Enrico Biancardi · Tetsuo Tamada
Editors

Rhizomania

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ISBN 978-3-319-30676-6 ISBN 978-3-319-30678-0 (eBook)
DOI 10.1007/978-3-319-30678-0

Library of Congress Control Number: 2016946022

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG Switzerland

“To my wife Donatella, who accepted to spend her life not only with me but also with the genus Beta”

—Enrico Biancardi

“To my wife Sachiko and my daughters Machiko and Chieko who gave warm support to my BNYVV work”

—Tetsuo Tamada

Foreword

An unknown disease of sugar beet was detected in Italy more than 50 years ago. Soon the new syndrome displayed devastating effects on yield. This greatly concerned the Italian sugar beet growers and processors, especially considering that the syndrome had spread to the most important Italian cultivation areas.

It was the start of a memorable enterprise for pathologists, breeders, and agronomists. The spontaneous and unusual synergy created among the universities, research stations, seed companies, and grower associations led not only at the first very appropriate attempts of prophylaxis measures but also to an awareness that the only possible management would be through the use of resistant varieties. In this phase, the *Beta maritima* germplasm selected at Rovigo and later at Salinas began to display its value against the new disease called "rizomania." Some resistant varieties were released, thanks to enhanced knowledge of the pathogenic agents (beet necrotic yellow vein virus and *Polymyxa betae*) obtained in Japan and Germany.

It also was the beginning of countless research projects and collaborations worldwide, which, in a relatively short time, led to almost complete control of the disease. There are perhaps few other diseases, even affecting more important crops, on which so many papers have been published. It should be noted that the most significant results in the discovery of rhizomania resistance traits were obtained by public research stations, often without any specific funding.

The future of sugar beet currently is endangered by the development of resistant strains in the virus, among other things. I believe that it also will be possible to overcome these new obstacles with the help of the powerful tools provided by molecular investigation and following the knowledge carefully collected in this very useful book, the first devoted exclusively to rhizomania.

The issue was much more difficult 50 years ago, when no one knew anything about the syndrome and the researchers only had their eyes to see, a microscope to look closer, and a pencil to take notes.

Alma Mater Studiorum
Bologna, Italy
May 2016

Antonio Canova

Preface

This book is the result of an international enterprise among researchers involved in past and present studies on rhizomania, a relatively new and devastating disease of sugar beet. In less than 50 years, the disease has become the most damaging biotic factor affecting the crop worldwide. Moreover, its spread is still ongoing in every cultivated area. Because the traditional management systems were almost ineffective, it was soon evident that the employment of genetic resistances was the only chance for limiting the economic damage. The discovery of the pathogenic agents and the release of the first resistant varieties are described by some of the researchers directly involved.

The breeding efforts led to both the current satisfactory management of the disease and to the survival of the beet sugar industry in several areas. The cooperation between the Italian and American Experimental Stations, born spontaneously about 80 years ago and still continuing today, should be remembered. The friendly collaboration led to the employment of genetic traits extracted from *Beta maritima,* which became the sole source so far of the resistances available against the disease.

The introduction briefly describes sugar beet cultivation, the more common diseases, and the damage caused by rhizomania. This is necessary because the book also is addressed to readers who are not directly involved with sugar beet. Without these brief explanations, some parts of the text would not be fully comprehensible. The following chapters refer to the molecular physiology of the disease agents and their interactions with the environment and the host-plant. The knowledge of ecology and epidemiology of rhizomania is, above all else, necessary to understand the means and practices valuable to avoid or at least delay the further spread of the disease into healthy soils. Some promising methods of control using concurrent but not damaging viruses, bacteria, and fungi are in progress. They could help the action of the genetic resistances, which are not completely effective. The integrated protection is useful, especially in the even more frequent occurrences of resistance-breaking strains of BNYVV, where the known types of resistance seem to have partially lost their original efficacy. Some almost immune transgenic varieties are already awaiting release. For traditional breeding, further efforts will be needed in

search of new resistances in the wild species of the genus *Beta*. The availability of large collections of *Beta* germplasm collected all over the world should ensure further success in this direction. The target will be gained by means of conventional selection methods, assisted by updated techniques for genome analyses. Finally, perspectives are described to not only reduce the current damages but also to avoid further spread and noxious evolutions of rhizomania agents.

By means of interdisciplinary approaches, this book was edited above all to provide a broad, comprehensive, and updated overview of the various aspects of rhizomania, now scattered in countless publications. The outlook should be valuable for farmers, extension services, students, and researchers committed to ensuring the future of the sugar beet crop.

Rovigo, Italy — Enrico Biancardi
Naganuma, Japan — Tetsuo Tamada

Acknowledgments

Antonio Canova must be recognized for his encouragement and convincing and enthusiastic support of this enterprise. The editors are grateful to Bob Lewellen, Lee Panella, and Mitch McGrath for their contributions and critical reading and revisions of the text and proofs. Special thanks go to Piergiorgio Stevanato for his help in translating and organizing the manuscript. Together with Mauro Colombo, he collaborated on the digital drawing of tables and figures. Many thanks are also addressed to Kelley Richardson for editing Chap. 12. Loving thanks are given by Biancardi to his wife for the original watercolor printed in the first pages of the book. Tamada is grateful to his colleague Hideki Kondo for generous help with tables and figures in parts of this book. His long-term research on rhizomania was carried out at Hokkaido Central Agricultural Experiment Station and Institute of Plant Science and Resources, Okayama University, and financially supported by Hokkaido Sugar Beet Associations.

The editors express also their gratitude to the numerous colleagues for the confidence given when the prospects for this book were still in the early stages of discussion and development. Finally, grateful memory must be addressed to the Italian researchers who firstly contributed both to the discovery of the disease agents and to plant-host resistance, which allowed survival and development of the sugar beet crop and the connected sugar industry.

Contents

Author's Contributions to Rhizomania Research

Michael J.C. Asher Studied the ecology and epidemiology of *Polymyxa betae* and sources of resistance in wild *Beta* species, identifying two genes carrying the trait. Contributed to models predicting the development and spread of rhizomania and to the development of molecular markers for novel sources of resistance.

Enrico Biancardi Classified as "Alba type" the multigenic rhizomania resistance carried by old Italian genotypes. Collected sea beet populations in the Po River Delta, from which new sources of resistance were identified and developed. Lead author and editor in books, reviews, papers on aspects of research on rhizomania and *Beta maritima*.

Antonio Canova In 1966, hypothesized the connection "virus A"-*Polymyxa betae* as cause of the "low sugar content syndrome". *Polymyxa betae*, identified and classified a few years before by Keskin, plays the role of carrier, while the real pathogen is the virus. He named the disease "*rizomania*", later anglicized to rhizomania.

Sotaro Chiba Collaborated with Tamada and identified amino acids of the p25 protein for induction of the resistance response in leaves of *Beta vulgaris* and found function of the p25 protein as an avirulence factor. He analyzed worldwide isolates of BNYVV and obtained information on the global biogeography, evolution, virulence, and spread of BNYVV.

Marco De Biaggi In 1978, together with Biancardi, discovered and selected rhizomania resistance traits in cercospora leaf spot resistant genotypes. In 1985, released the first monogenic resistant variety endowed with the "Rizor type" resistance. He was among the first to apply ELISA techniques in screening beets for mass selection. In collaboration with Stevanato and Biancardi, he recently verified the similarity between the resistances Rizor and Holly (Rz1).

David Gilmer Studies molecular biology of BNYVV looking for RNA and protein structure-function relationships. Aims to understand BNYVV viral cycle.

Luciano Giunchedi Collaborated with Canova on the etiology of rhizomania, and later with De Biaggi in characterizing the mechanisms of the Rizor resistance in reducing the damages caused by BNYVV.

Hideki Kondo Collaborated with Tamada since 1995. He studied with Andika the molecular mechanisms of RNA silencing in roots and root-specific suppression of RNA silencing. He analyzed genetic diversity of BNYVV and evolution of benyviruses, and discovered benyvirus replicase-related sequences integrated into the genomes of diverse eukaryotic organisms.

Robert T. Lewellen Determined inheritance and named the gene *Rz* (the "Holly type" resistance) after discovery by Erichsen and his subsequent release of the variety "Rhizosen" in 1990. Resistance to BNYVV was found in *Beta maritima* accessions collected in Europe. Released lines, as C48, C79, and R740, were used internationally to identify Rz2, Rz3, Rz4, and Rz5 and incorporated into commercial resistant varieties. With Wisler devised disease severity scale and its correlation with concentration of BNYVV. More recently, with Liu, discovered evidence of resistance-breaking strains of BNYVV and used *Beta macrocarpa* as systemic host to incorporate specific virus strains with *Polymyxa betae*. With Biancardi and Panella, published extensively on rhizomania research and resistance, particularly from *Beta maritima*.

J. Mitchell McGrath Has a long-standing interest in the genetics, genomics, and germplasm enhancement of sugar beets. He actively releases sugar beet germplasm with novel genetic characters and investigates the organization of the beet genome. Resistance gene structure and function, including rhizomania genes, is a recent interest afforded by a complete genome sequence.

Peyman Norouzi Identified some molecular markers linked to rhizomania resistance genes and selected many pollinators, OType lines, and populations accordingly. He co-authored papers published by Stevanato regarding the identification of SNP markers linked to rhizomania resistance genes. He collaborated to develop transgenic lines resistant to rhizomania and other traits.

Leonard W. Panella In collaboration, with Biancardi and Lewellen, co-authored the book "*Beta maritima*: the origin of beets". Collected seed of *Beta maritima* and other wild beets, including *Beta nana* and *Beta patellaris* (*Patellifolia patellaris*), in many parts of the world. In collaboration with Stevanato and colleagues, developed molecular genetic markers for resistance to BNYVV. Developed enhanced germplasm that combined resistances to rhizomania, cercospora leaf spot, and rhizoctonia root rot.

Claudio Ratti Studied the epidemiology of BNYVV in Italy and in Europe developing molecular methods for detection and characterization of BNYVV types. Together with Gilmer, currently studies the biology of members of family

Benyviridae by reverse genetic approach with particular attention to BNYVV and beet soil-borne mosaic virus (BSBMV).

George N. Skaracis and **Ourania I. Pavli** Authored several publications regarding conventional and molecular breeding methods, with emphasis on genetic engineering, to develop durable rhizomania resistance.

Piergiorgio Stevanato Collected seed of sea beet populations in the countries bordering the Adriatic Sea. Authored several publications together with Panella, Lewellen, Biancardi, De Biaggi, Pavli, and Skaracis. Improved the molecular methods for identifying and more rapidly increase the resistances to rhizomania, bolting, fusarium yellows, and *Heterodera schachtii*.

Tetsuo Tamada Identified the causal agent of rhizomania and named it "beet necrotic yellow vein virus" (BNYVV) in 1973. Since then, he worked with many colleagues on the characterization of virus, vector transmission, detection and diagnosis, ecology, and control. In 1995, he moved to Institute of Plant Science and Resources of Okayama University and continued to work on biological and molecular properties of BNYVV. Identified viral genes involved in vector transmission, disease development, and genetic resistances. Published extensively on the results.

Abbreviations

2x, 3x, 4x (or 2n, 3n, 4n)	Diploid, triploid, tetraploid genotypes
A	Alanine
aa	Amino acids
AD	Activation domain
AGO	Agronatute
AK	Alkalinity coefficient
AMOVA	Analysis of molecular variance
ANB	Italian Sugar Beet Growers Association
ARS	Agricultural Research Service
ASSBT	American Society of Sugar Beet Technologists
Avr	Avirulence
BaYMV	Barley yellow mosaic virus
Bb	Annuality (alleles)
BBSV	Beet black scorch virus
BCTV	Beet curly top virus
BdMoV	Burdock mottle virus
BMYV	Beet mild yellowing virus
BNYVV	Beet necrotic yellow vein virus
BOLV	Beet oak leaf virus
BRLS	Benyvirus replicase-related sequence
BSBMV	Beet soil-borne mosaic virus
BSBV	Beet soil-borne virus
BSMV	Barley stripe mosaic virus
BVQ	Beet virus Q
°Bx	Brix (refractometer degree or optical density)
BWYV	Beet western yellows virus
BYV	Beet yellows virus
C	Cysteine
Cas9	CRISPR-associated protein 9
CAV	Chara australis virus
CC	Coiled-coil

CCC	Copyright Clearance Center (www.copyright.com)
cDNA	Complementary DNA
CdTe	Cadmium telluride
CEC	Cation exchange capacity
CHS	Chalcone synthase
CLS	Cercospora leaf spot
cM	Centimorgan
CMS	Cytoplasmic male sterility
CP	Coat protein or capsid protein Cross-protection test CP-mediated protection
CRA-CIN	Centro per la Ricerca in Agricoltura – Centro per le Colture Industriali
CRISPR	Clustered regularly interspaced short palindromic repeats
CRP	Cysteine-rich protein
CWR	Crop wild relatives
D	Aspartic acid
DAS-ELISA	Double-antibody sandwich enzyme-linked immunosorbent assay
DCL	Dicer-like
D-D	Mixture dichloropropene + dichloropropane
DI	Disease index
d_N	Non-synonymous substitution
DNA	Deoxyribonucleic acid
DPI	Days post inoculation
d_S	Synonymous substitution
dsRNA	Double-stranded RNA
dT	Deoxythymidine
E	Glutamic acid
E, N, Z	Production ability of sugar beet varieties
ed, eds	Editor, editors
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
EPPO	European-Mediterranean Plant Protection Organization
ER	Endoplasmic reticulum
F	Phenylalanine
F-box	Cyclin F motif-containing protein
FLIM	Fluorescence lifetime imaging
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
GM	Genetically modified
GMO	Genetically modified organism
GO	Argonaute

GORV	Gentian ovary ring-spot virus
Gp-1a, GP1b, GP-2, GP-3	Primary, secondary, and tertiary *Beta* gene pool
GSP	Gene-specific primer
GST	Glutathione S-transferase
H	Histidine
Hel	Helicase
HR	Hypersensitive response
HrpZ	Harpin Z
Ibid.	Published in the formerly cited paper
IBPGR	International Board Plant Genetic Resources
IIRB	International Institute of Sugar Beet Research
IMP	Immunodominant membrane protein
ISEM	Immunosorbent electron microscopy
ITS	Internal transcribed spacers
IWGPVFV	International Working Group on Plant Viruses with Fungal Vectors
kDa	Kilodalton
kPa	Kilopascal
KWS	Kleinwanzlebener Saatzucht (seed company)
L	Leucine
LAI	Leaf area index
LSCS	Low sugar content syndrome
LSD	Least significant difference
MAPK	Mitogen-activated protein kinase
ML	Maximum likelihood
Mm	Monogermity (alleles)
mM	Millimoles
MP	Movement protein
MPN	Most probable number
mRFP	Monomeric red fluorescent protein
mRNA	Messenger RNA
MS	Male sterility
MT	Methyltransferase
N	Normal cytoplasm
NB-LRR	Nucleotide-binding site-leucine-rich repeat
NCR	Noncoding region
ncRNA	Noncoding RNA
NES	Nuclear exporting signal
NGS	Next-generation sequencing
NLS	Nuclear localization signal
NoLS	Nucleolar localization signal
nPCR	Nested PCR
nt	Nucleotides
NTPase	Nucleoside triphosphatase
NVMV	Nicotiana velutina mosaic virus

OA	Origin of assembly
ORF	Open reading frame
O-Type	CMS maintainer
p, pp	Page, pages
PCR	Polymerase chain reaction
PCV	Peanut clump virus
Pd	Plasmodesmata
PDR	Pathogen-derived resistance
pH	Acidity/basicity of aqueous solution
PMTV	Potato mop-top virus
ppm	Parts per million
PR	Pathogenesis-related
Pro	Protease
Psph	*Pseudomonas syringae* pv. *phaseolicola*
PTGS	Posttranscriptional gene silencing
PVX	Potato virus X
QD	Quantum dot
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
QTL	Quantitative trait locus
RB	Resistance-breaking
RdDM	RNA-directed DNA methylation
rDNA	Ribosomal DNA
RdRp	RNA-dependent RNA polymerase
Rep0/3/5/III	Replicon 0, 3, 5 or III
RFLP	Restriction fragment length polymorphism
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen specie
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RS	Rizomania Signal (in German)
RSNV	Rice stripe necrosis virus
RT	Read-through
RTD	Read-through domain
RT-PCR	Reverse transcriptase polymerase chain reaction
Rz1; Rz2; Rz3; Rz4; Rz5	Resistances to rhizomania
Rz1rz1; Rz2rz2; Rz3rz3 etc.	Alleles of rhizomania resistances
S	Sterile cytoplasm
°S	Polarization or sugar content (% w/w)
SBWMV	Soil-borne wheat mosaic virus
scFv	Single-chain antibody fragment
SDS	Sodium dodecyl sulfate

SES	Societè Europeenne des Semences (seed company)
siRNA	Small interfering RNA
SNP	Single-nucleotide polymorphism
SP	Signal peptide
sp.	Species (singular)
spp.	Species (plural)
SSCP	Single-strand conformation polymorphism
ssRNA	Single-stranded RNA
subsp.	Subspecies
T	Tyrosine
TA	Transcriptional activation
TAS-ELISA	Triple-antibody sandwich enzyme-linked immunosorbent assay
TEM	Transmission electron microscopy
TEV	Tobacco etch virus
TGB	Triple gene block
TIR	Toll/interleukin-1 receptor
TMV	Tobacco mosaic virus
TRV	Tobacco rattle virus
USDA	US Department of Agriculture
UTR	Untranslated region
V	Valine
VIGS	Virus-induced gene silencing
VLRA	Virus-like RNA assembly
VSR	Viral suppressor of RNA silencing
VY	Virus yellows
w/v	Weight unit of solute in volume units of solution
w/w	Weight units of solute in weight units of solution
WB	Wild beet
WSSMV	Wheat spindle streak mosaic virus
XRN	Exoribonuclease
XxZz	Male sterility (alleles)
µl	Microliters

List of Boxes

Part I
Historical Background

Chapter 1
Introduction

Enrico Biancardi and Robert T. Lewellen

Abstract The use of beets as a sugar-producing crop is rather recent, dating to a little over two centuries ago. However, domestication of beets dates back to prehistoric times. The history of the crop is summarized and represents an outstanding example of agricultural accomplishment. Sugar beet is subjected to a number of biotic and abiotic factors that more or less severely limit both sugar yield and processing quality. As it is for industrial purposes with specific requirements, sugar beet cultivation has been always more difficult than other crops. It is believed that agricultural innovation was introduced to accommodate cropping systems (e.g., crop rotation, row cropping) and technology to improve the sucrose production and its extraction (e.g., progeny testing). Among the diseases affecting the crop, rhizomania is certainly the most dangerous. Currently, the cropping of sugar beet would be difficult without the availability of some source of rhizomania resistance. The economic damage caused by rhizomania and its rapid spread across the world are described.

Keywords Sugar beet • Rhizomania • BNYVV • *Polymyxa betae* • Genetic resistances

Farming sugar beet began in Germany just over two centuries ago. Within a few decades, the crop assumed increasing importance in a number of European countries. Despite higher cultivation costs and care needed to grow a successful crop, farmer's income was improved by including sugar beet in the rotation. Moreover, the industry was based, often for the first time, in the countryside and every sugar factory provided employment for hundreds of workers. The crop rapidly became a hub of the economy and technical evolution of agriculture. Intended to counteract the monopoly of sugar produced by cane, sugar beet had to survive times of trouble (social, economic, political) due not only to the frequent world overproduction and consequent low prices, but also to the spread of serious diseases. Rhizomania has been one of these for around half a century, becoming rapidly widespread all over

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E. Biancardi, T. Tamada (eds.), *Rhizomania*, DOI 10.1007/978-3-319-30678-0_1

the world with rare exceptions. So far, breeding research has allowed satisfactory control of the damages by means of resistant varieties. Despite this, the disease is still rapidly expanding.

1.1 Beets and Sugar Beets

The wild ancestor was similar to the current sea beet [*Beta vulgaris* L. subsp. *maritima* (L.) Arcang.] (Fig. 1.1), now classified as a subspecies (subsp.) together with different types of cultivated beets, *Beta vulgaris* (*Beta vulgaris* L. subsp. *vulgaris*) (Ford-Lloyd et al. 1975; Ford-Lloyd 2005). The subspecies *vulgaris* and *maritima* belong to the species *vulgaris*, included in the section *Beta* (*Vulgares*), genus *Beta*, and family Amaranthaceae (formerly Chenopodiaceae). The other species and subspecies of the genus *Beta* (Box 1.1), usually named "wild beets," do not have commercial value. However, with the expected progress in molecular biology and gene transfer, they could become future sources of traits useful for the cultivated varieties. As crosses with wild beets of other sections of the genus *Beta* are difficult using traditional means, the best results so far have been obtained with *B. maritima*.

Fig. 1.1 *Beta maritima* living in very difficult conditions near Porto Levante, Italy. From beets collected at the same site in 1909, resistances to cercospora leaf spot and rhizomania have been obtained (Biancardi et al. 2012)

Box 1.1: Taxonomy of Genus *Beta*

Based on molecular phylogenetics, a modified taxonomy of the genus *Beta* was proposed by Kadereit et al. (2006) and Hohmann et al. (2006). More recently, Kadereit et al. (2006) suggested the reintroduction of the subfamily *Betoideae*, first proposed by Ulbrich (1934), because it better explains the position of the genus *Beta* inside the family or "alliance" Chenopodiaceae/Amaranthaceae and fits better with the analysis of the nuclear ribosomal ITS1 sequences. According to this proposal, the taxonomy of the genus *Beta* was revised by moving the section IV *Procumbentes* into another genus due to the differences between it and the species in the section I *Beta.* To do this, Kadereit et al. (2006) proposed the introduction of a separate genus *Patellifolia*, including the species *procumbens*, *patellaris*, and *webbiana* (Table 1.1). They also suggested the elimination of the section III *Nanae*, incorporating *Beta nana* (the lone species in that section) into section *Corollinae*. Hohmann et al. (2006), according to Kadereit et al. (2006), included only two sections (*Beta* and *Corollinae*) in the genus *Beta*. The family Amaranthaceae belongs to the order *Caryophyllales* (McGrath and Townsend 2015).

Table 1.1 Comparison of the taxonomy of the genus *Beta* proposed by Ford-Lloyd (2005) and Kadereit et al. (2006)

Ford-Lloyd (2005)	Kadereit et al. (2006)
Genus *Beta*	**Genus *Beta***
Section I *Beta* (*Vulgares*)	Section I *Beta*
Beta vulgaris	*Beta vulgaris*
subsp. *vulgaris* (cultivated forms[a])	subsp. *vulgaris* (cultivated forms[a])
subsp. *maritima*	subsp. *maritima*
subsp. *adanensis*	subsp. *adanensis*
Beta macrocarpa	*Beta macrocarpa*
Beta patula	
Section II *Corollinae*	Section II *Corollinae*
Beta corolliflora	*Beta corolliflora*
Beta lomatogona	*Beta lomatogona*
Beta intermedia	*Beta trigyna*
Beta trigyna	*Beta nana*
Section III *Nanae*	
Beta nana	
Section IV *Procumbentes*	**Genus *Patellifolia***
Beta procumbens	*Patellifolia procumbens*
Beta patellaris	*Patellifolia patellaris*
Beta webbiana	*Patellifolia webbiana*

[a]Cultivated forms include sugar beet group, leaf beet group, fodder beet group, and garden beet group (Lange et al. 1999)

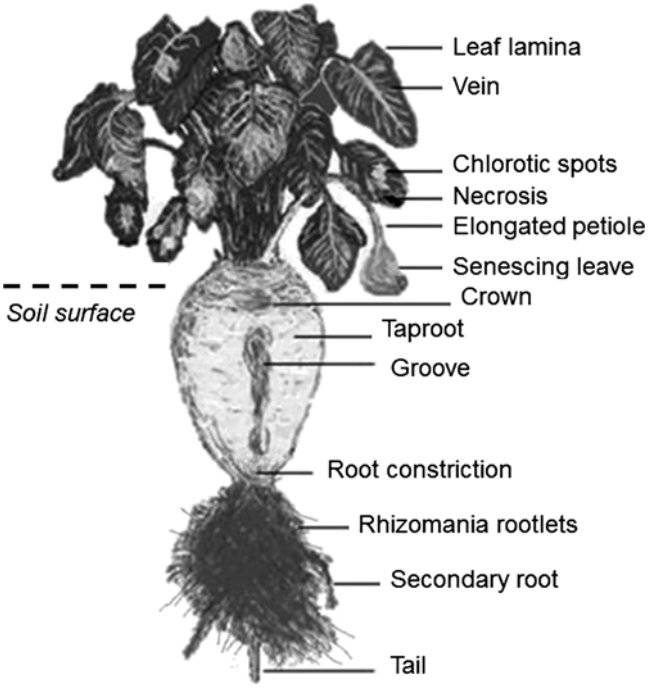

Fig. 1.2 Drawing of sugar beet with the common name of the parts cited in the text

Since prehistoric times, beet leaves have been used as a vegetable and more rarely as a drug (von Lippmann 1925). Beet cultivation likely began at the same time as the domestication of other important crops, such as wheat and barley. At the beginning of the Roman Empire, a second type of beet was cultivated, with round and often red-colored roots, petioles, and leaves, suitable to be used as a food after cooking. During the Middle Ages, another type appeared in central Europe, developing large, variously shaped, and colored roots suitable as winterfeed for cattle (von Lippmann 1929; Biancardi et al. 2012). At the end of the 1700s, a fourth utilization was achieved after cycles of selection to increase the sucrose content in the taproot (Fig. 1.2). Simultaneously, an industrial system for sugar beet processing was developed, first put into operation at Cunern (Germany) in 1802 (Fischer 1989). With some exceptions, the crop and the related industry remained confined to Europe for the next 100 years (Winner 1993).

In spite of the outstanding improvements in sugar yield and in genetic resistances to diseases such as beet curly top virus (BCTV), beet yellows virus (BYV), cercospora leaf spot (CLS), etc. (Box 1.2), until around 1950 and the advent of

Box 1.2: Tolerance or Resistance?

