cells (Cunningham et al. 2018). However, it requires regeneration from protoplasts, which is highly challenging because of the limited number of plant species amenable to protoplast regeneration. Thus, plant transformation presents a major bottleneck for GE technology. Conventional biomolecule delivering technologies are time-consuming and involve complicated protocols. Thus, low efficiency of gene transmission, narrow species range for application, limited cargo types, and tissue damage are the critical drawbacks of conventional delivery methods.

Nanotechnology advancements have created opportunities to overcome the limitations of the above conventional methods. Nanoparticles (NPs) are promising for the species-independent passive delivery of various genetic cargo [DNA, RNA, proteins (site-specific recombinases or nucleases), and ribonucleoprotein (RNP)] across plant systems (Cunningham et al. 2018). Use of NPs for the transfer of biological molecules into plant cells overcome all the issues that are previously hindering the success of GE and is emerging as a promising technique for improving the effectiveness, robustness and flexibility of GE. Development of genetically modified (GM) organisms (GMOs) through nanotechnology involving the use of Nanoparticles (NPs) as nanocarriers by constructing a binding complex with biomodifier molecules (eg. CRISPR/Cas system) signifies a powerful technique for transgene delivery into plant cells (Demirer et al. 2021).

The unmatched potential of the NP-based delivery of biomolecules to plant cells has revolutionized the GE delivery process (Ahmer et al. 2021). The NP-bound GE nuclease can be efficiently transferred to plant cells without resultant damage to the target tissue. Thus, the use of NP-based methods for genetic cargo delivery has emerged as a cutting-edge technology with new insights and a robust GE (Cunningham et al. 2018). Smaller size of NPs facilitate them to transverse the cell wall and overcome the obstacles to delivering biomolecules to plant tissues without the species- and tissue-specific limitations. NPs can also be engineered to facilitate cargo delivery to any subcellular parts such as mitochondrial or chloroplast DNA that AMT cannot target.

NPs commonly used for biomolecule delivery in both animal and plant systems are classified according to the base material used: bio-inspired, carbon-based, silicon-based, polymeric, and metallic/magnetic (Cunningham et al. 2018). Each of the NP types delivers different genetic cargos. For example, carbon nanotubes (CNTs) can carry RNA and DNA, but metallic NPs can only deliver DNA as genetic cargo. Silicon-based NPs can deliver DNA and proteins, and polymeric NPs (PEG and polyethyleneimine) transfer RNA, DNA, and proteins into cells (Silva et al. 2010). Cationic NPs are preferred for gene delivery into plants as they can bind to the plant

cell wall (negatively charged) and perform gene transfer, whereas CNT NPs have been used to deliver plasmid DNA into various crops. Several NPs can penetrate the cell wall (eg. CNTs and mesoporous silica), whereas other NPs such as gold NPs and magnetic NPs (MNPs) require additional physical methods (e.g., magnetoinfection and electroporation) for genetic cargo delivery into the cells.

NP-mediated cargo delivery can be through physical and non-physical means. Creation of transient pores in the cell membrane with electric fields, soundwaves, or light, magnetofection, microinjection, and biolistic particle delivery are some of the physical methods. Utilisation of cationic carriers, incubation, and infiltration are nonphysical methods. Optimizing the use of NPs in different plant species in respect of their dose and spatiotemporal tuning is essential as NPs behave differently in specific plant cells (Ahmar et al. 2021). NPs considered to enable an efficient transformation of plants because of their ability to protect the genetic cargo from cellular enzymatic degradation (Ahmar et al. 2021).

Literature on NP–tobacco systems is mainly found to focus on genetically modified tobacco mosaic virus-based metallic nanomaterial synthesis, NP as pesticides, NP uptake, effects on plant growth, biomolecule delivery systems etc. (Burklew et al. 2012; Love et al. 2014, 2015; Wang et al. 2016; Tirani et al. 2019). Delivery of DNA into *Nicotiana tabacum* plants via biolistic delivery of 100–200-nm gold-capped mesoporous silica NPs MSNs was first demonstrated in 2007 by Torney and colleagues. Fu et al. (2012) used Zinc NPs to deliver DNA plasmid into tobacco. By contrast, NPs, such as silicon carbide whiskers (SCW) and MSN, have been effectively used to transfer genes into tobacco without using other physical methods (Golestanipour et al. 2018). However, the disadvantage with SCW method compared to other NP-mediated plant transformation is that an adequate protocol is required for plant regeneration from cell cultures. Silva et al. (2010) introduce siRNA into tobacco protoplasts using polymer NPs as an alternative gene knockout mechanism. Meanwhile, NP-mediated passive delivery of DNA plasmids has been reported with tobacco through CNTs (Burlaka et al. 2015; Kwak et al. 2019) and dsDNA through clay nano sheet NPs (Mitter et al. 2017). Demirer et al. (2019) have recently achieved passive delivery of DNA plasmids and protected siRNA using functionalized CNT NPs for transient silencing of constitutively expressed gene in transgenic *N. benthamiana* leaves with 95% efficiency.

In spite of significant advantages, few challenges are hampering the successful use of NPs in GE. The first relates to nanophytotoxicity effect of NPs on plant growth, causing damage to either the plant or the environment because of the subsequent release of NPs up to a toxic level (Ahmar et al. 2021). In general, significantly less, nontoxic level in terms of both the environment and the plant, amount of engineered NPs are required as genetic cargo. However, cell structural stability, metabolic pathway disturbance, deposition and dispersal of NPs to other plant cells after application necessitate further research to improve the use of NPs as genetic cargo. Another issue requiring attention for improving NP-mediated GE's efficacy relates to efficiency in binding of NPs to biomolecules and the breakdown of the binding complex in plant cells. Different biomolecules have a different binding affinities with various NPs based on their chemical composition, structure, surface area, and charge, making

them suitable for a bioconjugation complex. However, optimization of biomolecules specific binding requires further research to increase their versatility as genetic cargo.

10.12 Brief on Genetic Engineering for Resistance Traits

Tobacco is used widely as a model in transgenic research of plant for several reasons due to its well-studied molecular genetics, nearly complete genomic mapping, readily achievable genetic transformation, its survival ability under in vitro and greenhouse conditions, and large biomass yielding potential (Jube and Borthakur 2007). Transgenic tobacco plants are also ideal model organisms for the study of basic biological functions, such as plant-pathogen interactions, environmental responses, growth regulation, senescence, etc. In view of this, number of studies have been undertaken incorporating genes from bacteria, animals and other plant species into tobacco and their functional roles validated. Genetic engineering of tobacco plants for resistance related traits are reviewed here.

10.12.1 Target Traits and Alien Genes—Biotic Stress Resistance

Tobacco transgenics incorporated with transgenes from bacterial, other plants and virus were validated to confer resistance to various pests and diseases. These transgenic studies clearly proved that tobacco cultivars having resistant to biotic stresses can be successfully developed for commercial cultivation.

Number of bacterial genes found to confer resistance to diseases viz. *P. syringae* pv. *phaseolicola* (argK-ornithine carbamoyl transferase from *P. syringae*), *P. syringae* pv. *tabaci* (bO-Bacterio-opsin from *Halobacterium halobium*), *P. parasitica* var. *nicotianae* (popA-PopA protein from *R. solanacearum*), *E. carotovora* (expI-*N*-oxoacyl-homoserine lactone from *E. carotovora*; aiiA-Acyl-homoserine lactonase from *Bacillus* spp.), *Helicoverpa* spp. And *Spodoptera* spp. (cry genes-Crystal proteins from *B. thuringiensis*), boll weevil larvae (choM-ChoM protein from *Actinomyces*), tobacco hornworm (ipt-Cytokinin isopentenyl transferase from *A. tumefaciens*), etc. (Jube and Borthakur 2007). Further, tobacco transgenics having bacterial genes found to tolerance herbicides viz. Bialaphos (Bar gene—producing PPT acetyl transferase from *Streptomyces hygroscopicus*), Glyphosate (aroA-M1-EPSPS from *E. coli*), Phenmedipham (pcd-PMPH from *Arthrobacter oxydans*), 2, 4-D (tfdA-2, 4-D monooxygenase from *Ralstonia eutrophus*), Paraquat (pqrA-Paraquat resistant protein (PqrA) from *Ochrobactrum anthropic*), etc. (Jube and Borthakur 2007).

Exhaustive list of tobacco transgenics validating the effectiveness of plant pathogen related genes, lectins, proteinase inhibitors, trypsin inhibitor, transcription factor etc. from various plant sources conferring resistant to *Spodoptera*, *Heliothis*,

aphids, TMV, phytopathogens and nematodes are available in the literature (Sane et al. 1997; Luo et al. 2009b; Malone et al. 2009; Priya et al. 2011; Guo et al. 2013). Plant derived antifungal proteins found to give protection from fungal pathogens. Animal derived Antimicrobial magainin analogs, avidins, gamma-aminobutyrate (GABA), proteinase inhibitor etc. found to enhance resistance of tobacco to diseases, pests and nematodes (Jach et al. 1995; Li et al. 2001; Burgess et al. 2002; Christeller et al. 2002; McLean et al. 2003).

Transgenic tobacco plants showing resistant to CMV, TMV, TLCV were developed by the transfer of transgenes from virus, plant or other origins (Prins et al. 2008). The approaches such as viral coat protein mediated resistance, replicase protein, movement, proteases, and antisense sequences 'R'-genes from plants, plantibodies, double stranded RNA (dsRNA) etc. were used in conferring virus resistance in tobacco (Powell-Abel et al. 1986; Day et al. 1991; Audy et al. 1994; Xiao et al. 2000; Hofius et al. 2001; Spassova et al. 2001; Kalantidis et al. 2002). In a large number of transgenics developed using transgenes of viral origin, resistance is found to be conferred by post-transcriptional gene silencing (Kalantidis et al. 2002).

10.12.2 Review on Achievements of Transgenics

Tobacco has served as a model plant for producing large number of transgenics having pest and disease resistant and other economically important genes. However, no genetically transformed tobacco varieties (transgenic cultivars) are released for commercial cultivation in any of the countries, in view of the opposition faced by GM tobacco in the global market (Bowman and Sisson 2000). Though GM Approval Database of International Service for the Acquisition of Agri-biotech Applications (ISAAA) reports two GM tobacco events viz. (1) oxynil herbicide tolerance and (2) nicotine reduction, antibiotic resistance (GM approval database 2021), none of them are cultivated on commercial scale in any of the countries. In contrast, millions of hectares of genetically engineered soybean, corn, cotton and canola are being grown throughout the world (ISAAA 2019). Thus, tobacco breeding efforts lag behind those of other crops in genetic engineering. In addition, the strong opposition from the European countries to genetically modified organisms (GMOs) is also acting as hindrance in transgenic tobacco breeding. Thus, genetic engineering of tobacco cultivars is on hold until the trade related obstacles are alleviated. However, this methodology holds great promise for improving tobacco cultivars in terms of disease and pest resistance, and possibly health-related constituents in the cured leaf.

10.12.3 Organelle Transformation

Manipulation of nuclear genome through genetic engineering is performed widely in most economically important plant species. However, nuclear transformation has

several drawbacks including unpredictable expression of the gene of interest and gene silencing due to the random location of transfer DNA integration and/or position effects (Meyers et al. 2010). As organelles containing genetic materials in small DNA genomes, plastids (chloroplasts) and mitochondria provide an opportunity for transformation in plants (Butow and Fox 1990; Rascon-Cruz et al 2021).

Plastid genomes of tobacco are typically 150 kb, and codes for about 140 genes. Plastids are seat for some of the important biosynthetic pathways and processes that include photosynthesis, photorespiration, metabolism of amino acids, lipids, starch, carotenoids, other isoprenoids, phenol compounds, purines, pyrimidines, isoprenoids, starch, pigments, vitamins synthesis, and also are implicated in the metabolism of phytohormones such as cytokinins, abscisic acid, and gibberellins (Kuchuk et al. 2006; Rascon-Cruz et al. 2021). Compared with conventional nuclear genetic engineering, plastid genome transformation offers several advantages (Kuchuk et al. 2006; Li et al. 2021). High level of transgene expression is possible with chloroplasts as there are about 100 chloroplasts per cell, each containing about 100 copies of genome. Thus, there is possibility of 10,000 copies of transgenes per cell due to plastid transformation. Gene silencing or position effects were not defined for plastid genes. Thus, the level of expression is much more predictable. Unlike integration into the nuclear genome, integration of heterologous DNA through homologous recombination mechanism into a plastome allows very precise genetic manipulations. There exists the possibility of multigene engineering through stacking transgenes in synthetic operons in a single transformation event. Maternal inheritance of plastomes in majority of crop species reduce the risk of uncontrolled transgene release into the environment (Kuchuk et al. 2006; Li et al. 2021).

Stable transformation of the plastome was achieved first in unicellular alga, *Chlamydomonas reinhardtii* in 1988, and two years later for the dicotyledonous seed plant tobacco (*N. tabacum*) (Svab et al. 1990). Over the years, plastid transformation in tobacco has become more and more routine with a transformation efficiency equivalent to nuclear transformation (Svab and Maliga 1993; Daniell et al. 2016; Li et al. 2021). Plastids of *N. tabacum* var. Petit Havana (Svab et al. 1990), *N. benthamiana* (Davarpanah et al. 2009) and *N. sylvestris* (Maliga and Svab 2010) are transformed by different workers. The plastid transformation technology has lead transgene expression, genome editing, and RNA editing analysis in plastids.

The first biotechnological application of transplastomic technology for pest control was the expression of cry1A(c) gene from *B. thuringiensis* (Bt) in the tobacco plastid. The tobacco transplastomic plants accumulates higher amounts of the Bt insecticidal protein (3–5% of Total Soluble Proteins-TSP) and displayed high levels of resistance to herbivorous insects (McBride et al. 1995). When cry2Aa2 was transformed as an operon along with two small open reading frames, the Cry2Aa2 protein accumulated up to 45% of TSP and led to the formation of crystals (De Cosa et al. 2001). Higher expression levels of cry9Aa2 (10% of TSP) in tobacco plastid genome results in severe growth retardation of the transplastomic plants (Chakrabarti et al. 2006) indicating that the transgene expression level need to be cautiously optimized for providing sufficient protection without a yield penalty. The advances made in other crops clearly indicate that developing tobacco with high levels of resistance to

insects, bacterial, fungal and viral diseases, and different types of herbicides is quite possible with plastid transformation (Adem et al. 2017).

The comparison between plastids and mitochondria make it possible to transform mitochondrial genome with suitably designed constructs. However, reliable methods for the transformation of mitochondria using a biolistic device currently exist only for yeasts (Johnston et al. 1988) and green algae (Remacle et al. 2006) and no successful transformation of mitochondria in plant systems has been reported to date (Li et al. 2021). Most of the plant mitochondrial genomes composed of non-coding repeated sequences, gene spacing sequences and introns. A system for genetic transformation of plant mitochondria would facilitate functional analyses of the mitochondrial genome and its products, and also open the way for modification of mitochondrial metabolism, or to introduce cytoplasmic male sterility (CMS) into new crops and varieties (European Commission 1989; Wang et al. 2020).

10.12.4 Biosynthesis and Biotransformation

10.12.4.1 Biosynthesis

Alkaloids are important compounds synthesized in *Nicotiana* plants and essential in establishing the commercial quality of tobacco as well as its defense against herbivores (Zenkner et al. 2019). Both wild and domesticated forms of *Nicotiana* spp. accumulate nicotine, the content and composition of which vary among species. Nicotine, in general, is produced in tobacco roots and translocated to leaves. Some *Nicotiana* wild species produce *N*-acyl-nornicotine, an alkaloid with more potent insecticidal properties than nicotine. The regulation of nicotine biosynthesis has been considered a complex physiological response, and many TFs are directly or indirectly involved in its regulation (Kajikawa et al. 2017; Xu et al. 2017; Qin et al. 2020). Six TFs (from three TF families) found to affect nicotine metabolism, with two basic helix-loop-helix genes positively regulating the jasmonate activation of nicotine biosynthesis (Todd et al. 2010). Metabolic engineering for the biosynthesis of nicotine and its more potent *N*-acyl-nornicotine in tobacco without compromising the commercial quality of tobacco could be an option in developing biotic stress resistant cultivar. The developed high nicotine yielding cultivars can be used to extract nicotine for use as a pesticide.

10.12.4.2 Biotransformation

Biotransformation of applied xenobiotic (pesticides, herbicides etc.) chemicals for controlling biotic stresses is essential to maintain tobacco quality and reduction of pesticide residues, and ultimately for health of the tobacco users. Tobacco transgenics

having herbicide tolerance viz. bialaphos (having *Bar* gene—producing PPT acetyltransferase from *S. hygroscopicus)*, glyphosate *(aroA*-M1-EPSPS from *E. coli)*, phenmedipham *(pcd*-PMPH from *A. oxydans)*, 2,4-D (*tfdA*-2,4-D monooxygenase from *R. eutrophus*), paraquat *(pqrA*-paraquat resistant protein (PqrA) from *O. anthropic)*, etc. found to reduce the effect of herbicides on tobacco through their biotransformation in tobacco plant (Jube and Borthakur 2007). This ability enables to use these herbicides in controlling weeds in tobacco field without affecting the mail crop. Biotransformation of pesticides to their less toxic forms in a reasonable time frame in tobacco will assists in reducing pesticide residues. Research in this direction is essential in future for adding more number to the list of crop protection agents that can be used on tobacco.

10.12.5 Metabolic Engineering Pathways and Gene Discovery

"The improvement of cellular activities by manipulation of regulatory, enzymatic, and transport functions of the cell with the use of recombinant DNA technology" is defined as Metabolic engineering (Bailey 1991). Metabolic engineering is motivated by commercial applications by which one can improve the developing strains for production of useful metabolites. It is basically meant for altering the metabolic pathways for the production of chemicals, pharmaceuticals, fuels, and medicine. A metabolic pathway can be defined as any sequence of feasible and observable biochemical-reaction steps connecting a specified set of input and output metabolites. The rate at which various input metabolites are processed to form output metabolites is known as pathway flux. Metabolic engineering involves useful alteration of metabolic pathways to better understand and utilize the cellular pathways. This involves overexpression or down regulation of certain proteins in a metabolic pathway in such a way that the cell produces a new product.

First step for successful engineering requires the complete understanding of metabolic pathway and genes involved in the path way and host cell for genetic modifications (Fuentes et al. 2018). The engineering of metabolic pathways in plants frequently requires the concerted expression of more than one gene involved in that pathway. With traditional transgenic approaches, the expression of such multiple transgenes has been a challenge. Recent progress in transformation techniques has making it possible to integrate multiple transgenes into host genomes. New technological options include combinatorial transformation (large-scale co-transformation of the nuclear genome) and transformation of the chloroplast genome with synthetic operon constructs (Bock 2013). Metabolic pathway engineering of plastid (chloroplast) genome offers significant advantages, including straight forward multigene engineering by pathway expression through operons, higher levels of transgene expression, and transgene containment due to maternal inheritance. Further, it allows

direct access to the large number of diverse metabolite pools in chloroplasts and other non-green plastid types.