In the case of viral diseases, the use of the terms "tolerance" and "resistance" is quite controversial. There are actually major differences between the two words indicating the best behavior of plants if compared to the more affected ones. "Tolerance" means the plant's ability to hinder the action of the disease agents that cause yield reductions (sometimes without displaying visual symptoms) in plants of the same crop (Clarke 1986). "Resistance" indicates the ability to reduce the multiplication, spread, or concentration of the pathogenic agent inside the plant (Fraser 1990). In order to define the latter, it is necessary to determine the concentration of the pathogen in the diseased parts, whereas data regarding growth and/or yield are required to define tolerance. It is therefore possible to speak of genetic resistance when the pathogen is present in the plant in lower concentration if compared with the susceptible ones. In resistant plants, the virus can multiply and move inside the plant, but at a slower rate than in susceptible individuals. The highest level of resistance is named "immunity," whereas the intermediate stage between resistance and susceptibility is wrongly referred to as "tolerance." It is not always easy to find a precise limit between the terms tolerance and resistance, because the parameters used for defining them often coexist, and it is almost impossible to identify the prevailing one. In the case of rhizomania, the presence of twin pathogenic agents, BNYVV and *P. betae*, increases the difficulty in choosing the right term. Since the diffusion of the virus also happens in resistant genotypes, the term "partial resistance" is sometimes used as is "field resistance" or "field tolerance," which means the better sugar yield of resistant or tolerant genotypes if compared to a set of susceptible ones (Tuitert 1994). These differences, collected by means of field trials, should be the safest method for quantifying the level of tolerance displayed by a given variety (Winner 1988). In the case of rhizomania, the analyses of the virus concentration in rootlets or taproots are positively correlated with the plants' ability to avoid the disease consequences on sugar yield. Moreover, the BNYVV multiplication is possible only in *P. betae*, which assumes the role of host plant, thus partially confirming the better correspondence of the word "resistance" (Büttner and Mangold 1998). In conclusion, and even if neither of the term "resistance" nor "tolerance" seems fully satisfactory in the case of rhizomania, the first one will be used here, as is prevalently done in the literature (Giunchedi et al. 1985, 1987). In the case of abiotic stresses, i.e., in the absence of living pathogens, the word "tolerance" will be used. The better results of rhizomania-resistant varieties are often the result of a higher proportion of healthy plants than diseased ones. Consequently, these varieties should be designated as field resistant (Graf 1987). In this book, the types of resistance to rhizomania are indicated by the names commonly used at the time (Alba, Rizor, Holly, etc.) or by the respective codes (Rz1, Rz2, etc.); if written in italics, *Rz1*, *Rz2*, *Rz3*, etc., indicate the dominant alleles of resistance. See also the schemes in Appendices 1 and 2, which summarize the current terminology in plant pathology.

hybrid varieties, both cultivation methods and the genetic structure of open-pollinated varieties remained nearly unchanged. Only a few cropping practices, like soil tillage, spraying devices for disease treatments, and some harvest operations, were aided by machinery. The use of synthetic fertilizers, pesticides, and herbicides was still in its infancy in many countries, and sugar beet was one of the first crops protected by agrochemicals against parasites and weeds (Winner 1993).

Cultivated beets are usually 18 chromosome diploids (2n = 2× = 18). Tetraploid families having twice (2n = 4× = 36) the normal number of chromosomes are obtained, using the mutagenic properties of colchicine (Rasmusson and Levan 1939). The 4× genotypes were characterized by better root shape and bolting resistance (Box 1.3) (Fig. 1.3) and fewer and larger leaves than diploid (2×) beets. The

Box 1.3: Annual and Bolting Beets

All cultivated beets are biennial, i.e., they require a vernalization period (overwintering with low temperature and increasing day length, also referred to as photothermal induction) to enter the reproductive phase (Fig. 1.4) (Letschert 1993). Under vernalizing weather conditions after the normal sowing time, biennial beets may give rise to bolting plants, which flower and produce viable seed (Smit 1983). Normally in commercial fields, a small proportion of beets (usually less than 0.1 %) bolts and flowers. It is always advisable to cut the stalk before flowering, especially in districts of sugar beet seed production, because the pollen produced by bolted plants carries the bolting tendency (annuality) and can be particularly damaging (Box 8.2) (Smit 1983). Despite the complexity of flowering physiology in biennial beets, depending also on genotype x environment interactions of variable intensity, continuous mass selection has improved bolting resistance. Early sowing is effective for inducing bolting in the more sensitive beets, allowing further selection. Since early sowing is not always possible in field conditions, different greenhouse systems with combined photothermal treatments were developed. Bolting resistance was quite easily improved using progeny testing (McFarlane 1971). Due to strong genotype x environment interactions, progress in bolting resistance is possible by selecting in the district where the improved variety will be sown (Smit 1983). The use of spring varieties with improved bolting resistance enables earlier sowing, resulting in a longer growth period and in a slightly improved sugar yield. Bolting resistance is controlled by several genes acting through different mechanisms, but the precise genetics are as yet undetermined (McFarlane et al. 1948; Le Cochec et al. 1989; Jolliffe 1990; Sadeghian and Johansson 1992; Leiva-Eriksson et al. 2014). Pin et al. (2010, 2012) explained a molecular mechanism involved in changing the annual to biennial cycle during beet domestication. It is also imperative with the advent and use of GMO varieties that bolters be prevented or removed before pollen release (anthesis).

Fig. 1.3 Bolting beets growing inside a spring-sown field (Ancona, Italy) (Courtesy, Stevanato et al. 2014)

2× and 4× families can be crossed to produce triploid (2n = 3× = 27) or anisoploid mixtures of 2×, 4×, and triploid seed. Selected triploid hybrids produced higher root and sugar yield than the comparable diploid or tetraploid parents and hybrids.

Anisoploid varieties were grown mainly in Europe after 1950 (Coons et al. 1955). Hand thinning of seedlings was necessary because "seed" (botanically a fruit) was composed of two to five or more fruits fused together, each carrying a single seed, with the potential to germinate and develop as many tightly packed plantlets. Around 100–150 person-hours per hectare were required in order to thin plants to the desired stand of 80,000–100,000 beets per hectare. This expensive operation was eliminated with the discovery and use of genetic monogerm seed (Savitsky 1950) and the use of precision sowing equipment. The release of 100 % hybrid varieties became possible after the discovery of genetic-cytoplasmic male sterility (CMS) (Box 1.4) (Owen 1942, 1945). The monogerm 2× CMS seed bearers were crossed with 2× or 4× pollinators, originating 2× or 3× hybrid varieties, respectively.

These new hybrid varieties became commercially available in the early 1960s and rapidly replaced the multigerm open-pollinated varieties. At the same time, seed pelleting improved sowing precision and chemical protection against seedling diseases (Leach and Bainer 1942; Winner 1993). The last 50 years have been marked by the discovery of new and more powerful genetic resistances to rhizomania, virus yellows, beet cyst nematode, nonselective herbicides, fusarium yellows, etc. Further improvements in sugar yield became possible through the use of molecular biology

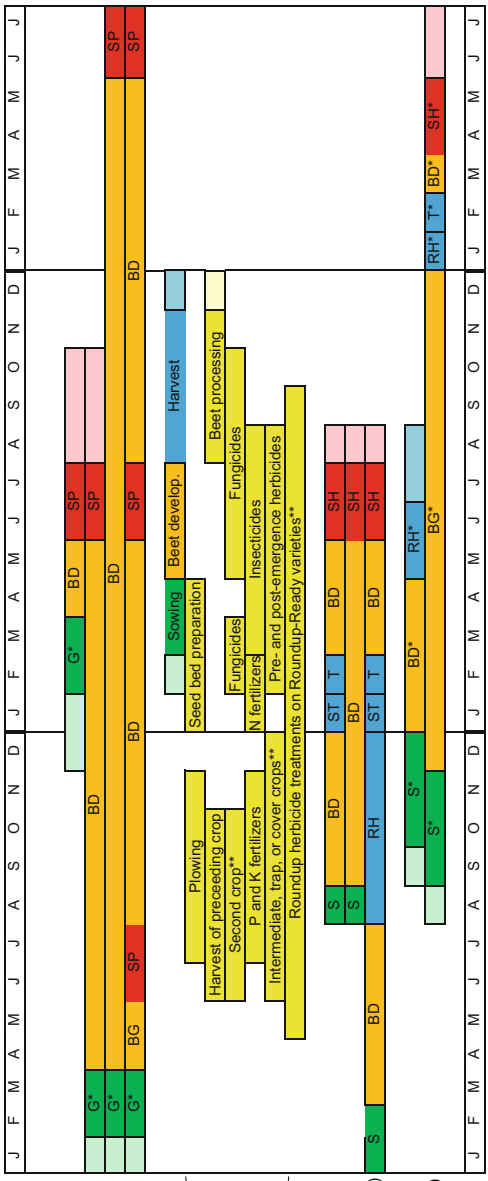

Fig. 1.4 Cycles of wild and cultivated beets. The normal timetable of operation on spring crop is highlighted in yellow. Wide variations are possible according to the local conditions. S sowing, *BD* beet development, *SP* seed production, *RH* root harvest, SH seed harvest, ST storage, *T* transplanting, *Variable according to local situations; **possible: iteroparous = perennial genotype producing seed every year; semelparous = perennial genotype producing seed one time before death; bright colors = extended time for germination, seed production, and root harvest

Box 1.4: O-Type and CMS
The release of fully hybrid sugar beet varieties became viable after the discovery of the combined effects of genetic and cytoplasmic factors. It was thus possible to obtain the sugar yield improvement ensured by the heterosis existing between the male parent (pollinator) and the male-sterile (or female) seed bearer, carrying the citoplasmic male sterilty (CMS) traits. The sterile cytoplasm (S) induces sterility only in the presence of the alleles *Xx* and *Zz* in homozygous and recessive condition (S *xxzz*). All the remaining combinations produce fertile or partially fertile offspring, whereas the normal (N) cytoplasm always develops fertile progenies. The reproduction of CMS lines requires lines bearing the N cytoplasm and the genes *x* and *z* in homozygous recessive condition (*xxzz*). The maintainer lines N (*xxzz*) were called O-Type (Owen 1942, 1945; McFarlane 1971). The monogerm trait (*mm*) is incorporated into CMS, O-Type lines.

techniques, which began to be available around 1980. The timetable of the most important progresses in sugar beet crop is shown in Table 1.2.

Sugar beet supplies 36 Mt raw sugar yearly, which is around 17 % of the world production/consumption (Source: Faostat, http://faostat.fao.org and https://www.commoditybasis.com/sugar_prices). The current per capita amount of 23.5 kg sugar is quite rapidly increasing by 0.150 kg annually: this means that every year, more than one additional Mt sugar should be available (Tilman 1999; Tilman et al. 2002). Because the cultivated surface of sugar cane (Box 1.5) is not always expandable due to its high water consumption, at least 15 % of future demand should continue to be supplied by sugar beet. Sugar beet crop has recently undergone massive acreage reductions or relocations, especially in Europe and the USA. This change is partially counteracted by the rise in production per unit area of land. Sugar yield is still increasing. In Germany, for example, sugar yield grows at a rate of 1.4 % yearly mainly due to genetic improvements (Märländer et al. 2003). In the USA, according to Panella et al. (2014), the gain in sugar yield per hectare was about 0.42 % yearly during the last century.

Much potential gain in yield still exists for sugar beet. Beet produced 23.70 t ha^{-1} of sugar in 2004 near Brawley, Imperial Valley, California. This is about four to five times the current world average (Rush et al. 2006; Jansen and Stibbe 2007). In the same district of about 10,100 ha, the mean sugar production is around 11 t ha^{-1} (Panella et al. 2014), which is quite above the normal target in Europe. The former data are surprising because 30 years ago, the sugar beet crop in Imperial Valley was almost totally lost due to rhizomania (Rush et al. 2006).

Table 1.2 Chronology of the most important progresses in sugar beet crop (from different sources)

1802 – First sugar beet variety (Achard 1803)
1803 – First sugar beet factory at Cunern, Germany (Fischer 1989)
1850 – Employment of progeny selection method (De Vilmorin 1856)
1833 – Employment of polarimetry in breeding and beet processing (De Vilmorin 1856)
1891 – Crosses *Beta maritima* x sugar beet (Rimpau 1891)
1909 – *Beta maritima* seed sampling in the Po Delta, Italy (Munerati 1946)
1937 – Release of cercospora leaf spot-resistant varieties (Munerati 1946)
1938 – Tetraploid lines (Schwanitz 1938)
1942 – Self-fertility (Owen 1942)
1942 – Release of resistant variety to curly top (Murphy 1946)
1945 – Discovery of cytoplasmic male sterility (Owen 1945)
1950 – Genetic monogerm seed (Savitsky 1950)
1950 – Anisoploid varieties in Europe
1960 – Hybrid monogerm varieties (Owen 1945)
1960 – Sampling of *Beta maritima* at Kalundborg Fjord, Denmark (Doney and Whitney 1990)
1968 – Release of multigenic rhizomania-resistant variety (Gentili and Poggi 1986)
1969 – Release of resistant varieties to virus yellows (Russell 1969)
1976 – Release of resistant varieties to root rots (Hecker and Ruppel 1975)
1977 – Biotechnology (ELISA)-assisted breeding methods (Clark and Adams 1977)
1985 – Release of monogenic rhizomania-resistant variety Rizor (De Biaggi 1987)
1990 – Release of monogenic rhizomania-resistant variety Rhizosen (Lewellen and Biancardi 1990)
1997 – Release of composite crosses including Rz2, Rz3, Rz4, and Rz5 resistances (Lewellen 1997)
2005 – Release of resistant variety to Roundup® (www.monsanto.com)
2006 – Selection of monogenic resistant variety to cyst nematode (Lewellen 2006)
2006 – Selection of powdery mildew-resistant breeding lines (Lewellen and Schrandt 2001)
2012 – Molecular mechanism of bolting (Pin et al. 2012)
2014 – Hs-Bvm-1 monogenic source of cyst nematode resistance (Stevanato et al. 2014)

1.2 Abiotic Stresses

Abiotic stresses limit sugar beet production through lack or excess of factors such as water, temperature, soil composition, and so on. Surveys on commercial sugar beet and wild relatives (Box 1.6) have shown the existence of genetic variability for tolerance to some abiotic factors such as drought, low or high temperatures, soil salinity, etc. Generally, these traits appear to be inherited through quantitative mechanisms subjected to genotype x environment interactions and hence hard to be improved.

The production of beet seed requires always great attention to prevent unwanted outcrossing. This is especially true for beets, which is one of the few cases where the wild ancestor and the derived major crop grow in the same geographic areas. Undesired gene flow in both directions is therefore possible (Box 1.7).

Box 1.5: Sugar Beet and Sugar Cane

Although they are grown to produce the same molecule (sucrose), there are huge differences between sugar beet and sugar cane beginning with their botanical taxonomy, structure, and physiology (Blackburn 1984). Beet is a broad-leafed dicotyledonous species with C3 photosynthesis, and sucrose is stored in the taproot. Cane is a monocotyledonous plant, tropical grass with C4 photosynthesis, transplanted, and lasts several years (ratoons) in the same field. The canopy of cane usually shades the soil and weed control is simplified. In sugar beet, weed control is often the most expensive production factor. Roots of cane are deeper, more than 4 m according to Smith et al. (2005), thus allowing the better uptake of soil nutrients and water. However, the water requirement is about sevenfold higher than beets (Martinelli and Filoso 2008). Cane is harvested by hand or with machines that separate the leaves from the stalk. In the factory, the stalks are squeezed to express the juice. Beetroots are sliced, and sucrose is removed by diffusion. At this point, the product of both is concentrated and purified using similar procedures. The fibrous residue (bagasse) of cane is used to produce energy, which is usually more than enough to operate the factory. The pulp of beetroot is dried and produces a high-quality animal feed. Beet sugar is generally produced in economically advanced countries in the temperate zone. Sugar cane is usually cultivated in third world and emerging countries. The production price of cane sugar is currently about 50 % that of beet sugar largely reflecting differential labor and energy costs.

Box 1.6: Crop Wild Relatives

The ancestors of the currently cultivated crops are defined as "crop wild relatives" (CWRs). Many wild relatives, including *B. maritima* – the ancestor of the *Beta* crops – are exposed to reduction, degradation, and modifications of their natural habitat and fitness, which may be responsible for losses of genetic resources and variability. Maxted et al. (2006) subdivided the species of the genus *Beta* into gene pools (GPs), according to the difficulty of intercrossing suitable traits for sugar beet. The primary gene pool is split in two subgroups: GP-1A,including the cultivated forms, the wild and weed beets; and GP-1B, where the remaining species and subspecies of section *Beta* are placed. The secondary gene pool (GP-2) includes the less closely related species, from which gene transfer to the crop is difficult but possible using conventional breeding techniques. The remaining species of the genera *Beta* and *Patellifolia*, from which gene transfer to the crop is very difficult or requires sophisticated techniques, are inserted in the tertiary gene pool (GP-3).

Box 1.7: Gene Flow

The dispersion of genes through seed, pollen, or vegetative reproducing parts of a given species is termed "gene flow" (McDermott and McDonald 1993; Bartsch 2010). In the genus *Beta*, seed and pollen have particular ability for long-distance spread (Bartsch et al. 1999; Biancardi et al. 2012). Moreover, in the subspecies *vulgaris*, there are different forms of wild, weed (infesting the crop), and feral beets (developing outside the crop) (Fig. 1.5) that cross easily with the cultivated ones during their flowering stage (bolting beets and seed production) (Sukopp et al. 2010). These genotypes, all included in the "*Beta vulgaris* complex" (Hautekèete et al. 2001; Viard et al. 2004), or in the wider "section *Beta* complex," also live close to the more important sugar beet seed production districts in France and Italy. Two types of risks arise: The first regards the seed production fields (1 in Fig. 1.5), where every type of pollen different to that released by the specific pollinator is a genetic contaminant, carrying, among other things, the ability to flower the first year. The second risk comes from the conservation of the genetic resources. In this case, the crosses of wild populations (2 and 3 in Fig. 1.5) with any type of pollen coming from outside should be avoided.

The major danger is represented by the transmission of the *B* annual gene into the commercial seed. Crop-to-weed gene flow promotes the evolution of potentially more aggressive weeds if the former carries disease resistances (Ellstrand and Schierenbeck 2000), while it could induce survival disadvantages and unwanted genetic drift in wild beet populations (Ellstrand and Elam 1993). Crop-to-crop pollination (i.e., sugar beet x fodder beet) is also very

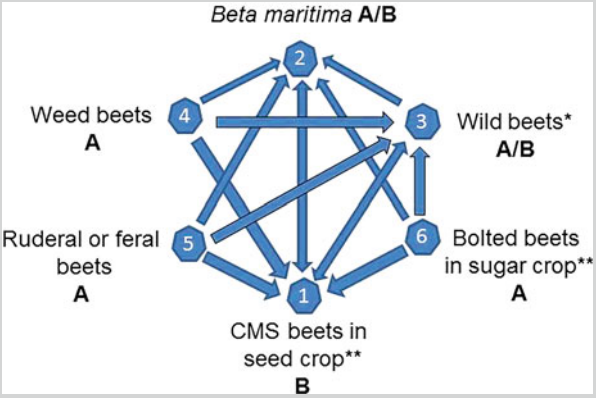

Fig. 1.5 Gene flow via pollen in *Beta* section *Beta* complex. * *Beta vulgaris* subsp. *adanensis*, *Beta patula*, and *Beta macrocarpa*; ** Sugar, leaf, garden, fodder beet groups. Gene flow among these listed beet crops is also very dangerous. The thickness of the arrows is approximately proportional to the damage. A normally annual, B normally biennial

(continued)

Box 1.7 (continued)
damaging. The release of the first GM beet varieties has raised questions about their potential impact on crop-to-wild gene flow and thus on biodiversity (Colwell 1994; Gepts and Papa 2003). Until now, the fitness advantage for hybrids between modified crops and the wild relatives seems to be not significant (Arnaud et al. 2009). Therefore, the introduction of GM traits should have no immediate consequences on the physiology of wild populations (Bartsch and Schuphan 2002; Saccomani et al. 2009). Gene flow from GM crop-to-weed populations is probable, and could give rise to difficulty in weed beet control if pollen carries the glyphosate resistance, for instance. The rhizomania-resistant crop-to-wild gene flow should not confer any survival advantage on the hybrids inside their populations, but these crosses should anyway be avoided.

In the last decades, there is concern about the effects that global climate change (Box 1.8) will (or might) have on crop production, especially through rising temperatures and the reproduction speed of the rhizomania pathogenic agents (Hofmeester and Tuitert 1989; Ziska and McConnell 2015). Because of the variability and lack of precipitations, which is also becoming frequent in formerly rainfed districts, the presence of some degree of drought tolerance which can ameliorate the consequences of moderate dry periods on sugar beet is desired (Peltonen et al. 2010). Accessions of *B. maritima* and other species of the genus *Beta* have been evaluated in drought conditions (Frese 2004). Some entries differed significantly in sugar yield if compared to the normal checks (Ober and Rajabi 2010). Ober et al. (2005) also found moderate variability in commercial varieties, likely caused also by different growth patterns of the root system (Ober and Luterbacher 2002; Stevanato et al. 2010).

In the sugar beet crop, low temperatures frequently occur in early stages of development in normal spring sowing or before plants are harvested in October or later. Preliminary surveys on breeding lines showed variability in frost resistance. An increase in sugar content was observed in the progenies of plants that survived frost. It was noted also that cold sensitivity and susceptibility to CLS were correlated, suggesting a possible common mechanism of action for tolerance/resistance to both stresses (Wood 1952). In a warm climate, sugar beet is frequently subjected to thermal stress. High temperatures (35–45 °C) coupled with dry winds originate stress conditions with subsequent reduction in sugar yield. Srivastava (1996) demonstrated the existence of sufficient genetic variability of reactions to thermal stress. A test based on chlorophyll fluorescence measurement has been proposed for selection of heat-tolerant genotypes (Clarke et al. 1993). As above mentioned, rhizomania multiplication rate in sugar beet is strictly correlated with temperature.

Box 1.8: Climate Change
According to several international sources, the mean global temperature is increasing slowly but continuously. It is suspected that this has been caused by the massive use of fossil fuels for power and heat production and by the consequent emission of greenhouse gasses. Agriculture should be mainly affected by the following climatic phenomena:

- Increasing temperature especially in the higher limits
- More intense and lasting drought over larger areas
- Increased frequency of heavy rains

These hypothesized changes will enhance some pathogens and hinder others (Martin and Sauerborn 2013). As far as rhizomania is concerned, the rising temperature will lengthen the favorable period both for beet inoculation and *Polymyxa*/BNYVV multiplication, whereas drought should limit the root infection.

There is growing interest regarding the physiology of salt tolerance in crops due to the shrinking supply of freshwater worldwide and the increasing soil salinization in irrigated crops due to the use of water with too high salt content (Koyro and Huchzermeyer 1999). The physiological basis of salt tolerance has been studied by Koyro (2000) and by Bor and Özdemir (2003) in *B. maritima* (Box 1.9). Wild beets frequently grow in soil with high content of sodium chloride (Shaw et al. 2002). Because the most useful sources of resistance to rhizomania were found in *B. maritima* populations growing very close to seashores and salty water, it was hypothesized that salt tolerance could also have been developed in *B. maritima* in response to natural selection (Bartsch and Brand 1998). Stevanato et al. (2013) have recently investigated and identified traits in sea beet useful for future selection (Fig. 1.6).

Two or more diseases or stresses frequently occur at the same time in the same field. Genotypes endowed with resistance/tolerance to multiple diseases or stresses would be useful, especially in the case of soil-borne diseases (McFarlane 1971; Francis and Luterbacher 2003; Harveson and Rush 2002). In sugar beet, CLS-resistant varieties have been crossed with genotypes bearing resistance to rhizomania and/or nematodes (see Chap. 2) (Biancardi et al. 2005). The term "multiple resistances" also means the combination in the same breeding line or variety of different types of resistance to a single disease. The mixture of diverse sources of resistance, when available, decreases the effects of the disease with complementary mechanisms and delays the natural selection of resistance-breaking strains in the pathogen (Lewellen and Biancardi 1990; Pferdmenges et al. 2009). Only in the case of rhizomania are dual sources of resistance currently available and commercially used in sugar beet.

Bolting (stem elongation and flowering before the normal period) in sugar beet is caused by climatic factors interacting with the genotype and as such is a natural process and not a typical abiotic stress. Biennial cultivated beets take on annual behavior under sufficient accumulation of low temperatures and increasing photoperiod, particularly during the early growth phases (Smit 1983). Bolted beets may affect harvest

Box 1.9: *Beta maritima*

Beta maritima, also named sea beet, grows along the shores of the Mediterranean Sea and the European North Atlantic Ocean. It does not normally develop in inland localities (Arnaud et al. 2009). The wild plant has had and will have invaluable economic and scientific importance. Indeed, it is considered the ancestor of the cultivated beets, as recently confirmed by molecular analyses (Biancardi et al. 2012). Sea beet crosses easily with the cultivated types, thus facilitating the transmission of genetic traits partly lost after domestication. In fact, the selection procedures aimed only at increasing the features useful to farmers, processors, and consumers may have unconsciously caused the loss of traits potentially suitable for the future development of the crop. Therefore, as in the case of several crop wild relatives, *B. maritima* has been successfully used for recovering old traits suitable, for example, to improve the genetic resistance against diseases. In the case of rhizomania, the sugar beet cropping would currently be quite difficult without the recovery of several qualities preserved in the *B. maritima* germplasm (Biancardi et al. 2012) (Figs. 1.1 and 1.6).

Fig. 1.6 *Beta maritima* showing the narrow shape of the taproot and the plentiful development of leaves. Numerous secondary roots are emerging perpendicularly to the taproot (De Vilmorin 1923)

and yield showing fangy and woody roots, low root weight, and low sugar content. If numerous, the stalks cause difficulties during harvest. Since the bolting percentage is normally less than 0.1 % (100 beets per hectare), there are no significant consequences on harvest and sugar production. More problematic might be the pollen released by flowering beets and the viable seed shattered into the soil (Box 1.9).

Varieties differ in their response to vernalization and the genetics of bolting is of great interest (Pin et al. 2010; Saccomani et al. 2009; Leiva-Eriksson et al. 2014; Stevanato and Biscarini 2015). It was shown that additive dominance could explain the bolting resistance in crosses between susceptible and resistant inbred lines (Le Cochec et al. 1989). A high polymorphism in bolting trait was found within *B. maritima* germplasm. This has enabled earlier sowing, resulting in a prolonged growing season and in improved sugar yield (Westerdijk and Tick 1991). The use of molecular markers will likely facilitate the selection of more resistant varieties to be grown also as winter crops (Fig. 1.4).