In contrast to most structural genes, TFs tend to control multiple pathway steps and hence, facilitates for the engineering of complex metabolic pathways for higher levels of metabolites (Broun 2004; Grotewold 2008). The TFs are often exists as gene families and regulate target genes in tissue- and species-specific patterns (Bovy et al. 2002). In most of the cases, detailed studies have not been made on the specificity of the regulatory genes. Studies on the variations in transcriptomes and metabolomes assists in understanding the regulation by transcription factors in heterologous systems. Flavonoids are a group of compounds involved in several aspects of plant growth and development, such as pigment production, pollen growth, seed coat development, pathogen resistance and UV light protection (Harborne 1986). Hence, manipulation of phenylpropanoid pathway responsible for flavonoid production can be a strategy for biotic and abiotic stress resistance.

Metabolic engineering using three monoterpene synthases from lemon altered fragrance of tobacco plants (Lucker et al. 2004). Engineering of synthetic operon constructs comprising three genes for the key enzymes of vitamin E (tocochromanol) biosynthesis resulted in an increase of up to tenfold in total tocochromanol accumulation in transplastomic tobacco (Lu et al. 2013). Astaxanthin content in the transplastomic tobacco plants was enhanced through plastid transformation of a synthetic operon consisting of three genes that redirect lycopene into the synthesis of β-carotene and ultimately astaxanthin, a high-value ketocarotenoid (Lu et al. 2017). Grafting of transplastomic tobacco onto the non-transformable *Nicotiana glauca* enabled the horizontal transfer of the transgenic chloroplast genomes through the graft junction (Lu et al. 2017). Thus, grafting may be helpful in the transplastomic engineering of plant species that are otherwise not amenable.

Metabolic engineering of artemisinic acid biosynthetic pathway provided a proof of concept for combining plastid and nuclear transformation to optimize product yields from complex biochemical pathways in chloroplasts (Fuentes et al. 2016). Transplastomic tobacco for two synthetic operons expressing the core artemisinic acid biosynthetic pathway accumulates only low levels of the metabolite. However, super transformation of the trasplastomics lines using the COSTREL (combinatorial super transformation of transplastomic recipient lines) approach, increased the artemisinic acid content up to 77-fold.

Photorespiration could be reduced in tobacco through the introduction of three distinct alternative glycolate metabolic pathways into tobacco chloroplasts (South et al. 2019). Coupling the reduced expression of a glycolate and glycerate transporter to limit glycolate flux out of the chloroplast with alternative photorespiratory pathway could raise the biomass productivity by >40% under field conditions (South et al. 2019). In this study, about 17 constructs were designed for nuclear transformation; these multienzyme pathways could effectively be introduced into the chloroplast in the form of operons.

Possibility of metabolic engineering for pest resistance was obtained with the modulation of transcriptome and metabolome of tobacco (*Nicotiana tabacum*) by *Arabidopsis* transcription factor, AtMYB12 (Misra et al. 2010). Expression of

AtMYB12 in tobacco resulted in enhanced expression of genes involved in the phenylpropanoid pathway, leading to severalfold higher accumulation of flavonols. The tobacco transgenic lines developed resistance against the insect pests *S. litura* and *H. armigera* due to enhanced accumulation of one of the flavonol, rutin. This study clearly indicates that metabolic engineering can be successfully employed in developing stress tolerant tobacco genotypes.

10.12.6 Gene Stacking

Gene stacking or Gene pyramiding or multigene transfer refers to incorporation of two or more genes of interest into a single plant. The combined traits obtained from this process are called stacked traits. A biotech crop variety that bears stacked traits is called a biotech stack or simply stack (ISAAA 2020). A biotech stack may be a plant transformed with two or more genes that code for proteins having different modes of action on a pest or a hybrid plant expressing both insect resistance and herbicide tolerance genes derived from two genetic sources. Biotech stacks are engineered to overcoming the myriad of problems that includes insect pests, diseases, weeds, and environmental stresses in order to increase farm level productivity. Insect resistance based on multiple genes confers stable resistance than single gene which may breakdown due to co-evaluation of pests. Gene stacks can be are generated through methods such as (i) the simultaneous introduction of transgenes through co-transformation, and (ii) the sequential introduction of genes using re-transformation processes or the sexual crossing of separate transgenic events.

Pyramiding of multiple genes found to impart resistance to insects and pathogens resistance and herbicide tolerance in tobacco. Stacking of genes conferring insect-resistance and herbicide (glyphosate) tolerance (Sun et al. 2012), dsRNAs silencing of chitin biosynthesis pathway genes for root-knot nematode resistance are reported in tobacco. Stacked insect resistant cry1Ac and cry2A genes (Bakhsh et al. 2018) and three codon optimized *cry2Ah1* genes (Li et al. 2018) found to confer resistance to lepidopteran insects. Pyramiding of two pathogenesis-related genes imparted resistance to three filamentous fungus in tobacco (Boccardo et al. 2019). Stacking of protease inhibitors from sweet potato and taro found to impart resistance to both insects (*H. armigera*) and pathogens (damping-off disease caused by *P. aphanidermatum* and bacterial soft rot caused by *E. carotovora*) (Senthilkumar et al. 2010). Through, stacked products are promising and technically feasible in tobacco, till date, none of the stacks produced, through genetic engineering, are approved for commercial cultivation in tobacco mainly because of their transgenic tag. Gene pyramiding events in tobacco are mainly used as proof of concepts or for gene function and interaction studies.

Regulatory principles and procedures for approval and release of biotech stacks differ globally (ISAAA 2020). In USA and Canada, no separate or additional regulatory approval is required for commercialization of hybrid stacks that are products of

crossing a number of already approved biotech lines. This policy is based on the assertion that interactions between individual trait components in a stack that have not been shown environmental or health hazard would not give rise to new or altered hazards. The combinations of "plant incorporated protectants" or PIPs (eg. Bt insecticidal proteins) may give rise to higher or altered toxicity, hence, the US Environmental Protection Agency calls for a separate safety review of a stack in case of identification of any such hazard. Contrary to this, stacks are considered new events in Japan and European Union (EU) countries, even though individual events have commercial approval, and must pass through a separate regulatory approval process, including risk assessment of their safety, similar to mono-trait biotech events (ISAAA 2020). Risk assessment is focused on the identification of additional risks that could arise from the combined genes.

10.12.7 Gene Silencing

Gene silencing is the regulation of gene expression in a cell to prevent or reducing the expression of a certain gene. Gene silencing is used as a means for developing species-specific pest control methods that are alternatives to potentially harmful chemical methods in plants. RNA interference (RNAi) is a promising method for controlling insect pests and diseases by silencing the expression of vital pest and disease-causing organism genes to interfere with development and physiology. RNAi technology is based on the expression of dsRNA that shares nearly 100% sequence homology with a desired target gene for optimal silencing. The mechanism of RNAi mediated gene silencing is based mainly on the exogenous production of short interfering RNAs/microRNAs (siRNAs/miRNAs) in an organism to control gene expression. Expression or introduction of dsRNA in eukaryotic cells can trigger sequence-specific gene silencing of transgenes, endogenes, and viruses. Transgenic plants producing dsRNAs with homology to pest or viral sequences are likely to exhibit pathogen-derived resistance to the pests and diseases. *Tobacco Rattle Virus*–based virus-induced posttranscriptional gene silencing (termed virus-induced gene silencing or VIGS) found to be the widely used method to downregulate the expression of a target plant gene (Bachan and Dinesh-Kumar 2012; Senthil-Kumar and Mysore 2014). Temporal and spatial control of gene silencing could be achieved through inducible (ethanol) expression of double-stranded RNAs in tobacco (Chen et al. 2003). *In planta*-expressed dsRNA synthesized within the plastids are more effective than nuclear expressed circumventing the native RNAi paths in eukaryotes. *M. sexta* (tobacco hornworm) genes could be effectively silenced through plastid transformation of dsRNA genes targeting the hornworm genes (Burke et al. 2019).

As a model organism number of studies conducted in tobacco for silencing genes of various pest and disease-causing organisms there-by reducing their growth and development leading to resistance. Transgenic tobacco plants expressing a hairpin RNA (hpRNA) targeting a root-knot nematode (*M. javanica*) putative zinc finger transcription factor effectively suppressed the growth of nematodes feeding on the

roots of the transgenic plants (Fairbairn et al. 2007).Vietnamese scientists succeeded in breeding tobacco for virus resistance using gene silencing or RNAi technology using three expression vectors carrying single gene (TMV, TSWV etc.) or multiple genes (TMV, TSWV, CMV and TYLCV) (http://agrobiotech.gov.vn/NewsDetail.aspx?ID=821&CatID=7). Expressing of dsRNA homologous to pest genes found to enhance host plant resistance to whitefly (Thakur et al. 2014; Malik et al. 2017), *H. armigera* and *S. exigua* (Zhu et al. 2012), nematodes (Mani et al. 2020) and CMV (Kalantidis et al. 2002) in tobacco.

10.12.8 Prospects of Cisgenics

In cisgenesis, as in transgenesis, extra DNA is stably built into the plant DNA. The major difference between transgenesis and cisgenesis is the origin of the DNA (Schouten and Jacobsen 2008). With cisgenesis, the extra DNA originates from a plant with which the acceptor plant (the plant that will receive the extra DNA) can cross-breed. 'Cis' refers to within the same crossable group (Schouten et al. 2006a, b). Cisgenesis approach combines both traditional breeding techniques with modern biotechnology and dramatically speed up the breeding process. Cisgenic plants are presumably considered safer than those produced through conventionally bred plants because of the lack of linkage drag (Hou et al. 2014). Cisgenesis introduces just desired genes without the undesirable genes and hence, prevents hazards compared to induced translocation or mutation breeding (Telem et al. 2013). Through cisgenesis various biotic and abiotic stress resistance genes can be pyramided to provide wider and long-lasting forms of resistance. Several backcrossed generations are required to get rid of undesired genes in conventional hybridization programs, hence takes long time (Telem et al. 2013). Whereas the time taken for introducing a single gene or more so with multiple genes can be drastically reduced in cisgenesis due to incorporation of merely target traits.

Introduction of exogenous transfer process related genes through cisgenesis can be avoided through the use of new transformation protocols without bacterial selection markers (de Vetten et al. 2003; Schaart et al. 2004) and species-specific P-DNAs instead of bacterial T-DNAs for insertion of isolated genes (de Vetten et al. 2003; Rommens et al. 2004). Further, application of new methods, such as promoter trapping and RNA fingerprinting for the isolation of native regulatory elements can now be exploited for the precise expression of the desired traits (Meissner et al. 2000; Trindade et al. 2003). Majority of the methods for production of cisgenic crops without exogenous genes including removal of selectable marker genes, use of pDNAs and segregation of independently integrated T-DNAs have been patented, therefore scientists need either to use the existing patents or design new protocols to eradicate the undesired DNA sequences from host genomes (Holme et al. 2013).

Vast prospects exists for cisgenesis in tobacco crop. Availability of large number of wild species and germplasm resources, genome sequence information of cultivated

tobacco and few wild relatives, and comparative genomic techniques, the development of efficient gene isolation techniques like map- based cloning and allele mining are opening the avenues for identification and cloning of resistant traits from tobacco and their wild relatives. The cisgenes thus isolated can be used for transferring and imparting resistance for biotic stresses in tobacco. Cisgenesis was successfully taken up in tobacco through gene editing techniques, such as ZFNs (Townsend et al. 2009), TALENs (Zhang et al. 2013) and CRISPR-Cas (Upadhyay et al. 2013; Ali et al. 2015) induce targeted alleles in tobacco (Hou et al. 2014). Identification, cloning and transferring single or multiple biotic stress resistant cisgenes into tobacco can be achieved in future with the ever-improving gene technologies.

Though both transgenesis and cisgenesis employ the same genetic modification techniques for introducing gene(s) into a plant, cisgenesis use only genes of interest from the plant itself or from a crossable species which otherwise could also be transferred by traditional breeding techniques. The release of cisgenic plants into the environment doesn't evoke any environmental risk and they are as safe as that of traditionally bred plants. Hence, compared to transgenesis, cisgenesis is more similar to traditional plant breeding and may be considered as non-transgenic, in spite of use of molecular biology and plant genetic engineering methods in their development. Restrictions on any form on cisgenesis could delay further research on development of improved crop varieties, particularly at a time when a greater number of genes from crops and their crossable wild relatives are being isolated and are amenable to cisgenesis. Hence, it is necessity to discriminate cisgenesis from transgenesis.

Common people are found to be much satisfied with cis/intragenic crop than transgenic crops. Surveys indicated cisgenic plants are more likely to be acceptable to the public than transgenic plants (Viswanath and Strauss 2010; Gaskell et al. 2011; Mielby 2011). However, currently, GMO regulations to prevent any negative effects on the environment or human health are based on transgenic organisms and do not distinguish transgenic plants from cisgenic plants in majority of the countries. Canada has adopted a product-based regulation system instead of process-based one and making it legally possible to control cisgenic plants less strictly than transgenic plants. As per the Australian Gene Technology Regulations, "a mutant organism in which the mutational event did not involve the introduction of any foreign nucleic acid" is not specified as GMO and thus treats cisgenic plants differently (Russell and Sparrow 2008). European Food Safety Authority (EFSA 2012) states that cisgenic plants are similar to the traditionally bred plants and safe in terms of environment, food and feed.

Though, biotic stress resistant trasgenics are available, they could not be used for commercial cultivation due to worldwide GMO regulations. In case cisgenics are treated differently, that will be advantageous to tobacco in which GMOs are not acceptable. Lesser regulations on cisgenics will boost the cisgenesis research in tobacco for improving tobacco yields and resistant factors.

10.13 Brief Account on Role of Bioinformatics as a Tool

Recent technological advances have resulted in the accumulation of large volumes of biological data in terms of nucleic acid sequences and various details of biomolecules produced in tobacco and other crops under different situations and life stages. In order to store and analyze these data, number of general and crop specific databases were created. The databases may have the information related to one or more of omics types in an integrated way. The information pertaining to tobacco are being stored and accessed through quite a number of databases, globally. However, to name a few, some of the key databases that are covering most of the data related to tobacco are discussed here under.

10.13.1 Gene and Genome Databases

Ever-improving sequencing technologies, gene mapping and tagging projects, and phylogenetic studies have resulted in accumulation of large volumes of genomic data in tobacco. Computer databases are an increasingly necessary tool for organizing such vast amounts of biological data generated and for making it easier for researchers to access and analyze relevant information. The Genomic databases serve as hubs for storing, sharing and comparison of data across research studies, data types, individuals and organisms.

Among the various databases, the key genome databases harboring *Nicotiana* genome and gene information are NCBI Genome, Sol Genome Networks (SGN), Kyoto Encyclopedia of Genes and Genomes (KEGG genome), EnsemblPlants, *Nicotiana attenuata* data hub (NaDH), The International Nucleotide Sequence Database Collaboration (INSDC), Gramene etc. (Table 10.13). At present, genome sequences of 12 *Nicotiana* spp. viz. *N. tabacum, N. tomentosiformis, N. sylvestris, N. attenuata, N. undulate, N. otophora, N. suaveolens, N. glauca, N. stocktonii, N. repanda, N. amplexicaulis and N. debneyi* at scaffold or contigs level, chloroplast genomes of five species and mitochondrial genomes of three species are available with one or the other databases. Further, a total of over more than two lakh records of gene sequences belonging to 12 *Nicotiana* species are existing at various data bases. NCBI and SGN together are the principal databases that cover all the currently available information on genomes and genes of various *Nicotiana* spp. These databases are sharing the stored information with other databases and providing extensive tools for the analysis of sequences and annotation. INSDC is a long-standing foundational initiative that operates between DNA Databank of Japan (DDBJ), European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) and NCBI and covers the spectrum of data raw reads, from assemblies and alignments to functional annotation. Other databases mentioned above provide access to the *Nicotiana* resources, mostly, through collaboration with other databases along with additional analysis tools existing with their databases. *Nicotiana attenuata* data hub (NaDH) covers

Table 10.13 Some of the main genomic resource databases for *Nicotiana* species

S. No.	Name	Salient features	URL
1	National Center for Biotechnology Information (NCBI)	• An integrated database for gene loci, genomes, functional genomics data, gene expression, sequence sets from phylogenetic and population studies • Offers tools for genome, gene and protein analysis and VIGS Tool to design VIGS constructs	http://www.ncbi.nlm.nih.gov/
2	Sol Genomics Networks (SGN)	• An integrated database that stores information on genome, genes, loci and phenotype data for the Solanaceae family and close relatives • Provides analysis tools for browsing genomes, expression and map data viewers, community annotation system, QTL analysis and to improve VIGS constructs etc	http://solgenomics.net/
3	*Nicotiana attenuata* Data Hub (NaDH)	• NaDH maintains genomic, transcriptomic and metabolomic data and combines different data sources for their analysis relevant to *N. attenuata*	http://nadh.ice.mpg.de
4	Kyoto Encyclopedia of Genes and Genomes (KEGG genome)	• An integrated database resource with 16 databases that includes genes, proteins, metabolites and other chemical substances, biochemical reactions, enzyme, disease-related network variations etc. • Provide tools for understanding high-level functions and utilities of the biological system covering genomic and molecular-level information related to an ecosystem, organism and cell	https://www.genome.jp/kegg/

(continued)

Table 10.13 (continued)

S. No.	Name	Salient features	URL
5	EnsemblPlants	• An integrative resource presenting genome-scale information for sequenced plant species including *Nicotiana* • Data includes genome sequence, gene models, functional annotation, and polymorphic loci etc. and tools for their analysis	https://plants.ensembl.org
6	Gramene	• A curated, integrated data resource for comparative functional genomics in crops and model plant species	https://www.gramene.org/
7	VISTA	• A comprehensive suite of programs and databases for comparative analysis of genomic sequences	https://genome.lbl.gov/
8	The Gene Ontology (GO) Resource	• Largest source of information on the functions and cellular locations of genes along with processes they may carry out	http://geneontology.org/
9	Gene Expression Omnibus (GEO)	• A functional genomics data repository and accepts Array- and sequence-based data	https://www.ncbi.nlm.nih.gov/geo/
10	ArrayExpress (AE)	• Repository that archive functional genomics data from microarray and sequencing platforms	https://www.ebi.ac.uk/arrayexpress/
11	Genomic Expression Archive (GEA)	• A database of functional genomics data such as gene expression, epigenetics and genotyping SNP array	https://www.ddbj.nig.ac.jp/gea/index-e.html
12	Universal Protein Resource (UniProt)	• Provide a comprehensive, high-quality and freely accessible resource of protein sequence, annotation data and functional information	https://www.uniprot.org/

(continued)

Table 10.13 (continued)

S. No.	Name	Salient features	URL
13	Pfam database	• Large collection of protein families, each represented by multiple sequence alignments and hidden Markov models (HMMs) • Provides tools for protein alignments and annotation, domain organisation of a protein sequence etc.	http://pfam.xfam.org/
14	SolCyc Biochemical Pathways	• A collection of Pathway Genome Databases (PGDBs) for Solanaceae species generated using pathway tools	https://solcyc.solgenomics.net/
15	REACTOME	• A manually curated and peer-reviewed pathway database • Offers intuitive bioinformatics tools for the visualization, interpretation and analysis of pathway knowledge to genome analysis, modeling, systems biology, etc.	https://reactome.org/
16	The Golm Metabolome Database (GMD)	• A metabolome database and exchange platform to develop and improve metabolomics by multidisciplinary cooperation • Provides access to custom mass spectral libraries, metabolite profiling experiments and tools with regard to methods, spectral information or compounds	http://gmd.mpimp-golm.mpg.de/
17	MoNA (Massbank of North America)	• A centralized and collaborative database of metabolite mass spectra, metadata and associated compounds	https://mona.fiehnlab.ucdavis.edu/
18	Database for Annotation, Visualization, and Integrated Discovery (DAVID)	• An integrated database for functional genomic annotations with intuitive graphical summaries	https://david.ncifcrf.gov/

(continued)

Table 10.13 (continued)

S. No.	Name	Salient features	URL
19	The International Nucleotide Sequence Database Collaboration (INSDC)	• INSDC covers the spectrum of data raw reads, through alignments and assemblies to functional annotation, enriched with contextual information relating to samples and experimental configurations	https://www.insdc.org/
20	The BioStudies Database	• A resource for encapsulating all the data associated with a biological study • Accept and archive "multi-omics". data generated in experiments	https://www.ebi.ac.uk/biostudies/

the exclusive information on *N. attenuata* and its similarities with other *Nicotiana* species and 11 published dicot species. A website of Boyce Thompson Institute's for *N. benthamiana* resources provide access to *N. benthamiana* genomic resources existing at SGN including gene and protein data, markers, genes to phenotypes database etc. (https://btiscience.org/our-research/research-facilities/research-resources/nicotiana-benthamiana). It is also providing tools for alignment, annotation, designing siRNAs for VIGS, CRISPR designing etc.

In addition to above databases, the Gene Ontology resource database provides access to scientific information about the molecular functions of genes (or, more properly, the protein and non-coding RNA molecules produced by genes) from many different organisms, from humans to bacteria, their cellular locations and processes those gene products may carry out (Table 10.13). Currently, 25,761 genes and gene products are found to be associated with the term *Nicotiana* in The Gene Ontology resource database.

Most of the gene and genomic databases provide tools for searching, alignment and comparison of sequences with other *Nicotiana* spp. Solanaceous crops and other crops, annotation, marker designing, QTL analysis, VIGS Tool to design virus-induced gene silencing constructs, CRISPR designing etc. Genome analysis aims to describe the functions of genes and proteins, as well as the relationship that exists between a given genotype and phenotype. Gene and gene sequence alignments helps in the identification of inherited genetic variations like SNPs and alterations in gene sequences and their relationship to resultant phenotypes. Apart from analysis of genome sequence data, various genome databases are facilitating the analysis of gene variation and expression, prediction of gene/protein structure and function, prediction and detection of gene regulation networks, etc.

10.13.2 Comparative Genome Databases

The increasing availability of genomic sequence from multiple organisms has provided biomedical scientists with a large dataset for orthologous-sequence comparisons. The rationale for using cross-species sequence comparisons is to identify biologically active regions of a genome based on the observation that sequences that perform important functions are frequently conserved between evolutionarily distant species, distinguishing them from nonfunctional surrounding sequences. This is most readily apparent for protein-encoding sequences but also holds true for the sequences involved in the regulation of gene expression. However, examination of orthologous genomic sequences from several vertebrates has shown that the inverse is also true.

Genome sequences can be compared using pairwise and multiple whole-genome alignments and based on these alignments, synteny, sequence conservation scores and constrained elements can be determined. Comparison of whole-genome sequences at the level of nucleotide or protein provides a highly detailed narration of how organisms are related to each other at the genetic level. Comparative genome studies on genomic variations will identify the types of genes, gene families, and their location, as well as provide clues on the history of evolutionary gene rearrangements and duplications that might be responsible for the recognized genetic variations. By carefully comparing genome characteristics that define various organisms, researchers can pinpoint regions of similarity and difference. This information can be used to identify putative genes and regulatory elements for various traits that may lead to their cloning and further utilization.

A variety of tools to compare the complete genome sequences of within or among the different species are made available by different databases. All most all the gene and genome databases of tobacco namely NCBI, SGN, NaDH etc. are offering the tools for such comparative genome analysis. VISTA provides a comprehensive suite of programs and databases for comparative analysis of genomic sequences. There are two ways of using VISTA—one can submit their own sequences and alignments to VISTA servers for analysis or examine pre-computed whole-genome alignments of different species.

Gramene, a knowledgebase founded on comparative functional analyses of genomic and pathway data for model plants and major crops including tobacco. The current release, #64 (September 2021), hosts 114 reference genomes, and around 3.0 million genes from 90 plant genomes with 3,256,006 input proteins in 123,064 families with orthologous and paralogous classifications. Comparative genomics contains around 340 pairwise DNA alignments and 80 synteny maps. Plant Reactome portrays pathway networks using a combination of manual biocuration and orthology-based projections to 106 species. The Reactome platform enables comparison of reference and projected pathways, gene expression analyses and overlays of gene–gene interactions. Gramene integrates information extracted from plant-focused journals on genetic, epigenetic, expression, and phenotypic diversity; ontology-based protein structure–function annotation; and gene functional annotations.

Many online/web applications can be effectively used for comparative analyses at genomic and genic levels. Apart from tools provided by various genomic databases, applications such as BRIG (Alikhan et al. 2011), Mauve (Darling et al. 2004)), Artemis Comparison Tool (ACT) (Carver et al. 2005), geneCo (Jung et al. 2019) etc. can be used for comparative genomics. At *Nicotiana attenuata* data hub, genes of 11 published dicot species were compared and found to cluster into 23,340 homologous groups (HG) based on their sequence similarity with at least two homolog sequences. The phylogenetic trees were also constructed for all these HG.

Comparative analyses of *Nicotiana* plastid genomes among themselves and with currently available Solanaceae genome sequences indicated the existence of similar GC and gene content, codon usage, simple sequence and oligonucleotide repeats, RNA editing sites, and substitutions (Asaf et al. 2016; Mehmood et al. 2020). Such analysis also revealed that *N. otophora* is a sister species to *N. tomentosiformis* within the *Nicotiana* genus, and *Atropha belladonna* and *Datura stramonium* are their closest relatives (Asaf et al. 2016).

Comparision of whole nuclear and plastid genomes facilitated the identification and confirmation wild progenitor species and their relative genome contributions in the evoluation of *N. tabacum* and *N. rustica* genomes (Murad et al. 2002; Lim et al. 2005; Leitch et al. 2008; Sierro et al. 2014, 2018; Edwards et al. 2017). Whole-genomic sequence comparison indicated that the genomes of *N. sylvestris* and *N. tomentosiformis* contributes 53 and 47%, respectively to the genome of N. *tabacum* confirming a larger biased reduction of T genome (Sierro et al. 2014). Comparison of chloroplast genomes revealed that *N. tomentosiformis* is a sister species and *Atropha belladonna* and *Datura stramonium* are closest relatives to *N. otophora* (Asaf et al. 2016). Further, it also revealed that the maternal parent of the tetraploid *N. rustica* was a common ancestor even for *N. paniculata* and *N. knightiana*, and the later species is more closely related to *N. rustica.*

Comparative studies of *Nicotiana* genome sequences provided insights into how speciation impacts plant metabolism, and in particular alkaloid transport and accumulation. In a genome evolution study, Sierro et al. (2018) found that 41% of allotetraploid genome of *N. rustica* originated from the paternal donor (*N. undulata*) and 59% from the maternal donor (*N. paniculata*/*N. knightiana*). Gene clustering analysis revealed the commonality of 14,623 ortholog groups among the *Nicotiana* species and 207 specific to *N. rustica.* It was speculated from the results the higher nicotine content of *N. rustica* leaves is due to the combination of progenitor genomes and higher active transport of nicotine to the shoot. Comparative genomics of pests and diseases is helping in distinguishing isolates, races and strains (Hou et al. 2016).

10.13.3 Gene Expression Databases

With technological advancements large volumes of data is being generated on gene expression patterns in tobacco from seed to senescence and under different growing conditions including during the incidence of biotic stresses. The expression of genes

in plants are measured at transcriptome levels. In tobacco, the efforts of the Tobacco Genome Initiative (TGI) resulted in enrichment of the sequence information for the transcriptionally active regions of the tobacco genome. The information generated in the form of Expressed Sequence Tags (ESTs), short, single pass sequence reads derived from complementary DNA (cDNA) libraries and methyl filtered Genome Space Sequence Reads (GSRs) laid foundation for gene expression analysis in tobacco. Kamalay and Goldberg (1980) measured the extent to which structural gene expression is regulated in an entire tobacco plant. Later, Matsuoka et al. (2004) analyzed the change of gene expression during the growth of tobacco BY-2 cell lines and found 9200 EST fragments corresponding to about 7000 genes. Rushton et al. (2008) reported 2513 TFs representing all of the 64 well-characterized plant TF families and used to create a database of tobacco transcription factors (TOBFAC). Edwards et al. (2010) designed Affymetrix tobacco expression microarray from a set of over 40 k unigenes and measured the gene expression in 19 different tobacco samples to produce the Tobacco Expression Atlas (TobEA). TobEA provides a snap shot of the transcriptional activity for thousands of tobacco genes in different tissues throughout the lifecycle of the plant. Expression profiling of tobacco leaf trichomes resulted in the identification of putative genes involved in resistance to biotic and abiotic stresses (Harada et al. 2010; Cui et al. 2011). Differentially expressed long noncoding RNAs (lncRNAs) found to be involved regulating jasmonates (JA) mediated plant defense against *M. sexta* attack (Li et al. 2020).

Storing and integrating different types of expression data and making these data freely available in formats appropriate for comprehensive analysis is essential for their effective utilization. Analysis of gene expression data provide hints towards understanding various aspects of plant development and resistance to biotic and abiotic stresses along with defining the molecular and genetic pathways associated with these processes. The expression databases host the transcript/ RNA/ probe information of different genes under varied native or test conditions along with the relevant software tools for analysis and retrieval of the data. EST databases constructed in the years ago for this purpose has metamorphosed to host microarrays and RNA sequences with the advent of technology. ESTs provide an insight into transcriptionally active genes in a biological sample under a given set of conditions and is relatively expensive and time consuming. Microarrays, however, offer a faster and less expensive alternative for simultaneously measuring the expression of thousands of genes that can be easily and reproducibly applied to identify genes showing specific expression patterns or responses across a broad range of conditions or treatments.

At present the Gene expression data have been archived as microarray and RNA-seq datasets in the public databases such as Gene Expression Omnibus (GEO), ArrayExpress (AE) and Genomic Expression Archive (GEA) (Table 10.13). These databases act as useful resources for the functional interpretation of genes. GEO hosts around 4,860 curated gene expression data sets as well as original series and platform records of 11 *Nicotiana* spp. (Table 10.8; https://www.ncbi.nlm.nih.gov/gds). ArrayExpress database have gene expression profiles of around 75 experiments including *N. attenuate*, *N. benthamiana*, *N. langsdorffii* x *N. sanderae* and *N. tabacum*. Genomic Expression Archive has 205 gene expression records related to 11

Nicotiana species. SGN maintaining two transcript libraries of *N. sylvestris* and 39 of *N. tabacum*. Further, there are exclusive expression databases for *Nicotiana attenuata* (NaDH) and *N. benthamiana* (https://btiscience.org/our-research/research-facilities/research-resources/nicotiana-benthamiana) along with the Sol genome networks for expression analysis among solanaceous members.

10.13.4 Protein or Metabolome Databases

Proteins are the end products of some of the expressed genes in an organism. While, a proteome is the set of proteins thought to be expressed by an organism in its life cycle. The metabolome can be defined as the complete complement of all small molecule (<1500 Da) metabolites found in a specific cell, organ or organism. The metabolome of a species is the link between its genotype and phenotype. It indicates the stage specific and organ specific response of the plants through gene expression in response to its environment. Metabolomics can influence both the gene expression and the protein function of the plant, hence, make it a central component in elucidating cellular systems and deciphering gene functions.

Proteomics and metabolomics approaches play pivotal role in functional genomics and have been essentially required for understanding plant development and stress tolerance abilities. Proteome and Metabolome profiling is an attractive tool for phenotyping plants confronted by environmental changes and biotic stresses. Such studies contribute significantly to the study of stress biology by distinguishing different compounds such as auxiliary products of stress metabolism from biosynthetically unrelated pathways, stress induced signal molecules, molecules that are part of plant acclimation process etc. The resultant metabolic compounds could be further studied by direct measurement or correlating with the changes in transcriptome and proteome expression during stress condition and can be confirmed by mutant analysis. Thus, metabolome study aid in unravelling the different pathways related to plant development and response to stresses. With the advent of high-throughput based systems, proteome and metabolome profiling was extensively carry out in the model plant like tobacco to examine stress signaling pathways, cellular and developmental processes.

Principal databases hosting tobacco proteome information are UniProt, Pfam, KEGG, SGN, NCBI, etc. and metabolome databases are SolCyc, REACTOME, The Golm Metabolome Database (GMD), MoNA (Massbank of North America), etc. The salient features of various proteome data bases are briefed below.

The proteomic data generated globally in various organisms is being stored and accessed through the Universal Protein Resource (UniProt), a comprehensive resource for protein sequence and annotation data. UniProt was established as a collaboration between the European Bioinformatics Institute (EBI), the Swiss Institute of Bioinformatics (SIB), and the Protein Information Resource (PIR) and consists of three databases that are optimized for different uses. The UniProt Knowledgebase (UniProtKB) is the central access point for providing information on extensively curated proteins including their function, classification and cross-references. The

UniProt Reference Clusters (UniRef) pools closely related sequences into a single record to speed up sequence similarity searches. The UniProt Archive (UniParc) is a comprehensive repository of all protein sequences and consisting only of unique identifiers and sequences. UniProt provides several sets of proteins assumed to be expressed by organisms whose genomes have been completely sequenced, termed "proteomes". There are 73,606 protein entries associated with proteome N. tabacum and a total of 154,728 entries for all Nicotiana species as on 30.09.2021.

The Pfam database contains a large collection of protein families, each represented by multiple sequence alignments. This database provides tools for protein alignments and annotation, domain organization of a protein sequence etc. There are about 4970 unique results for the search term Nicotiana in this data base as on 30.09.2021 indicating the protein entries in the database.

KEGG database in addition to protein info, projects biological processes from various organisms onto pathways consolidated in large network schemata. The KEGG database, at present, contains the information of annotated proteins of *N. tabacum* (61,780 No.), *N. tomentosiformis* (30,989) *N. sylvestris* (33,816) and *N. attenuata* (34,218). In the Sol genomics network database also providing the data of proteins annotated based on the draft genome sequences of *N. tabacum*, *N. benthamiana* and *N. attenuata.* Protein sequences collection of around 275,000, from several sources, are available for 20 *Nicotiana* spp. at NCBI along with annotated reports of four *Nicotiana* spp. (Tables 10.8 and 10.11).

SolCyc contains a collection of Pathway Genome Databases (PGDBs) related to Solanaceae species that are generated using Pathway Tools. It is a database hub at SGN for the manual curation of metabolic networks and includes annotated metabolic, regulatory and signaling processes in Solanaceous plants based on Omics data obtained from multiple resources. It has species-specific databases for Tomato (LycoCyc), Potato (PotatoCyc), Pepper (CapCyc), Coffee (CoffeaCyc), Petunia (PetCyc), *N. tabacum* (K326Cyc), *N. attenuata* (NattCyc), *N. sylvestris* (NiSylCyc), *N. tomentosiformis* (NiTomCyc), *N. benthamiana* (BenthaCyc); and multi-species databases for Combined *Nicotiana* genus (NicotianaCyc) and Combined Solanaceae database (SolanaCyc). In addition to proteomic data, NaDH is also providing metabolome data of *N. attenuata* with analysis tools and facilities for the search of metabolites and fragments based on annotation and measured values.

REACTOME offers intuitive bioinformatics tools for the visualization, interpretation and analysis of pathway knowledge to support basic and clinical research, modeling, genome analysis, systems biology and education. GMD is an open access metabolome database and provides public access to custom mass spectral libraries, metabolite profiling experiments along with additional information and tools. MoNA (Massbank of North America) is a centralized and collaborative database of metabolite mass spectra, metadata and associated compounds. At present, MoNA contains over 200,000 mass spectral records from experimental and *in-silico* libraries besides from user contributions.

The proteomic studies in tobacco revealed the interesting facts about the different stress responses (Amme et al. 2005). Analysis of the proteome of glandular trichomes revealed the enrichment of proteins belongs to components of stress defense

responses. In another study comparative proteomics of tobacco mosaic virus-infected *N. tabacum* plants identified major host proteins involved in photosystems and plant defense (Das et al. 2019). Metabolome study under water stress in tobacco identified a useful marker for drought stress for members of Solanaceae (Rabara et al. 2017).

10.13.5 Integration of Different Data

Analysis of a single omics data (e.g. genome, proteome, transcriptome and metabolome) provides biological understanding at a specific molecular layer. However, many agronomic and quality traits embrace complex crosstalk between various molecular layers viz., genome, transcriptome, proteome, and metabolome. These four 'omes' collectively generate the building blocks of systems biology. An integrative analysis of multiple layers of molecular data or system biology helps to discover and elucidate underlying molecular mechanisms of complex traits and thus, provide clues for genome designing. In the holistic study of the complex biological processes, it is imperative to have an integrative approach that combines multi-omics data to highlight the interrelationships of the involved biomolecules and their functions. Advent of high-throughput techniques and accessibility of multi-omics data from a large set of samples, a number of promising methods and tools have been developed for data integration and interpretation. Most of the biological databases collects and integrate data from different sources.

Databases namely INSDC, NCBI, SGN, NaDH, KEGG genome, EnsemblPlants, DAVID (Database for Annotation, Visualization, and Integrated Discovery), The BioStudies Database etc. are some of the integrated databases and resources that are collecting and integrating the omics data from different plants and tobacco (Table 10.13). As a collaborative foundational initiative, INSDC covers the spectrum of data raw reads, through alignments and assemblies to functional annotation, enriched with contextual information relating to samples and experimental configurations. NCBI in addition to providing wide-ranging tobacco basic data in its various databases, offers tools for integration of structural and functional genomic data and their annotations. The curated proteome data of *Nicotiana* species in Uniport, and metabolome data from different resources are being integrated in new datahubs like SGN, KEGG, NaDH etc., to provide holistic information from gene to pathway for the researchers. KEGG is an integrated database resource consisting of sixteen databases that includes genes and proteins, metabolites and other chemical substances, biochemical reactions, enzyme, disease-related network variations etc.