1.3 Pests and Diseases

Sugar beet is subjected to a number of diseases, which cause more or less severe metabolic disorders, losses in sugar yield, and lowering of processing quality (Box 8.1). Pathogens do not distinguish among beet crops. Therefore, what is first discovered against the diseases of sugar beet due to its major economic importance may be subsequently transferred to leaf, garden, fodder beet, etc. (McGrath et al. 2007). An incomplete list of the more common pests and diseases affecting sugar beet is shown in Table 1.3.

Infection may start in any part of the plant and during all stages of development, from the growth of the seed on the seed bearers until harvest. Harvest (topping, lifting, transporting) and postharvest diseases may also occur (Campbell et al. 2008) (Box 8.2). The economic losses depend on a number of factors (Table 1.4). In the case of severe and poorly controlled diseases, the geography and acreage of cultivation areas have been modified. In California, the 40 % surface reduction in sugar beet crop between 1983 and 1995 and the related closure of sugar factories occurred mainly after the spread of rhizomania (Mahmood and Rush 1999). The same happened in Italy two decades previously (Lewellen and Biancardi 1990).

Chemical control commonly works well against fungi and insects, but the economic and environmental costs are continuously increasing, together with the threats for human health (Zimmermann and Zeddies 2000). In addition, the insurgence of resistant strains of pathogens has been detected to several pesticides. Due to increasing costs and difficulties in registration procedures, research into new agrochemicals is slowing down. The future pesticides should be more selective against one or more pathogens, possibly without any side effects for man, other organisms, and the environment. Adequately long-lasting crop rotations are effective in reducing a number of sugar beet diseases, but the availability of genetic resistances is often the only reliable possibility for protection. As with the use of agrochemicals, some resistance-breaking strains have been detected in diseases continuously limited in their development by the same source of genetic resistance.

Table 1.3 Diseases affecting sugar beets

Pests and diseases (common names)	Causal organisms (vector)	Diseased part (growing stage)	Diffusion (in particular)	Damages[a]	Control[b]	Efficacy[c]
Viruses (acronym)						
Beet curly top (BCTV)	Beet curly top virus *(Circulifer tenellus)*	Leaves	USA, Canada, Mexico, Turkey, Iran	***	GR, CT (vector)	**
Beet leaf curl (BLCV)	Beet leaf curl virus *(Piesma quadrata)*	Leaves	Central Europe	*	CT (vector)	*
Beet yellows (BYV)	Beet yellows virus *(Myzus persicae* and *Aphis fabae)*	Leaves	Widespread	**	CT (vector), PIC	**
Beet mild yellows (BMYV)	Beet mild yellows virus *(Myzus persicae)*	Leaves	Widespread	*	PIC (vector)	
Beet western yellows (BWYV)	Beet western yellows virus *(Myzus persicae)*	Leaves	Widespread	**	GR, AM, PIC (vector)	**
Beet chlorosis (BChV)	Beet chlorosis virus *(Myzus persicae)*	Leaves	Widespread			
Beet mosaic (BMV)	Beet mosaic virus *(Myzus persicae* and *Aphis fabae)*	Leaves	Widespread	*	GR, PIC (vector)	*
Beet soil-borne mosaic (BSBMV)	Beet soil-borne mosaic virus (*Polymyxa betae*)	Mainly root	USA	*	GR?	* ?
Rhizomania (BNYVV)	Beet necrotic yellow vein virus *(Polymyxa betae)*	Mainly root	Widespread, excluding some cool areas	***	GR	***
Beet soil-borne (BSBV)	Beet soil-borne virus *(Polymyxa betae)*	Root	Widespread	*?	GR?	* ?
Beet black scorch (BBSV)	Beet black scorch virus *(Olpidium brassicae)*	Root	Widespread	*	GR?	*?
Beet virus Q (BVQ)	Beet virus Q (*Polymyxa betae*)	Root	Europe	*	GR?	* ?

(continued)

Table 1.3 (continued)

Pests and diseases (common names)	Causal organisms (vector)	Diseased part (growing stage)	Diffusion (in particular)	Damages[a]	Control[b]	Efficacy[c]
Beet oak-leaf virus (BOLV)	Beet oak-leaf virus (*Polymyxa betae*?)	Root leaves	USA	*	GR?	* ?
Bacteria						
Bacterial vascular necrosis and rot	*Erwinia carotovora* ssp. *betavascularum*	Root	USA, Europe	°	GR, CR	**
Yellow wilt	*Rickettsia*-like organism *(Paratanus exitiosus)*	Leaves	Argentina, Chile	*	GR, CR	**
Bacterial leaf spot	*Pseudomonas syringae*	Leaves	Widespread	*	CR	*
Fungi						
Cercospora leaf spot	*Cercospora beticola*	Leaves	Widespread, except cool or cold areas	***	GR, CT, CR	**
Powdery mildew	*Erysiphe betae*	Leaves	Widespread	***	CT, GR	***
Downy mildew	*Peronospora schachtii*	Crown	Mostly in irrigated areas	*	GR, CR	**
Fusarium yellows	*Fusarium oxysporum*	Root	Widespread (China)	**	CR?, AM	*
Root rots	*Rhizoctonia solani*, *Pythium* spp., *Phoma betae*	Root	Widespread in humid soils	*	CR, PIC, AM	**
Damping-off	*Rhizoctonia s.*, *Pythium* spp., *Phoma b.*, *Aphanomyces c.*	Germinating seed	Widespread	**	CR, PIC	**
Southern sclerotium root rot	*Sclerotium rolfsii*	Root	Hot and dry areas	***	CR?	* ?
Black root or black leg	*Aphanomyces cochlioides*	Germinating seed	Widespread	*	CR. PIC	**
Rhizoctonia root and crown rot	*Rhizoctonia solani*	Root, crown	Widespread	**	CR, AM, AB	*
Phoma leaf spot	*Phoma betae*	Leaves, seed stalk	Widespread	*	CR, CT	**

(continued)

Table 1.3 (continued)

Pests and diseases (common names)	Causal organisms (vector)	Diseased part (growing stage)	Diffusion (in particular)	Damages[a]	Control[b]	Efficacy[c]
Alternaria leaf spot	*Alternaria alternata*	Leaves	Widespread in cold temperate areas	*	CT, PIC	***
Ramularia leaf spot	*Ramularia beticola*	Leaves	Widespread in cold temperate areas	*	CT	***
Beet rust	*Uromyces betae*	Leaves	Widespread	*	CT	***
Nematodes						
Cyst nematode	*Heterodera schachtii*	Root	Widespread	***	CR, GR	***
Root-knot nematode	*Meloidogyne* spp.	Root	Widespread	**	CR, GR?	**
Insects and mites						
Green peach aphid	*Myzus persicae*	Leaves	Widespread	**	CT, GR?, PIC	**
Black aphid	*Aphis fabae*	Leaves	Widespread	**	CT, GR?, PIC	**
Root aphid	*Pemphigus betae*	Roots	Widespread (USA)	**	CR, GR?	*
Spinach leaf miner	*Pegomya hyoscyami*	Leaves	Widespread	*	CT	**
Mangold flea beetle	*Chaetocnema concinna*	Leaves (plantlet)	Widespread	**	CT, PIC	**
Pigmy mangold beetle	*Atomaria linearis*	Leaves (plantlet)	Widespread	*	CT, PIC	**
Tortoise beetle	*Cassida vittata*	Leaves	Widespread	*	CT	**
Sugar beet armyworm	*Mamestra brassicae*	Leaves, crown	Widespread	*	CT	**
Sugar beet root maggot	*Tetranops myopaeformis*	Root, crown	Widespread	*	CT, CR, AM, GR?	*
Flea beetles	*Chaetocnema tibialis*	Leaves, crown	Widespread	*	CT, GR?	**
Spider mites	*Tetranychus urticae*	Leaves	Widespread	*	CT, GR?	*

[a]Damages: *generally low (locally severe); **medium; *** generally severe

[b]Control by means of CT chemical treatments, CR crop rotation, GR genetic resistance, PIC pellet incorporated chemicals, GR? genetic resistance not yet released, SS seedling stage, AM agronomic measures, AB antagonistic bacteria

[c]Efficacy: * low; ** medium; ***good; ? = under evaluation

Table 1.4 Ranking of sugar beet diseases in terms of economic losses. The ranking in the top positions matches similar evaluations made by Richard-Molard and Cariolle (2001)

1.	Beet necrotic yellow vein virus (BNYVV) + *Polymyxa betae* (rhizomania)
2.	*Cercospora beticola* (cercospora leaf spot)
3.	*Heterodera schachtii* (cyst nematode), *Meloidogyne* spp. (root-knot nematode)
4.	*Rhizoctonia solani*, *Pythium* spp., *Phoma betae*, *Aphanomyces cochlioides* (damping-off, root rots)
5.	*Sclerotium rolfsii* (southern sclerotium root rot), *Fusarium oxysporum* (fusarium yellows)
6.	Beet curly top virus (BCTV), beet yellows virus (BYV), beet mosaic virus (BMV)
7.	*Aphis fabae* (black aphis), *Myzus persicae* (green aphis), *Pemphigus betae* (root or gray aphis)
8.	*Peronospora solani* (downy mildew), *Erysiphe betae* (powdery mildew)
9.	*Tetranops myopaeformis*, *Lixus junci*, *Temnorhinus mendicus* (root maggot)
10.	*Alternaria alternata* (alternaria leaf spot), *Ramularia beticola* (ramularia leaf spot)

1.4 Rhizomania and Economic Damages

Rhizomania is ranked as the most damaging disease in sugar beet (Scholten and Lange 2000). This is due to its still continuous diffusion in almost all sugar beet-producing countries (McGrann et al. 2009), to the very long persistence in the soil, and to the potential damage that can reduce sugar beet yield by 80 % or more (Fig. 1.6) as compared to healthy conditions (Peltier et al. 2008). In trials in diseased and rhizomania-free fields, there was a 24 % reduction in leaf area index (LAI) with attendant 57 % loss in dry root matter (Rezaei et al. 2014). In Germany, the estimated 15 % reduction in sugar yield caused an economic loss of around 5.5 M$ (Schäufele 1983). In Austria, cultivation of rhizomania susceptible varieties in 1981, 1982, and 1983 was estimated to result in losses between 1.5 and 3.5 M$ (Graf 1984). If resistant varieties have been sown, the losses could have been reduced by 0.3–1.5 M$, respectively.

An attempt has been made to roughly quantify the worldwide cost of losses caused by rhizomania. It was assumed that at least half of the crop is more or less affected by the disease. The use of resistant varieties reduces the potential losses significantly, but not completely. It is also important to consider:

- The frequent occurrence of unforeseen disease after sowing
- The reducing but still existing gap between resistant and susceptible varieties sown in healthy soil
- The production gap between healthy fields sown with normal varieties and rhizomania-diseased field cultivated with resistant varieties
- The reluctance of farmers to change from susceptible varieties
- The increased losses during harvest
- The losses in sugar content and the reduced processing quality caused by the disease

Major expenses for the factories are caused by the wider areas from which beets are supplied, because the closer fields are more likely to be diseased by short rotations. Where prolonged root storage is possible (up to 200 days in some cold areas such as Minnesota and North Dakota), susceptible varieties affected by rhizomania are subject to greater respiration rate and loss of sucrose, which means lower sugar content and extraction rates. This happens in presence of very low rhizomania infections not detected by the analysis routinely made before the beet download to the factory. Resistant varieties minimize these losses (Campbell et al. 2008; Strausbaugh et al. 2009). Besides, for sugar industry, it is important to take into account:

- The treatment or the separate disposal of infected tare soil
- The costs of the quarantine measures
- The employment of traditional varieties advised in supposed rhizomania-free conditions (Francis and Luterbacher 2003)

It must be remembered that the seed of rhizomania-resistant varieties is almost everywhere more expensive (around 5 %) than the susceptible varieties. In the advent of GM resistance, seed cost will involve an expensive technology fee. The overall damage resulting from the factors listed above can be estimated as equivalent to a loss of 10 % in world beet sugar production (around 36 Mt a year. Source: http://www.sucden.com/statistics/1_world-sugar-production) corresponding to about 3.6 Mt loss. The economic value of the damages may be roughly quantified at 1,300 M$ yearly (world price of white sugar 380 $ t^{-1}. Source: http://www.investing.com/commodities/london-sugar-technical).

Since the discovery of the rhizomania causal agents, many attempts have been made to limit the disease with soil fungicides (D'Ambra and Mutto 1975) or fumigant treatments (Bongiovanni 1965; Alghisi and D'Ambra 1966), but these means were ineffectual or not economical and/or ecologically adequate. In addition, prolonged rotation cycles for sugar beet crops are inactive in controlling the disease, since the *P. betae* viruliferous resting spores remain viable in the soil for decades (Pferdmenges 2007). Thus, once infected by BNYVV, the field could be considered almost permanently infected (Asher 1993). In practice, the only way to reduce the incidence of the disease is possible by means of genetic resistances, firstly identified in some Italian sugar beet genotypes (see Sect. 9.2). However, these resistant beets are subjected to virus infection, although they exhibit moderate rootlet proliferation and limited damages.

Ranking sugar beet diseases from their economic damage potential is shown in Table 1.4. This ranking takes into account the respective world distribution, damage, and variability at least in Europe, the USA, China, and Japan. The ranking in the top positions matches similar evaluations made by Richard-Molard and Cariolle (2001).

The future of sugar beet has never been easy. In addition to eruptive pests, diseases, resistance-breaking strains in BNYVV, etc., there are complex and serious problems to be considered and solved:

- The increasing competition with sugar cane
- The improvement of the crop sustainability
- The effects of climate change

As always, continuous attention and research will be required.

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Chapter 2
History and Current Status

Antonio Canova, Luciano Giunchedi, and Enrico Biancardi

Abstract Research into the etiology of the syndrome later called "rizomania" and measures to limit the considerable reductions in sugar yield required a large number of studies in different disciplines. The traditional methods for reducing the damages and the spread of the disease resulted without any effect or were too expensive. Since some degrees of genetic variability were soon observed in commercial varieties, the search of genetic resistance appeared the sole possibility of success. At this stage, the unofficial collaboration among universities and research institutes proved instrumental. Around 20 years after the first observations, it was discovered that the syndrome was caused by a virus, transmitted by the plasmodiophoral protist *Polymyxa betae*. The identification of the causal agents leads to considerable progress toward genetically resistant varieties and a satisfactory control of the economic consequences of rhizomania. Also in this case, the mentioned collaborations turned out decisive.

Keywords Sugar beet • Rhizomania • BNYVV • *Polymyxa betae* • History • Genetic resistances

Around 1950, a strong reduction in sugar content was reported in sugar beet fields located near some volcanic hills among the provinces of Padua, Vicenza, and Rovigo (Italy), especially in crops grown in fields poorly cultivated or with compact soils, subjected to waterlogging, and after a too short sugar beet rotation (Donà Dalle Rose 1954, 1956). This was probably the earliest written documentation of the disease, firstly named "low sugar content syndrome" (LSCS) or "soil sickness" (Fig. 2.1). To the best of our knowledge, the first photo of LSCS diseased beets is reproduced in Fig. 2.2.

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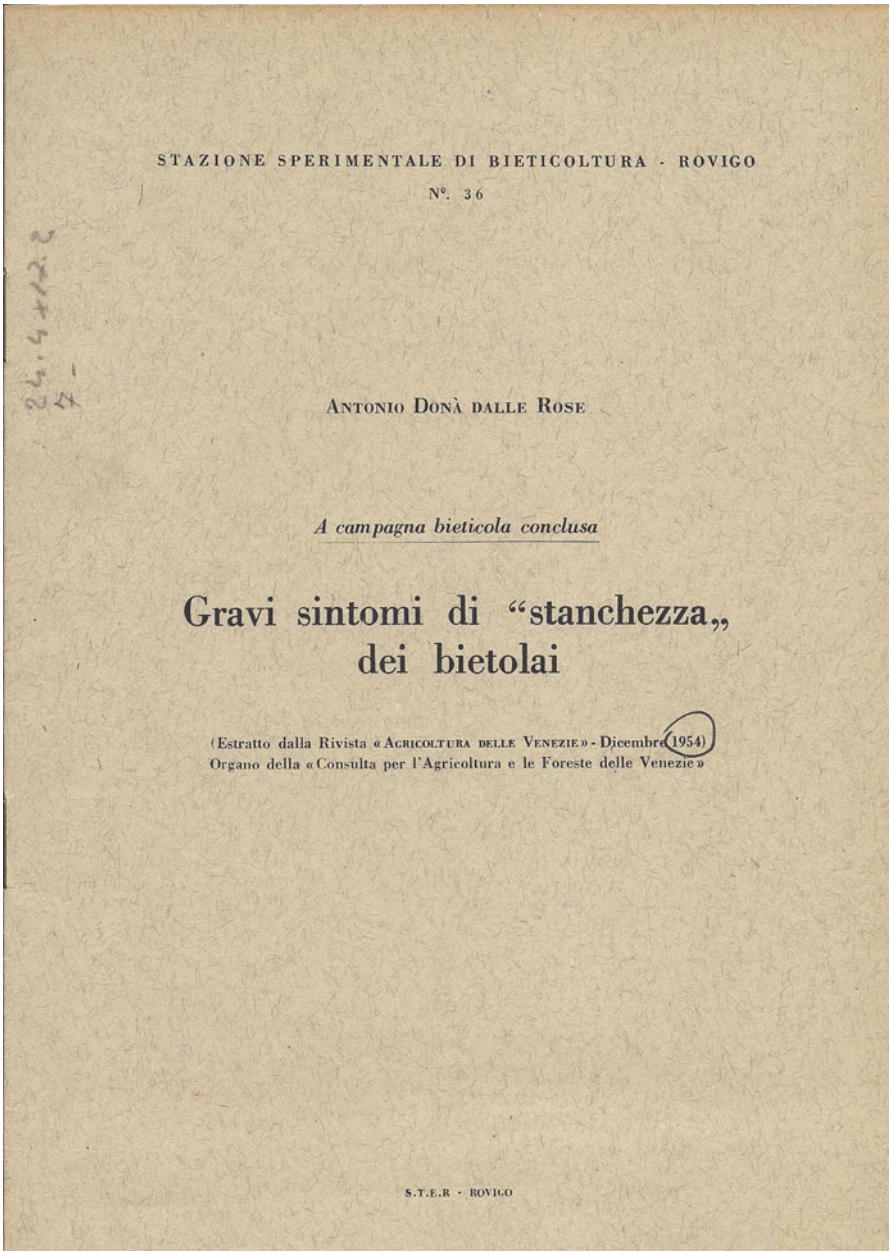
E. Biancardi, T. Tamada (eds.), *Rhizomania*, DOI 10.1007/978-3-319-30678-0_2


STAZIONE SPERIMENTALE DI BIETICOLTURA - ROVIGO

N°. 36

ANTONIO DONÀ DALLE ROSE

A campagna bieticola conclusa

Gravi sintomi di "stanchezza„ dei bietolai

(Estratto dalla Rivista «AGRICOLTURA DELLE VENEZIE» - Dicembre 1954)
Organo della «Consulta per l'Agricoltura e le Foreste delle Venezie»

S.T.E.R. - ROVIGO

Fig. 2.1 Cover of the first paper describing some symptoms of the new disease, which was named "low sugar content syndrome" (Donà Dalle Rose 1954)

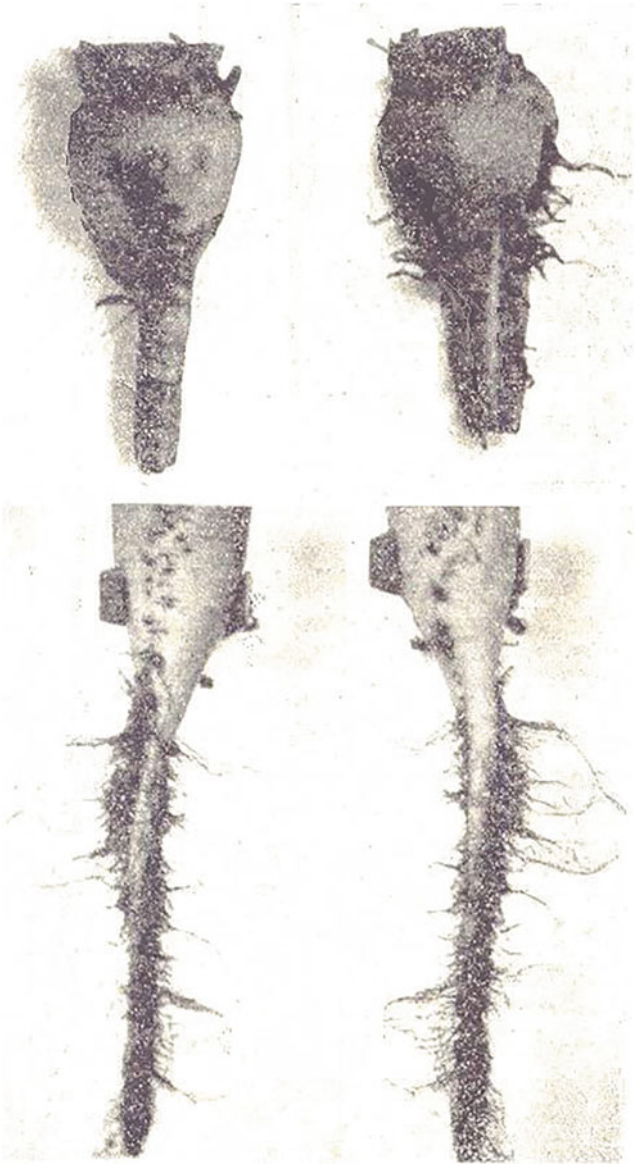


Fig. 2.2 The above beets, showing the typical wineglass shape, are surely diseased by rhizomania. The abnormal rooting of the below ones may be caused by cyst nematode. The picture was taken in 1951 by Donà dalle Rose and should be, at the best of our knowledge, the first one regarding the new disease

2.1 First Observations and Investigations

The unknown disease was also linked to the flood in 1951, when most of the lower Po Valley was submerged for several days (Koch 1976). The structure of the clay soils was modified for years, giving rise to the development of some latent soil-borne pathogens like beet cyst nematode *Heterodera schachtii* (Koch 1976). A few years after the first report, LSCS was associated with significant losses in root yield and severe lateral rootlet proliferation above all evident after rainy seasons (Piolanti et al. 1957). The real damage of LSCS on sugar production was quantified in three field trials planted in 1957 (Table 2.1). The spread of the disease soon appeared very rapid in neighboring areas.

According to the first preliminary investigation organized by the Italian Sugar Beet Growers Association (ANB), the unknown disease was moderately diffused in the northern part of the Po Valley (Bongiovanni 1960). A second more extensive survey, conducted 4 years later, provided worrying data. In fact, LSCS was recorded on about 10,000 ha, which was 5 % of the domestic sugar beet acreage (Bongiovanni 1964). Considering traditional crop rotations of 3–4 years, this meant that at least double or triple the surface area was potentially contaminated. Furthermore, most of the affected fields were located in the eastern Po Valley, the areas with the most long-standing sugar beet cultivation and the highest Italian concentration of sugar factories (Fig. 2.3). A lower intensity of disease was detected in the provinces of Ravenna and Bologna (Northern Italy) and Ancona (Central Italy) (Bongiovanni 1964; Bongiovanni and Lanzoni 1964).

The rapid spread of the infection was certainly due mainly to the practice of returning the soil residues from sugar factories to the farms. In many cases, the first appearance of LSCS in the fields was clearly linked to those patches where the tare soil was discharged. From these small infected areas, the disease spread throughout the farm with the physical movement of contaminated soil carried mainly by machinery.

Table 2.1 Yield differences observed in 1957 between healthy and diseased fields in the province of Padua, Italy (Bongiovanni 1964). Modified

Locality	Field	Root yield (t/ha)	Sugar content (%)	Sugar yield (t/ha)
Sant'Urbano	Healthy	3.97	12.05	4.78
	Diseased	1.24	6.69	0.86
	LSD ≤ 0.01	*1.77*	*2.24*	*2.77*
Vighizzolo	Healthy	3.97	11.30	4.48
	Diseased	2.02	10.18	2.06
	LSD ≤ 0.01	*1.40*	*0.98*	*2.33*
Battaglia Terme	Healthy	5.35	12.26	6.56
	Diseased	1.93	9.06	1.75
	LSD ≤ 0.01	*8.50*	*0.76*	*0.95*

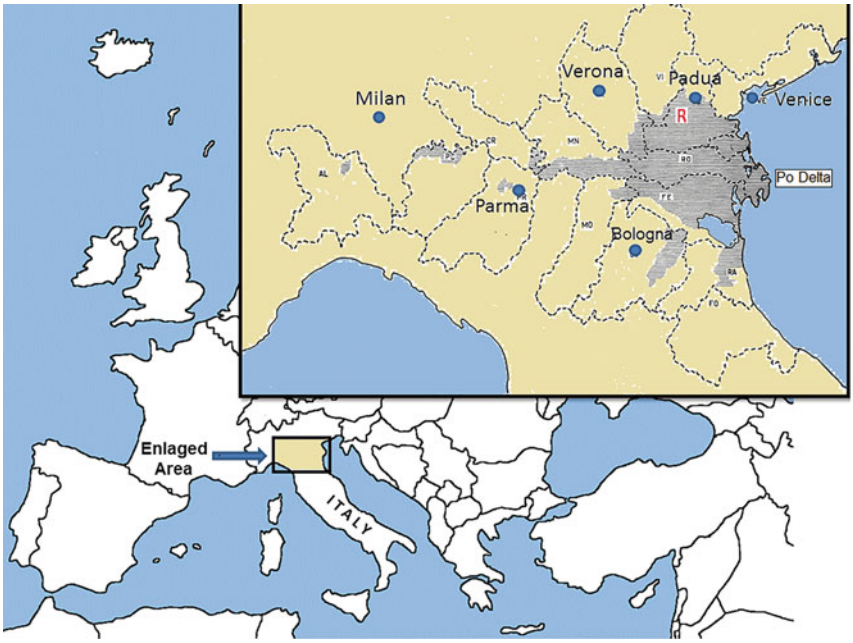


Fig. 2.3 Diffusion of LSCS (rhizomania) in the provinces of the Po Valley in 1963 (shaded area). The provinces of Rovigo (RO) and Ferrara (FE) are almost completely included in the diseased area (Bongiovanni 1964) (Modified). R = first localization of the disease

In subsequent years, the cyst nematode *H. schachtii* and various soil-borne fungi were considered as possible causal agents, forgetting that in the former case, the typical cysts among the rootlets are very evident. However, since some early symptoms of LSCS may easily be confused with those caused by *H. schachtii*, Bongiovanni (1960) explained the main differences at the different stages of crop development (Table 2.2).