EnsemblPlants is an integrative resource presenting genome-scale information for a growing number of sequenced plant species (currently 33). It provides data on genome sequence, gene models, polymorphic loci, and functional annotation. Also provide additional information on variation data comprising phenotype data, population structure, individual genotypes, and linkage. In each of its release, comparative analyses are performed on whole genome and protein sequences, and genome alignments and gene trees are made available showing the implied evolutionary

history of each gene family. DAVID is a web-accessible database that integrates functional genomic annotations with intuitive graphical summaries. According to shared categorical data for Gene Ontology, protein domain, and biochemical pathway membership, lists of gene or protein identifiers are rapidly annotated and summarized. Numerous public sources of protein and gene annotation have been parsed and integrated into DAVID database. This database currently contains information on over 1.5 million genes covering more than 65,000 species. European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) is building and maintaining the BioStudies Database as a resource for encapsulating all the data associated with a biological study. One of the goals of BioStudies is to accept and archive data generated in experiments that can be characterized as "multi-omics".

These efforts of building archives, databases, and analysis tools in an integrated approach have been successful at enabling a better understanding as well as comparison of the omic resources of tobacco.

10.14 Brief Account on Social, Political and Regulatory Issues

10.14.1 Concerns and Compliances

Tobacco is one of the important high value commercial crops and is valued for its potential to generate higher farm income and employment to farmers and farm labors, and revenue to national governments. Today tobacco sector is reeling under the whirlpool of diametrically conflicting concerns relating to the livelihood security of those associated with tobacco production, processing and marketing on one hand and the serious health risks to consumers on the other. Use of huge quantities of forest wood as source of energy for flue-curing of FCV tobacco, environmental pollution caused by tobacco smoking and associated spitting habits of chewing tobacco are issues of serious concern to tobacco production. Further, climate change impacts, emerging biotic and abiotic stresses, pesticide residues, consumer preferences and tobacco regulatory policies are becoming increasingly complex and represent future challenges for tobacco cultivation (ICAR 2015).

WHO-Framework Convention on Tobacco Control (FCTC), with overwhelming membership of 182 countries, envisages non-price, price and tax measures to reduce the supply and demand for tobacco in the world. The FCTC signatory countries are under obligation to support the measures for reduction of supply and demand for tobacco. On May 31st every year, the world observes World No Tobacco Day (WNTD) promoted by the World Health Organization (WHO) with a primary focus on encouraging users to refrain from tobacco consumption and its related products for a period of at least 24 h.

10.14.2 Patent and IPR Issues

Researchers are developing various management strategies for minimizing crop yield losses at field level due to biotic stresses. This includes development of biotic stress resistant varieties through conventional breeding and biotechnological interventions. The advances in biotechnology and bioinformatics generated various genome-based tools, techniques, genes and gene constructs in the field of stress resistance (Dangl and Jones 2001). Intellectual property rights (IPR) for plants help protect investments made in research and development of new tobacco varieties (CORESTA 2005). In turn, this encourages further investment and helps continue the development of new varieties that increases economic returns throughout the tobacco supply chain. Over the past 25 years, an increasing number of governments and international organizations have enacted laws, regulations, or policies that acknowledge the need for IPR. While these provide protection to inventors or breeders, some of them may also recognize a farmer's exemption for saving seed for their own use.

Patents offer the owner the exclusive right to make, use, sell, offer for sale, or import for those purposes a patented product. It also offers the rights to a patented process and to make, use, sell, offer for sale, or import for those purposes the direct product of the patented process. The ability to patent plant varieties is recognized in some countries, but in many countries like India it is disallowed. There have been few patents granted for tobacco varieties in the world (CORESTA 2005). Court decisions in the United States have permitted the use of utility patents to protect plant varieties, and to date the United States Patent and Trademark Office has issued over 200 utility patents with claims to seeds. European countries that are members of the European Patent Office (EPO) ban the patenting of plant varieties, but recent determinations support patent claims directed to plants of more than one variety. Thus, a utility patent and the ruling by the EPO may suggest a type of broad protection for a novel plant trait that is not recognized under plant variety protection (PVP) legislation. Rights granted under patents are also distinguishable from PVP in that exceptions are not allowed for using a patented variety for experiments, breeding purposes, or for private or non-commercial purposes. On the other hand, a patent provides explicit information on the processes and methods used to develop the variety, and it is often tied to a public notification of seed availability once the patent expires. The term of a patent varies across the world, but in the US a utility patent is valid for 20 years after the application date.

Recent breakthroughs in crop science, especially in the area of molecular biology, and plant genomics have opened unlimited number of opportunities in the way the tobacco plants can be utilized for commercial and medicinal uses. Genetic improvements in agronomic traits of crops such as yield attributes and, pest and disease resistance through molecular and plant genomic approaches has been accompanied by the great abundance of new patents issued in these fields. There is a scope for patenting of novel methods of making biotic stress resistant tobacco genotypes, methods of introgressing nucleic acid molecules associated with biotic stresses, genes conferring resistance to various tobacco pests, viruses, bacteria, fungi and nematodes (Hefferon

2010). Patenting activity in resistance genes in tobacco is initiated in 1992 and there is considerable progress in patenting from the year 2000 and it was more prominent from 2010 onwards (Prabhakararao et al. 2016). Majority of these patent documents (around 60%) are in the jurisdiction of USA and China.

The intellectual information generated in the frontier areas is available in non-patent and patent literature. Non-patent literature (NPL) consists of peer reviewed scientific paper, publications such as conference proceedings, databases (DNA structures, gene sequences, chemical compounds, etc.) and other literature (translation guides, statistical manuals, etc.). Nearly 80% of all the technical information available in the world is hidden in the patent documents and other IP assets (Prabhakararao et al. 2016). Patent mapping helps in retrieving and exploring the information protected in the intellectual property documents. Collections of patent documents are available in a number of patent information databases (https://guides.library.queensu.ca/patents/databases**).** Most patent offices provide free access to patent documents via public databases and some of the largest and most popular patent databases are given at Table 10.14. Patent information can be used to decide the patentability of an invention, avoid re-invention and infringement, provide the current state of the art in a given field of technology, find the latest trends in R and D being pursued by the peers and competitors etc.

Table 10.14 Patent databases

I	Patent office databases	
S. No	Databases	Producing patent office
1	Canadian Patents Database	Canadian Intellectual Property Office (CIPO)
2	DEPATISnet	German Patent and Trademark Office (DPMA)
3	Espacenet	European Patent Office (EPO)
4	JP-PlatPat	Japan Patent Office (JPO)
5	PatentScope	World Intellectual Property Organization (WIPO)
6	US Patent Databases	United States Patent and Trademark Office
II	Databases that index patent literature	
S. No	Database	Literature covered
1	FreePatentsOnline (FPO)	Patents from the European Patent Office, Germany and the US, Japanese patent abstracts and published Patent cooperation Treaty (PCT) applications
2	Google Patents	Full-text patent documents from Canada, China, EPO, Germany, PCT and the US
3	Patent Lens	Around 100 million patent documents from 90+ national and regional patent offices, and full-text EP, US and WO documents

10.14.3 Disclosure of Sources of GRs, Access and Benefit Sharing

Genetic Resources (GRs) are a key source of numerous biotechnology innovations (Steward 2018). Historical studies reveals that less than 1% of species have provided the necessary basic resources for the progress of all civilization so far. Therefore, unexplored GRs expected to have certain potential value in further advancement of civilization. The main characteristic of bioprospecting is uncertainty as it is seldom possible to forecast which genes, species or ecosystems will turn out to be valuable in the future. With an aim to improve the sustainable use of GRs to protect biodiversity, and support benefit sharing with originating countries Access-Benefit Sharing (ABS) systems/regulations have been developed in the recent decades. 'Convention on Biological Diversity' (CBD) of 1992 serves as starting point in many countries for biodiversity conservation and use. As a supplementary agreement to the CBD, The Nagoya protocol of 2010 is aimed at improving the fair and equitable sharing of benefits arising out of the utilization of genetic resources. ABS systems vary widely from country to country. GR-rich countries tend to organize their ABS systems more strictly and focus on securing an equitable share of the benefits from the products developed through the use of GRs. In order to enhance the ABS compliance, over the years, several governments have introduced disclosure requirements (DRs) in their patent systems. However, the study conducted by Steward (2018) in Brazil and India indicated that DRs might enhance the R&D costs with increased uncertainty and delay in using GRs. The uncertainty may relate to unclear definitions of GRs (Ex.: Brazil, India) and to the fact that the content of the Disclosure Requirements is not verified by the IP authorities in these countries. This may provides ample scope for challenging patents on ABS conditions even after approval. The extent of the DR-effects on R&D cycles depends largely on local market conditions and (efficiency of) ABS legislation (Steward 2018).

10.14.4 Farmers' Rights

Since the dawn of agriculture, farmers around the world have been the custodians and innovators of agricultural biodiversity (FAO 2017; Craig 2004). Farmers are responsible for collecting the best seeds and cultivating different types and species of tobacco throughout the world. Through the careful selection of their best seeds and propagating material, and exchange with other farmers, it became possible to develop and diversify crop varieties. Over thousands of years of constant management and innovation by farmers, a small number of initial crops and varieties have progressed into an incredible treasure of plant diversity for food and farming. In certain countries like India, households traditionally raising different tobacco landraces in their kitchen gardens since generations from the seeds collected from their own crops, thus maintaining and protecting biodiversity.

Farmers' access to seed and propagating material and opportunities for exchanging the planting material are strongly influenced by seed regulations (variety release and seed marketing regulations), legislation linked to intellectual property rights (patents and plant breeders' rights), and regulations concerning the bio-prospecting of genetic resources. Farmers' Rights are the rights arising from the contributions of farmers in conserving, improving, and making available plant genetic resources, mainly those in the centers of origin/diversity, in the past, present and future.

The notion of Farmers' Rights was developed during the early 1980s to counter increased demands for Plant Breeders' Rights (PBR) being voiced in international negotiations. The aim was to draw attention to the unremunerated innovations of farmers, which were seen as the foundation of all modern plant breeding. The concept first emerged in international negotiations within FAO in 1986. Already in 1987, practical solutions were being proposed, serving as the foundation for all further negotiations on Farmers' Rights, and providing substantial input to the framing of current understanding of the issue. In 1989, Farmers' Rights gained formal recognition by the FAO Conference. In 1991, the Conference decided to set up a fund for the realization of these rights, but this has never materialized. In May 1992, the Convention on Biological Diversity (CBD) was adopted with a resolution on the interrelationship between the CBD and the promotion of sustainable agriculture. Through this resolution, FAO was advised to initiate negotiations for a legally binding international regime on the management of plant genetic resources, and in this context, to resolve the question of Farmers' Rights. Agenda 21, a dynamic program approved at the UN Conference on Environment and Development held in Rio de Janeiro in 1991, had voiced similar demands. In November 1996, Global Plan of Action for the Conservation and Sustainable Utilization of Plant Genetic Resources for Food and Agriculture (hereafter referred to as the Global Plan of Action) was endorsed by the FAO Council, by the Conference of the Parties to the CBD, and by the World Food Summit at FAO acknowledges the need to realize Farmers' Rights. The Second Global Plan of Action, prepared under the aegis of the Commission on Genetic Resources for Food and Agriculture, was adopted by the FAO Council on 29 November 2011 contains a set of recommendations and activities intended as a framework, guide and catalyst for action at community, national, regional and international levels. The International Treaty on Plant Genetic Resources for Food and Agriculture (hereafter referred as the International Treaty) adopted in 2001 addressed the issue of Farmers' Rights in its Article 9, and in its Preamble. This Treaty recommends the Contracting Parties to protect and promote Farmers' Rights in agreement with their national laws. In Article 9, the Contracting Parties of the International Treaty recognize the enormous contribution that farmers of all regions of the world have made, and will continue to make, for the conservation and development of plant genetic resources as the basis of food and agricultural production throughout the world. The responsibility for implementing Farmers' Rights lies with national governments, and that they can choose the measures to do so as per their needs and priorities. Measures covering the protection of traditional knowledge, benefit-sharing and participation in decision-making are suggested. Also, the farmers rights to save, use, exchange and sell farm-saved seeds and planting material are addressed, but without giving any specific direction

for execution. In addition, Farmers' Rights are addressed in the preamble, and other articles in the Treaty clearly support these rights, albeit not explicitly (for example, the provisions on conservation and sustainable use and on benefit sharing). There are no legally binding provisions in the International Treaty on how to implement Farmers' Rights at national level.

10.14.5 Traditional Knowledge

Indigenous peoples and local communities have to cope with extreme weather and adapt to environmental change for centuries in order to survive as often they live in harsh natural environments (Swiderska et al. 2011). They use their traditional knowledge (TK) generated using long standing traditions and practices in relation to adaptive ecosystem management and sustainable use of natural resources. Hence, the diversity of traditional varieties sustained by farmers around the world is increasingly valuable for adaptation to climate changes and to cope with emerging new pests and diseases, particularly as modern agriculture relies on a very limited number of crops and varieties. Landraces or traditional varieties are genetically more diverse than recent varieties and considered to be good sources of resistance to biotic stresses (https://www.cbd.int/traditional/what.shtml). Local communities use wild foods to supplement their diets and thus conserve wild species which are valuable sources of stress resistant genes. The tradition farmers are well placed to identify resilient crop species and resistant varieties for biotic stresses with the available accumulated TK, and in view of their long experience in cultivating crops under changing climates. Traditional Knowledge about resilient properties, such as drought and pest resistance traits and biotic stress resistant varieties and wild crop relatives (Jarvis et al. 2008) can be a valuable information in developing biotic stress tolerant varieties in tobacco also.

10.14.6 Treaties and Conventions

International agreements like Convention on Biological Diversity (CBD), International Treaty for Plant Genetic Resources for Food and Agriculture (ITPGRFA), The International Union for the Protection of New Varieties of Plants (UPOV) are of special significance in the context of agricultural sector in general and biotechnology particular. They are briefly discussed below.

10.14.6.1 Convention on Biological Diversity (CBD)

The Convention on Biological Diversity (CBD), known informally as the Biodiversity Convention, is a legally binding international treaty and was ratified in 1992 at the Rio

earth summit (https://www.cbd.int/convention/). The convention currently having 196 Parties (168 Signatures). The three main goals of the Convention are conservation of biological diversity (or biodiversity), sustainable use of its components and fair and equitable sharing of benefits arising from genetic resources. In other words, its objective is to develop national strategies for the conservation and sustainable use of biological diversity. It is considered as the key document with regards to sustainable development.

The convention, for the first time, recognized in the international law that the conservation of biological diversity is "a common concern of humankind" and is an integral part of the development process. All the ecosystems, species, and genetic resources are covered in the agreement. It associates traditional conservation efforts to the economic goal of sustainably using biological resources. It has put in place the principles for the fair and equitable sharing of the benefits arising from the use of genetic resources, notably those destined for commercial use.

The Contracting Parties shall promote international technical and scientific cooperation in the field of conservation and sustainable use of biological diversity, wherever necessary, through appropriate international and national institutions. In pursuance of the objectives of this Convention and in accordance with national legislation and policies, the Contracting Parties shall, encourage and develop methods of cooperation for the development and use of both indigenous and traditional technologies. For achieving this purpose, the Contracting Parties shall also promote cooperation in the training of personnel and exchange of experts.

In respect of handling of biotechnology and distribution of its benefits, Article 19 of the Convention states—

- Each Contracting Party shall take legislative, administrative or policy measures, as appropriate, to provide for the effective participation in biotechnological research activities by those Contracting Parties, especially developing countries, which provide the genetic resources for such research, and where feasible in such Contracting Parties.
- Each Contracting Party shall take all practicable measures to promote and advance priority access on a fair and equitable basis by Contracting Parties, especially developing countries, to the results and benefits arising from biotechnologies based upon genetic resources provided by those Contracting Parties.
- The Parties shall consider the need for and modalities of a protocol setting out appropriate procedures, including, in particular, advance informed agreement, in the field of the safe transfer, handling and use of any living modified organism resulting from biotechnology that may have adverse effect on the conservation and sustainable use of biological diversity.
- Each Contracting Party shall, directly or by requiring any natural or legal person under its jurisdiction providing the organisms referred to in paragraph 3 above, provide any available information about the use and safety regulations required by that Contracting Party in handling such organisms, as well as any available information on the potential adverse impact of the specific organisms concerned to the Contracting Party into which those organisms are to be introduced.

CBD has three Protocols viz. The Nagoya Protocol on Access and Benefit-sharing, The Cartagena Protocol on Biosafety and The Nagoya – Kuala Lumpur Supplementary Protocol on Liability and Redress to the Cartagena Protocol on Biosafety under CBD. The essence of these protocols is given below.

10.14.6.2 Cartagena Protocol on Biosafety

The Cartagena Protocol on Biosafety to the Convention on Biological Diversity is an international treaty governing the movements of living modified organisms (LMOs) resulting from modern biotechnology from one country to another (http://bch.cbd.int/protocol). It was adopted as a supplementary agreement to the Convention on Biological Diversity on 29 January 2000 and came in force on 11 September 2003. Currently, it has 173 Parties (103 Signatures). The objective of the Protocol is to contribute to ensuring an adequate level of protection in the field of the safe transfer, handling and use of 'living modified organisms resulting from modern biotechnology' that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health, and specifically focusing on trans-boundary movements. The Protocol provides for Parties to enter into bilateral, regional and multilateral agreements and arrangements regarding intentional trans-boundary movements of living modified organisms.

The Protocol establishes a Bio-Safety Clearing -House to: (a) Facilitate the exchange of scientific, technical, environmental and legal information on, and experience with, living modified organisms; and, (b) Assist Parties to implement the Protocol, taking into account the special needs of developing country Parties, in particular the least developed and small island developing States among them, and countries with economies in transition as well as countries that are centres of origin and centres of genetic diversity.

10.14.6.3 Nagoya Protocol

The Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization (ABS) to the Convention on Biological Diversity is a supplementary international agreement (https://www.cbd.int/abs/). The Protocol was adopted on 29 October 2010 in Nagoya, Japan and entered into force on 12 October 2014. Currently, it has 131 Parties (132 ratifications) (92 signatories). The protocol aims at fair and equitable sharing of benefits arising from the utilization of genetic resources, there-by contributing to the conservation and sustainable use of biodiversity. The protocol offers a transparent legal framework for the effective implementation of one of the three objectives of the CBD: the fair and equitable sharing of benefits arising out of the utilization of genetic resources. The Nagoya Protocol applies to genetic resources that are covered by the CBD, and to the benefits arising from their utilization. It also covers traditional knowledge (TK) associated with genetic resources that are covered by the CBD and the benefits arising from its utilization.