It was observed that in the presence of cyst nematode, sugar content is slightly lower than normal, as is alpha-amino N, while potassium and sodium are quite normal. The main damage is due to root yield reduction. In the case of LSCS, the quality pattern is completely different, with a striking drop in sugar content and increase in sodium. The potassium concentration remains around the normal values, whereas alpha-amino N decreases.

Canova (1959) described the symptoms of LSCS more precisely, confirming that the anomalies had been observed for a long time in North Italy, especially in soils with water drainage problems. Based on the abnormal rootlet production, he proposed the name "rizomania" (root madness: rootlets development in abnormal parts of the plant) which, since then, has been adopted in its English version "rhizomania" (Box 2.1). It should be pointed out that, at the time, the infective etiology of the disease was still unknown.

Table 2.2 Differences of symptoms between cyst nematode and the still unknown LSCS (Bongiovanni 1960). Modified

Traits	Beet cyst nematode (*Heterodera schachtii*)	Rizomania (formerly LSCS)
Look of the crop in field condition	Diseased plants with reduced canopy distributed in patches or rows. At the border, very diseased and healthy plants are close	More even distribution. Gradual reduction of symptoms in the border between diseased and healthy soil
Leaf color	Green as normal, sometimes darker in the central rosette	Yellowing is quite frequent; more rarely chlorotic spots and vein discoloration develop on the leaves
Canopy	Leaves less developed often wilting in the hotter hours and/or with low water supply	Wilting of the leaves in the hotter hours with low water supply. Elongated petioles
Shape of taproot	Quite normal	Sometimes normal in case of late infection, frequently wineglass shaped or strangled
Secondary roots	Not frequent	Frequent
Rootlets	Long, braided, but quite separated. Presence in the second half of the campaign of little pearl-white ovoidal cysts of the nematode visible to the naked eye	Shorter, not branched, but more strictly braided forming a sort of beard. Some new rootlet along the taproot. Bearding is musty smelling and firstly developing on the grooves
Root weight	Always less than normal	Quite normal in case of late infection. Very low in the presence of severe infection
Root analyses	Sugar content slightly lower. Quite normal-quality parameters except α-amino N, which is lower than normal	Very low sugar content. High Na and K, low α-amino N contents

In heavily contaminated fields, LSCS symptoms appeared soon after beet singling, firstly with upright and poor development of leaves, light green in color and subject to wilting in the hotter hours. Occasionally, the leaves showed typical bright yellow spots along the veins or yellowing veins (Bongiovanni 1964) (Fig. 2.4). Root symptoms consisted of a continuous production of rootlets, at first along the grooves (Figs. 1.2 and 2.7b) and then over the entire distal part of the taproot, secondary roots, and tail. In case of *H. schachtii*, the development of rootlets is more limited.

The excess production of rootlets originated from characteristic surface tumor-like nodules on the taproot, which could be seen clearly on cross- and longitudinal root cuttings. Other symptoms included the browning of the vascular system, severe constriction of the taproot, and necrosis at the tail (Figs. 2.7a and 2.7b). In the advanced stages of the disease, the growth of the taproot was severely limited, and sometimes the entire root became necrotic, brown, and rotten. The impressive effects of the disease compared with a healthy beet are shown in Fig. 2.8 (Bongiovanni 1965). In the case of late infections in Italian conditions, rootlet proliferation was limited to the distal end of the tap and secondary roots, but here again beets displayed

Box 2.1: *Rizomanìa* or Rhizomània (From Biancardi et al. 2002)
The word "rhizomània" is composed by the Latin parts "*rhizo*" and "*manĭa*", in their turn derived from the Greek "*ρίζα*" and "*μανία*" meaning, respectively, "root (radical)" and "abnormal trend (madness)." The original sense of the composite word refers to an atypical and/or pathological development of roots and rootlets (Lindley and Moore 1866) also in improper position (Canova 1980). The disorder, in terms of intensity and location on the plant, may arise from non-biotic causes or due to attacks from viruses, phytoplasmas (mycoplasms), fungi, bacteria, and nematodes (Canova 1959, 1980). In the first paper the author, referring to an outbreak on sugar beet roots, chose the Italian word "*rizomanìa*" and then widely used it in plant pathology (together with its synonyms in other languages). As far as beets are concerned, the first use of the word "*rizomanìa*" seems to have been by Munerati et al. (1913) to describe three sea beets, one of which displaying an excessive proliferation of the rootlets (Munerati and Zapparoli 1915). On the basis of the illustration reported in the paper (Fig. 2.5), it seems unlikely that the visible symptoms were caused by the disease in today's meaning. Previous use of the word "rhizomania" in other plants (vine, ivy, fig, common laurel, etc.) is reported by Lindley and Moore's *Treasury of Botany* (1866) (Figs. 2.6a and 2.6b). The word including h (like the versions in English and French) is formally correct given its Latin origin. Today, the word "*rizomanìa*" survives in papers written in Italian, German, Spanish, Greek, Portuguese, etc. Some additional explanation on the terms currently used in plant pathology is given in Appendix Fig. A1 and Fig. A2.

Fig. 2.4 Leaf with the typical symptoms of systemic infection of rhizomania. The chlorotic spots became necrotic after some days (Courtesy, Ghedini 2015)

a conspicuous reduction in sugar content, which was also in relation to the leaf regrowth in late August–September (Bongiovanni 1964).

Yield differences between varieties considered to have some degrees of resistance and a normal one (Saros) were reported by Bongiovanni and Lanzoni (1964)

Fig. 2.5 Root of *Beta maritima* with abnormal development of rootlets caused by unknown agents and named "rizomania" by Munerati and Zapparoli (1915)

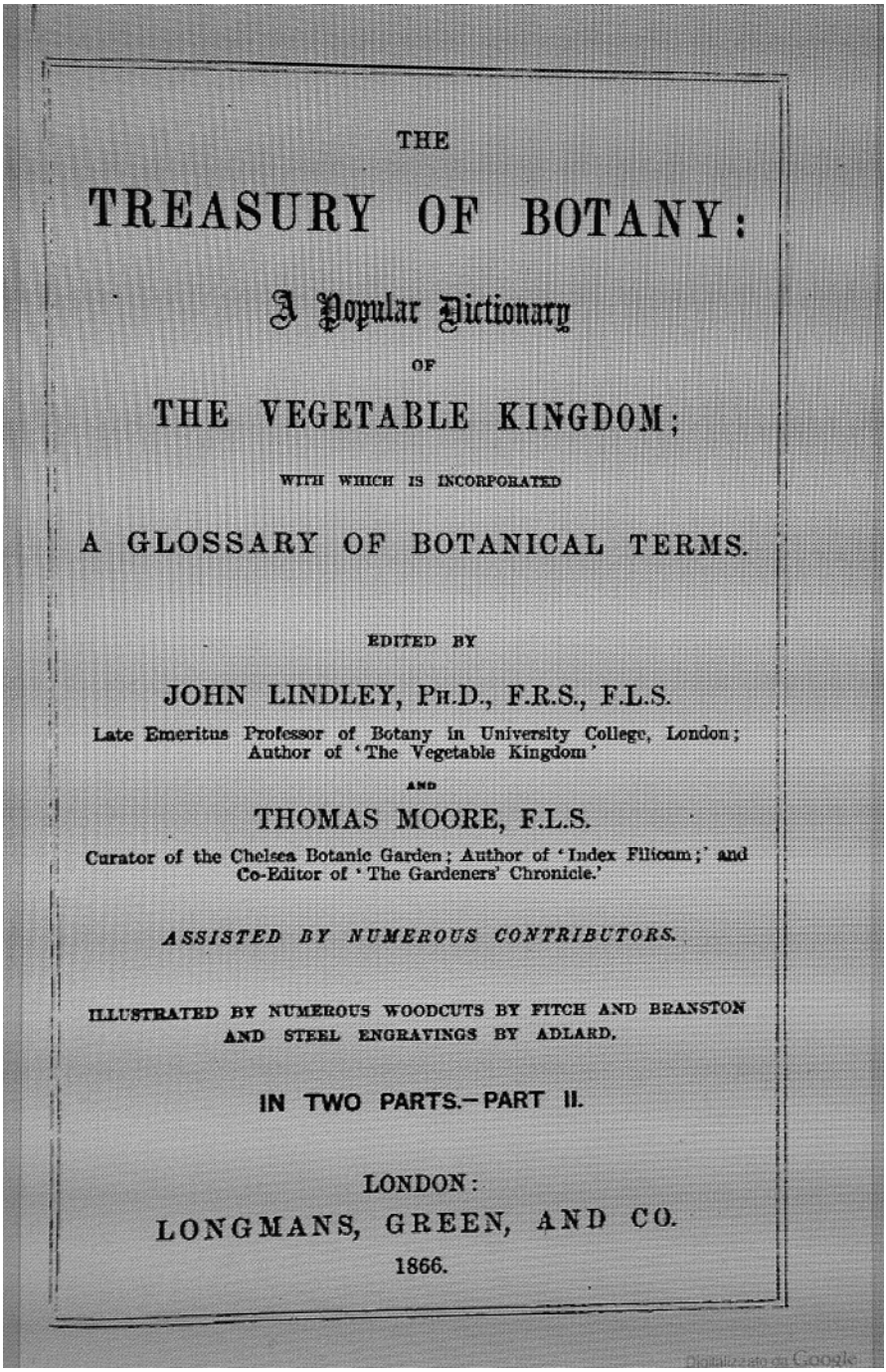
(Table 2.3). In the late sampling, the "NZ" and "Z" varieties endowed with cercospora leaf spot (CLS) resistance appeared to be slightly better than the "N" and "E," as later confirmed by Magro et al. (1975) and by Rosso and Bimbatti (1985) (Box 2.2).

2.2 Etiology

In 1962, the easy diffusion of LSCS through infected soil was confirmed (Figs. 2.9a and 2.9b), as the biotic etiology of the disease through field and glasshouse experiments (Bongiovanni 1964). Other experiences made it possible to ascertain:

- The absence of symptoms in beets grown in infected soil after sterilization at 135 °C (Alghisi et al. 1964)
- The lower incidence of the disease in fields treated with methyl bromide and D-D soil fumigants (Alghisi et al. 1964; Bongiovanni 1964)
- That excess water had a moderate effect on the disease development (Alghisi et al. 1964; Bongiovanni 1965)

On the basis of these observations, the ANB suggested some measures to prevent the spreading of LSCS (Bongiovanni 1964):



THE

TREASURY OF BOTANY:

A Popular Dictionary

OF

THE VEGETABLE KINGDOM;

WITH WHICH IS INCORPORATED

A GLOSSARY OF BOTANICAL TERMS.

EDITED BY

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AND

THOMAS MOORE, F.L.S.

Curator of the Chelsea Botanic Garden; Author of 'Index Filicum;' and Co-Editor of 'The Gardeners' Chronicle.'

ASSISTED BY NUMEROUS CONTRIBUTORS.

ILLUSTRATED BY NUMEROUS WOODCUTS BY FITCH AND BRANSTON AND STEEL ENGRAVINGS BY ADLARD.

IN TWO PARTS.—PART II.

LONDON:

LONGMANS, GREEN, AND CO.

1866.

Fig. 2.6a Front page of *Treasury of Botany* (Lindley and Moore 1866)

togamic Botany, p. 514. [M. J. B.]

RHIZOMANIA. An unnatural development of roots. Many plants, as ivy, screw-pines, figs, &c., without any indication of disease whatever, send out roots from various parts in the same way as trees so commonly produce adventitious buds. In the common garden fig, wherever it is nailed to the wall, roots are sent out within the band which surrounds the stem. Their development, however, as in the vine and common laurel, usually indicates something wrong about the ordinary roots, in consequence of which sufficient moisture is not supplied to the stems and leaves, and rootlets are thrown out from the stem in search of it. In the laurel this generally ends in death. Roots again are frequently developed in little bundles on the stems of apple-trees; and as they retain moisture amongst them, and their tips in process of time decay, decomposition is set up, extending inwards till canker is produced. [M. J. B.]

RHIZOMORPHA. A spurious genus of *Fungi*, comprising a great number of root-

Fig. 2.6b Description of rhizomania in its botanical and pathological meaning. Sugar beet is not included in the list of the affected species (Lindley and Moore 1866)

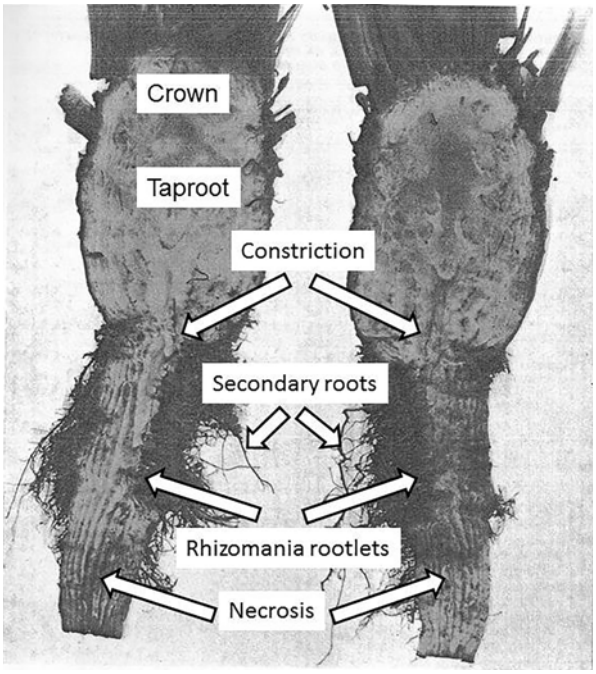

Fig. 2.7a The longitudinal section of beet severely diseased by rhizomania shows the browning of the vascular vessels and the disordered growth of the tissues, causing the restriction of the taproot, which takes the typical wineglass shape (Alghisi et al. 1964)

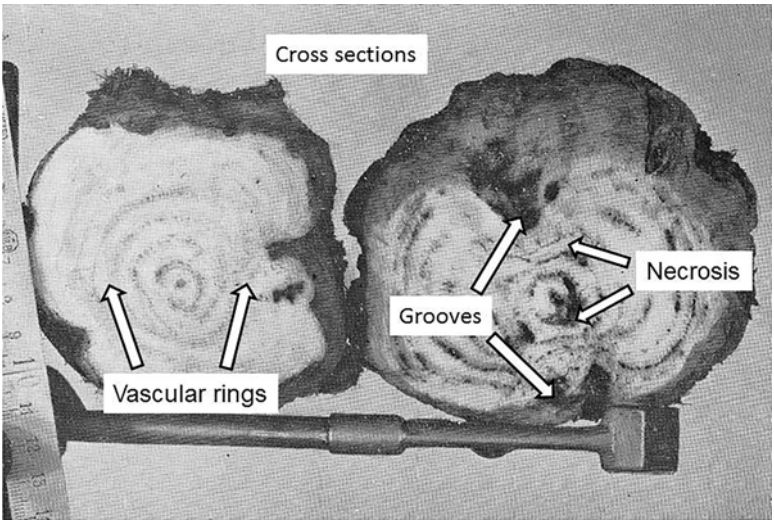

Fig. 2.7b Cross section of beets differently diseased by rhizomania. The necrosis of the vascular rings in the distal parts of the taproot is the first evident symptom of the disease (Donà Dalle Rose 1956)

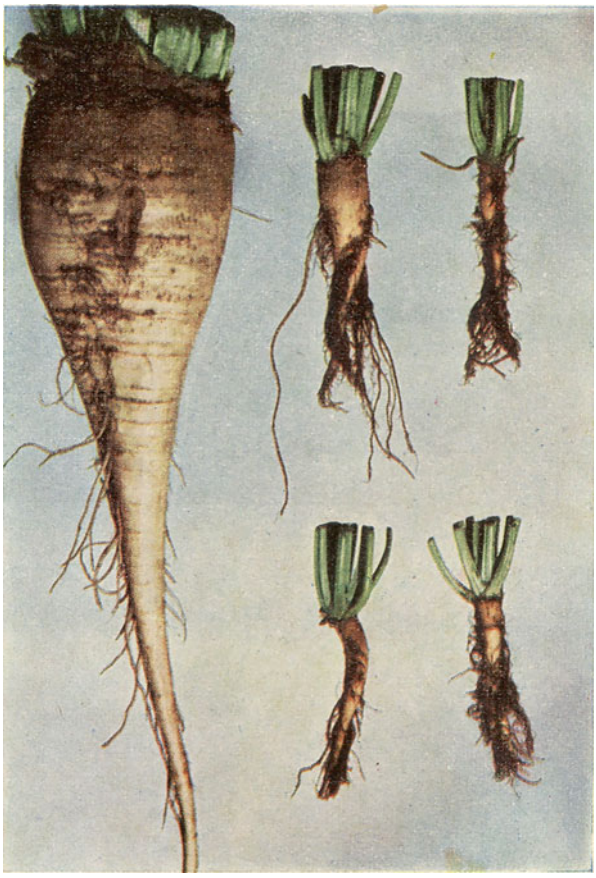

Fig. 2.8 Beets harvested in different parts of the same field at Adria, Italy. The remarkable differences are only due to absence (left) or presence of LSCS (Bongiovanni 1964)

Table 2.3 Trials sown in diseased field the year before and for this reason showing very low sugar yield. It was recognized for the first time the existence of some genetic variability among the commercial varieties (Bongiovanni and Lanzoni 1964). Modified

		Early Harvest			Late Harvest		
Locality (province)	Variety	Roots (t/ha)	Sugar content (%)	Sugar yield (t/ha)	Roots (t/ha)	Sugar content (%)	Sugar yield (t/ha)
Cona (Venice)	Alba P[a]	2.07	9,24	0.19	2.50	6,34	0.16
	Buszczynski CLR[b]	1.68	9.98	0.16	2.00	6,86	0.13
Cavarzere (Venice)	Alba P	1.65	6,60	0.11	2.68	6,42	0.17
	Saros H9N[c]	0.74	5,06	0.03	0.92	4,06	0.03
Vighizzolo (Padua)	Alba P	1.69	8,12	0.13	1.33	5,28	0.07
	Mezzano NP[d]	1.48	10,30	0.15	1.68	7,64	0.13

[a]Italian NZ- and CLS-resistant variety
[b]Polish Z- and CLS-resistant variety
[c]Hungarian variety with unknown traits
[d]Italian N-NZ- and CLS-resistant variety

Box 2.2: E, N, and Z Varieties

Root yield per hectare and sugar content are the more important data for farmers. There is a high negative correlation between the two values. Traditionally, varieties are classified according to their ability to produce relatively more root weight and low sugar content or vice versa. The former are named "E" (Ertrag = yield in German), the latter "Z" (Zucker = sugar). The varieties with intermediate traits are called "N" (normal). This simple classification was subsequently refined with additional types (EE, E, NE, N, NZ, Z, ZZ). The different types suit particular environmental situations and cultivation requirements. Usually the Z varieties are slightly more resistant to some diseases (CLS, root rot, etc.), have better processing quality, and are harvested late in the season, and their transport per sugar weight unit costs less. For the latter reason, sugar beet processors prefer the NZ-Z varieties even if they yield slightly less than the E type in most cultivation areas.

- Avoid waterlogging.
- Sow the beets early and only in the better and not formerly diseased fields.
- Use Italian varieties with high sugar content.
- Harvest early in diseased fields.
- Avoid the spreading of the disease through contaminated soil adhering to farm machinery and coming from sugar factories.
- In the event of worsening epidemics, sugar beet should be postponed at least until the discovery of the disease agent.

Fig. 2.9a Healthy plot inoculated with infected soil in August 1956 (Bongiovanni 1964)

Fig. 2.9b The same plot in June 1957, showing the typical symptoms of the disease later called "rizomania" (Bongiovanni 1964)

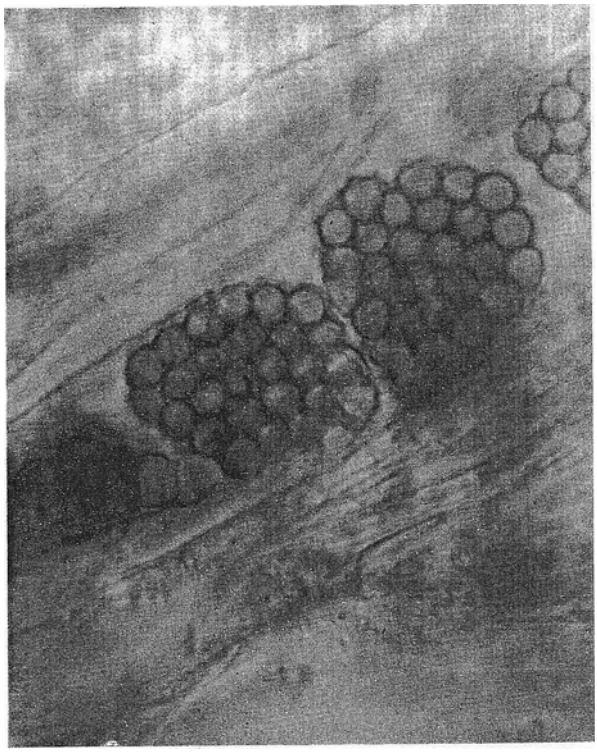

Fig. 2.10 Cystosori of *P. betae* at 1300× (Ghillini et al. 1965)

During the same period, the soil-borne fungus *Polymyxa betae* Keskin, at the time considered a fungus and now classified in the *Protozoa* kingdom, was detected for the first time in Germany in healthy sugar beet roots (Keskin et al. 1962; Keskin 1964). *Polymyxa betae* (Fig. 2.10) was also consistently found in Italy in beets naturally and artificially infected by LSCS (Ghillini et al. 1965; D'Ambra and Keskin 1966). At the same time, two viruses were also found in the diseased roots, which, in the presence of *P. betae*, induced overall symptoms very similar to those of LSCS (Canova 1966). This was the first indication that the disease could be due to some interaction or association between virus and fungus (Canova 1966). Conversely, *P. betae* alone only caused a minor development of rootlets, which remained functional for a longer time (Canova 1966).

It should be pointed out that around the same period, an association was demonstrated between *Polymyxa graminis*, a species morphologically identical to *P. betae*, and a wheat virus disease caused by soil-borne wheat mosaic virus. This disease was common in Italy, Japan, and the USA and had epidemiological properties analogous to those attributed to LSCS (Canova 1966; Estes and Brakke 1966). The LSCS was named "rizomania" as earlier proposed (Canova 1959) (Box 2.1).

Some years later, abnormal sugar beet growth was reported in Japan in fields and experimental plots where sugar beet was cropped as monoculture (Masuda et al. 1969). In a few years, the disease spread rapidly to the entire Hokkaido district as a

consequence of transplanting beets in paper pots sometimes filled with soil recovered from sugar factories. In the region, the use of paper pots in spring is effective to lengthen the vegetative cycle of sugar beet in colder climate.

Tamada et al. (1971) observed the constant presence of *P. betae* and a rod-shaped virus in the diseased beets. They produced specific rhizomania symptoms by inoculating young sugar beets with the virus alone, thus confirming the viral etiology of the disease (Tamada 1975; Fujisawa and Sugimoto 1976), while *P. betae* was considered the probable vector. The virus was named beet necrotic yellow vein virus (BNYVV), because of the vein yellowing and necrosis that occasionally appeared on sugar beet leaves when the virus became systemic (Fig. 2.4) (Tamada and Baba 1973). In Italy, around the same time, the disease was associated with a rod-shaped virus similar to BNYVV, presumably vectored by *P. betae* (Faccioli and Giunchedi 1974).

Furthermore, the demonstration that *P. betae* was the vector of BNYVV was obtained with both resting spores and zoospores in the absence of other root parasitic fungi (Giunchedi and Langemberg 1982). Other studies established that the virus is maintained within the zoospores and resting spores of *P. betae* (Abe and Tamada 1986; Fujisawa and Sugimoto 1976; Rysanek et al. 1992; Tamada 1975) and potentially by 10–15 % of the latter (Tuitert 1990) (Fig. 6.1). BNYVV is now considered to be the vector of other viruses infecting sugar beet such as beet soil-borne virus (BSBV), beet soil-borne mosaic virus (BSBMV), (BSBV), beet soil-borne mosaic virus (BSBMV), beet oak-leaf virus (BOLV), and beet virus Q (BVQ). Based on its host range, *P. betae* can be divided into three main *formae speciales*: f.s. *amaranthi*, f.s. *betae*, and f.s. *portulacaceae* (Abe and Ui 1986; Barr and Asher 1992).

2.3 Histological Observations

At histological level, the alterations in rhizomania-affected roots consist of lateral root necrosis at the point of origin in the peripheral growth ring. This may be triggered by necrosis of the growing region where *P. betae* zoospores penetrate and BNYVV infection mainly occurs. Lateral root necrosis stimulates the production of new lateral roots, normally not connected to the taproot vessel system. This is followed by regression of affected cortical cells to tumorlike disorganized meristematic tissue, with few vascular elements and smaller than normal cells with thin walls, which differentiate to root primordia with a marked rhizogenic activity. In susceptible varieties, the areas of cell proliferation, although partially isolated from normal tissues by a layer of phellogen, may expand deep into the cortex to the central cylinder, with consequent interruption of several growth rings and stop of radial growth (D'Ambra et al. 1972; Poggi Pollini and Giunchedi 1989). In the rootlets, the virus particles are present in the xylematic tissues and a few epidermal cells in contact with plasmodia, zoosporangia, and *P. betae* cystosori, while in the taproot, they are limited to a few xylem vessels (Giunchedi and Poggi Pollini 1988; Dubois et al. 1994). These histopathological alterations may explain some symptoms of the

disease. Thus, the constriction of the taproot at the point of severe rootlet proliferation may be the consequence of growth ring interruption. Similarly, leaf wilting during the warmest hours of the day may be mainly related to the absence of connections between the newly formed rootlets and the root vessel system, while the low sugar content may be the result of the high-energy requirements of the meristematic tissue due to its rhizogenic activities (D'Ambra et al. 1972). Analyses of root tissues have shown a strong increase in free indole-3-acetic acid in rhizomania-affected tissues, as compared to unaffected tissue, which may be directly or indirectly responsible for beet reactions to the disease (Poggi Pollini et al. 1990). Other analyses have shown an induction of phytohormones, such as abscisic acid and auxin, which may be involved in the abnormal rootlet production (Larson et al. 2008).

2.4 Spreading of the Disease

Following Italy and Japan, rhizomania was detected in Eastern France (Putz and Vuittenez 1974), West Germany (Hamdorf et al. 1977), and Greece (Kouyeas 1979), and it has since been reported in most European sugar beet-growing areas. In 1978, it was first reported in China (Gao et al. 1983; Li et al. 2008) and in California in 1983, where, 6 years later, the disease had spread over 35,000 ha (Duffus et al. 1984; Harveson et al. 1996). The chronology of first reports is given in Table 2.4 (Asher 1993; Rush et al. 2006; McGrann et al. 2009).

The sudden outbreak of rhizomania across the main sugar beet-growing areas of Europe and other more distant countries, in the absence of contact with areas already infected, may imply that the BNYVV-*P. betae* complex was already present several years before its first detection. It can be noted that at such a low level, the disease was not economically relevant and passed unobserved until a change in some epidemiological factor increased the severity and damages. This observation may be supported by the evidence of the worldwide diffusion of non-viruliferous *P. betae* in all cultivated soils (Gerik and Duffus 1988; Scholten and Lange 2000) and also by the presence of BNYVV-carrying *P. betae* in soils where sugar beet was never cropped (Chiba et al. 2011). For instance, BNYVV was first reported in the USA on soil samples from beneath the canopy of sweet cherry trees in Washington State (Al Musa and Mink 1981). Furthermore, Asher (1999) observed that the disease agent inoculum required two to three sugar beet crops before discovery and that it might be enhanced by some recently introduced cultivation techniques, such as irrigation. In addition, Heijbroek (1989) confirmed these speculations describing a rapid decrease in sugar production in diseased fields starting in the Netherlands after 1960, when the sprinkler irrigation began to spread. Thus, as what happened in Italy and Japan, in most of the infected European areas, rhizomania was found after a long period of too short sugar beet rotation cycles (Sutic and Milovanović 1981) or repeated beet cropping, as was the case of the first record of rhizomania in England in 1987 (Hill and Torrance 1989), while in Greece and in California (Duffus et al.

Table 2.4 Chronology of the first "official" detection of rhizomania in the world (from different sources)

Country	Year	Country	Year
Italy	1953	Switzerland	1983
Japan	1965	Belgium	1984
Croatia	1971	UK	1987
France	1971	Spain	1988
Greece	1972	Sweden	1997
Germany	1974	Syria	1998
Czech Republic	1978	Slovenia	1999
China	1978	Denmark	2000
Slovakia	1978	Chile	2001
Austria	1979	Turkey	2001
Kazakhstan	1979	Poland	2002
Romania	1979	Lithuania	2002
Russia	1979	Canada	2002
Ukraine	1979	Egypt	2003
USA	1981	Iran	2004
Hungary	1982	Morocco	2005
Bulgaria	1983	Brazil (red beet)	2015
The Netherlands	1983	South Africa (red beet)	2015

1984), it was found following an unusually rainy spring. The relatively delayed appearance of rhizomania in the colder (Denmark, Canada, etc.) and hotter or dryer cultivation areas (Morocco, Egypt, etc.) is likely due to the unsuitable temperature and soil moisture during the early phase of the *P. betae* root inoculation.

In addition, it should be considered that the rapid diffusion of rhizomania coincided with the introduction of genetic monogerm varieties, which began in Europe around 1963. It was suggested that the use of the new genotypes may have replaced or reduced some hidden or unknown resistance traits carried by the previously cropped multigerm varieties (Biancardi et al. 2005). This trait could have been a sort of resistance to the virus or, more likely, to *P. betae* which was completely lost by the monogerm seed-bearers during the selection procedures (Asher et al. 2009).

In most situations, the rhizomania symptoms display several years after the first BNYVV infection. This delay limits the efficacy of the measures adopted to prevent the rhizomania diffusion, since their efficacy was hardly verifiable in such a long time. About 10 years after the first identification, notwithstanding the prophylaxis and quarantine measures, there were an estimated 5,930 ha of infected fields in France (Putz and Richard-Molard 1983) and around 24,000 ha in Germany (Schäufele 1983). In 1990, according to Richard-Molard and Cariolle (2001), 15 % of the 1.6 Mha cropped area in the EU was affected by rhizomania. The acreage increased to 36 % in 2000 and is expected to reach 56 % in 2020 (Richard-Molard and Cariolle 2001). However, there is still divergence of opinion as regards the

speed of the disease spreading, apparently due to the variability of factors affecting the dynamics of inoculum and reproduction of BNYVV.

Since Italy is one of the leading producers of sugar beet seed, the diffusion of the disease was initially suspected to be carried by the seed of commercial varieties. This has proven to be hardly possible because of the harsh treatments given to the monogerm seed and the inclusion of fungicides in the pellet surrounding the seed (Hess et al. 1984; Schäufele 1989a, b).

It is thought that the currently hypothesized climate change (Box 1.6), with the consequent rising of temperature, could favor the multiplication of the viruliferous *P. betae*. In Belgium, for instance, the rapid spread of rhizomania between 1984 and 2002 in the main growing areas was associated with an increase in temperature and rainy springs that could have been conducive to an increase in inoculum in the soil (Legrève et al. 2005).

Molecular analysis of BNYVV has revealed the presence of four distinct types: A, B, P, and J. The genome of types A and B consists of four distinct RNA species, whereas the types P and J contain an additional RNA species (RNA5). The P and J types are considered to be more aggressive in susceptible cultivars than the A and B types and also more infectious on cultivars containing the *Rz1* resistance gene. Chiba et al. (2011) hypothesized that BNYVV originated from East Asia, and the A-type BNYVV expanded first to Europe and then the USA. The A type is now distributed throughout Europe and it is prevalent in southern, eastern, and extreme western countries (Koenig et al. 1995). The B type has a minor diffusion in Germany, France, and Iran. The P type was first detected in a limited area around Pithiviers (France) and is only present in few small areas of Kazakhstan and the UK (Koenig and Lennefors 2000; Harju and Richard-Molard 2002). The J type, similar to the P type, was detected in Japan and China (see Sect. 5.3).

Since the greatest genetic diversity of the virus was found in Chinese and Japanese isolates, it was hypothesized that the ancestral form of BNYVV could have appeared there, vectored *P. betae* or similar fungi reproducing in native hosts, such as some species of the Chenopodiaceae family well before the introduction of sugar beet cultivation (Chiba et al. 2011). The diffusion in Europe and the rest of the world may be explained by the commercial exchange of plants, drugs, animals, etc. between Europe and the Far East. The P-type BNYVV was probably introduced to the Pithiviers area from China through soil adhering to mulberry tree plantlets imported for multiplication and used for feeding silkworms (Meulemans et al. 2003). The same area has long been renowned for the cultivation of saffron; thus, it could have been introduced with imported bulbs (Harju and Richard-Molard 2002). These hypotheses seem plausible, taking into account the very long persistence of the cystosori and the rapid multiplication rate of the BNYVV-*P. betae* complex in the presence of sugar beet or other host plants (Chiba et al. 2011). The timetable of the more important research advances in rhizomania control is reported in Table 2.5 (see Chap. 5).

Table 2.5 Timetable of the more important facts and progresses regarding rhizomania

Rhizomania chronology
≈1866 – Meaning of the word "rhizomania" (Lindley and Moore 1866)
1909 – Sampling of *Beta maritima* at Porto Levante, Italy, subsequently the source of rhizomania resistances (Munerati 1946)
1915 – The word "rizomania" used firstly for sugar beet (Munerati and Zapparoli 1915)
≈ 1945 – Rapid spread of "soil sickness" or "low sugar content syndrome" (LSCS) in the eastern Po Valley (Donà Dalle Rose 1954)
1954 – First written reference and B&W pictures of LSCS (Donà Dalle Rose 1954)
1954 – Beginning of systematic observations about LSCS (Bongiovanni 1960)
1959 – The LSCS was called "rizomania" (Canova 1959)
1960 – Sampling of *Beta maritima* at Kalundborg Fjord, Denmark, source of some rhizomania resistances subsequently coded Rz2 and Rz3 (Doney and Whitney 1990)
1964 – Some degree of tolerance/resistance to rhizomania in cercospora-resistant varieties (Bongiovanni and Lanzoni 1964)
1964 – The plasmodiophoral fungus *Polymyxa betae* found in diseased sugar beet (Keskin 1964)
1964 – Established the biotic origin of rhizomania (Ghillini 1964)
1964 – First known distribution map of the disease (Bongiovanni 1964)
≈1966 – First breeding activity on diseased fields (Gentili and Poggi 1986)
1966 – Possible viral etiology of rhizomania (Canova 1966)
≈1968 – Release of multigenic rhizomania-resistant varieties
1973 – Presence of rod-shaped virus named beet necrotic yellow vein virus (BNYVV) in diseased beets (Tamada and Baba (1973)
1976 – Demonstration that *Polymyxa betae* is the vector of BNYVV (Fujisawa and Sugimoto 1976)
1978 – Sampling of *Beta maritima* source of Rz5 rhizomania resistance at Porto Levante, Italy (Grimmer et al. 2007)
1980 – Discovery of monogenic resistance in SES-Italy germplasm (De Biaggi 1987)
≈1983 – Employment of molecular techniques in rhizomania breeding
1983 – Discovery of resistance traits in Holly germplasm (Lewellen et al. 1985), subsequently coded Rz1 (Scholten and Lange 2000)
1985 – Release of monogenic resistant variety Rizor (De Biaggi 1987)
1986 – Release of Holly CMS-/OT-resistant lines (Lewellen et al. 1987);
1990 – Release of Rz1-resistant variety in Europe
1997 – Release of monogenic rhizomania-resistant alleles subsequently coded Rz2, Rz3, Rz4, and Rz5 (Lewellen 1997)
≈2000 – Discovery of rhizomania resistance-breaking strains (Liu et al. 2005)
2005 – Monogenic rhizomania-resistant alleles coded *Rz*3, *Rz*4, and *Rz*5 (Gidner et al. 2005)
2006 – Release of double monogenic (Rz1 + Rz2) rhizomania-resistant variety Angelina (Rush et al. 2006)
2015 – Rhizomania resistances "Rizor" and "Holly" (Rz1) have the same origin (Stevanato et al. 2015)

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Part II
The Virus

Chapter 3
General Features of Beet Necrotic Yellow Vein Virus

Tetsuo Tamada

Abstract Beet necrotic yellow vein virus (BNYVV), identified as the causal agent of rhizomania of sugar beet in the early 1970s, is transmitted by the soil-borne protist *Polymyxa betae* and by inoculation of sap to most species of the family Chenopodiaceae and several species of a few other families. BNYVV, the type member of the genus *Benyvirus* in the family *Benyviridae*, has rod-shaped particles and four to five single-stranded, positive-sense RNA genomes. RNA1 and RNA2 encode the essential elements for replication, assembly, transmission, and cell-to-cell movement; smaller RNA segments, RNA3, RNA4, and RNA5, are associated with vector-mediated infection and disease development in sugar beet roots. RNA3 is required for development of rhizomania symptoms in sugar beet, whereas RNA4 is important for efficient vector transmission. RNA5 is associated with the severity of symptom development in sugar beet roots, but is dispensable for BNYVV survival. Synergistic effects on symptom development and vector transmission efficiency have also been found between RNA3 and RNA4 and between RNA5 and RNA4. Symptom expression has been suggested to involve auxin-induced genes during BNYVV infection. BNYVV is usually confined to root systems of sugar beet. This restricted distribution of the virus in the root is attributed to barriers that block virus movement between vascular and nonvascular tissues in roots. Enzyme-linked immunosorbent assay (ELISA) is the best method for rapid diagnosis of large numbers of samples. Reverse transcriptase polymerase chain reaction (RT-PCR) and improved-RT-PCR methods are more sensitive and specific for distinguishing BNYVV strains. Other soil-borne RNA viruses can be present in sugar beet roots, some of which are occasionally associated with rhizomania.

Keywords Sugar beet • Rhizomania • BNYVV • Benyvirus • Multipartite RNA viruses • *Polymyxa betae*

Rhizomania of sugar beet, characterized by the abnormal proliferation of rootlets, was first recorded in Italy in the mid-1950s. Canova (1959) was the first to attribute

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E. Biancardi, T. Tamada (eds.), *Rhizomania*, DOI 10.1007/978-3-319-30678-0_3

the disease to an association between a virus and a soil-borne fungus. The fungus was then identified and named *Polymyxa betae* by Keskin (1964). *Polymyxa* spp. are obligate biotrophs belonging to the plasmodiophorids, which were previously considered to be fungi, but now are classified as protists. Resting spores of *P. betae* were always detected in the rootlets of diseased sugar beet plants, from which a new virus originally designated as "virus A" was detected (Canova 1966). Around the same time in Japan, a disease reported by Masuda et al. (1969) was described as "abnormal sugar beet growth due to succession cropping." Kanzawa and Ui (1972) found that *P. betae* was prevalent in the rootlets of such abnormal sugar beet plants, suggesting some similarity to rhizomania reported in Italy. Tamada et al. (1971) found that a rod-shaped virus was frequently detected from roots of rhizomania-affected plants and only from leaves with a yellow vein symptom, a rare symptom of rhizomania. This rod-shaped virus was subsequently named beet necrotic yellow vein virus (BNYVV) and was distinct from any other virus described (Tamada and Baba 1973). They also proved that the main pathogen of rhizomania in sugar beet was BNYVV and that *P. betae* played a role as a vector of BNYVV based on the lack of rhizomania symptoms in sugar beets with BNYVV-free *P. betae* infection (Tamada 1975; Fujisawa and Sugimoto 1976).

Since the first naming of the causal virus of rhizomania in Japan, Putz and Vuittenez (1974) identified a French isolate of BNYVV from rhizomania-affected sugar beet plants in Alsace, based on serological and biological properties and particle morphology. Faccioli and Giunchedi (1974) also found BNYVV in beets with rhizomania disease in Italy. A decade later in Strasbourg in France, the research group of Ken Richards determined the complete nucleotide sequences of the BNYVV genomes (four RNA species, RNA1 to RNA4) (Bouzoubaa et al. 1985, 1986, 1987), which has greatly contributed to subsequent molecular biological studies on BNYVV, such as genome organization, genome-wide function, viral evolution, and virus–host interactions (see Chaps. 4 and 5).

In Europe, since the first report of the disease in Italy in the 1950s, BNYVV has spread from central and southern Europe to eastern and northern Europe in the 1970s to 1990s (see Table 2.4 in Chap. 2). Outside Europe, BNYVV was first found on the island of Hokkaido, Japan, in 1964. In China, it was first found in Nei Menggu (Inner Mongolia) in 1978 (Gao et al. 1983) and in many districts along the Yellow River, Heilongjiang, and Xinjiang Uyghur in the 1980s. In the United States, BNYVV was first recorded in California in 1983 (Duffus et al. 1984) and in Texas in 1987 (Duffus and Liu 1987). Between 1992 and 1994, BNYVV was also found in Colorado, Idaho, Nebraska, and Wyoming (Rush and Heidel 1995). In the Middle East, BNYVV was found in Turkey, Iran, Egypt, and Syria in the 2000s. So BNYVV has now been detected in most sugar beet-growing areas of Europe, Asia, and the United States (Asher 1993; Tamada 1999; McGrann et al. 2009). Most recently, a rhizomania-like disease of red table beet (*B. vulgaris* subsp. *vulgaris* cv. Boro) caused by BNYVV was found in Brazil (Rezende et al. 2015) and South Africa (Roberts et al. 2015) in the Southern Hemisphere.

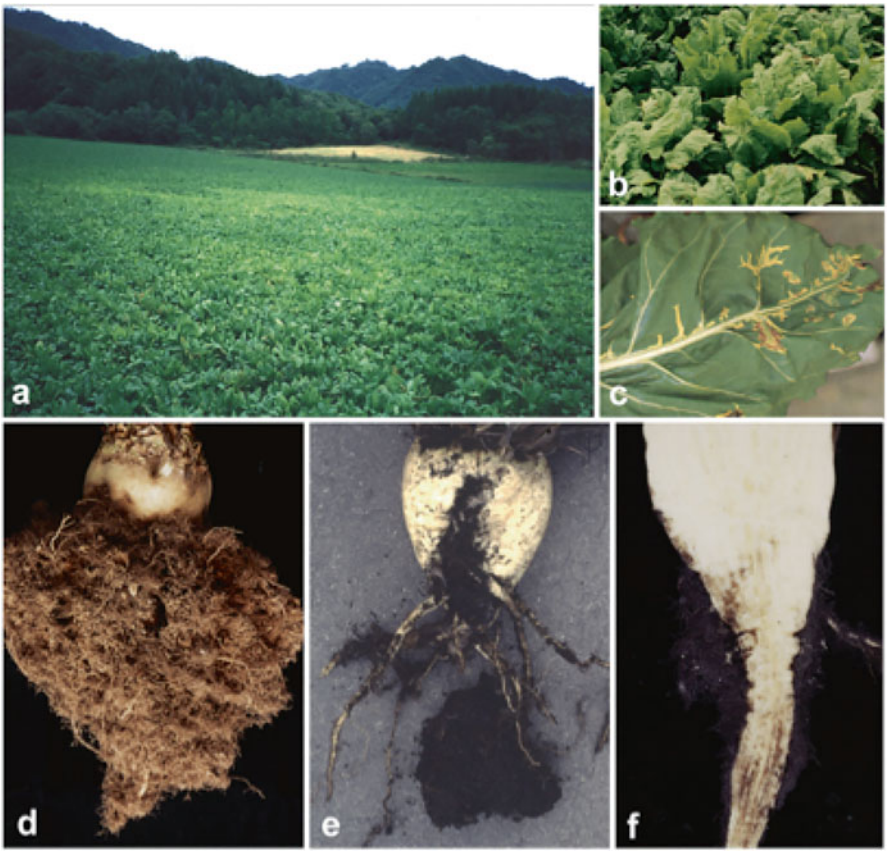

Fig. 3.1 Symptoms on sugar beet plants naturally infected by BNYVV in Japan (Hokkaido) (**a**, **d**, **e**, and **f**, from Tamada (2002), with permission of AAB). (**a**) Yellow patches of infected sugar beets in the field; (**b**) pale yellowing and slight upright growth of plants; (**c**) striking yellow vein on leaf of systemically infected plant; (**d**) abnormal rootlet proliferation of taproot with heavy infection; (**e**) abnormal rootlet proliferation on lateral roots with slight level of infection; (**f**) vascular browning on tip of taproot (longitudinal section)

3.1 Host Range and Symptoms

In nature, BNYVV infects sugar beet (*Beta vulgaris* subsp. *vulgaris*), fodder beet, Swiss chard (*Beta vulgaris* var. *cicla*), and spinach (*Spinacia oleracea*). The disease is usually distributed as foci of chlorotic patches in the sugar beet field (Fig. 3.1a). Obvious leaf symptoms are visible at the end of the growing season; leaves become pale yellow, with an elongated petiole and more upright growth (Fig. 3.1b). The major symptoms are root stunting and abnormal proliferation of lateral roots and taproots (Fig. 3.1d, e), and a yellow-brown discoloration of vascular bundles (Fig. 3.1f), causing infected plants to wilt during the day due to insufficient water

uptake by damaged roots, although the plants can recover at night. In the case of severe infection (e.g., infection at the seedling stage), plants become stunted, wilt, and eventually die. Taproots remain small, with severe proliferation of lateral roots and rootlets (Fig. 3.1d). When these symptoms are present, the bright yellow symptom along the veins (hence the virus name necrotic yellow vein) sometimes develops (Fig. 3.1c). When infection is later and mild, the taproot is more developed and often has a turnip-like shape and rootlet proliferation. Very slight infection may produce no obvious foliage or root symptoms.

BNYVV has a narrow host range. It is transmitted by mechanical inoculation with sap to most species of the family Chenopodiaceae and to several species belonging to the Aizoaceae, Amaranthaceae, Caryophyllaceae, and Solanaceae (Tamada and Baba 1973; Tamada 1975, 2002; Kuszala and Putz 1977; Horváth 1994; Hugo et al. 1996). Many of these plants are hosts of the vector *P. betae* (Abe and Tamada 1986; Abe and Ui 1986; Barr and Asher 1992, 1996; Hugo et al. 1996). The virus tends to be restricted to the inoculated leaves of these host plants; however, it can spread systemically in some plant species such as *Beta macrocarpa*, *Beta vulgaris* subsp. *maritima* M8, *Spinacia oleracea*, and *Nicotiana benthamiana* (Tamada 1975, 2002, 2007). Symptoms on diagnostic host plants inoculated mechanically are described next:

- *Beta vulgaris* (sugar beet). Inoculated leaves develop chlorotic lesions 6–8 days after rub-inoculation with BNYVV. The lesions then become bright yellow (Fig. 3.2a), enlarge, and tend to coalesce, spreading along the veins. In rare cases under favorable conditions, some plants become systemically infected (Fig. 3.2b); symptoms can include yellow vein (Fig. 3.2c), vein necrosis, chlorotic or yellow spotting, leaf distortion, wilting, and stunting (Fig. 3.2b).
- *Beta macrocarpa* and *B. vulgaris* subsp. *maritima* M8. Inoculated leaves develop yellowish local lesions, followed by systemic yellow mottle or yellow flecks with severe stunting (Fig. 3.2d, e) from 10 to 14 days after inoculation.
- *Nicotiana benthamiana*. Systemic symptoms (mottling, leaf distortion, and downward curling of leaves) appear about 10 days after inoculation. Bright yellow chlorosis is not observed on this host.
- *Chenopodium quinoa* and *C. amaranticolor*. Chlorotic or necrotic lesions appear on inoculated leaves 5–7 days after inoculation. They are not systemically infected. *C. quinoa* is a good local lesion assay host and is also used for assessing pathogenicity of BNYVV.
- *Tetragonia expansa*. Inoculated leaves usually develop bright yellow lesions after 10–14 days after inoculation (Fig. 3.2f). The majority of symptoms are bright yellow lesions, but necrotic or chlorotic lesions appear occasionally as mixtures. These phenotypes can be separated by single lesion isolation (Fig. 3.2g, h). Thus, this species can be useful for distinguishing pathogenicity of virus isolates, strains, or mutants as described later.

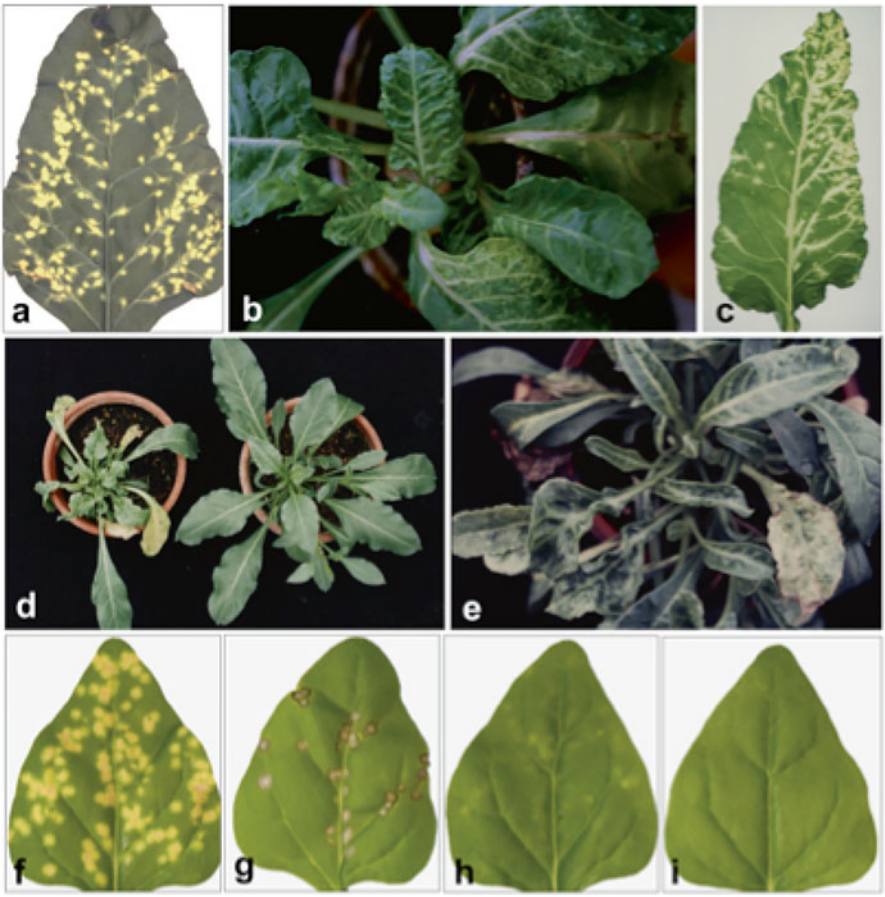

Fig. 3.2 Symptoms on mechanically BNYVV-inoculated plants (**a**, **c**, and **e**, from Tamada (2002), with permission of AAB). (**a**) Bright yellow lesions on an inoculated leaf of sugar beet (*Beta vulgaris* subsp. *vulgaris*); (**b**) systemically infected sugar beet plant; (**c**) bright yellow in leaf veins of a systemically infected sugar beet; (**d**) systemically infected *Beta macrocarpa* plant (*left*) and non-inoculated plant (*right*); (**e**) systemically infected *B. macrocarpa* plant showing yellow mottle or yellow flecks with severe stunting. Different lesion types produced by single lesion isolates of BNYVV on inoculated leaves of *Tetragonia expansa*; (**f**) yellow spots; (**g**) necrotic spots; (**h**) chlorotic spots; (**i**) non-inoculated healthy leaf

3.2 Strains

No serological differences are found among BNYVV isolates. However, most isolates are classified into two major groups (A and B types) using restriction fragment length polymorphism (RFLP) or single-strand conformation polymorphism (SSCP) patterns of RNA1, RNA2, RNA3, and RNA4 (Kruse et al. 1994; Koenig et al. 1995). The sequence diversity between A- and B-type BNYVV is low, with nucleotide

identity in the range 96–99 % (Saito et al. 1997; Koenig and Lennefors 2000). For example, the coat protein (CP) nucleotide sequences of the A and B types differ by 3.5–4.8 % (three to five amino acid changes). The A and B types can be discriminated by the same amino acid changes at three positions (Miyanishi et al. 1999).

A-type BNYVV is distributed worldwide in most European countries, the United States, Middle East, China, and Japan (Kruse et al. 1994; Schirmer et al. 2005), whereas the B type occurs in limited areas of Europe (Germany, France, and Sweden) (Kruse et al. 1994; Koenig et al. 2008).