The Nagoya–Kuala Lumpur Supplementary Protocol on Liability and Redress to the Cartagena Protocol on Biosafety

This Supplementary Protocol entered into force on 5 March 2018 and currently, it has 49 Parties. Adopted as a supplementary agreement to the Cartagena Protocol on Biosafety, it aims to contribute to the conservation and sustainable use of biodiversity by providing international rules and procedures in the field of liability and redress relating to living modified organisms (http://bch.cbd.int/protocol/supplementary/). The Supplementary Protocol requires that response measures are taken in the event of damage resulting from living modified organisms which find their origin in a transboundary movement, or where there is sufficient likelihood that damage will result if timely response measures are not taken. This Protocol gives a definition of 'damage', referring to an adverse effect on the conservation and sustainable use of biological diversity that is measurable or otherwise observable and significant, taking also into account risks to human health. The Protocol requires that a causal link between the damage and the living modified organism be established. Along with imposing a requirement for response measures, the Protocol obliges Parties to continue to apply existing legislation on civil liability or to develop specific legislation concerning liability and redress for material or personal damage associated with damage to the conservation and sustainable use of biological diversity.

10.14.6.4 The International Treaty on Plant Genetic Resources for Food and Agriculture

This International Treaty was adopted in 2001 (FAO 2009). The objectives of the International Treaty on Plant Genetic Resources for Food and Agriculture are the conservation and sustainable use of all plant genetic resources for food and agriculture and the fair and equitable sharing of the benefits arising out of their use, in harmony with the Convention on Biological Diversity, for sustainable agriculture and food security. This legally-binding Treaty is in harmony with the Convention on Biological Diversity and covers all plant genetic resources relevant for food and agriculture. The Treaty is vital for ensuring the continuous availability of the plant genetic resources for feeding people from different countries. The Treaty recognizes the enormous contribution that the local and indigenous communities and farmers of all regions of the world and takes measures to protect and promote Farmers' Rights. The Contracting Parties agree to establish a multilateral system, which is efficient, effective, and transparent, both to facilitate access to plant genetic resources for food and agriculture, and to share, in a fair and equitable way, the benefits arising from the utilization of these resources, on a complementary and mutually reinforcing basis. The treaty takes care of (a) protection of traditional knowledge relevant to plant genetic resources for food and agriculture; (b) the right to equitably participate in sharing benefits arising from the utilization of plant genetic resources for food and agriculture; and (c) the right to participate in making decisions, at the national level, on matters related to the conservation and sustainable use of plant genetic resources for food and agriculture.

10.14.6.5 The UPOV Convention

For the protection of plant variety, The UPOV Convention was adopted in Paris in 1961 and entered into force in 1968. It was first revised slightly in 1972 and more considerably in 1978 and 1991. The 1978 Act entered into force in 1981, and the 1991 Act in 1998 (www.upov.int). The Convention established an inter-governmental organization called the Union Internationale pour la Protection des Obtentions Végétales (UPOV). Internationally, the most prevalent means of protecting plant varieties is through Plant Variety Protection (PVP), which is enabled in the 77 countries that are members of the International Union for the Protection of New Varieties of Plants which is headquartered in Geneva, Switzerland. The stated mission of UPOV's is "To provide and promote an effective system for plant variety protection, with the aim of encouraging the development of new varieties of plants for the benefit of society". The current act of the convention was adopted in 1991 and recognizes breeder's rights to a variety if the variety is: (1) new; (2) distinct; (3) uniform; and (4) stable. The breeder's rights in the 1991 Act require authorization of the breeder to perform the following: (1) production or reproduction (multiplication) (2) conditioning for the purpose of propagation, (3) offering for sale, (4) selling or marketing, (5) exporting, (6) importing, (7) stocking for any purpose mentioned in (1)–(6) above. Breeder's rights to a variety remain in effect for a period of 20 years from the date on which the rights were granted.

For the first time, the Act of 1991 included protection against "essentially derived" varieties, which are derived from the protected variety, that is not clearly distinguishable from the protected variety, or which requires repeated use of the protected variety for production purposes. Examples of the manner in which an essentially derived variety could be developed from a protected variety include: (1) the selection of a natural or induced mutant, or of a somaclonal variant, (2) the selection of a variant individual from plants of the initial variety, (3) backcrossing, (4) transformation by genetic engineering. Exceptions to breeder's rights were granted for: (1) Acts done privately and for non-commercial purposes, (2) Acts done for experimental purposes (3) Acts done for the purpose of breeding other varieties, except for the generation of essentially derived varieties.

10.14.7 Participatory Breeding

Conservation of diversity and selection of naturally occurring high yielding and stress resistant variants by cultivators was the principal method of tobacco improvement, for thousands of years prior to 1800s, during and after the domestication of *Nicotiana* species. The systematic varietal improvement started by scientists in the later period in established research organizations in different countries led to release of number of biotic stress resistant tobacco varieties using conventional plant breeding techniques. This has resulted in erosion of genetic diversity in the farmers' fields due to large

scale cultivation of few improved varieties. The resultant genetic vulnerability to diseases and pests necessitates the development of stress resistant cultivars.

In view of the limitations to formal breeding and the threats to farmers' seed systems, participatory plant breeding (PPB) emerged as a means to bring farmers back into the breeding process as active participants (Greenberg 2018). The role played by farmers in agricultural biodiversity conservation and use is taken as an advantage while making them partners in breeding tobacco varieties. In the development of improved biotic stress resistant varieties, PPB ensures the improvement of adapted local genetic materials using the diversity available either with them or public gene banks to suit the farmer needs having resistance to races and biotypes prevailing under their conditions. This also empower the farmer in terms of technical and organizational skills in preserving and evolving materials under their control, on-farm management, and local creativity/innovation. PPB involves the active participation of farmers in few or all of the set of sequenced breeding program activities viz. priority setting, genetic materials acquisition and selection, crossing, selection at early stages and advanced stages, in situ experimentation/testing, and production and sharing of genetic materials and knowledge. However, PPB may be a substitute for station-based research or scientist-managed on-farm trials; rather than a complementary breeding process (Hardon et al. 2005; Aguilar-Espinoza, 2007; Ceccarelli et al. 2009).

10.15 Future Perspectives

10.15.1 Potential for Expansion of Productivity

The genetic potential for increasing the crop yield through conventional approaches is still obtainable in tobacco (Sarala et al. 2016). Combination of traditional breeding techniques with genome designing strategies can further accelerate the genotype improvements in tobacco for increasing and stabilizing yields. Further advances in genomic research in terms of genomic, transcriptomic, proteomic and metabolomics and their integrated analysis would assist in designing of appropriate genome assisted breeding strategies for genome designing of tobacco for attaining maximum potential yields with good quality and pests and disease resistance in a short span of time.

10.15.2 Potential for Expansion into Nontraditional Areas

The genus *Nicotiana* with well-defined group of species of which tobacco (*N. tabacum L.)* is an important agricultural crop plant that plays a significant role in the economies of many countries (FAO 2019). *Nicotiana* species are also used in the elucidation of various principles related to disease resistance, synthesis of secondary metabolites and basic aspects of plant physiology. In view of its higher level of

biomass accumulation, tobacco is considered to be a promising crop for the production of commercially important substances (e.g., medical drugs and vaccines) through molecular farming and cultivation of tobacco for its valuable native phyto-chemicals viz. nicotine, solanesol, proteins and organic acids (Sarala 2019).

Tobacco is considered to be one of the most important model systems in plant biotechnology till date and going to continue further. In view of its easy transformation ability, tobacco plant serving as an experimental system various pilot studies on the expression of novel transgenes that are later being used in important food crops (Krishnamurthy et al. 2008), for the study of polyploidy, and for investigation of secondary product biosynthesis (Wang and Bennetzen 2015). Scientific research in *Nicotiana* going to be accelerated in a wide range of areas with the availability of entire genome sequences and transcriptomic profiles. As *Nicotiana* species are found to be one of the best plants for transient transgene expression in leaf via simple and *A. tumefaciens* infiltration, better knowledge of the *Nicotiana* genome will provide necessary raw material for studying the function of any transformed gene.

Both wild and domesticated forms of tobacco accumulate a wide variety of alkaloids and other phytochemicals, the content and composition of which vary among species. The work done so far has brought out the tremendous scope for exploiting the crop for extraction of many valuable native phytochemicals viz. nicotine, solanesol, organic acids, proteins (green leaf) and seed oil having pharmaceutical and industrial importance (Chakraborty et al. 1982; Chida et al. 2005; Narasimha Rao and Prabhu 2005; Patel et al. 1998). Solanesol is a major component of tobacco (from traces to 4.7%) and is used as an intermediate in the production of valuable pharmaceuticals viz. co enzyme Q9, co enzyme Q10, Vitamin K2, Vitamin E and N-solanesyl-N, N′-bis (3,4-dimethoxybenzyl) ethylenediamine (SDB) which has the potential for use in the treatment of migraines, osteoporosis, neurodegenerative diseases, hypertension, cardiovascular diseases, anti-aging, to improve brain health and as dietary supplement for type 2 diabetics (Sarala 2019). While, Nicotine is the principal alkaloid synthesized in roots and accumulated in the leaf (0.1–5%). Nicotine can be used as a pesticide for controlling many agriculturally important pests and in pharma industry as smoking cessation products. Fraction-1 protein is the most abundant protein in tobacco and constitutes about 50% of soluble protein and 25% of total protein. This can be used for manufacturing food supplements. Tobacco leaf contains malic acid (4.0–4.5%) and citric acid (0.5–2.0%). These acids can be extracted from leaf and can be used for solubilisation of Rock phosphate and as foods and beverages. Tobacco seed contains 32–42% oil which can be used as edible oil and for paint and soap industry. Thus, with the potential to generate considerable volumes of biomass per unit area, tobacco genotypes can be bred for higher amounts of native phytochemicals viz. nicotine, solanesol, organic acids, proteins (green leaf) and seed oil for pharmaceutical and industrial purposes.

Tobacco has an established history as a routine system for molecular farming and often is chosen as a production platform due to its easy genetic modification. Other advantages of tobacco are established technology for gene transfer and expression, higher biomass yield, potential for rapid scale-up in view of prolific seed production, and availability of required infrastructure for processing. Although many tobacco

cultivars produce high levels of toxic alkaloids, there are low-alkaloid ones that can be utilized for the production of pharmaceutical proteins (Ma et al. 2003).

Tobacco is the first and probably only plant, where plastid transformation is successfully established as a routine (Svab and Maliga 1993; Daniell et al. 2002). High level of transgene expression is possible with chloroplasts and hence, there is a remarkable potential for large scale production of active proteins trough molecular farming. TMV genome can be rapidly manipulated and can be used as a vector as it has the ability to rapidly infect the tobacco plant. The transgenic tobacco plants express the target protein transiently. Molecular farming in tobacco hairy roots is another option where it triggers the secretion of a pharmaceutical antibody. Generally recombinant pharmaceutical proteins expressed in hairy root cultures can be secreted into the medium to improve product homogeneity and to facilitate purification.

A transgenic tobacco was the first plant used to be used for recombinant human antibody production (in 1989); this was quickly followed by production of human serum albumin (1990). Furthermore, it is not used as food or feed and therefore it is easier to manufacture active compounds in tobacco without fear of these compounds mixing with food or feed. Important pharmaceutical proteins viz. Human Growth hormone, Human serum albumin, Erythropoietin, Human-secreted alkaline phosphatise, Collagen, Protein C, Granulocyte–macrophage colony-stimulating factor, Epidermal growth factor, Hepatitis B virus envelope protein, Escherichia coli heat-labile enterotoxin, Diabetes autoantigen Disease, Cholera toxin B subunit, Immunoglobulin G1, Immunoglobulin M and Secretory immunoglobulin A etc. have been successfully found to produce in Tobacco (Thomas et al. 2002; Ma et al. 2003). In a review, Ashraful et al. (2014) reported that the bio-diesel from tobacco seed oil could be successfully used to run diesel engine that gave excellent performance and most effective regulated emissions. Tobacco genotypes with high biomass and seed yield can be identified and tailored to biosynthesize and accumulate of plant derived storage lipids such as triacylglycerol that can acts as a sustainable, carbon–neutral alternative biofuels (Carlsson et al. 2011). Considering various potential use of tobacco in farming of important biomolecules, tailoring tobacco for molecular farming going to be an important objective for tobacco improvement programs.

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Chapter 11
Biotechnology Approaches in Breeding for Biotic Stress Resistance in Yam (*Dioscorea* spp.)

Paterne A. Agre, Jean M. Mondo, Alex Edemodu, Ryo Matsumoto, Olufisayo Kolade, Lava P. Kumar, Robert Asiedu, Malachy Akoroda, Ranjana Bhattacharjee, Melaku Gedil, Patrick Adebola, and Asrat Asfaw

Abstract Yam (*Dioscorea* spp.) is a major staple and cash crop in tropical and subtropical regions. However, biotic (fungus, viruses, tuber rots, nematodes, insects, etc.) and abiotic stresses (drought, low soil fertility, etc.) substantially impact the productivity and quality of yam crop in regions where it is majorly cultivated.

P. A. Agre (✉) · J. M. Mondo · A. Edemodu · R. Matsumoto · O. Kolade · L. P. Kumar · R. Asiedu · R. Bhattacharjee · M. Gedil · A. Asfaw
International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria
e-mail: p.agre@cgiar.org

J. M. Mondo
e-mail: m.mubalama@cgiar.org

A. Edemodu
e-mail: a.edemodu@cgiar.org

R. Matsumoto
e-mail: r.matsumoto@cgiar.org

O. Kolade
e-mail: o.kolade@cgiar.org

L. P. Kumar
e-mail: l.kumar@cgiar.org

R. Asiedu
e-mail: r.asiedu@cgiar.org

R. Bhattacharjee
e-mail: r.bhattacharjee@cgiar.org

M. Gedil
e-mail: m.gedil@cgiar.org

A. Asfaw
e-mail: a.amele@cgiar.org

J. M. Mondo
Institute of Life and Earth Sciences, Pan African University, University of Ibadan, Ibadan, Nigeria

Université Evangéliqueen Afrique (UEA), Bukavu, Democratic Republic of Congo

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Developing and deploying resilient cultivars is a cost-effective and environmentally sound approach to enhance productivity in stressful environments. Breeding initiatives in yam to develop improved cultivars have long relied on conventional or classical methods, which are time-consuming and labor-intensive. However, in recent years, biotechnological approaches are being successfully introduced into yam genetic improvement to shorten the breeding cycle, optimize parent selection, predict cross and progeny performances, identify seedling sex, and break interspecific hybridization barriers among yam species. The approaches include next-generation sequencing-based genotyping, transcriptomics, metabolomics, genetic transformation, gene editing, genome-wide association studies, genomic prediction, marker-assisted selection, in vitro culture, ploidy analysis, and somatic hybridization. Although several advances have been attained in yam research to identify regions controlling key traits for biotic stresses, there is low translation to widespread applications in yam cultivar development. This chapter reviews the status and prospects of resistance breeding for yam and discusses biotechnology approaches in breeding multiple-stress-resistant cultivars. In addition, it provides insights in to the broader implementation of biotechnological tools in yam breeding and research.

Keywords Marker technology · Anthracnose · Yam mosaic virus · Nematode · Variety development

11.1 Introduction

Yam is a generic name for ~600 species of the *Dioscorea* genus (Wilkin et al. 2005; Darkwa et al. 2020a). Of these species, 11 are widely cultivated. These include *D. alata* L., *D. rotundata* Poir., *D. esculenta* (Lour.) Burkill, *D. cayenensis* Lam., *D. bulbifera* L., *D. dumetorum* (Kunth) Pax, *D. trifida* L., *D. opposite* Thunb., *D. japonica* Thunb., *D. nummularia* Lam., and *D. pentaphylla* L. (Darkwa et al. 2020a). In addition to these cultivated species, there are semi-domesticated and wild species such as *D. burkilliana* J. Miège, *D. minutiflora* Engl., *D. praehensilis* Benth., *D. schimperiana* Hochst. Ex Kunth., *D. semperflorens* Uline, *D. mangenotiana* J. Miège, *D. smilacifolia* De Wild. & T. Durand, etc., grown on a subsistence basis or collected from the wild to fill the hunger gap during drought and lean periods (Adewumi et al. 2021). Based on its economic importance, yam ranks fourth among root and tuber crops following cassava, potato and sweet potato worldwide and the second to cassava in West Africa (Alabi et al. 2019). It is cultivated for starchy underground and aerial tubers rich in vitamin C, dietary fiber, vitamin B6, protein, potassium,

M. Akoroda
Department of Agronomy, University of Ibadan, Ibadan, Nigeria
e-mail: malachyoakoroda@gmail.com

P. Adebola
International Institute of Tropical Agriculture (IITA), Abuja, Nigeria
e-mail: p.adebola@cgiar.org

and manganese and low in saturated fat and sodium (Arnau et al. 2010). The crop provides 200 cal a day to ~300 million people in tropical and subtropical countries (Price et al. 2017, 2020). Some yam species are sources of secondary metabolites used for industrial and pharmaceutical purposes (Price et al. 2018).

West Africa is the major producer and consumer of yam. Six countries, namely Nigeria, Ghana, Côte d'Ivoire, Benin, Cameroon, and Togo, accounted for 92% (~67 million tons) of the global yam production in 2018 (FAO 2020). In these countries, referred to as "the African yam belt", the per capita consumption is high, ~40 kg per person per year with significant disparities (9–73 kg) among ethnic groups (Bricas and Attaie 1998). In this region, yam represents an opportunity for poverty alleviation as ~5 million people directly depend on its value chain for income (Mignouna et al. 2020). The yam production is also part of religious and socio-cultural events (Sartie and Asiedu 2011; Darkwa et al. 2020a; Obidiegwuet al. 2020).

Biotic (fungus, viruses, nematodes, insect pests) and abiotic (drought, low soil fertility, etc.) stresses are among the major yield-limiting factors in low input yam farming systems (Arnau et al. 2010; Frossard et al. 2017; Darkwa et al. 2020a; Matsumoto et al. 2021; Morse 2021). These factors keep the average yam yield at ~10 t ha^{-1}, far below its potential (40 and 50 t ha^{-1} for *D. rotundata* and *D. alata*, respectively) (Frossard et al. 2017; FAO 2020). In the last two decades (1998–2018), yam production in West Africa doubled from ~34 to 67 million tons, as a result of rapid expansion of cultivated lands (from ~3.6 to 8.1 million hectares). During the same period, the productivity oscillated between 8 and 12 t ha^{-1} with a decreasing trend (FAO 2020). The current extensive yam farming and the search for new fertile lands will soon reach the limit due to rapid population growth. Besides, expanding cultivated lands is often associated with deforestation, which could exacerbate climate change in the region. Table 11.1 provides an estimate of yield losses associated with major yam biotic and abiotic stresses. Fast population growth and climate change will most probably worsen these stresses in sub-Saharan Africa (Srivastava et al.

Table 11.1 Yield losses associated with major biotic and abiotic factors in yam production

Factors	Species	Yield loss (%)	Distribution	Reference
YMV	*D. rotundata*	40–50	West Africa	Adeniji et al. (2012)
YAD	*D. alata*	80–90	Worldwide	Penet et al. (2016)
Tuber rots	*D. rotundata*	25–40	West Africa	Bonire (1985), Acholo et al. (1997)
Nematode	*D. rotundata*	~40	West Africa	Atu et al. (1983), Kolombia et al. (2017)
Drought + heat	*D. alata*	18–33	West Africa	Srivastava et al. (2012)
Low soil fertility	*D. rotundata*	33–70	West Africa	Matsumoto et al. (2021)
Waterlogging	*D. alata & rotundata*	~57	West Africa	Igwilo and Udeh (1987)

YMV Yam mosaic virus, *YAD* Yam anthracnose disease

2012; Thiele et al. 2017; Friedmann et al. 2018). An extensive use of external inputs (fertilizers, pesticides, irrigation, etc.) to control these constraints is unpractical for the predominantly resource-poor farmers and harmful to the environment. There is, therefore, a need for developing high-yielding varieties with resistance to biotic and abiotic stresses and deliver them to farmers through a functional seed system (Friedmann et al. 2018; Mondo et al. 2021a). Breeding for resistance has several advantages over using chemicals or any other external input: it is cost-effective, practical, usually long-lasting, efficient, and safer for the environment and humans (Hua et al. 2020).

Yam breeding still largely relies on conventional or classical methods for variety development. These are, however, time-consuming and labor-intensive (Darkwa et al. 2020a). It takes more than ten years to get a variety released using conventional approaches. Therefore, a range of biotechnological approaches are being successfully introduced into yam research. These approaches include next-generation sequencing (NGS)-based genotyping procedures, transcriptomics, metabolomics, genetic transformation (or transgenics), gene editing, marker-assisted selection, ploidy analysis, etc. (Darkwa et al. 2020a). The approaches aimed at shortening the breeding cycle, optimizing the breeding program as well as fast developing yam varieties to meet end-user' preferences (Tamiru et al. 2017; Friedmann et al. 2018; Darkwa et al. 2020a).

Among the advanced approaches adopted by the International Institute for Tropical Agriculture (IITA) and research partners under Africa Yam and NSF-BREAD projects, Genome-wide association studies (GWAS) are currently underway. GWAS efforts are to determine quantitative trait loci (QTLs) linked to various traits such as yam mosaic virus (YMV), yam anthracnose disease (YAD) resistance, dry matter content, tuber browning index, plant sex, etc. in *D. rotundata* and *D. alata* and thus facilitate marker-assisted breeding in yam (Gatarira et al. 2020; Darkwa et al. 2020a; Sugihara et al. 2020; Mondo et al. 2021b). In vitro culture is routinely used for germplasm conservation; multiplication (meristem culture) of disease-free plants; embryo rescue for interspecific crosses and chromosome doubling of diploids (Aighewi et al. 2015; Babil et al. 2016). In addition, somatic hybridization and transgenesis activities have been reported (Arnau et al. 2010; Nyaboga et al. 2014; Manoharan et al. 2016; Syombua et al. 2021). Semi-autotrophic hydroponic (SAH) technology has been implemented in yam breeding at IITA and holds potential for reduced breeding cycle and rapid quality seed delivery in West Africa (Pelemo et al. 2019).

Although several advances have been made in yam research to identify genomic regions associated with key economic traits (www.africayam.org), their applications in yam breeding programs are limited. This chapter reviews current and prospective biotechnology approaches for breeding varieties that are resistant to biotic stresses.

11.2 Genetic Resources and Diversity Analysis for Yam Resistance Breeding

To put the biotechnological approaches in context, it is essential to appreciate the extent of diversity and understand the taxonomic and cytological complexity of yams. The world checklist in Royal Botanic Gardens, Kew includes 644 accepted species for the family Dioscoreaceae from five genera: *Dioscorea, Rajania, Tacca, Stenomeris,* and *Trichopus* (Govaerts et al. 2007). Yams belong to the genus *Dioscorea* L., which is the largest genus in the family Dioscoreaceae, in the order Dioscoreales. This genus is made of ~600 species and thus constitutes ~95% of the family species (Govaerts et al. 2007, 2017).

The genus *Dioscorea* is subdivided into five sections based on gross morphological characters. The section Enantiophyllum is the largest in terms of the number of species and includes the most important yam species such as *D. alata*, *D. rotundata,* and *D.cayenensis*. Other members of this section are *D. opposita*, *D. japonica,* and *D. transversa* (Bai and Ekanayake 1998). Members of this section are characterized by vines that twine to the right, i.e., in the clockwise direction when viewed from the ground upwards (Bai and Ekanayake 1998). The other sections containing cultivated yam species are Lasiophyton (*D. dumetorum* and *D. hispida*), Macrogynodium (*D. trifida*), Combilium (*D. esculenta*), and Opsophyton (*D. bulbifera*), which are characterized by vines twining to the left (Onwueme and Charles 1994; Bai and Ekanayake 1998).

Gene flow among these yam species is often constrained by pre-and post-zygotic barriers resulting from the evolutionary divergence among them (Mondo et al. 2020, 2021a). However, spontaneous and controlled interspecific hybrids of *D. rotundata* with its wild relatives (*D. abyssinica, D. burkilliana*, and *D. praehensilis*) and between *D. alata* and *D. nummularia* were reported (Akoroda 1985; Scarcelli et al. 2006; Arnau et al. 2010; Loko et al. 2013; Lebot et al. 2019a; Mondo et al. 2020, 2021a).These interspecific crosses allow broadening the genetic base of cultivated yam species and introgress resistance and adaptation trait genes. Reports showed that most failures in interspecific hybridizations were linked to differences in ploidy levels among *Dioscorea* species (Arnau et al. 2010; Mondo et al. 2020, 2021a). CIRAD-Guadeloupe had established embryo rescue techniques that hold the potential to facilitate interploidy crosses in yam breeding (Abraham et al. 2013).

In addition to the large genetic diversity in terms of species, there are several cultivars within each yam species and whose names vary greatly with local and national languages and their sources. This makes the assessment of the diversity of local landraces challenging, as more often, several names are used for the same clone or a single name may be allocated to several cultivars (Azeteh et al. 2019a; Kouakou et al. 2019; Adewumi et al. 2021; Bakayoko et al. 2021). Therefore, more elaborate and robust studies are necessary for West Africa to effectively determine the genetic diversity of yam within the region to facilitate the conservation efforts and use of existing variability in the crop improvement programs. In response to this, several genetic diversity studies have been conducted on yam worldwide and in West Africa in

particular. These included inventories and characterizations of local landraces using phenotypic traits, isozymes, and molecular markers (Darkwa et al. 2020a). Due to limitations of phenotypic markers (limited number, highly influenced by environment and plant developmental stages); molecular markers were introduced as stable and abundant across the genome. Molecular markers used in previous genetic diversity studies for yam include random amplified polymorphic DNA (RAPD) (Asemota et al. 1995), amplified fragment length polymorphism (AFLP) (Mignouna et al. 1998; Terauchi and Kahl 1999; Mignouna et al. 2002a, b), simple sequence repeat (SSR) (Arnau et al. 2009; Loko et al. 2017; Mulualem et al. 2018), inter simple sequence repeat (ISSR) (Ousmael et al. 2019), and single nucleotide polymorphism (SNP) markers (Agre et al. 2019, 2021a, b, c; Darkwa et al. 2020b; Bhattacharjee et al. 2020; Bakayoko et al. 2021). There is an increasing interest in combining phenotypic and genotypic information while dissecting functional genetic diversity in plants. The trend is explained by the fact that a large part of the variability discovered by DNA markers is non-adaptive while variations detected by phenotypic markers/characters are often under environmental influence (Arnau et al. 2017; Agre et al. 2019). The effectiveness of combined analysis vis-à-vis separate use of molecular or phenotypic markers in dissecting genetic diversity and defining genetic group has been reported (Sartie et al. 2012; Agre et al. 2019, 2021a, b, c; Darkwa et al. 2020b) and has been adopted. Despite the large number of yam diversity analysis studies, little research has been conducted in determining the sources of resistance to major biotic and abiotic stresses among breeding lines and landraces in West Africa. Most studies had a general focus and did not target specific traits in characterizing the germplasm.

Yam genetic diversity is seriously threatened by genetic erosion due to absent/poor germplasm conservation facilities and the lack of financial support for germplasm maintenance in most West African countries (https://www.iita.org/research/genetic-resources/). Even though accessions of *D. rotundata, D. alata, D. cayenensis, D. bulbifera,* and *D. dumetorum* are available in large quantity across the West and Central Africa, Azeteh et al. (2019a) warned that *D. esculenta, D. liebrechtsiana, D. schimperiana,* and *D. trifida*, are at high risk and are increasingly rare. Research and maintenance of existing diversity are still weak in most of the West African countries. Farmers only maintain the species and genotypes suitable to their needs, an attititude which accelerates genetic erosion in most countries (Adewumi et al. 2021). Semi-domesticated species, such as *D. praehensilis,* are threatened by bushfires and deforestation in countries like Ghana (Adewumi et al. 2021). Furthermore, only on-farm conservation is done in most countries without any backup in the form of in-vitro culture or cryopreservation, despite the exposure of conserved materials to environmental stresses exacerbated by climate changes (Adewumi et al. 2021). The few existing conservation initiatives often focus on cultivated species and neglect wild relatives, which are crucial in crop improvement programs as they possess genes for resistance to pests and diseases and are the source of genes for adaptation traits (Mondo et al. 2021a).

It is noteworthy to recognize the conservation efforts by IITA and its partners in West Africa. The IITA maintains the largest collection of yams in the world (Darkwa et al. 2020a). Its yam germplasm collections steadily increased from 3319 in 2010

to 5788 in 2018 and from 8 to 10 species during the same period (IITA 2018). These accessions were mainly collected from West and Central Africa, and *D. rotundata* constitutes ~68% of the collection. Other species in the IITA collection include *D. alata* (21.8%), *D. burkiliana* (6.2%), *D. abyssinica* (1.6%), *D. cayenensis* (1.5%), *D. dumetorum* (1.3%), *D. prehensilis* (1.2%), *D. bulbifera* (1.2%), *D. esculenta* (0.4%), *D. preusii* (0.17%) and *D. mangenotiana* (0.14%). All these accessions are grown annually in the field, but 1544 of these are also maintained as in vitro plantlets for conservation and research purposes. The entire IITA core collection is undergoing genotyping by sequencing (GBS) and detailed phenotyping for identifying sources of resistance genes to broaden the genetic base of currently used breeding populations as well as for cryo-conservation.

11.3 Highlights of Classical Genetics and Breeding

11.3.1 Cytogenetics and Yam Genome Size

Dioscorea is a problematic genus for cytogenetic investigations. Counting chromosomesis challenging due to their small size and their tendency to stick together. Besides, satellites of chromosomes are often as large as the chromosomes themselves (Bousalem et al. 2006). The basic number of chromosomes of *D. rotundata, D. alata,* and *D. trifida* (the three main cultivated yam species) is $x = 20$ (Arnau et al. 2010). *Dioscorea rotundata* is predominantly diploid ($2n = 2x = 40$); *D. cayenensis* is dominated by triploid males ($2n = 3x = 60$); while *D. alata*is polyploid with diploid, triploid, and tetraploid individuals ($2n = 4x = 80$). The ploidy status trends, as above-described, have recently been confirmed by Gatarira (2021) in the IITA core collection of eight species using three methods (chromosome counts, SNP marker, and impedance flow cytometry).

Previous reports showed that triploid and tetraploid yam cultivars are often more vigorous and productive compared with diploid counterparts (Lebot 2009; Arnau et al. 2010). Besides, there are reports demonstrating an association between a cultivar/species' ploidy level and its ability to flower (or sex of the flower it produces) or produce viable seeds in yam. For instance, triploids are either male or non-flowering (sterile) compared to diploid individuals which are highly fertile and form viable seeds (Abraham and Nair 1991; Girma et al. 2014, 2019). Besides, cross-pollination success is highly influenced by parents' ploidy statuses; such that inter-ploidy hybridization is seldom successful and when successful seedling survival rate is low (Lebot et al. 2019a). Studies manipulating/doubling the chromosome number using in vitro polyploidy induction have been successful (Babil et al. 2016).

11.3.2 Breeding Objectives and Farmers' Trait Preference Criteria

Although varying with regional priorities and the species involved, the main yam breeding objectives are:

- High and stable tuber yield
- Good tuber quality including low flesh oxidation rate (browning of the cut tuber), taste, texture, dry matter content, aroma, etc.
- Tuber characteristics that facilitate harvesting and meet consumers' needs (size, shape, culinary quality, tuber texture/smoothness)
- Plant architecture (e.g. dwarf types) that suppresses staking
- Resistance to abiotic (drought and low soil nutrients) and biotic stresses (virus, fungi, rots and nematodes).

These breeding objectives change over time and are influenced by farmers' and other end-user's local/regional preferences. These local trait preferences include short growth cycle, resistance to in-soil deformation, long storability of harvested tubers, and acceptable culinary qualities for both consumers and processors. Unfortunately, many of these traits are still missing in released varieties, and thus, explain their low market penetration (Darkwa et al. 2020a). Therefore, to boost the adoption of new varieties, the focus of yam breeding programs in West Africa should be led by farmers' and other end-user's expectations.

In West Africa, expectations from a variety vary significantly with the local preferences of each ethnic group. In general, farmers' preferences for yam varieties are driven by the culinary quality of tubers, productivity, market demand, seed propagation rate, quality of chips, maturity period (double/early harvested), post-harvest storage aptitude, resistance to biotic and abiotic factors, multiple roles as food and for ceremonies (Akoroda 1993; Adewumi et al. 2021). White color and elongated or round tubers are ideal traits for commercialization (Silva et al. 2017). Also, with the above traits, giant ceremonial tubers are used for socio-cultural events (Akoroda 1993). Consumers and processors mostly target yam varieties with superior eating quality such as mealiness, taste, and softness (Addy 2012). Besides, processors look for varieties with shorter processing time, gel strength and elasticity, low viscosity, and paste stability at low temperatures, pasting properties of flours that increase the range of options for consumers on the local and export markets (Addy 2012). Low moisture content is critical in yam export as this enhance storability and the yam shelf-life. Varieties with high dry matter and starch content are increasingly preferred to making flour used in many dishes such as the "Amala" in West Africa.

Efforts at modernizing yam processing in West Africa were hindered by the failure to design a single product meeting different ethnic groups' expectations. Besides, consumers are not paying for the extra cost incurred in new yam products; they perceive them as expensive compared to their advantages (Bricas and Attaie 1998).

11.3.3 Yam Breeding Challenges and Mitigation Methods

The complexity of yam reproductive biology and the expected low research investment returns have limited the attention given to yam breeding. The complexity lies mainly in its unpredictable and low flowering behavior. These sexual reproduction abnormalities result from continuous vegetative propagation following domestication (Mondo et al. 2020). Some genotypes do not flower at all or flower in some years and under particular conditions (Girma et al. 2019; Darkwa et al. 2020a). Besides, there are differences in flowering intensity among male and female plants, poor synchronization of flowering periods, low pollen viability, low stigma receptivity, low fruit and seed set, and low seed viability (Lebot et al. 2019a; Agre et al. 2020; Mondo et al. 2020, 2021c). Before understanding its cytology, some cultivated yam species, such as *D. alata*, were thought to be completely sterile, and thus, unable to undergo hybridization (Arnau et al. 2010).

Several breeding methods and techniques are used in yam improvement, including the domestication of wild species, introduction and selection, hybridization (intra- and inter-specific crosses), cytogenetic and mutation techniques, in vitro culture, transformation, and molecular breeding (Arnau et al. 2010; Darkwa et al. 2020a).

Despite its limitations (labor-intensive and time-consuming), conventional, also referred to as traditional breeding, is the major contributor of improved yam cultivars released in West Africa. Collaborative research between IITA, National Root Crops Research Institute (NRCRI, Umudike, Nigeria), and the Crops Research Institute of Ghana (CRIG) developed and released 15 *D. rotundata* clones in Nigeria and two in Ghana for the period of 2001–2016. With the advent of the AfricaYam project, several other *D. alata* and *D. rotundata* varieties have been released in Nigeria, Ghana, Benin, and Côte d'Ivoire (Table 11.2).

Up to date, no improved variety from molecular breeding has been reported (Darkwa et al. 2020a). Advances achieved in incorporating molecular markers in yam breeding programs in West Africa are discussed in Sect. 11.4 on the current status of yamomics resources.