Another group, named the P type that contains a fifth RNA (RNA5), was first isolated by Koenig et al. (1997b) from the Pithiviers area of France. The P-type virus was detected in small, limited areas of France (Schirmer et al. 2005), Germany (Koenig et al. 2008), and the United Kingdom (Ward et al. 2007). However, some Japanese RNA5-containing isolates that were previously described (Tamada et al. 1989) seem to belong to the A type, so the presence of RNA5 is not sufficient to designate an isolate as P-type BNYVV. The RNA5 sequences from P-type isolates share about 96 % identity with those from China and Japan (Koenig et al.1997b; Miyanishi et al. 1999; Ward et al. 2007). Sequence comparisons of A, B, and P types indicated that A and P types are much more closely related than they are to the B type (Koenig and Lennefors 2000; Schirmer et al. 2005). It was also notable that some RNA5-containing isolates from China and Japan have the *CP* gene of the B type but other genes are of the A type, suggesting that these isolates were generated from reassortants or recombinations by a mixed infection of A- and B-type strains in the past (Miyanishi et al. 1999; Li et al. 2008). Schirmer et al. (2005) referred to Asian BNYVV isolates as Japanese type (J type) to distinguish them from the A, B, and P types that were detected in Europe.

To resolve this complexity of BNYVV strains, Chiba et al. (2011) analyzed the four genes from worldwide isolates and showed that BNYVV isolates consist of eight strains that derived from at least four original lineages (A-I, A-II, A-III, and B). The details on original and current strains of BNYVV are described in Chap. 5.

3.3 Transmission by Vectors

No evidence of transmission by vectors other than *Polymyxa betae* (family Plasmodiophoraceae; protozoa) (Abe and Tamada 1986; Tamada 1975) (Fig. 3.3) or of seed transmission has been reported.

BNYVV can be present in the protozoan during all stages of its life cycle. When sugar beet plants are grown in infested fields, zoospores of *P. betae* (Fig. 3.3c) are released from resting spores (Fig. 3.3d) or from zoosporangia (Fig. 3.3b) and can infect root hairs or epidermal cells of the roots (Keskin 1964; Keskin and Fuchs 1969; Barr and Asher 1996). Infection of root cells by zoospores involves encystment on the cell surface followed by the direct injection of zoospore contents into the cell. At that time, rootlets seem to become infected with BNYVV, when *Polymyxa* zoospores carry the virus. After penetration of zoospores into the root

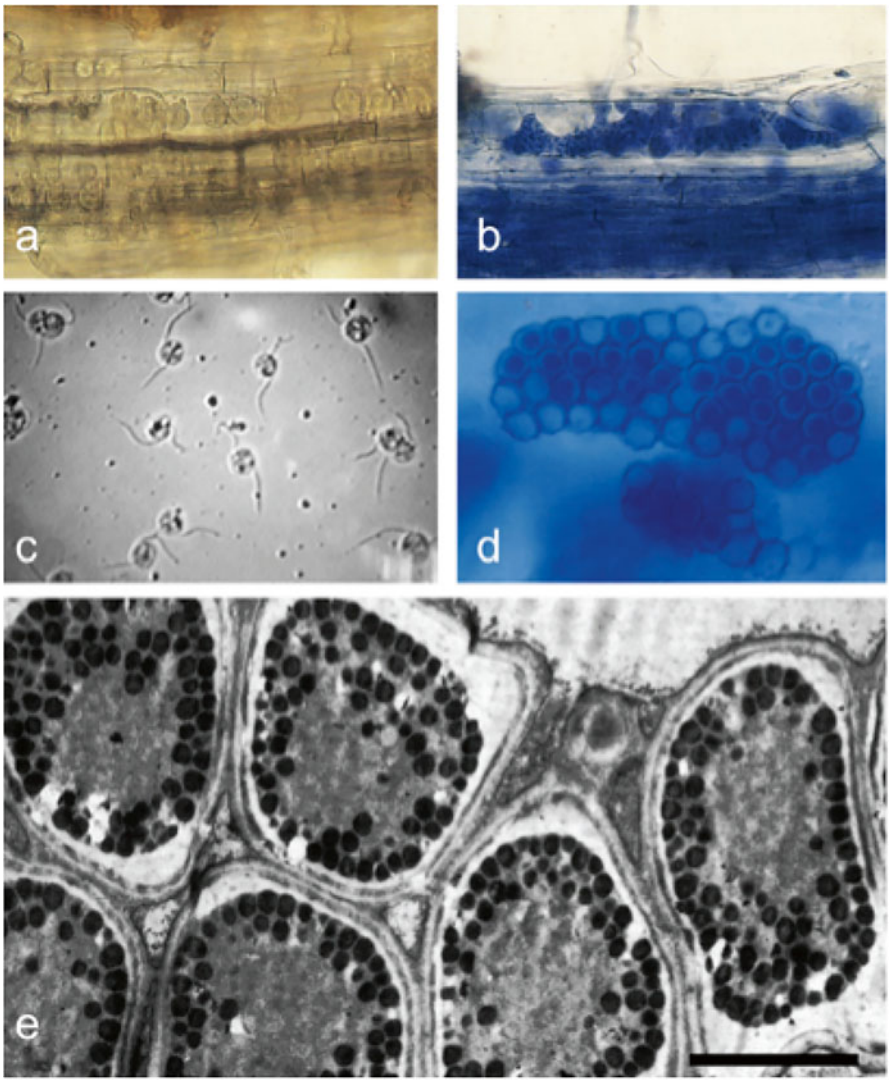

Fig. 3.3 *Polymyxa betae*, the vector of BNYVV (Courtesy, Hideo Abe). (**a**) Plasmodia; (**b**) zoosporangia (stained with cotton *blue*); (**c**) zoospores released from zoosporangia; (**d**) resting spore cluster (stained with cotton *blue*); (**e**) ultrathin section of resting spores in a root cell: Bar = 3 μm

cells, young sporangial plasmodia develop several septa, followed by several cycles of noncruciform mitotic nuclear divisions, and eventually develop into mature zoosporangia (Fig. 3.3a), from which secondary zoospores are released within a few days (Keskin 1964; Dirven and Peters 1995). During the other sporogenic phase of the life cycle, the nucleus of the plasmodium undergoes cruciform division, followed by meiosis. The sporogenic plasmodium is then cleaved, and aggregates of

unicellular resting spores are produced. Mature resting spores are usually grouped in clusters (Fig. 3.3d,e) and each resting spore releases a primary zoospore.

BNYVV can survive in soil within the long-lived resting spores of *P. betae*. The virus replicates in plant cells, but there is no evidence for its multiplication in *P. betae*. The virus is acquired by *P. betae* only *in vivo* from infected root cells, although its mechanism is unknown. The virus is present within rather than on the surface of the spores, because transmission is not reduced by treating viruliferous zoospores with virus-specific antiserum or by washing resting spores with acid or alkali (Abe and Tamada 1986). The viruslike particles can be observed within numerous vacuoles in young immature plasmodia or zoospores (Tamada 1975; Abe and Tamada 1986; Rysanek et al.1992), but this has not been demonstrated for resting spores. Whether *P. betae* is actually a host for BNYVV or simply a vector for virus transmission requires further study.

3.4 Properties of Virus Particles

3.4.1 Particle Morphology

BNYVV particles are hollow, rigid nucleoprotein rods, around 20 nm in diameter and have four to five different modal lengths (Fig. 3.4a), which encapsidate each single-stranded genome RNA component separately. BNYVV particles 390, 270,105, 90, and 80 nm long correspond to RNA1, RNA2, RNA3, RNA4, and RNA5, respectively (Tamada et al. 1989; Richards and Tamada 1992) (Fig. 3.4a, b).

The major coat protein (CP) species for BNYVV is 21 kDa (188 amino acids) (Putz 1977). The single-stranded right-handed helix has a 2.6 nm pitch with an axial repeat of four turns, involving 49 subunits of the CP (Steven et al. 1981). Each CP subunit binds four nucleotides on the RNA genome. The CP read-through (RT) protein (75 kDa) (Fig. 3.4c) is a minor component of virions and is located at the ends of some viral particles (Haeberle et al. 1994).

3.4.2 Antigenic Properties

BNYVV particles are moderately antigenic (Tamada 2002). BNYVV is serologically very distantly related to beet soil-borne mosaic virus (BSBMV, the genus-*Benyvirus*) (Wisler et al. 1994), but is not related to any other rod-shaped virus. Several rat or mouse monoclonal antibodies to purified virus particles have been obtained (Torrance et al. 1988; Koenig et al. 1990). The BNYVV CP was analyzed in detail using immunosorbent electron microscopy (ISEM) (Lesemann et al. 1990),

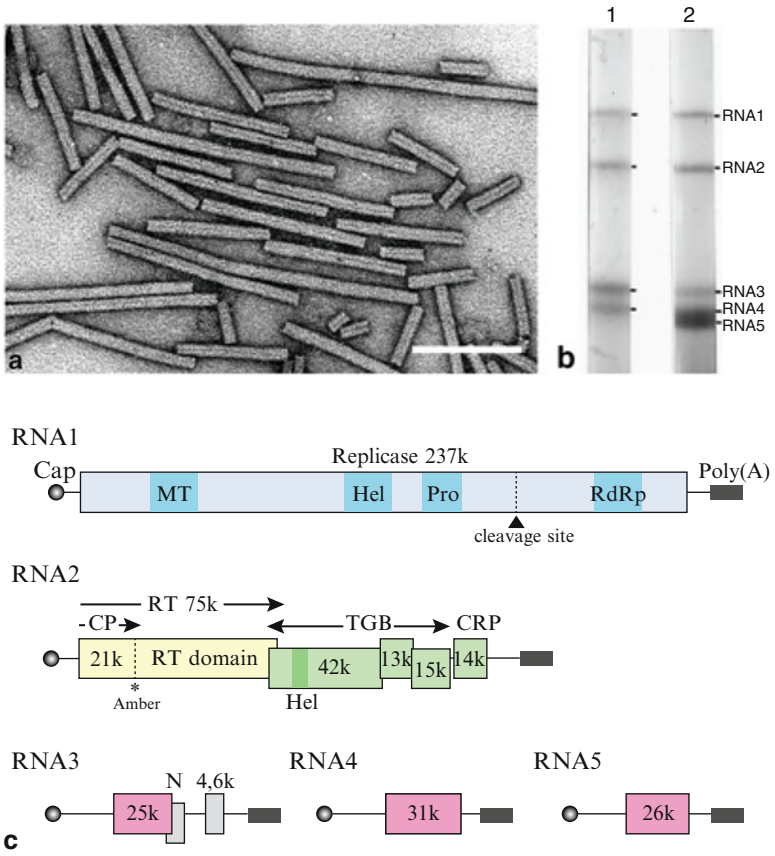

Fig. 3.4 Schematic representation of BNYVV particles and genome structure (**a**, from Tamada (2002), with permission of AAB). (**a**) TEM of purified virus particles of BNYVV, stained with 1 % uranyl acetate. Bar = 100 nm; (**b**) four (lane 1) and five (lane 2) RNA segments of BNYVV detected in agarose gel electrophoresis (stained with 0.005 % Stains-all); (**c**) genomic organization of BNYVV. Solid lines and open boxes denote noncoding regions (NCRs) and open reading frames (ORFs) on genomic RNAs, respectively. The methyltransferase (MT), RNA helicase (Hel), papain-like protease (Pro), and RNA-dependent RNA polymerase (RdRp) domains are labeled and shaded in blue. The arrowhead marks the cleavage site for the polyprotein. The asterisk in RNA2 indicates a single amber termination codon, which permits expression of a CP read-through fusion protein (CP-RT 75 k) by stop codon read-through (~10 % frequency). *CP* coat protein, *RT* read-through domain of CP, *TGB* triple gene block, *CRP* cysteine-rich protein. Cap (*gray circle*) and poly(A) (*thick gray bar*) indicate the 5′-cap structure and the 3′ poly(A) tail, respectively

trypsin treatment (Koenig et al. 1990), and synthetic overlapping peptides (Commandeur et al. 1994). Five different SDS-stable epitopes on the CP and at least two SDS-labile epitopes on the particles were identified using monoclonal antibodies (Commandeur et al. 1992).

3.4.3 Stability in Sap

When sap from BNYVV-infected leaves of sugar beet was diluted with 0.1 M phosphate buffer (pH 7.0), the dilution end point of the virus was about 10^{-4} (Tamada and Baba 1973; Kuszala and Putz 1977). The infectivity of the sap extract was retained for 5 days at 20 °C and for 8 days at 4 °C, but decreased greatly at −20 °C (Tamada and Baba 1973). The infectivity of the virus in sap is little affected by pH variation (pH 4–pH 9). In air-dried soil, the infectivity of BNYVV is maintained within *P. betae* for many years (Abe and Tamada 1986).

3.4.4 Purification

Tamada (2002) purified the virus from inoculated leaves of *T. expansa* or *C. quinoa* with well-developed lesions. The main problem in purifying the virus is a marked tendency for its particles to aggregate and to stick to plant cell debris, with the possible serious loss of virus particles at each cycle of low- and high-speed centrifugation. Such loss is minimized by using an extraction buffer (0.5 M borate buffer, pH 9.0, containing 1 mM Na_2EDTA) and a suspension buffer (0.05 M borate or 0.05 M Tris-HCl buffer, pH 8.0). Addition of Triton X-100 (2 %) to the supernatant is effective for clarification. The virus particles are then purified by differential centrifugation and sucrose density-gradient centrifugation. For antiserum production, a virus particle fraction is obtained by further density-gradient centrifugation in cesium chloride.

3.5 Properties of the Genome

3.5.1 General Features

BNYVV is the type member of the genus *Benyvirus* in the family *Benyviridae* (Gilmer et al. 2013). The five plus-strand RNA components of its genome in decreasing order of size are RNA1, 6.8 kb; RNA2, 4.7 kb; RNA3, 1.8 kb; RNA4, 1.5 kb; and RNA5, 1.45 kb (Fig. 3.4b). The genetic map of the viral RNA in Fig. 3.4c shows that all viral RNA components have cap structures at their 5′-termini and terminate in a 3′-poly(A) tail (Putz et al. 1983). Close sequence homology among the five RNAs is limited to the 5′-terminal eight to nine nucleotides (nt) and to the last 70 nt preceding the 3′-poly(A) tail (Richards and Tamada 1992).

An interesting feature of the BNYVV genome is the behavior of the three smaller RNA segments, RNA3, RNA4, and RNA5, in different conditions of virus propagation. In the roots of naturally infected sugar beets, RNA1 to RNA4 are always present, although RNA5 is not always present (Koenig et al. 1986; Lemaire et al. 1988;

Tamada et al. 1989). When extracts of infected roots are used to mechanically inoculate leaves of a local lesion host such as *T. expansa* or *C. quinoa*, only RNA1 and RNA2 are essential for infection (Koenig et al. 1986; Kuszala et al. 1986; Tamada et al. 1989). This finding suggested that RNA1 and RNA2 encode basic host-independent "housekeeping" functions, whereas the smaller RNAs intervene specifically during natural infection. In this regard, the smaller RNA species play an important role in vector-mediated infection of sugar beet roots, proliferation within the root system, and production of rhizomania symptoms (Richards and Tamada 1992).

3.5.2 RNA1

BNYVV RNA1 is 6746 nt long and contains one long open reading frame (ORF) encoding a polypeptide of 237 kDa (p237), which contains the domain necessary for replication of the viral genome (Bouzoubaa et al. 1987; Richards and Tamada 1992) (Fig. 3.4c). The p237 protein is autocatalytically cleaved into 150 kDa (p150) and 66 kDa (p66) proteins after translation. The p150 protein contains methyltransferase, NTP binding/helicase, and papain-like protease domains, whereas the p66 protein region contains an entire RNA-dependent RNA polymerase domain (Hehn et al. 1997).

3.5.3 RNA2

RNA2 is 4612 nt long and contains six ORFs (Bouzoubaa et al. 1986) (Fig. 3.4c). The 5′-proximal ORF encodes the 21 kDa CP. The CP cistron is separated from a long (54 kDa) inphase ORF by a single amber termination codon, which is suppressed about 10 % of the time during translation, resulting in a 75 kDa fusion protein referred to as the p75 read-through (RT) protein (Ziegler et al. 1985). The N-terminal half of the RT domain is involved in virus particle assembly (Schmitt et al. 1992). The C-terminal portion of the RT sequence is essential for vector transmission (Tamada and Kusume 1991; Tamada et al. 1996a), including a peptide motif (KTER at amino acid numbers 553–556) identified by alanine scanning mutagenesis (Tamada et al. 1996a). The p75 protein is a minor component of virions and is located at the end of rod-shaped viral particles (Haeberle et al. 1994). The BNYVV virion has a mitochondria targeting property, which is controlled by the p75 RT domain with a mitochondrial targeting sequence and transmembrane regions (Erhardt et al. 2001; Valentin et al. 2005).

The central portion of BNYVV RNA2 contains a cluster of three overlapping ORFs known as the triple gene block (TGB) (Fig. 3.4c), encoding proteins of 42 kDa (p42, TGBp1), 13 kDa (p13, TGBp2), and 15 kDa (p15, TGBp3) that are expressed from subgenomic RNAs (Gilmer et al. 1992). The TGB proteins have

amino acid sequence homologies and similar hydrophobicities to equivalent proteins involved in cell-to-cell movement of a number of other plant RNA viruses, including potex-, carla-, hordei-, pomo-, and pecluviruses. The p42 protein has sequence motifs characteristic of a superfamily I DNA or RNA helicase, including a "P-Loop" ATP/GTP-binding domain, and has nucleic acid-binding activity (Bleykasten et al. 1996). The p13 protein has two hydrophobic domains that are potentially membrane spanning and are separated by a hydrophilic sequence that contains a peptide motif highly conserved among homologues. Highly specific interactions are found among the TGB proteins, which are important for their function and/or stability (Lauber et al. 1998a). The p13 and p15 proteins localize to cell wall thickenings that are considered to represent degenerate plasmodesmata (Erhardt et al. 2005). Both proteins are required *in planta* to target p42 to punctate bodies that are associated with the plasmodesmata to enable virus movement between cells (Erhardt et al. 2000).

The 3′-proximal ORF of BNYVV RNA2 encodes a cysteine-rich, 14 kDa protein (p14) (Fig. 3.4c) that is expressed from subgenomic RNA (Gilmer et al. 1992). The p14 protein is shown to regulate the accumulation of RNA2 (Hehn et al. 1995) and acts as a viral suppressor of RNA silencing *in planta* (Dunoyer et al. 2002; Andika et al. 2012). The p14 suppresses RNA silencing more efficiently in roots than in shoots (Andika et al. 2012). It accumulates in the nucleolus and the cytoplasm, and its suppressive activity is correlated with long-distance movement (Chiba et al. 2013).

3.5.4 RNA3

BNYVV RNA3 is 1,775 nt long and encodes a 25 kDa protein (p25) (Bouzoubaa et al. 1985) (Fig. 3.4c). RNA3 is responsible for the induction of rhizomania symptoms in sugar beet roots and severe symptom expression in *T. expansa* and Chenopodiaceae hosts (Kuszala et al. 1986; Tamada et al. 1989; Koenig et al. 1991; Jupin et al. 1992). The p25 protein also acts as an avirulence factor in mechanically inoculated leaves of some BNYVV-resistant beet varieties and lines, and this interaction is controlled by single amino acid changes in p25 (Chiba et al. 2008). Thus, RNA3 is recognized as a pathogenicity determinant of BNYVV. An important role of the RNA3-encoding *p25* gene in BNVV evolution and resistance breaking is described in Chap. 5. The p25 protein is a nucleocytoplasm shuttle protein, containing nuclear localization and nuclear export signals and cysteine-rich region and acidic transcriptional activation domain (Vetter et al. 2004) (see Fig. 5.6a). Klein et al. (2007) obtained further evidence that the p25 amino acid 67–70 tetrad is involved in the development of necrotic symptoms in leaves of *T. expansa*.

The 3′-terminal 600 nt of RNA3 are easily detected *in vivo* as a possible subgenomic RNA (termed RNA3sub, unencapsidated), which encodes a 4.6 kDa polypeptide (p4.6) of unknown function (Bouzoubaa et al. 1991) (Fig. 3.4c). In addition, there is a short ORF (called N), which overlaps the C-terminus of the p25 ORF

(Jupin et al. 1992) (Fig. 3.4c). Interestingly, necrotic-type symptoms such as those in Fig. 3.2g are provoked by expression of this ORF N that is silent on full-length RNA3 but is translationally activated by the deletion, which has the effect of positioning ORF N near the 5′-terminus (Jupin et al. 1992).

RNA3 is essential for systemic (vascular) movement in *B. macrocarpa* and *B. vulgaris* subsp. *maritima* M8 (Tamada et al. 1989; Rahim et al. 2007), but this role may depend on an RNA3 sequence domain rather than an RNA3-coded protein (Lauber et al. 1998b). Peltire et al. (2012) showed that a specific, short, noncoding RNA (very similar to RNA3sub) is responsible for viral long-distance movement and that the 5′-terminus of this noncoding RNA contains the "coremin" motif, which is also present in BNYVV RNA5 and BSBMV RNA3 and RNA4.

3.5.5 RNA4

BNYVV RNA4 is 1,431 nt long and contains an ORF for a 31 kDa protein (p31) (Bouzoubaa et al. 1985) (Fig. 3.4c). RNA4 is required for efficient transmission of the virus by *P. betae* (Tamada and Abe 1989; Rahim et al. 2007). Rahim et al. (2007) showed that p31 is involved in other infection processes and that RNA4, but not RNA3, is associated with the development of severe symptoms in *N. benthamiana* plants. In addition, p31 has no activity in RNA silencing suppression in leaves, but p31 (or RNA4) enhances the ability of BNYVV to suppress RNA silencing specifically in roots without affecting viral RNA accumulation. A possible link between this trait of RNA4 and efficient vector transmission may be implicated (Rahim et al. 2007).

3.5.6 RNA5

BNYVV RNA5 is 1342–1347 nt long and contains a single ORF for a 26 kDa protein (p26) (Kiguchi et al. 1996) (Fig. 3.4c), which varies in length among BNYVV isolates (Koenig et al. 1997b; Miyanishi et al. 1999; Zhuo et al. 2015). RNA5 is involved in the severity of symptoms in roots (Tamada et al. 1996b). RNA5 is also involved in systemic infection in *B. macrocarpa*, but it is less effective than RNA3 (Tamada et al. 1989). The p26 protein is a nucleocytoplasmic protein that induces necrosis in inoculated leaves of *C. quinoa* (Link et al. 2005). Synergistic effects on symptom development and vector transmission efficiency have been found between RNA3 and RNA4 or RNA5 and RNA4 (Tamada et al. 1989; Richards and Tamada 1992).

3.6 Effect of Small RNA Species on Root Symptoms

As regards vector transmission of BNYVV, Tamada and Abe (1989) and Rahim et al. (2007) showed that an RNA4-containing isolate without RNA3 was about 100- to 1000-fold more efficiently transmitted by *P. betae* than an RNA3-containing isolate without RNA4 or an isolate lacking both RNA3 and RNA4. Similarly, isolates containing RNA5 but not RNA4 are transmitted inefficiently by *P. betae*, suggesting that RNA5 resembles RNA3 in its ability to aid vector transmission (Richards and Tamada 1992). Although RNA3 has no effect on transmissibility, this RNA species influences the ability of BNYVV to spread in root systems. Thus, there are synergistic interactions on virus–vector interactions between RNA4 and RNA3 or RNA5.

By using an inoculation system with the virus-carrying *P. betae* cultures described, the effects of the smaller RNA segments on root symptoms can be investigated in a glasshouse and in the field. *P. betae*-inoculated sugar beet seedlings were grown in quartz sand culture for about 1 month in a growth cabinet and then transplanted to soil in the glasshouse or in the field (Tamada et al. 1989, 1996b, 1999; Richards and Tamada 1992). The results are summarized as follows:

- In quartz sand culture, massive rootlet proliferation was observed with BNYVV isolates carrying RNA3 (alone or in combination with RNA4, or RNA5, or both RNA4 and RNA5) but not for isolates lacking RNA3. The degree of rootlet proliferation was not strictly correlated with virus content of the infected root tissue.
- *P. betae*-inoculated seedlings all displayed some browning (slight necrosis) of the rootlets, but a similar effect was induced by virus-free *P. betae*. The severity of the browning symptom was not correlated with the presence of RNA3 or with the virus content of the root tissue.
- In the glasshouse or in the field, BNYVV isolates containing RNA3 caused rhizomania symptoms and yield reduction, depending on the susceptibility of the sugar beet varieties. In contrast, virus isolates containing RNA3 mutants with internal deletion (p25 defective) or lacking RNA3 did not induce rhizomania symptoms. The mutant virus concentrations in roots were about tenfold less than those of the wild-type virus.
- BNYVV isolates that contain RNA4 and RNA5 but not RNA3 caused a slight reduction in root mass and sugar content, but did not cause rootlet proliferation. In some cases, such RNA5-containing isolates may induce a scab-like symptom on the surface of roots. The presence of RNA5 influenced the severity of root symptoms, although the effect was small compared with that of RNA3.
- BNYVV isolates with RNA3, RNA4, and RNA5 caused much more severe symptoms and sugar yield loss in roots than did normal isolates with RNA3 and RNA4.

3.7 Cytopathological Effects

3.7.1 Distribution and Movement of BNYVV in Plants

When virus-carrying zoospores of *P. betae* are used to inoculate sugar beet roots, BNYVV first infects a root hair cell or an epidermal cell of lateral roots. After that, the virus accumulates in the cortex of the rootlets, moves through the vascular systems in the roots, and then spreads toward the shoots. However, BNYVV infection is usually confined to the root system; very rarely does it move to shoots of plants and cause the typical yellow vein symptoms (Fig. 3.1c). Such systemic symptoms or virus multiplication in roots is greatly influenced by the infection stage of beet plants; the earlier the initial infection, the faster is viral spread (Dubois et al. 1994).

In ultrathin sections of BNYVV-infected tissues, virus particles are encountered in loose, small aggregates and scattered in the cytoplasm of infected leaves or roots (Tamada 1975; Putz and Vuittenez 1980; Giunchedi et al. 1981; Russo et al. 1981) (Fig. 3.5a). Particles are often in angle-layer aggregates, in which the angles between virus particles are 45 and 90° (Fig. 3.5b, c). There are no large well-ordered aggregates and no characteristic inclusion bodies. No association is observed between virus particles and cell organelles (Putz and Vuittenez 1980), but infected cells have an increase in the number of rough endoplasmic reticulum profiles (Salle et al. 1986). Erhardt et al. (2001) showed that virus particles localize to the cytoplasmic surface of mitochondria early in infection, but later relocate in the cytoplasm as semi-ordered clusters.