11.3.4 Yam Breeding Scheme

The yam breeding scheme is a cyclic and incremental process (Fig. 11.1). It starts with goal setting followed by creating and identifying variability, evaluation, and selection of superior variants in a target set of environments and final release into the production system. As the yam cycle is very lengthy from the parental selection to the release process, SAH techniques are optimized at the IITA for rapid multiplication of seed yam using explants such as nodal leaves (Pelemo et al. 2019). Correct product profiling and choice of desirable parents for crossing are stepping stones in the process. Parent choice is based on trait profiling and genetic merits' studies in breeders' working collections, gene bank accessions, landrace cultivars, and related

Table 11.2 List of released yam varieties across WestAfrica

SN	Clones	Species	Country of release	Year of release	Attribute traits	Disease reaction
1	TDr8902677	*D. rotundata*	Nigeria	2001	Stable yield, very good cooking and pounding qualities, cream non-oxidizing tuber flesh, 25% tuber dry matter	Tolerant to YMV
2	TDr8902565	*D. rotundata*	Nigeria	2001	Stable yield, very good cooking and pounding qualities, cream non-oxidizing tuber flesh, 35% tuber dry matter	Tolerant to YMV
3	TDr8902461	*D. rotundata*	Nigeria	2001	Stable yield, very good cooking and pounding qualities, cream non-oxidizing tuber flesh, 26.7% tuber dry matter	Tolerant to YMV
4	TDr8902665	*D. rotundata*	Nigeria	2003	Stable yield, very good cooking and pounding qualities, cream non-oxidizing tuber flesh, 35.3% tuber dry matter	Tolerant to YMV
5	TDr8901213	*D. rotundata*	Nigeria	2003	Stable yield, very good cooking and pounding qualities, white non-oxidizing tuber flesh, 29.8% tuber dry matter	Tolerant to YMV
6	TDr8901438	*D. rotundata*	Nigeria	2003	Stable yield, very good cooking and pounding qualities, white non-oxidizing tuber flesh, 29.3% tuber dry matter	Tolerant to YMV

(continued)

Table 11.2 (continued)

SN	Clones	Species	Country of release	Year of release	Attribute traits	Disease reaction
7	TDr9501924	*D. rotundata*	Nigeria	2003	Stable yield, very good cooking and pounding qualities, white non-oxidizing tuber flesh, 35% tuber dry matter	Tolerant to YMV
8	Drn20042	*D. rotundata*	Nigeria	2008	High yielding (35 t ha^{-1}), pests and diseases tolerant, very good for fufu, frying and boiling	Tolerant to YMV
9	TDa9801176	*D.alata*	Nigeria	2008	High yielding (26–30 t ha^{-1}), pests and diseases tolerant, very good for fufu, frying and boiling, suitable for rainy and dry yam production seasons	Tolerant to YAD
10	TDa9801168	*D. alata*	Nigeria	2008	High yielding (24–28 t ha^{-1}), pests and diseases tolerant, good for pounding and boiling	Tolerant to YAD
11	TDa9801166	*D. alata*	Nigeria	2008	High yielding (26–30 t ha^{-1}), pests and diseases tolerant, very good for fufu, frying and boiling, suitable for rainy and dry yam production seasons	Tolerant to YAD
12	TDr9519158	*D. rotundata*	Nigeria	2009	High yielding (29.4 t ha^{-1}), pests and diseases tolerant, good for pounding and boiling	Tolerant to YMV

(continued)

Table 11.2 (continued)

SN	Clones	Species	Country of release	Year of release	Attribute traits	Disease reaction
13	TDr8902602	*D. rotundata*	Nigeria	2009	High yielding (31.5 t ha^{-1}), pests and diseases tolerant, good for pounding and boiling	Tolerant to YMV
14	TDr8902660	*D. rotundata*	Nigeria	2009	High yielding (31 t ha^{-1}), pests and diseases tolerant, good for pounding and boiling	–
15	TDa0000194	*D. alata*	Nigeria	2009	High yielding (37.5 t ha^{-1}), pests and diseases tolerant, good for pounding and boiling	Tolerant to YAD
16	TDa0000104	*D. alata*	Nigeria	2009	High yielding (30 t ha^{-1}), pests and diseases tolerant, very good for fufu, frying, boiling and pounded yam	Tolerant to YAD
17	TDa0000364	*D. alata*	Nigeria	2010	High yielding (33.3 t ha^{-1}), good for "*Amala*", frying and boiling	Tolerant to YAD
18	TDr95/19177	*D. rotundata*	Nigeria	2010	High yielding (30 t ha^{-1}) under dry season planting	Tolerant to YAD
19	TDr8902475	*D. rotundata*	Nigeria	2010	High yielding (31 t ha^{-1}), very good for yam fufu, frying and boiling	Tolerant to YMV
20	TDr9800933	*D. rotundata*	Nigeria	2016	High yielding (39.8 t ha^{-1})	Tolerant to YMV
21	TDr98Amo064	*D. rotundata*	Nigeria	2016	High yielding (43.9 t ha^{-1})	Tolerant to YMV

(continued)

Table 11.2 (continued)

SN	Clones	Species	Country of release	Year of release	Attribute traits	Disease reaction
22	TDa1100316	*D. alata*	Nigeria	2019	High tuber yield (33 t ha^{-1}) and stability, high dry matter (30.5%), tuber flesh non-oxidation or browning after cut	Tolerant to YAD
23	TDa1100201	*D. alata*	Nigeria	2019	High tuber yield (34 t ha^{-1}) and stability, high dry matter (33.5%), tuber flesh non-oxidation or browning after cut	Tolerant to YAD
24	TDa1100432	*D. alata*	Nigeria	2020	High dry matter content, high yield (43 t ha^{-1}), excellent boiling and pounding quality	Tolerant to YAD
25	TDr0900067	*D. rotundata*	Nigeria	2020	Potential yield of 22 t ha^{-1} and high dry matter content (30.85%)	Tolerant to YMV
26	TDr10/00048	*D. rotundata*	Nigeria	2020	Potential yield of 24 t ha^{-1} and high dry matter content (30.9%)	Tolerant to YMV
27	MankrongPona	*D. rotundata*	Ghana	2005	Potential yield of 45–70 t ha^{-1} and 34.63% dry matter	Tolerant to YMV
28	CRI Pona	*D. rotundata*	Ghana	2005	Potential yield of 26–42 t ha^{-1} and 33.4% dry matter	Tolerance to YMV
29	CRI Kukrupa	*D. rotundata*	Ghana	2005	Potential yield of 42–50 t ha^{-1} and 33.42% dry matter	Tolerance for YMV
30	TDa0000003	*D. alata*	Ghana	2017	–	Tolerant to YAD
31	TDa0100029	*D. alata*	Ghana	2017	–	Tolerant to YAD
32	TDa0100004	*D. alata*	Ghana	2017	–	Tolerant to YAD

(continued)

Table 11.2 (continued)

SN	Clones	Species	Country of release	Year of release	Attribute traits	Disease reaction
33	TDa0000046	*D. alata*	Ghana	2017	–	Tolerant to YAD
34	TDa0100113	*D. alata*	Côte d'Ivoire	2021	Potential yield of 40 t ha^{-1} and good culinary qualities	Tolerant to YAD
35	TDr0102562	*D. rotundata*	Côte d'Ivoire	2021	Potential yield of 30 t ha^{-1} and good culinary qualities, multiple tubering	Tolerant to YMV
36	TDa0100018	*D. alata*	Côte d'Ivoire	2021	Potential yield of 25 t ha^{-1} and good culinary qualities	Tolerant to YAD

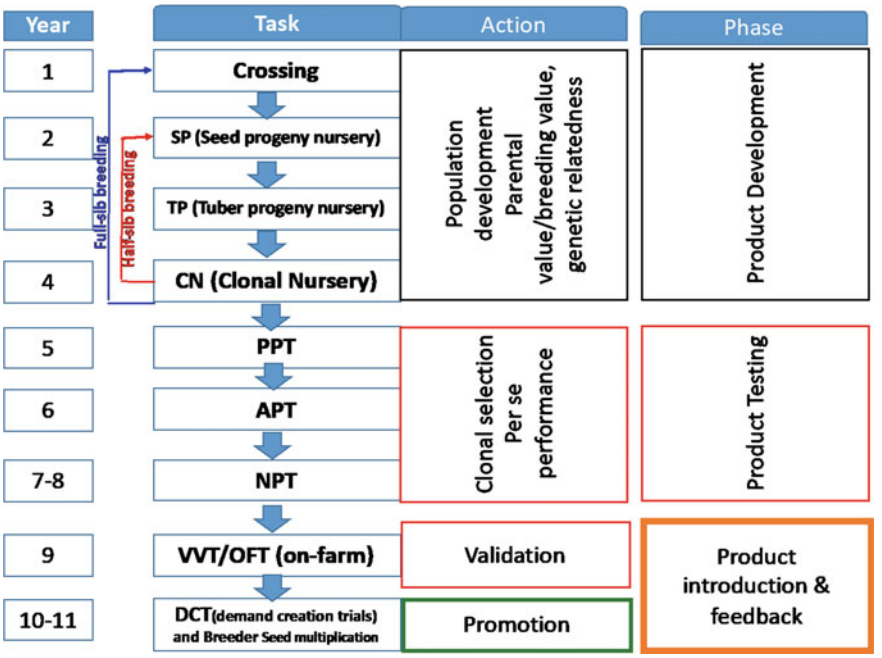

Fig. 11.1 Yam breeding scheme (*PPT* Preliminary performance trial; *APT* Advanced performance trial; *NPT* National performance trial; *VVT* Variety validation trial; *OFT* On-farm trial)

wild relatives. Parental selection for crosses considers diverse aspects: agronomic traits, tuber quality, ploidy status, flowering ability, cultivar sex, cross-combination ability, etc. (Arnau et al. 2010; Lebot et al. 2019a; Mondo et al. 2020).

Yam is mainly a dioecious plant with male and female flowers developed on different individuals (Tamiru et al. 2017; Agre et al. 2020; Mondo et al. 2020, 2021c). Thus, separate crossing blocks of males and females are required. When natural pollination is desired, male and female individual plants are grown close (1 × 1 m) to each other, and their vines are trained onto the same stakes. Polycross design (Fig. 11.2) is cheap and easy to conduct, especially when using fertile parents, although the male parent of the progenies is usually unknown (Arnau et al. 2010; Norman et al. 2018). Pedigree reconstruction of open-pollinated progenies is possible using molecular markers (Norman et al. 2020). The purity of crosses is ensured by hand pollination of parents grown in isolated plots/blocks. Although not systematically specified, the separation distance between male and female blocks usually ranges from 500 to 1000 m (Mondo et al. 2020). The same isolation distance is maintained between crossing blocks and wild environments to prevent unwanted pollen sources. Multiple planting dates are advised to increase the chances of synchronization of the flowering of male and female parents. It is noteworthy that male genotypes flower earlier than female counterparts, and thus, planting females 2–4 weeks before males will promote a synchronized flowering of parents (Mondo et al. 2020).

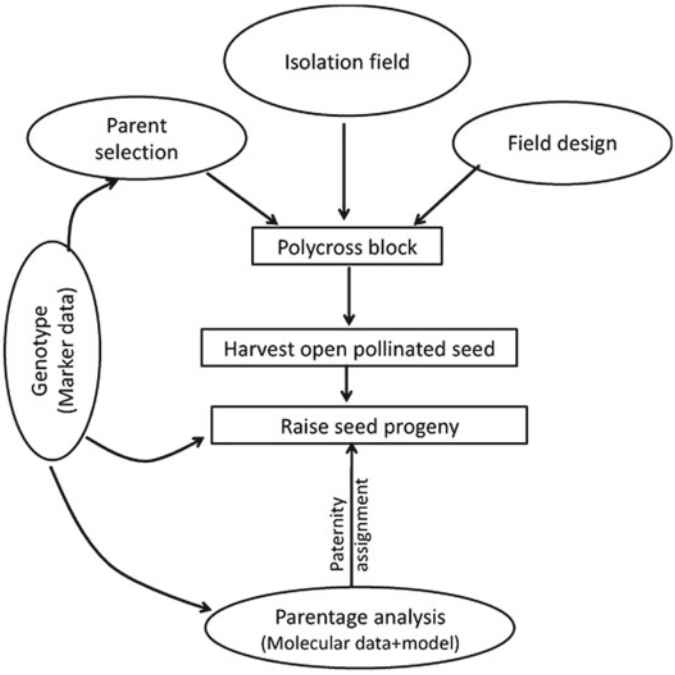

Fig. 11.2 The scheme used for yam polycross and parentage reconstruction

For controlled (hand) pollination, female inflorescences are bagged with thrip-proof cloth-bags 2–7 days before the flowers open, varying with the spike length (Darkwa et al. 2020a; Mondo et al. 2020, 2021c). Supervised hybridization through hand-pollination is recommended to ensure biparental crossing (Arnau et al. 2010). The male flower usually opens at noon, and the period of good pollen viability is rather short (2–4 h) (Mondo et al. 2020). Therefore, the indicated time for hand-pollination is 12 noon to 3 pm, after which the pollen viability decreases significantly, depending on the prevailing weather conditions (Arnau et al. 2010). It is noteworthy that female flowers are fully receptive for 6–10 days, although better results are achieved at least one day after anthesis. After pollination, flowers are kept covered for two weeks to ensure the purity of offspring from crosses (Arnau et al. 2010).

At physiological maturity when fruits start drying, botanical seeds are collected before they disperse from the capsules. These are then processed by releasing seeds, testing viability (using seed weight) and stored at appropriate temperature for about 3–4 months until the dormancy is broken (Abraham 1992). The following season, those seeds are grown in seedling trays or nursery beds (Fig. 11.3) which are filled with appropriate growing media such as carbonized rice husk and coco peat. Seed germination starts 10 days after sowing and continues for one month (Darkwa et al. 2020a). The seedlings are then transplanted to pots in a screenhouse or nursery bed in the field for single plant selections. Next steps include early clonal generation evaluation nursery, preliminary performance trial, advanced multi-location and multi-season performance trial, and on-farm variety validation trial for an official release and commercial deployment (Arnau et al. 2010; Darkwa et al. 2020a).

Only a limited number of traits are evaluated at the seedling (F_1) and the first clonal generations (C_1): flesh color, tuber oxidation, and disease symptoms (Darkwa et al. 2020a). Tuber yield is much stable from the second clonal generation (C_2),

Fig. 11.3 Yam seedlings established on seedling trays

with positive relationships with following generations. Therefore, selection for traits such as shoot vigor, disease severity, tuber shape, tuber yield, and other tuber yield components is best conducted from C_2 stage (Abraham 2002; Arnau et al. 2010).

11.4 Current Status of Yam Omics Resources

As mentioned in the introduction, various biotechnology tools are being introduced in yam breeding programs. Although there has been no report on the successful release of a yam variety using biotechnological tools (Darkwa et al. 2020a), several achievements in the path to its incorporation into yam breeding have been realized. These include the recent development of the reference genome sequences for *D. alata* (Cormier et al. 2019; Bredeson et al. 2021), *D. rotundata* (Tamiru et al. 2017; Sugihara et al. 2020), and *D. dumetorum* (Siadjeu et al. 2020), discovery of several molecular markers and genes through yam metabolomics and transcriptomics. Therefore, this section provides an overview of the use of biotechnology tools in yam breeding programs.

11.4.1 Reference Genome Sequences

Advances and decreased cost in genome sequencing through NGS technologies enabled the generation of millions of novel markers and high-density genetic maps in major food crops, including yam (Tamiru et al. 2017; Bhattacharjee et al. 2018; Cormier et al. 2019; Siadjeu et al. 2020; Bredeson et al. 2021).

Tamiru et al. (2017) developed and released the reference genome sequence of *D. rotundata* accession TDr96_F1. Its genome size is 594 Mb, out of which 76.4% is distributed among 21 linkage groups (http://genome-e.ibrc.or.jp/home/bioinformatics-team/yam). The results of gene prediction using the genome sequence showed a total of 26,198 genes in *D. rotundata.* Recently, an improved version of the *D. rotundata* sequence has been released, covering a total of 636.8 Mb and distributed on 20 linkage groups of the genome with an N_{50} of 137,007 bp (Sugihara et al. 2020) (https://www.pnas.org/content/pnas/suppl/2020/12/02/2015830117.DCSupplemental/pnas.2015830117.sapp.pdf).

Cormier et al. (2019) established the first high-density genetic map of *D. alata* using GBS. In that *D. alata* high-density map, 20 linkage groups were identified, and 1579 polymorphic markers were ordered. The consensus map length was 2613.5 cM with an average SNP interval of 1.68 cM. This corresponded with estimated genome coverage of 94% and thus, promoted further investigations on the inheritance of key traits and the development of molecular breeding tools. Recently, a reference genome for *D. dumetorum* has been developed, and the assembly represents 485 Mbp of the genome with an N_{50} of over 3.2 Mbp (Siadjeu et al. 2020). A total of 35,269 protein-encoding gene models and 9941 non-coding RNA genes were

predicted, and functional annotations were assigned. The establishment of these reference genome sequences in yam has opened a new avenue for exploitation and thorough understanding of yam genetics, genomics, and domestication, essential for successful yam breeding (Scarcelli et al. 2019; Darkwa et al. 2020a; Sugihara et al. 2020).

11.4.2 Molecular Marker Uses in Yam Improvement Programs

Cytogenetic techniques and different types of markers (isozymes, RFLP, RAPD, AFLP, SSR, and SNP) are relevant in yam breeding. These markers have been used, with different levels of reliability, in genetic diversity studies, phylogenetic relationships, estimation of population structures, cultivar fingerprinting, mapping of major effect genes and QTLs, identification of elite genotypes in crop breeding programs, and for validation of progenies originating from genetic hybridizations (Tamiru et al. 2015; Darkwa et al. 2020a). Molecular markers and genotyping systems in yam breeding have recently been reviewed by Darkwa et al. (2020a). Early use of markers in yam was mainly for diversity studies, parentage analysis, origin and phylogenetic studies, and identification of genes controlling major diseases such as YAD and YMV (Arnau et al. 2010).

From 2015, there was a shift from the predominance of genetic diversity studies to QTL analysis with the start of the AfricaYam project. IITA and its partners are making substantial efforts to develop diverse molecular markers both for Guinea and water yams (Tamiru et al. 2015, 2017; Cormier et al. 2019, 2021; Darkwa et al. 2020a). For instance, Tamiru et al. (2015) developed 90 SSR markers from an enriched genomic library of yellow Guinea yam (*D. cayenensis* Lam.) with assumption that these SSRs could be successfully transferred to the two major cultivated species (*D. rotundata* and *D. alata*). A higher level of transferability to *D. rotundata* (94%) was reported due to its proven relatedness with *D. cayenensis* (Dansi et al. 2013), while it was low with *D. alata* (57%).

The AfricaYam Project has made significant efforts to develop genomic resources to transform yam breeding in West Africa (https://africayam.org). It developed markers for major traits such as plant vigorand sex, flowering intensity, number of tubers per plant, tuber yield, flesh tuber oxidation, disease resistance (mostly anthracnose and viruses), tuber appearance, and spines on tuber surface. Different regions controlling numerous traits were identified through different gene model actions and need validation to implement marker-assisted selection in yam breeding (https://africayam.org) fully. The application of these novel methods will enhance yam breeding efforts and ensure quick delivery of high-yielding, nutrient-dense, and climate-resilient varieties to farmers in West Africa.

Effective integration of marker-assisted selection in yam breeding will allow this crop to be efficiently and quickly improved by drawing on genomic advances reported

in other crops such as maize, cassava, rice, potato, beans, etc., for which the molecular research is more advanced. Among the advantages is shortening the breeding cycle by speeding up the identification and transfer of desirable genes. In fact, markers will make possible the selection at early growth stages for traits that could only be assayed at late stages such as flower sex, tuber yield, and quality, etc. (Mignouna et al. 2008; Desta and Ortiz 2014; Hickey et al. 2017; Friedmann et al. 2018; Agre et al. 2020). Besides, the use of markers will significantly reduce the cost that could otherwise be spent in phenotyping large numbers of plant materials as done in conventional breeding. Furthermore, reliability in selection will be improved by controlling inconsistent year-to-year symptom phenotypic data that hinder conventional yam breeding (Arnau et al. 2010; Saski et al. 2015; Tamiru et al. 2015; Cormier et al. 2019). In contrast to phenotypic descriptors, molecular markers are insensitive to environmental effects. Moreover, using molecular markers will provide a deeper understanding of genes controlling the expression of traits of interest in opposition to conventional breeding. Another key advantage of molecular markers in yam breeding is to facilitate the pyramiding of genes from different sources of resistance for more durable resistance to major diseases (Arnau et al. 2010).

11.4.3 Other Genomic Tools in Yam Improvement

Many other novel genomic tools are being introduced in yam breeding including next-generation-based genotyping procedures, transcriptome sequencing, and metabolomics (Darkwa et al. 2020a).

Genotyping by sequencing (GBS) is a next-generation genotyping procedure which helps to unravel the magnitude of genetic similarity and diversity within and between cultivated species and their wild relatives (Spindel et al. 2013). The GBS procedure is based on minimizing genome complexity with restriction enzymes, coupled with multiplex NGS for high-density SNP discovery (Elshire et al. 2011). A successful application of GBS in Guinea yam breeding is the case study by Girma et al. (2014). Using 2215 SNP markers, this study elucidated the nature of genetic diversity within and between *D. rotundata* and *D. cayenensis* and five wild relatives (*D. mangenotiana, D. praehensilis*, *D. togoensis*, *D. burkilliana*, and *D. abyssinica*). Furthermore, Siadjeu et al. (2018) and Bhattacharjee et al. (2020) showed the potential of the GBS to unlock genetic diversity and population structure in *D. dumetorum* and *D. rotundata* accessions, respectively.

Transcriptome sequencing uses genome-wide differential RNA expression to better understand biological pathways and molecular mechanisms that control important but complex traits in plants. Narina et al. (2011) successfully used transcriptome sequencing in water yam (*D. alata*) to investigate gene expression by the large-scale generation of ESTs from a susceptible (TDa950310) and two resistant (TDa8701091 and TDa950328) yam genotypes infected with the anthracnose (*C. gloeosporioides*).

Gene expression of flavonoid content (purple flesh color) to characterize the transcriptomes of tubers from a purple-flesh and a white flesh variety of *D. alata* tubers is another application of the transcriptome sequencing procedure (Wu et al. 2015). Besides, SuperSAGE transcriptome profiling identified flowering and sex-related genes in *D. rotundata* (Girma et al. 2019). A total of 88 tags were expressed in male, female, and monoecious plants. Among these tags, 18 matched with genes for flower development and sex determination previously identified in many plant species. Siadjeu et al. (2021) used transcriptome sequence to reveal candidate genes involved in the post-harvest hardening of *D. dumetorum* and thus opened an avenue for improving the storability of this yam species.

Metabolomic techniques produce extensive biochemical phenotypes that can be indicative of quality traits. In fact, desirable quality traits are often directly linked with metabolite composition, thus providing a path to metabolite-marker-based breeding (Bino et al. 2004; Fernie and Schauer 2009). This explains the increasing interest in metabolomics in complement to genomics in yam studies. Price et al. (2016, 2017, 2018, 2020) and Lebot et al. (2019b) are the most relevant reports on the potential for application of metabolomic technology in yam breeding. Metabolite profiles provided enormous insight into biochemically related species and revealed *Dioscorea* species as potential sources of essential compounds such as shikimic acid (Price et al. 2016). Besides, a large number of unknown metabolites highlighted the understudied nature of the genus *Dioscorea.* Price et al. (2017) identified a subgroup of metabolites useful for accurate species classification and emphasized the possibility of predicting tuber composition from leaf profiles. Metabolic differences were accession-specific and usually confined to compound classes and, therefore, support trait-targeting for metabolite markers. Price et al. (2018) investigated the cross-species carotenoid profiling of 46 yam accessions belonging to five species (*D. alata, D. bulbifera, D. cayenensis, D. dumetorum,* and *D. rotundata*). They found non-significant differences between the *D. rotundata* and *D. alata* accessions on β-carotene content and provitamin A activity. Besides, they elucidated the absence of a link between yellow tuber flesh color and provitamin A content in yam, as opposed to reports on cassava and sweet potato. Linking biochemical signatures with several agronomic and sensory characters offers potential to expedite the selection and consequently the breeding cycle. Lebot et al. (2019b) developed and optimized a high-performance thin-layer chromatography (HPTLC) protocol for rapid quantification of individual sugars, allantoin, phenolic acids, catechins, and saponins in yam tuber flours. This technique was successfully used for the rapid quantification of compounds related to tuber flour quality of 522 accessions from eight *Dioscorea* species.

Genome-wide association studies (GWAS) were used to identify and understand the genetic architecture of genes responsible for complex traits by exploiting linkage disequilibrium. As opposed to QTL analysis which assays only allelic diversity that segregates between the parents, GWAS uses natural populations (collection of individual varieties or inbred lines) and thus increases the power to dissect historical recombinations. This technology is currently implemented at IITA (under AfricaYam

and NSFBREAD projects), to determine QTLs linked to various traits in *D. rotundata* and *D. alata*, to facilitate marker-assisted breeding in yam (Darkwa et al. 2020a; Gatarira et al. 2020; Mondo et al. 2021b; Agre et al. 2021b).

11.4.4 Genetic Transformation and Tissue Culture

Efforts in establishing an efficient genetic transformation system of *D. rotundata* were reported by Nyaboga et al. (2014) and were intended to open up many avenues to produce disease-resistant yams through pathogen-derived resistance strategies that would not be possible using conventional breeding approaches. Zhu et al. (2009) used the *Agrobacterium tumefaciens*-mediated transformation of *D. zingiberensis,* with leaves and calli as explants, in developing a method to produce transgenic *D. zingiberensis* plants. The application of the CRISPR/Cas9-based genome-editing system in *D. zingiberensis* (Feng et al. 2018) and *D. rotundata* (Syombua et al. 2021) has been successful. Zhao-wei (2012) tested the callus-cultivating effects of different *D. opposita* explants to establishan efficient plant regeneration system for further use in the genetic transformation of that yam species.

11.5 Biotechnology Approaches in Breeding for Biotic Stress Resistance in Yam

Yam is subject to pests and pathogens throughout the growing season, from the seedling stage to post-harvest storage (Morse 2021). These diseases and pests result in reduced yield and low tuber quality, decreasing the tuber's market value substantially. The most important diseases affecting yam production and storage are anthracnose, viruses, tuber rots, and nematodes. The most important pests are weevils, termites, beetles, mealy bugs, and aphids (Korada et al. 2010; Kolombia et al. 2020; Adewumi et al. 2021). In this book chapter, we are only focusing on efforts done in breeding for resistance to YMV and YAD, as they arethe most economically damaging diseases of major yam species (*D. alata* and *D. rotundata*) worldwide. Due to significant losses during yam storage, a brief discussion is included on yam nematodes.

11.5.1 Genetic Engineering for YMV Resistance

YMV is the most economically important and widespread *D. rotundata* disease (Azeteh et al. 2019b; Kumar et al. 2021).YMV is caused by an aphid-transmitted poty virus that infects several *Dioscorea* species (Azeteh et al. 2019b). It is also transmitted mechanically and perpetuated across generations through planting materials (Ita et al.

2020; Nkere et al. 2020). Infected plants usually show inter-veinal mosaic, curling, molting, and stunted growth (Thouvenel and Dumont 1990; Adeniji et al. 2012; Azeteh et al. 2019b). These symptoms result in decreased photosynthetic ability and significant yield losses (40–50%) (Adeniji et al. 2012; Bömer et al. 2016; Mignouna et al. 2019). Infected plants are thus less vigorous and may produce few small tubers with less starch content. The most common YMV symptoms are shown in Fig. 11.4.

Effective control measures rely on healthy planting materials (Amusa et al. 2003). Sources of resistance and tolerance to yam viruses have been identified. This allowed the development and release of several tolerant *D. rotundata* varieties by IITA and partner national yam breeding programs (Arnau et al. 2010; Darkwa et al. 2020a). However, these efforts in developing resistant cultivars are hindered by the high variability in African YMV isolates and the rapid pathogen evolution, generating genetic variants that can overcome the host plant's resistance. Cases of resistance

Fig. 11.4 Yam plant showing symptoms of severe YMV

breakdown have been reported (Bousalem et al. 2000; Ayisah and Gumedzoe 2012). Pyramiding of genes from different sources could provide more durable resistance. However, pyramiding genes through conventional breeding is a challenging and time-consuming target. Biotechnology and molecular tools were then introduced to speed up the variety development process as well as add precision in the identification, transfer, and pyramiding of resistance genes.

Mignouna et al. (2002b) developed the first *D. rotundata* mapping population to determine chromosomal regions with genes or QTLs for YMV resistance. Furthermore, a genetic linkage map of *D. rotundata* was developed based on 341 co-dominantly scored AFLP markers, segregating in an intraspecific F_1 cross. One QTL for YMV resistance was associated with the marker P16/M16-126 on linkage group 1 and explained up to 24% of the total phenotypic variance (Mignouna et al. 2002a, b, c). Two other QTLs were linked to P14/M22-418 and P17/M22-238 on linkage group 8 and explained 22 and 35% of the phenotypic variance on the maternal linkage group, respectively. Two QTLs for YMV were also detected on the paternal linkage group 4 and were associated with the markers P12/M19-241 and P16/M15-81 that explained 13 and 16% of the phenotypic variation, respectively (Mignouna et al. 2002a, b, c). With the ongoing AfricaYam project, several genomic regions linked with YMV have been identified alongside some putative genes involved in plant defense mechanisms (Agre et al. 2021b). The effort is ongoing for the conversion of SNP markers into KASP for MAS application.

11.5.2 Molecular Breeding Tools for Yam Anthracnose Disease (YAD) Tolerance

YAD is caused by a fungus, *Colletotrichum gleosporoides* Penz., and is recognized as one of the most devastating diseases of yam. Although more important on *D. alata* (Abang et al. 2003; Penet et al. 2016; Lebot et al. 2019a), YAD is also a threat to *D. rotundata* farmers in West Africa (Kwodaga et al. 2020). Yam anthracnose is characterized by discrete leaf necrosis before expanding to dieback of emerging stems, shoots, and extensive defoliation (Penet et al. 2016). These symptoms affect the crop's photosynthetic activity, which translates into a reduction in yield (Abang et al. 2003). Depending on the growth stage when the crop is infected and prevailing weather conditions, yield losses can be as high as 80–90% in West Africa (Nwankiti and Ene 1984; Mignucci et al. 1988; Green 1994). Furthermore, yam anthracnose leads to genetic erosion in large-scale field collections of susceptible yam varieties (Orkwor and Asiedu 1995). Characteristic symptoms of YAD are illustrated in Fig. 11.5.

The use of genetically resistant planting materials is a cost-effective and environmentally sound control measure. Several sources of resistance to anthracnose were identified in Ilesde Caraïbesand Guadeloupe germplasm collections and provided opportunity for resistance breeding (Arnau et al. 2010). In IITA and in WestAfrica, the effort has been concentrated on identifying stable sources of resistance to YAD

Fig. 11.5 Yam plants with symptoms of yam anthracnose disease

as no immune varieties were reported (Darkwa et al. 2020a). However, conventional breeding for YAD resistance is negatively affected by the pathogen's genetic diversity (heterogeneous population) due to its ability to undergo sexual recombination (Abang 1997; Abang et al. 2003). A more durable resistance would be achieved by pyramiding resistance genes from different sources into a single genotype (Arnau et al. 2010; Saski et al. 2015; Tamiru et al. 2015; Cormier et al. 2019). This is a time-consuming and uncertain target with conventional breeding approaches.

First efforts in integrating molecular tools in YAD resistance breeding identified a major dominant gene "*Dcg-1*" as the gene controlling resistance to the most predominant Nigerian virulent strain (Mignouna et al. 2002a). Petro et al. (2011) constructed an intraspecific genetic linkage map of *D. alata*using 523 polymorphic AFLP markers and nine putative QTLs. These QTLs were identified for YAD resistance on five different linkage groups. The phenotypic variance explained by each QTL ranged from 7.0 to 32.9%, while all significant QTLs accounted for 26.4–73.7% of total phenotypic variance depending on the isolate (Petro et al. 2011). In the search for more markers, Saski et al. (2015) utilized the NGS techniques such as expressed sequence tags (EST) sequencing, de novo sequencing, and GBS profiles on two *D. alata* genotypes, viz. TDa9500328 (resistant to anthracnose) and TDa9500310 (susceptible to anthracnose). They developed a comprehensive set of EST-SSRs, genomic SSRs, whole-genome SNPs and reduced representation SNPs for resistance to YAD. Further, a genetic linkage map of *D. alata* was developed from 380 EST-SSRs on 20 linkage groups to identify QTLs controlling YAD resistance (Bhattacharjee et al. 2018). Linkage analysis found that a robust QTL on linkage group 14, at a position interval of 71.1–84.8 cM, explained 68.5% of the total phenotypic variation. The high-density genetic map of *D. alata* developed by Cormier et al. (2019) using GBS had opened new avenues for further investigations on the inheritance of key traits such as disease resistance and the development of molecular breeding tools.

Narina et al. (2011) successfully used transcriptome sequencing in *D. alata* to investigate gene expression by the large-scale generation of ESTs from a susceptible

(TDa 95/0310) and two resistant (TDa 87/01091 and TDa 95/0328) yam genotypes infected with YAD. Transcriptome analysis was also used by Hua et al. (2020) to understand the defense mechanisms and the function of ethylene against *Botrytis cinerea* and *Colletotrichum alatae* in *D. alata*. This study showed a high accumulation of endogenous ethylene levels in the resistant cultivar.

Agre et al. (2021c) used SNP-based GBS sequencing platform to genotype 204 *D. alata* full-sib offsprings in developing a high-density genetic linkage map with 3182 SNP markers. The total length of the genetic map was 1460.93 cM with an average of 163 markers per chromosome, and thus, represented the most saturated *D. alata*genetic map todate. Four QTLs were detected for YAD resistance on three chromosomes. The proportion of the phenotypic variance explained by these QTLs ranged from 29.54 to 39.40%. In addition, plant defense response genes including GDSL-like Lipase/Acylhydrolase, Protein kinase domain, and Fbox protein were also detected within the QTL regions.

11.5.3 Development, Validation, and Deployment of Trait-Linked Markers for YMV and YAD

Marker discovery for YMV and YAD resistance is ongoing at IITA and other research institutions in collaboration with several international partners and national agricultural research programs across sub-Saharan Africa and beyond. The next step is the conversion of already identified QTLs to diagnostic SNP markers. These markers will then go through verification and subsequent deployment in breeding programs. The application of these novel methods will enhance yam breeding efforts and ensure quick delivery to farmers of varieties combining high yield potential, disease and pest resistance, and climate resilience in West Africa.

11.5.4 Yam Nematode Resistance Breeding

Yam nematode is caused by a range of species, including *Meloidogyne* spp., *Scutellonema* spp., and *Pratylenchus* spp. Nematode symptoms include galling and "crazy root" syndrome on tubers, distorting tubers, dry rot, and cracking, which reduce the tuber quality (Kolombia et al. 2020). Depending on the level of infection, nematodes can cause high levels of loss during storage, reduce harvestable yield and seed tuber viability, and predispose tubers to secondary rots and rapid deterioration (Coyne et al. 2006; Nyaboga et al. 2014). The severity of nematode damage is generally proportional to the nematode population. Nematode populations build up in the soil if yams are grown in the same place in successive seasons (O'Sullivan 2010). This might be accentuated by short fallows, as currently observed in West Africa. During a fallow,

nematode populations decline through both the lack of appropriate host plants and by direct antagonism from other soil organisms (O'Sullivan 2010).

The use of resistant varieties can be an effective strategy in controlling yam nematodes, although no varieties are known to be tolerant to nematodes (Nyaboga et al. 2014). These authors argued that transgenic plants would be an alternative approach to improve the nematode resistance in yam. In fact, several transgenes have been used to confer plant resistance to both tropical and temperate plant-parasitic nematodes (Nyaboga et al. 2014). However, no conventional or biotechnological approach is reported in breeding yam for nematode resistance in West Africa or

Table 11.3 Biotechnological applications in yam breeding for biotic and abiotic stresses

Species	Technology	Stress	Objectives	References
D. alata	Transcriptome analysis	*Botrytis cinerea, Colletotrichum alatae*	Understanding the defense mechanism and the function of ethylene	Hua et al. (2020)
D. alata	Hormonal regulations of dioscorin genes	High-temperature, low-temperature, and drought	Elucidate the regulatory mechanisms of dioscorin gene *Da-dio5* expressions	Liu et al. (2017)
Dioscorea spp.	Metabolomics	Diseases and abiotic stresses	Inventory of metabolites with biomarker potential in abiotic and disease resistance	Friedmann et al. (2019), Price et al. (2020)
D. alata	Tissue culture	Salinity	Development of protocol for in vitro salt tolerance screening	Wheatley et al. (2003)
D. alata	EST-sequencing	Anthracnose	Identification of QTLs for resistance	Bhattacharjee et al. (2018)
D. alata, D. rotundata	–	YAD, YMV	QTL identification	Mignouna et al. (2003)
D. alata	Transcriptome sequencing	Anthracnose	Germplasm characterization	Narina et al. (2011)
D. rotundata	*Agrobacterium*-mediated transformation	Field and storage pests and diseases	Developing transformation and regeneration system	Nyaboga et al. (2014)
D. alata, D. rotundata	Genome-wide association studies	YAD, YMV	Identification of genome regions controlling resistance	IITA (Unpublished), Agre et al. (2021b)
D. alata	Whole-genome sequencing	Anthracnose	QTL mapping	Saski et al. (2015)
D. rotundata	Genome-editing using CRISPR/Cas9	–	–	Syombua et al. (2021)

elsewhere. Table 11.3 provides some of the biotechnological tools used for biotic and abiotic resistance breeding in yam.

11.6 Conclusions

Molecular and biotechnology approaches provide a deeper understanding of genes controlling biotic expressions at a genotype or population level. Efforts in their integration as routine tools in yam breeding programs are ongoing to implement modern yam breeding programs following a recent initiative to modernize crop breeding led by the Excellence in Breeding (EiB) platform of the CGIAR. Genetic information derived from heterotic group mapping has been employed to classify progenitors for elite population development. Three product profiles for early, intermediate, and late maturity white and water yams have been developed as a useful guide for current and future genetic improvement efforts for yam. The adoption of the electronic phenotypic data capturing process using the field book in addition to the development and application of digital disease phenotyping app and the management and storage of generated data on the Yambase have also yielded significant improvements in yam breeding. Rapid cycle genomic selection and prediction along with complementary molecular and biotechnological approaches and accurate phenotyping for biotic stress in yam breeding will result in more efficient and accelerated improvement of this vital crop which is imperative in light of the exponential human population growth, food demand, and climate change challenges. These technologies will particularly be useful in breeding for biotic resistance as they will facilitate the pyramiding of resistance genes from different sources for a more durable resistance effectively. Besides, these tools will facilitate broadening the genetic base of existing breeding populations by breaking interspecific incompatibility barriers among yam species and wild relatives.

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