As for virus distribution in root tissue, Scholten et al. (1994) showed by immunogold–silver labeling that, in rootlets of sugar beets infected with *P. betae*, the virus was detected in the epidermis, cortex parenchyma, endodermis, and interstitial parenchyma, but usually not in the vascular tissue. Only a limited number of epidermal cells contained virus, while several cortical parenchyma cells appeared to be infected, suggesting that the virus does not spread laterally in the epidermis, but that it spreads from the epidermis toward the vascular tissue, where the virus was detected only occasionally (Scholten et al. 1994). Kaufmann et al. (1992) also showed by tissue print immunoblotting that the virus is occasionally detected in xylem vessels, while Giunchedi and Poggi-Pollini (1988) detected virus almost exclusively in xylem vessels. Such nonuniform distribution of BNYVV in roots of sugar beets is related to limited long-distance transport of the virus, which leads to reduced virus transport from rootlets to tap roots.

Dubois et al. (1994) further found that a few differentiated cells of the cortex and of the xylem parenchyma were highly susceptible to virus multiplication. The spread of virus infection through such differentiated cells, however, was slow. Virus particles were frequently found in immature and mature vessel elements and xylem parenchyma, but were rare in sieve elements. These observations suggested that the viruses reach vessels by infecting their progenitor cells before or during differentiation. Virus particles were abundant in the xylem tissue of the primary root, but they were not detected in the hypocotyls or leaves (Dubois et al. 1994).

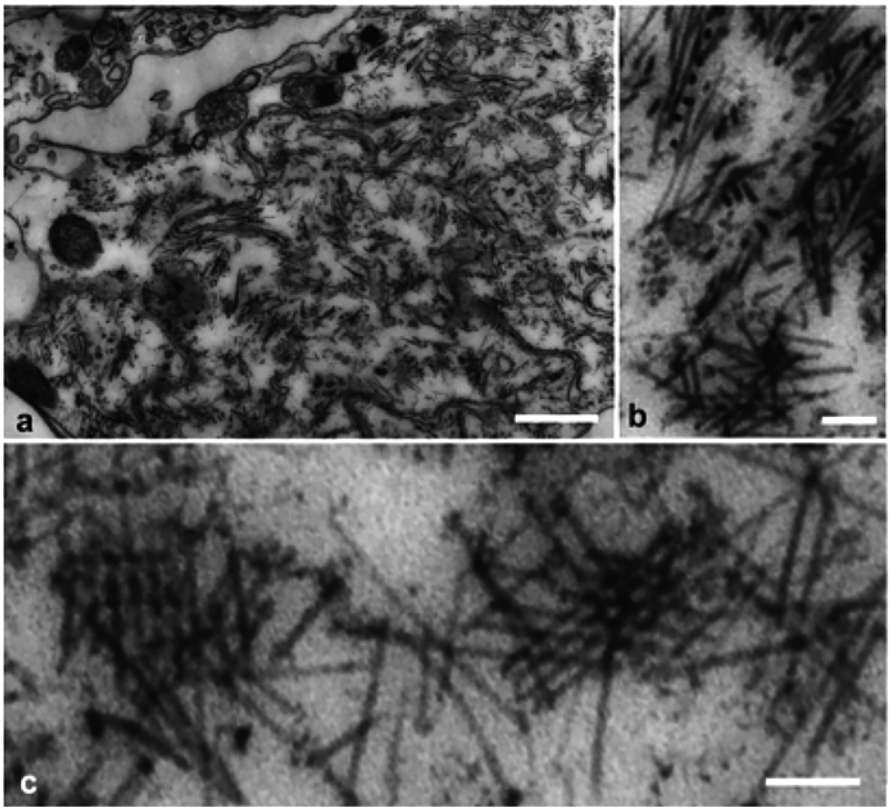

Fig. 3.5 Ultrathin section of BNYVV-infected sugar beet leaf. (**a**) Virus particles are in loose, small aggregates and scattered in the cytoplasm. Bar = 500 nm; (**b**, **c**) angle-layer aggregates of particles at 45° and 90° angle. Bar = 100 nm

On the other hand, Tamada et al. (2013) examined the pattern of systemic movement of BNYVV in the systemic host *B. vulgaris* subsp. *maritima* M8 using green fluorescent protein (GFP)-tagged virus (BNYVV-GFP). This GFP virus was constructed for RNA2 to express a p75-GFP fusion protein (Valentin et al. 2005) (Fig. 3.6). When leaves were rub-inoculated with BNYVV-GFP, GFP fluorescence first appeared in the basal part of the shoot apex (Fig. 3.6a), followed by an extensive, rapid spread of GFP fluorescence in the vascular tissue and the surrounding cortical tissue of the shoot (Fig. 3.6b). Subsequently, small spots of GFP fluorescence were detected in vascular tissues of hypocotyls and primary roots (Fig. 3.6c). Five days after the first symptoms appeared, GFP fluorescence was observed to spread from cell to cell within the vascular tissue and to move to inner tissues such as cambial cells and xylem elements, but less into outer cortical cells (Fig. 3.6d). This direction of movement suggested that there is a barrier between vascular and nonvascular tissues against virus movement within hypocotyls and primary roots. In lateral roots, however, BNYVV-GFP was readily detected in both the cortical and vascular

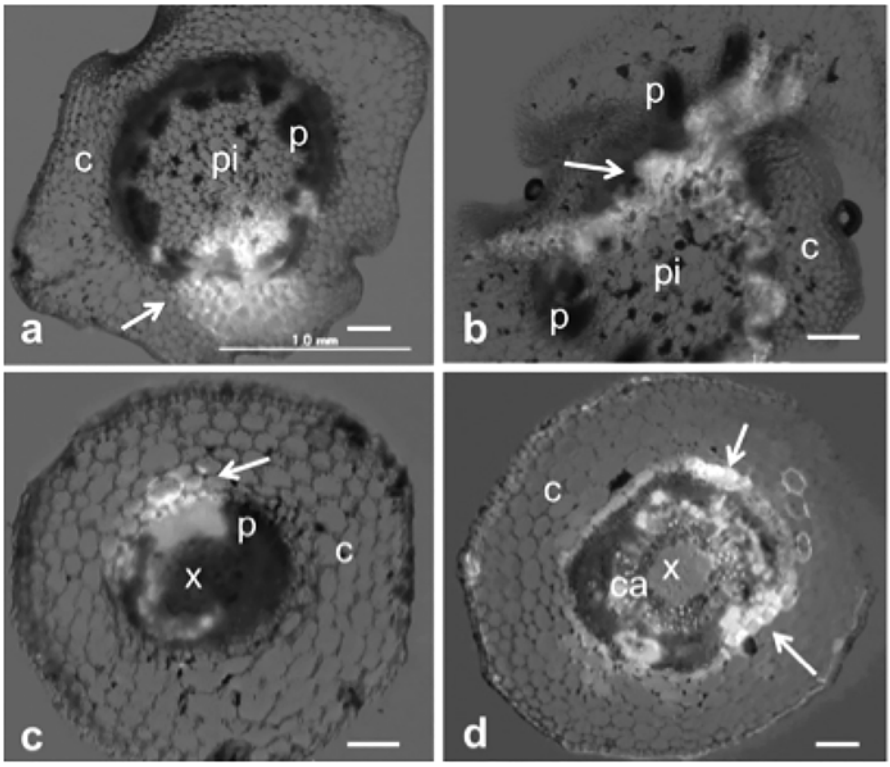

Fig. 3.6 Distribution and accumulation of p75-GFP fusion protein in various parts of *B. vulgaris* subsp. *maritima* M8 plants after rub-inoculation with BNYVV-GFP (p75-GFP fusion in RNA2) (Reproduced and modified from Tamada et al. (2013)). (**a**) Basal part of shoot at 8 days postinoculation (dpi); GFP fluorescence spread from phloem to cortical and pitch tissues; (**b**) basal part of shoot at 9 dpi; GFP fluorescence has spread as the shoot grew; (**c**) primary root at 9 dpi; GFP fluorescence in vascular tissue; (**d**) hypocotyl at 15 dpi; GFP fluorescence in vascular tissue containing cambium and xylem; inside the figures: *c* cortex, *ca* cambium, *p* phloem, *pi* pith cells, *x* xylem: Bar = 200 μm; *arrows* indicate GFP fluorescence

tissues. By contrast to the foliar rub-inoculation, in roots of *B. vulgaris* subsp. *maritima* M8 that were inoculated with virus-carrying *P. betae*, BNYVV movement from roots to shoot was very slow (Tamada et al. 2013). These results, with observations of Dubois et al. (1994), suggest the presence of several barriers that block upward virus movement from lateral roots via the taproot to the plant shoot. Although BNYVV particles (including also BNYVV-GFP) are present in xylem vessels of root tissues, long-distance movement of virus through the xylem vessels is not likely to be frequent.

3.7.2 Physiological Effects

BNYVV induces abnormal rootlet proliferation in sugar beet roots, which is quite unique for a plant virus. BNYVV RNA3-encoded p25 is essential for such symptom expression (Tamada et al. 1999). Virus-induced histological changes and exclusive increase of free auxin in root hairs of susceptible sugar beet plants has been reported (Poggi-Pollini and Giunchedi 1989; Poggi-Pollini et al. 1990). Recently, several molecular studies have reported that on auxin-induced genes are among those that are deregulated after BNYVV infection (Larson et al. 2008; Schmidlin et al. 2008; Thiel and Varrelmann 2009; Peltier et al. 2011). After extensive transcriptome analysis (restriction fragment differential display polymerase chain reaction), Schmidlin et al. (2008) reported virus- and plant-specific expression of candidate genes that are involved in cell development, metabolism, defense signaling, and oxidative stress response. Larson et al. (2008) conducted proteomic analysis with multidimensional liquid chromatography on different sugar beet accessions induced by BNYVV and identified 50 putative sugar beet proteins that were either up- or down-regulated in response to BNYVV infection. Using mass spectrometry, Webb et al. (2014) identified a total of 203 proteins, predominantly associated with photosynthesis and energy, metabolism, and response to stimulus. Many proteins are typically associated with systemic acquired resistance and general plant defense responses. Thiel and Varrelmann (2009) used a yeast two-hybrid screen of a resistant sugar beet cDNA library to identify sugar beet proteins that physically interacted with BNYVV p25; these proteins contained several factors involved in auxin, jasmonate, and ethylene responses as well as with proteasome components. Interestingly, Peltier et al. (2011) generated p25 protein-expressing transgenic plants of *Arabidopsis thaliana* that developed abnormal root branching and increased auxin levels, even though *A. thaliana* is not a susceptible host for BNYVV infection. Microarray analyses on *p25*-transgenic *A. thaliana* roots revealed differential expression of genes involved in cell wall modification that could corroborate the root-branching phenotype (Peltier et al. 2011).

3.8 Detection and Diagnosis

3.8.1 Visible and Biological Methods

For diagnosis of BNYVV in the field, foliage symptoms such as pale yellowing, wilting, and stunting (Fig. 3.1a, b) are rough indicators. A handheld chlorophyll meter is used to evaluate the yellowing intensity of sugar beet leaves infected with BNYVV (Uchino and Kanzawa 1995). Root symptoms of rootlet proliferation and necrosis of vascular bundles (Fig. 3.1d–f) are considered to be reliable indicators of infection. Cutting the root shows browning of vascular rings or of the whole tip of the root. However, foliar and root symptoms of BNYVV on sugar beet plants are

easily confused with other causes such as some soil-borne fungal pathogens, nematodes, insect-transmitted viruses, and poor soil conditions. For example, foliage symptoms are especially confused with nitrogen deficiency. The discoloration (orange or reddish brown) of the central stele may be confused with a symptom of *Fusarium* root rot (Rush and Heidel 1995). The yellow vein symptom (Fig. 3.1c) in leaves is an indication of BNYVV infection, although it is very rare in the field.

BNYVV is detected by mechanical inoculation of indicator plants such as *C. quinoa* and *T. expansa* using sap from systemically infected leaves; the inoculated leaves develop chlorotic or necrotic lesions (Tamada and Baba 1973; Tamada 1975, 2002). Using sap from infected roots is less effective as inoculum because the virus concentration is low. However, samples from infected roots can be partially purified and then used for mechanical inoculations (Tamada et al. 1989).

3.8.2 Serological Methods

Because foliar and root symptoms are unreliable as just described, the most efficient and accurate diagnosis is by means of an enzyme-linked immunosorbent assay (ELISA) using either polyclonal antiserum, which is available in a commercial kit form, or monoclonal antibodies for enhanced specificity (Torrance et al. 1988). In naturally infected sugar beet plants, BNYVV is generally confined to the root and generally present at relatively low concentrations. A higher titer of virus is found in the fibrous lateral roots than in the taproot (Giunchedi et al. 1987; Büttner and Bürcky 1990). In the developing taproot, the highest concentration is found near the tip. Therefore, samples should be extracted from fibrous rootles or from the tip of the lateral roots. The sensitivity threshold of the ELISA is 1–10 ng of virus per gram of tissue. BNYVV can easily be detected by immunosorbent electron microscopy (Putz et al. 1988). Immunogold–silver labeling (Giunchedi and Poggi-Pollini 1988; Poggi-Pollini and Giunchedi 1989; Scholten et al. 1994) or a tissue print immunoassay (Kaufmann et al. 1992) is useful for examining localization and distribution of the virus in sugar beet roots.

3.8.3 Nucleic Acid-Based Methods

Nucleic acid hybridization assays can be applied to detect and distinguish different RNA species of BNYVV (Richards et al. 1985; Koenig et al. 1986; Putz et al. 1988; Saito et al. 1997). Reverse transcriptase polymerase chain reaction (RT-PCR) has proved to be a very powerful tool for detection and diagnosis of BNYVV (Fenby et al. 1995; Henry et al. 1995). This RT-PCR method is more sensitive than ELISA. Morris et al. (2001) improved the sensitivity of RT-PCR for BNYVV detection by developing a nested PCR (nPCR), in which primers were designed to anneal internally to the amplicon produced from standard PCR. The sensitivity of the nPCR was

100–1000 times greater than the standard RT-PCR. The use of the nPCR assay is recommended when greater sensitivity than in the standard RT-PCR is necessary. The RT-PCR can also be used to amplify immunocaptured virions (immunocapture RT-PCR), but this method is slightly less sensitive than amplification from total RNA extracts (Morris et al. 2001). Ratti et al. (2005) developed a multiplex (m) RT-PCR assay for discriminating between A- and B-type BNYVV strains. Harju et al. (2005) further developed real-time RT-PCR assays (using TaqMan, Applied Biosystems) for the specific detection of BNYVV; one is broad spectrum to detect RNA2 from all other types, and the other detects BNYVV containing RNA5. Sensitivity comparisons showed that for the detection of RNA5, TaqMan was 10,000 times more sensitive than the conventional RT-PCR assay. Further improvements were made to the test procedure by using post-ELISA virus release, as an alternative to RNA extraction (Harju et al. 2005). This increases the speed of processing samples and is laborsaving.

3.8.4 Detection from Soils Using Bait Plants

Biological tests to detect BNYVV in free or adherent soil have also developed. Rootlets of beet bait plants that were grown in the suspected soil are tested by ELISA (Beemster and de Heij 1987; Büttner and Bürcky 1990) or by RT-PCR (Meunier et al. 2003). Pre-grown sugar beet seedlings can also be used as bait plants to estimate the level of infestation in soils (Tuitert 1990; Ciafardini 1991) and to calculate potential yield losses.

Overall, many of the serological (e.g., ELISA) and nucleic acid-based (e.g., RT-PCR) methods are adequate for BNYVV detection and should be chosen to the suit for the intended purpose according to specificity, sensitivity, ease and speed of operations, and cost of equipment and consumable supplies. BNYVV detection is helpful to identify infested fields, to determine appropriate field management strategies such as the selection of resistant variety or crop rotation, and to identify rhizomania-resistant sugar beet lines in breeding programs.

3.9 Other Soil-Borne Viruses Associated with Rhizomania

Although BNYVV is the major causal agent of rhizomania, other soil-borne viruses are frequently, but not always, detected in rhizomania-affected sugar beet roots. These include beet soil-borne mosaic virus (BSBMV), beet soil-borne virus (BSBV), beet virus Q (BVQ), and beet black scorch virus (BBSV). BSBMV, BSBV, and BVQ are all transmitted by the same vector *P. betae*, whereas BBSV is transmitted in the soil by the chytrid fungus *Olpidium brassicae*.

3.9.1 Beet Soil-Borne Mosaic Virus

Beet soil-borne mosaic virus (BSBMV) was first found in Texas, USA, and is now widely distributed in the country (Rush and Heidel 1995). It has not been identified from other countries. In greenhouse tests, BSBMV is less virulent than BNYVV (Heidel et al. 1997). Systemic foliar symptoms caused by BSBMV are slight leaf distortion, mottling, and yellow veinbanding, and they appear more frequently than those caused by BNYVV. BSBMV and BNYVV may be found in the same fields and even in the same plants in rhizomania-infested areas.

BSBMV is a member of the genus *Benyvirus* and is very similar to BNYVV in transmission, host range, particle morphology, and genome organization. The two viruses are distinguished serologically (Rush et al. 1994; Wisler et al. 1994; Rush and Heidel 1995). Ratti et al. (2009) reported that the BSBMV RNA3-encoded p29 sequence is more similar to BNYVV RNA5-encoded p26 than to BNYVV RNA3-encoded p25. Moreover, BSBMV RNA3 can be replicated and encapsidated by the BNYVV RNA1 and RNA2, but the presence of BNYVV RNA3 impairs BSBMV RNA3 amplification due to competition (Ratti et al. 2009). This result confirms the complexity of the interaction between BNYVV and BSBMV, which show a high degree of reciprocal cross-protection when infecting the same beet plant, a phenomenon that usually occurs between strains of the same virus (Mahmood and Rush 1999).

3.9.2 Beet Soil-Borne Virus

Beet soil-borne virus (BSBV) was first found by Ivanovic and MacFarlane (1982) in England and later described by Henry et al. (1986). BSBV is very common in sugar beet fields throughout the world (Lindsten 1989) and may be separate from the rhizomania disease complex in some fields. BSBV is restricted to roots of sugar beet and usually causes no obvious symptoms on either roots or leaves.

BSBV belongs to the genus *Pomovirus* (the family *Virgaviridae*) and consists of three RNA species (Koenig et al. 1996, 1997a; Koenig and Loss 1997). RNA1 and RNA2 encode the replicase-associated proteins and the coupled CP and RT protein, respectively, whereas RNA3 codes for the movement proteins (such as TGB). In part of the BSBV genome, there is considerable sequence variability between different isolates from the same soil sample (Koenig et al. 2000). BSBV can infect many *Chenopodium* species via mechanical inoculation, producing local lesions in inoculated leaves (Henry et al. 1986).

3.9.3 *Beet Virus Q*

Beet virus Q (BVQ, genus *Pomovirus* in the family *Virgaviridae*) was originally considered to be a serologically distinct strain of BSBV (Lesemann et al. 1989), but Koenig et al. (1998) referred to it as distinct from BSBV based on its genome sequences. BVQ is very similar to BSBV in transmission, host range, particle morphology, and genome organization. It has been detected in several European countries (Meunier et al. 2003) and in Iran (Farzadfar et al. 2005) and apparently is not as widespread as BSBV. It frequently occurs with BNYVV and BSBV in the same soil samples and in the same roots (Meunier et al. 2003).

3.9.4 *Beet Black Scorch Virus*

Black scorch of leaves and necrosis of roots of sugar beet caused by beet black scorch virus (BBSV) was first reported in China (Cao et al. 2002). BBSV belongs to the genus *Necrovirus* in the family *Tombusviridae* (Cao et al. 2002) and is transmitted by the chytrid fungus *Olpidium brassicae*. In China, a satellite RNA (615 nt, no sequence homology with its helper virus) was found to be associated with BBSV isolates from Xinjiang province (Guo et al. 2005). BBSV was isolated from sugar beet plants with rhizomania-like symptoms in a field near Greeley, Colorado, United States (Weiland et al. 2006, 2007). BBSV is widespread in sugar beet fields in Western Europe (perhaps in other countries). There are two putative strains of BBSV in Europe, but there is no clear evidence for its association with rhizomania-like symptoms (González-Vazquez et al. 2009).

Acknowledgments I thank Drs. Hideki Kondo, Sotaro Chiba, and Mike Asher for helpful comments in the text and Dr. Beth E. Hazen for editing the chapter.

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Chapter 4
Molecular Biology and Replication of Beet Necrotic Yellow Vein Virus

David Gilmer

Abstract Rhizomania disease biology is closely linked to the replication and expression of the beet necrotic yellow vein virus (BNYVV) genome. Understanding viral processes within infected cells and tissues allows fundamental biological discoveries and could inspire new antiviral strategies. BNYVV amplification involves the direct translation of genomic RNAs to produce the viral machinery (replicase), which in turn recognizes genomic RNAs for their specific replication. Nonetheless, the production and expression of subgenomic messenger RNAs are also required to complete the viral life cycle. Whereas certain nonstructural proteins are dedicated to replicase formation and viral movement, other components specifically interact with cellular proteins to overcome innate defense mechanisms and to induce cellular reprogramming. These interactions permit completion of the viral life cycle and allow transmission of the virus. All these processes need to be orchestrated within a coordinated expression pattern to insure optimal viral amplification. In this chapter, the known properties of viral products will be described, and hypotheses about the regulatory mechanisms driving BNYVV biology will be presented. Among regulatory elements, the structure of genomic RNAs plays an essential role in regulating BNYVV protein expression, encapsidation, and movement.

Keywords Sugar beet • Rhizomania • Benyvirus • Pathogenicity • Noncoding RNA

At the end of the 1980s, molecular biologists acquired molecular tools that allowed them to manipulate RNA, either as cDNA copies or as RNA molecules. High-quality reverse transcriptases and recombinant RNA polymerases from bacteriophages (e.g., T7, T3, or Sp6) allowed the initiation of reverse genetic experiments on beet necrotic yellow vein virus (BNYVV) genomic RNAs (Quillet et al. 1989) and established the structure-function relationships of housekeeping genes. Thereafter, *Arabidopsis thaliana* genome sequencing and its manipulation and use as a genetic tool allowed huge steps forward in many host-virus interactions. However, the complexity of *Beta* species genomes (Dohm et al. 2014) and the limited genetic tools available for *Beta* species manipulation (see chapters 8.11) still restrict our findings

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E. Biancardi, T. Tamada (eds.), *Rhizomania*, DOI 10.1007/978-3-319-30678-0_4

and knowledge on BNYVV/*B. vulgaris* (and *Polymyxa betae*) pathosystem(s) because BNYVV does not multiply in *A. thaliana*. The BNYVV genome consists of four to five positive-strand RNAs (RNA1 to RNA4 and additional RNA5) that resemble cellular messenger RNAs (mRNAs). In this sense, together with cileviruses, benyviruses constitute the only viral family having a segmented genome with termini characteristic of cellular mRNAs (i.e., 5′ Cap and 3′ polyA).

Usage of in vitro transcribed RNAs demonstrated the essential requirements of RNA1 and RNA2 for the establishment of a minimal infection in certain host plants. Protoplast infection experiments then demonstrated that RNA1 is the sole viral component required for the production of a functional, minimal replication machinery that allows the amplification of RNA1 itself and other RNA species when present (Gilmer et al. 1992a). Thereafter, protoplast infection combined with host plant leaf inoculation established the foundation for molecular studies of BNYVV biology using reverse genetic tools. The comparative behavior of transcripts carrying mutations in genes or in putative *cis*-acting sequences allowed the determination of their respective roles in viral replication, encapsidation, and cell-to-cell movement. One peculiarity of BNYVV is the conditional requirement of small genomic RNA species (i.e., RNA3, RNA4, and RNA5) that could serve as reporters for the characterization of RNA elements required for RNA amplification and packaging. Derivatives of such small genomic RNAs have been used as expression vectors (described in Sect. 4.1.3).

4.1 Replication and *Cis* Elements

Bioinformatic studies suggested replicative functions for the protein encoded by BNYVV RNA1, particularly by identifying an RNA-dependent RNA polymerase (RdRp) signature present in its C-terminal region (Bouzoubaa et al. 1987; Poch et al. 1989; Koonin and Dolja 1993). Protoplast infection with in vitro transcripts corresponding to RNA1 confirmed its role in the amplification of viral RNA species (Gilmer et al. 1992a), acting *in trans* to facilitate replication of the other viral genomic RNAs (Quillet et al. 1989). *In vitro* and *in vivo* (in the viral context) expression studies of RNA1 revealed its efficient translation into a 237 kDa polyprotein, which is then cleaved into p150 and p66 proteins (Hehn et al. 1997). Thanks to domains identified in each cleavage product, p150 and p66 were thought to participate in the replication complex formation that is required for the amplification of viral genomic RNA species and also for the production of subgenomic RNAs. The p150 protein corresponds to the N-terminal part of p237 and contains the methyltransferase (MT) domain required for Cap incorporation at the 5′ termini of genomic and subgenomic RNAs and a helicase (HE) domain possessing an nucleoside triphosphatase (NTPase) motif required for unwinding RNA secondary structures. Furthermore, p150 carries a papain-like cysteine protease domain necessary for p237 auto-cleavage (Koonin and Dolja 1993; Hehn et al. 1997), but this domain

could also perform deubiquitination as hypothesized recently (Pakdel et al. 2015). The p66 protein corresponds to the C-terminal part of p237 and harbors the RdRp domain. Up to now, no extensive mutagenesis studies have been performed on the replicase-forming units of benyviruses. However, recent work indicated that cleavage products interact together to create a replication complex in close association with endoplasmic reticulum (ER)-derived membranes (Pakdel et al. 2015).

4.1.1 p237 and Replication Complex Formation

Recent advances in the characterization of plant replication complex machineries have been made possible by *in vivo* imaging facilities such as laser-scanning confocal microscopy. Until now, all the replication complexes that have been identified and localized for eukaryotic cell-infecting viruses have been found in close association with cellular membranes (Den Boon et al. 2010; Romero-Brey and Bartenschlager 2014). It was known for decades that benyvirus replication occurs in the "cytoplasm" of the infected cell without particular knowledge of the membranous anchor of its viral machinery. By studying BNYVV RNA1 replicating alone in protoplasts, dsRNA replicative intermediates were found to be cytoplasmically distributed in an ER-like pattern. This ER association was then confirmed by transmission electron microscopy (TEM) of immunogold-labeled dsRNA replicative intermediates and p237 protein in *Chenopodium quinoa*-infected cells (Pakdel et al. 2015). Because the p237 polyprotein is cleaved during translation from RNA1 (Hehn et al. 1997), the released cleavage products need to interact together to establish a functional replicase complex. To get insights into the protein domains involved in replicase complex formation, yeast two-hybrid interaction, co-immunoprecipitation, and fluorescence resonance energy transfer and fluorescence lifetime imaging (FRET-FLIM) analyses were conducted. These studies confirmed an ER association for the two replicase components (i.e., the p150 N-terminal and the p66 RdRp C-terminal cleavage products), which interact with an unknown stoichiometry throughout the ER network. Remarkably, no major ER reorganization was observed in infected cells, presumably because the multipartite BNYVV genome needs more than one replication focus to amplify all the genomic components. Production of a huge viral factory would favor a competition and a loss of "nonessential" genomic RNA species. Based on the accumulation levels of viral RNAs in protoplasts, this is apparently not the case. However, competition between quasi-identical RNA species has been observed, suggesting that genome integrity checkpoints do exist (see below). This latter question is currently under investigation; together with Dr. C. Ratti, we are studying RNA-RNA interactions that would maintain genome integrity via the formation of a genomic RNA network complex, similar to those existing in orthomyxoviruses (Gavazzi et al. 2013). Such complexes could be important for viral movement as well as for the fine-tuned regulation of protein expression (see Sect. 4.6).

4.1.2 Cis-*Active Sequences Required for Viral RNA Amplification*

Once delivered into the cell cytoplasm, viral particles disassemble to release positive-strand genomic RNAs. Translation of BNYVV RNA1 produces the viral replication complex. Because viral RNAs mimic mRNAs, *cis* essential sequences are required to insure the specific amplification of the genome and the production of subgenomic RNAs, which in turn serve as mRNAs for the expression of internal open reading frames (ORFs) present on RNA2 genomic species. Like all single-stranded RNA genomes, the replicase complex recruits viral RNAs by the specific recognition of their 3′ termini. The replicase complex not only acts *in cis* to amplify RNA1 but also *in trans* for the amplification of RNA2, RNA3, RNA4, and RNA5 species.

The remarkable ability of BNYVV RNA1 and RNA2 to fulfill housekeeping functions of replication, packaging, movement, and RNA silencing (or interference, RNAi) suppression has considerably helped the identification of *cis*-acting elements necessary for the amplification of smaller RNA species. Indeed, on local host plants, these small genomic RNAs are unnecessary for the establishment of infection foci after mechanical inoculation with *in vitro*-synthesized RNA1 and RNA2. Thus, RNA3 can be used or engineered to express a reporter protein to characterize RNA3 promoter sequences in the context of BNYVV infection without impairing housekeeping functions. Recurrent deletion mapping performed on the RNA3 sequence, starting from the inner coding sequence, led to the identification of the 5′ and 3′ untranslated regions (UTRs) containing *cis* elements. These *cis*-acting sequences were first localized within the first 300 and the last 70 nt of RNA3 (Jupin et al. 1990). These two UTRs are sufficient and essential for the amplification and packaging of an RNA3-derived species, called a replicon (Rep), which was further used as a viral expression vector (see Sect. 4.1.3).

Focusing on the 5′ leader sequence of RNA3, mutagenesis approaches identified and confirmed the existence of base-paired sequences required for appropriate folding and amplification of the positive-strand RNA (Gilmer et al. 1992b, 1993). In other words, rather than the folding of the 3′ extremity of the complementary strand, the 5′ structure of the positive-strand RNA was identified as the *cis*-acting determinant for replication, a characteristic feature also identified for brome mosaic virus (Pogue and Hall 1992). Therefore, positive-strand RNA3 and, presumably, the other BNYVV RNA species harbor all the functional *cis* elements required for their amplification at both their 3′ and 5′ extremities. Secondary structure characterization was determined in solution using chemical and enzymatic probes. The identification of exposed and protected nucleotides allowed a representation of the secondary structure for the first 330 nucleotides (nt) of BNYVV RNA3, within which base pairings identified by mutagenesis were retrieved (Gilmer et al. 1993). Base pairing involved in the formation of such secondary structures was further confirmed with the study of beet soil-borne mosaic virus (BSBMV) RNA3. Compensatory base substitutions were found in the 5′ UTR of this benyvirus RNA species, which can be replicated

and packaged by the BNYVV machinery (Ratti et al. 2009). A similar approach was used to characterize the 3′ extremity of RNA3. Computational secondary structure predictions (Jupin et al. 1990) were validated both by probing and mutagenesis (Lauber et al. 1997) and then later confirmed by phylogenetic sequence comparisons with the BSBMV RNA3 sequence (Ratti et al. 2009).

4.1.3 Replicons as Virus-Derived Expression Vectors Uses and Limits

The ability of the 5′ and 3′ UTRs to direct BNYVV RNA amplification has been exploited to remove the viral RNA3 coding region and thereby create a replicon vector called Rep0 or Rep3, allowing sequences of choice to be inserted, such as enhanced green fluorescence protein (eGFP) or monomeric red fluorescent protein (mRFP) coding regions, or chimeric constructs of these reporters fused to viral elements (Jupin et al. 1990; Hehn et al. 1995; Bleykasten-Grosshans et al. 1997; Lauber et al. 1998a, 2001, 2005; Erhardt et al. 2005). Other replicon species carrying RNA1 or RNA2 UTRs behave like defective interfering RNAs (Hehn et al. 1994), leading to a limited number of functional replicons. Only viral expression vectors carrying RNA3, RNA4 (Gilmer et al. 1992b), or RNA5 (Schmidlin et al. 2005) UTRs were amplified without effect on RNA1 and RNA2 accumulation. No expression vector derived from RNA4 has been developed due to the overlap between the 5′ coding sequence of p31 and *cis*-acting sequences required for its amplification (Gilmer et al. 1992b). Co-inoculation of Rep3 together with its cognate genomic RNA3 generally ends with the loss of the replicon due to a competition between the two species. This competition is due to the sequence similarity of the 5′ UTRs of the RNAs (Lauber et al. 1999). Persistence of replicons possessing the same 5′ sequence is possible, however, when each RNA encodes for a gene product essential for the viral life cycle, such as movement proteins (Lauber et al. 1998a). To overcome such competition for replication and expression, an RNA5-based expression vector called Rep5 has been developed to express proteins in a viral context and in the presence of RNA3, RNA4, or Rep3 (Schmidlin et al. 2005; Guilley et al. 2009). Later, a replicon derived from an infectious cDNA clone of BSBMV RNA3 (RepIII) confirmed that molecules having similar 5′ UTRs compete with one another during viral amplification. Indeed, inoculation of BNYVV (RNA1 and RNA2) and Rep3-RFP together with RepIII-GFP produced local lesions expressing only one kind of fluorescent protein or displayed a segregated (sectored) expression of the reporter proteins on the inoculated leaves (Ratti et al. 2009). The absence of a mixed RNA population (Rep3-RFP and RepIII-GFP) within infected cells strongly suggests that a specific recognition of the viral genome content is intrinsic to the infection process.

Two different hypotheses could be proposed to explain the specific recognition of viral genome content. The first would be that the virus expresses a protein

machinery that can recognize and maintain the viral formula. The second hypothesis is based on an RNA-RNA interaction network in which viral genomic RNAs interact with each other to form a complex that facilitates cell-to-cell and, perhaps, long-distance transport of viral RNA (see Sect. 4.6). This RNA-RNA interaction network could involve riboswitches that fine-tune protein expression and/or RNA replication.

This latter hypothesis could be emphasized by a requirement of the maintenance of viral formula within infected tissues, while virus progresses from cell to cell. If one considers viral particles as a moving material and a requirement of all four (or five) rod-shaped particles to maintain the genome integrity, identity determinant allowing distinction of RNA1 to RNA4 (RNA5) will be masked. In this case, the only possibility to overcome the loss of a genomic component is to increase the number of moving particles to statistically get at least one of each encapsidated genomic RNA in the distant cells. Nonetheless, if we consider a moving material as a modular ribonucleoprotein complex, the necessity for RNA1 binding to RNA2 for an efficient minimal transport, that could then be completed with RNA3 and RNA4 interaction to RNA1 (or RNA2) to create a network, then only this particular RNA complex recognition is required for transportation of the entire genomic composition. This particular hypothesis is discussed within "a BNYVV life" at the end of this chapter.

4.2 Origin of Assembly

As described above for the efficient amplification of genomic RNA, a selective encapsidation of the BNYVV genome is also required to ensure virus stability, particularly for its transmission. Along with the genomic RNAs, subgenomic RNAs are expressed in the context of viral infection, but they are not encapsidated into stable particles. Subgenomic RNA sequences are coterminous with the 3′ part of the cognate genomic RNAs, suggesting that packaging signals reside instead within the 5′ UTRs of the viral RNAs. Taking advantage of this property of the BNYVV genome and the large collection of available deletion mutants, encapsidation of RNA3 deletion mutant species was tested using nuclease protection assays performed on RNA extracted from viral particles. This allowed the identification of an origin of assembly (OA) or packaging sequence located between nt 181–207 of RNA3 (Gilmer et al. 1992b). The absence of conserved RNA sequences suggests that this packaging signal corresponds to particular, transient structural features that are also present in benyvirus RNAs, because BSBMV RNA3 and RNA4 are also replicated and encapsidated by the BNYVV machinery (Ratti et al. 2009; D'Alonzo et al. 2012).

Nothing is known about a possible cotranscriptional encapsidation of viral RNAs. Such packaging linked to the replication process would allow a fine-tuned balance of viral RNA dedicated to expression in the presence of low amounts of capsid proteins (CP), movement, or transmission while CP accumulates. Here again, specific interactions between the replication complex and structural proteins should also be involved in a fine-tuned regulation as described for rubella virus (Sakata

et al. 2014). Unfortunately, no RNA3 mutant has yet allowed us to test the hypothesis that structural motifs are both required for replication and packaging.

The origin of assembly is a transient RNA structure that is recognized by viral structural proteins. Two different structural proteins produced from RNA2 species are recovered from viral particles, including CP (p21) and minor coat protein (p75), which is detected at the extremity of virions (Haeberle et al. 1994). The presence of this minor structural protein at one extremity of the viral particle was confirmed using a fluorescent-tagged construct (Erhardt et al. 2001). This experiment also led to the initial observation of BNYVV entities surrounding mitochondria (ibid). The presence of the virus around mitochondria is intriguing, because it does not conform to a model in which RNA protruding from the replication site is directly encapsidated. A mitochondrial targeting sequence and transmembrane regions within the N-terminal part of p75 protein were identified, demonstrating that association of BNYVV particles with mitochondria before their release into the cytoplasm (Valentin et al. 2005). This mitochondrial route during the BNYVV cycle may allow the fate of viral RNA to be regulated, with respect to its use for either protein expression or as encapsidated genomic RNA.

The major, 21 kDa structural component of BNYVV is directly translated from RNA2, while the minor, 75 kDa structural protein is produced by read-through (RT) of the CP stop codon (Ziegler et al. 1985), leading to the synthesis of p75 (or CP-RT). This RT mechanism requires a predicted secondary structure following the CP stop codon (Firth et al. 2011), which explains the absence of p75 expression from the Δ49 mutant missing nt 712–813 (Tamada et al. 1996b). Deletion mapping of the CP and CP-RT genes highlighted the essential role of the RT domain in the encapsidation process and for the viral transmission (see Sect. 6.5), although these two gene products are dispensable for the efficient accumulation of viral RNA2 species and viral cell-to-cell movement (Schmitt et al. 1992; Tamada and Kusume 1991). BNYVV mutants expressing the CP alone or missing the N-terminal part of the RT domain are unable to produce viral particles (Schmitt et al. 1992; Tamada et al. 1996b). By contrast, mutants that retain the N-terminal part of the RT domain produce viral particles but in lesser amounts than the wild type (Schmitt et al. 1992).

A putative model of BNYVV encapsidation has been suggested in which p75 specifically recognizes the OA, favoring recruitment of CP species that in turn bind cooperatively to the RNA. Taking into account the structural stability of the 5′ UTR of viral RNAs (particularly RNA3), this process should require energy to destabilize base pairings. Thus, packaging could either be linked to a replication process that provides unwinding activity to allow for specific recognition of viral RNAs containing OA sequences, or packaging could hijack a cellular component around the energy-producing mitochondria to fulfill such a process. These two hypotheses are not mutually exclusive, as the initial recognition could be linked to the migration of nascent full-length viral RNA toward mitochondria along with the p75 protein to complete the encapsidation process. Another explanation for the mitochondrial association of virions could be linked to the transmission of the virus by *P. betae* during the plasmodium formation, where mitochondria organelle(s) are recruited in each zoospore or resting spore.

4.3 Movement

After a first cell is infected following wounding or vector transmission, plant viruses need to overcome the cell wall barrier, which prevents budding and receptor interactions for secondary infections. To overcome this extracellular matrix, phytoviruses take advantages of plasmodesmata (Pd), which consist of a continuum of the ER between cells to allow nutrient transfer and molecular signaling (Heinlein 2015b). Two main mechanisms have been described by which virus-encoded proteins modify Pd structures to allow the transport of infectious material from one cell to others (Heinlein 2015a). One mechanism is the formation of viral tubular structures between adjacent cells to transfer viral particles (Van Lent and Schmitt-Keichinger 2006). Such structures require the expression of viral movement protein(s) as well as virus production; functional CPs are thus required. The second mechanism is the expression of one (e.g., tobacco mosaic virus, TMV) or more (e.g., three for potato mop-top virus, PMTV; four for potato virus X, PVX; or five for beet yellows virus, BYV) viral proteins, which are targeted to Pd. Some viral movement proteins are able to modify the Pd size exclusion limit, and all such movement proteins allow the active transport of nucleoprotein complexes from infected cells to healthy neighbors (Lucas 2006). Depending on the virus, this mechanism may require ancillary CPs (Lucas 2006; Verchot-Lubicz et al. 2010). The specialized and conserved triple gene block (TGB) overlapping gene module is utilized by diverse helical plant RNA viruses (see Fig. 5.1), including members of the *Benyviridae* family (Morozov and Solovyev 2003; Verchot-Lubicz et al. 2010).

4.3.1 Cell-to-Cell Movement

Benyvirus movement depends on the expression of the TGB movement proteins but does not require the CP for its cell-to-cell movement. BNYVV TGB movement proteins are the translational products of subgenomic RNA components, i.e., RNA2sub-a and RNA2sub-b (Gilmer et al. 1992a). RNA2sub-a encodes the most abundant TGB movement protein, TGB1, whereas the bicistronic RNA2sub-b encodes TGB2 and TGB3 proteins; TGB3 is translated by a leaky scanning of the TGB2 start codon in the ribosome. The relative accumulation of TGB1, TGB2, and TGB3 has been estimated to be 100:10:1 for hordeiviruses (Jackson et al. 2009), but these abundance ratios are unknown for benyviruses.

BNYVV TGB1 is a 42 kDa protein that cooperatively binds RNA via its N-terminal domain. TGB1 also possesses ATP binding and HE domains. Substitution of residues in the N-terminal domain or in the HE domain can prevent cell-to-cell movement (Bleykasten et al. 1996). TGB1 itself is not addressed to Pd as shown by transient expression studies of TGB1-GFP or GFP-TGB1. For its function, TGB1 acts in concert with a tightly regulated expression of TGB2 and TGB3; by contrast, unregulated BNYVV protein expression or “hordei-like” TGB3 protein expression

inhibits cell-to-cell movement of BNYVV (Bleykasten-Grosshans et al. 1997; Erhardt et al. 2000; Lauber et al. 2005). TGB2 and TGB3 are 13 kDa and 15 kDa proteins, respectively, which both carry N- and C-terminal hydrophobic domains, presumably required for membrane association (Niesbach-Klosgen et al. 1990; Erhardt et al. 2005). Point mutations destabilizing these hydrophobic domains or the conserved central motif sequence of TGB2 ($^{41}GD_{x5}F_xNGG_xY_xDG_xK/R_{x3}Y/F_{x2}N^{66}$) result in the absence of viral movement without affecting its replication (Lauber et al. 2001). Altogether, TGB proteins ensure the recruitment of viral RNAs to help target infectious material into neighboring cells. However, the TGB cluster can be functionally replaced with the TMV movement protein or with a "hordei-like" TGB cluster (e.g., peanut clump virus, PCV) (see Fig. 5.1) expressed from two expression vectors (Lauber et al. 1998a).

Similar to the amplification process in infected cells, genomic RNAs also need to be recognized by cell-to-cell movement proteins to ensure their active transport. One could hypothesize that a specific RNA motif is recognized by the TGB movement protein complex by means of a particular RNA-binding domain in TGB1. Although TGB1 possesses a nucleic acid-binding domain in its N-terminal domain, this domain does not show specificity for binding viral RNA (Bleykasten et al. 1996). Moreover, heterologous complementation was achieved using the PCV TGB cluster, which would imply that BNYVV and PCV would need to share conserved RNA motifs and RNA-binding domains. But this hypothesis is not supported by the fact that TMV movement protein can complement BNYVV movement in mutants for TGB proteins. Hence, one could presume that BNYVV movement is linked to the RNA amplification and translation where RNA network formation together with protein expression (e.g., replicase and TGB proteins) ensure the correct targeting of RNAs to adjacent cells.

4.3.2 Long-Distance Movement

For BNYVV viral species compatible for cell-to-cell movement, the capacity for long-distance spread depends on the host plant and the viral genome composition (Table 4.1). No systemic movement occurs in *C. quinoa* or *T. expansa* host plants. By contrast, an inoculum consisting of only RNA1 and RNA2 is sufficient to systemically infect *Spinacia oleracea* and *Nicotiana benthamiana* plants (Lauber et al. 1998b; Andika et al. 2005), the other viral components behaving as accessories. However, replicon-derived RNA3 (Rep3) does not move long distance in such hosts, an observation that supports the existence of an RNA interaction network between genomic RNAs, as stated before. That is, if long-distance movement is linked to the completion of encapsidation, then Rep3 should move long distance just like other viral particles.

However, evidence indicating the requirement for the presence of a functional CP in viral systemic spread comes from the CP mutant (B25) $R^{119}S$ (Quillet et al. 1989). This suggests that RNA-RNA complexes are also bound to structural

Table 4.1 The systemic movement of BNYVV depends on the host species and genomic RNA composition

Plant host	Systemic	BNYVV RNA requirement
Chenopodium quinoa	No	LL with RNA1+2 min
Tetragonia expansa	No	LL with RNA1+2 min
Chenopodium murale	Yes	RNA1 + RNA2
Spinacia oleracea	Yes	RNA1 + RNA2
Nicotiana benthamiana	Yes	RNA1 + RNA2
Beta macrocarpa	Yes	RNA1 + RNA2 + RNA3[a]/RNA5[a]
Beta vulgaris	Yes	RNA1 + RNA2 + RNA3[a]

LL local lesion
[a]Coremin motif requirement

proteins, but not necessarily as completely protected viral RNAs. The latter hypothesis comes from RNase protection assays used to study long-distance movement of the CP-RT deletion mutant (Tamada et al. 1996b).

Viral systemic spread in *Beta macrocarpa* absolutely requires the presence of full-length RNA3 species. Interestingly, the RNA3 translation product is not required for viral systemic spread, which instead depends only on the "core" nucleotide sequence between nt 1147 and 1477 of RNA3 (Lauber et al. 1998b). More specifically, systemic spread depends on the presence of the so-called "coremin" motif, which was identified recently by performing systemic spread complementation of BNYVV carrying BSBMV RNA3 (Ratti et al. 2009). If the "coremin" motif is retrieved in other viral genus, it is present on BNYVV RNA5 3′ UTR and could account for the systemic movement of BNYVV carrying RNA1, RNA2, and RNA5 in *B. macrocarpa*. Mutagenesis of the "coremin" motif confirmed its essential role in long-distance movement of BNYVV within *Beta* species. Northern blot analyses allowed a direct link to be shown between "coremin" sequence preservation and production of RNA3sub, a subgenomic RNA3 species (Bouzoubaa et al. 1991; Balmori et al. 1993). The RNA3 domain essential for systemic movement, described by Lauber et al. (1998b), overlaps *cis*-acting sequences required for production of RNA3sub, reported by Balmori et al. (1993). RNA3 expressed under control of cauliflower mosaic virus (CaMV) 35S promoter also led to the accumulation of RNA3sub with the same 5′ sequence as the normal viral species, which indicates that BNYVV replication is not required for RNA3sub production. Therefore, RNA3sub is not a subgenomic RNA species per se but rather a noncoding RNA (ncRNA) stabilized by a "coremin" motif (thus called as ncRNA3); this motif is retrieved at the 5′ extremity of ncRNA3, two nucleotides downstream of a 5′ monophosphate. The absence of a 5′ Cap structure on RNA3sub confirmed that it does not function as an mRNA (Peltier et al. 2012).

Nucleotide substitutions within the "coremin" motif prevented both systemic movement and ncRNA3 accumulation (Peltier et al. 2012). Ectopic expression of BNYVV RNA3 in *Saccharomyces cerevisiae* allows the production of ncRNA3 except in an *Xrn1*-deficient strain, which suggests that ncRNA3 is the product of a

5′-3′ exoribonuclease acting on RNA3. Exoribonuclease presumably stalls on the "coremin" containing RNA structure leading to ncRNA3 accumulation in vivo. ncRNA3 accumulation has been reproduced in vitro using purified and monophosphorylated RNA3 and a commercial *Xrn1* enzyme (Flobinus et al. in preparation). The viral function of ncRNA3 during the infection process is still under investigation.

The discovery of noncoding properties of "RNA3sub/ncRNA3," which is not an elongation product of the BNYVV replicase, suggests that RNA2 subgenomic promoters should also be studied in more detail.

4.4 Pathogenicity Determinants and Counter-Defense Mechanisms

BNYVV has the remarkable capacity to behave like a bipartite virus when mechanically inoculated onto host plant leaves (Quillet et al. 1989). Therefore, RNA1 and RNA2 must themselves harbor pathogenicity determinants that can bypass some of the host innate immunity. Besides its core RdRp function, nothing is known about counter-defense mechanisms linked to the RNA1-encoded p237 protein or its proteolytic cleavage products. If such mechanisms exist, uncovering them will require extensive efforts. A counter-defense function for the cysteine-rich, 14 kDa protein (p14) encoded by RNA2sub-c had been suspected initially, because null mutations introduced in the p14 ORF diminished accumulation of progeny RNAs by about 10 to 100-fold; its function as a viral suppressor of RNA silencing (VSR) was discovered later (Gilmer et al. 1992a; Hehn et al. 1995; Chiba et al. 2013). The p14 properties and its VSR functions are described in Sect. 4.5. During its natural life cycle, BNYVV consists of either four or five positive-strand RNAs, depending on the isolate. The study of the incidence of each RNA species has been successfully performed, thanks to in vitro transcripts produced from cDNA clones, which made it possible to study the behavior of BNYVV "strains" composed of RNA1 and RNA2 supplemented with various combinations of small genomic RNAs.

4.4.1 RNA5 and p26 Protein

Virulence of BNYVV depends on the RNA species combination. Although the root infection phenotype is similar between BNYVV isolates, RNA5-containing viruses are reported to be more aggressive, particularly when infecting tolerant crops. These viral species accumulate at higher levels and often provoke enhanced foliar symptoms (Tamada et al. 1996a; Heijbroek et al. 1999).

RNA5 is about 1350 nt long and encodes a 26 kDa protein (p26) that resembles the BSBMV RNA3-encoded p29 protein (Gilmer and Ratti 2012). Local host inocu-

lation with an artificial isolate derived from in vitro transcripts provoked local necrotic lesions, which were associated with the expression of the p26 protein as null mutants induced mild local symptoms (Link et al. 2005). The p26 protein has been localized in the nucleus and the cytoplasm of infected cells, suggesting a role for this protein in the modification of a nuclear process that contributes to the viral infection. In yeast two-hybrid assays, the p26 protein fused to a DNA-binding domain protein (i.e., Gal4BD or LexA) induced a strong transcriptional activation (TA) of reporter genes in the absence of any prey, suggesting that p26 domain contains a TA domain (Link et al. 2005). This TA domain has been localized within the first 17 amino acid residues of the protein (Covelli et al. 2009). Interestingly, p26 proteins impaired in TA were still able to induce necrotic symptoms, suggesting that p26 could be recognized as an avirulence (Avr) protein by cellular proteins in *C. quinoa* species.

To complete this short molecular description of the RNA5 properties, one should focus on the presence of a "coremin" motif within the 3′ UTR of RNA5. This repeated motif on RNA species may contribute to viral pathogenicity. Indeed, it appears that viral isolates possessing two "coremin" motifs within their genome possess higher fitness for systemic spread (Gilmer unpublished data). Similarly, the BSBMV genome harbors two "coremin" motifs, in RNA3 and RNA4, and some cucumber mosaic virus isolates possess the motif in all their genomic RNAs, where it is referred to as "Box 1" (Thompson et al. 2008).

4.4.2 RNA4 and p31 Protein

RNA4 is 1467 nt long and encodes a 31 kDa protein (p31). p31 is required for an optimal viral transmission and, therefore, RNA4 species appear essential for an efficient and complete viral life cycle (Tamada and Abe 1989; Rahim et al. 2007) (see Chap. 3). The presence of RNA4 in an inoculum also affects symptom expression with different outcomes depending on the host plant species and affected tissues (Rahim et al. 2007). Recent studies performed in *N. benthamiana* host revealed a TA of pathogenesis-related protein 10 (Wu et al. 2014), a gene product never found in other global molecular approaches, such as RNA profiling or proteomic studies, conducted on sugar beet (Larson et al. 2008; Schmidlin et al. 2008). Next-generation sequencing transcriptome profiling of tissues infected with BNYVV in the presence or absence of RNA4 uncovered a specific effect of RNA4 (and therefore p31 expression) on RNA silencing gene expression (*AGO4*, *AGO5*, *AGO10*, *RNase III-like protein 2*) and ubiquitin-mediated proteasome pathways (Fan et al. 2014). However, nothing was known about the effect of RNA4 in *Beta* species host, particularly in root cells, even though some related gene products were retrieved in other global approaches (Larson et al. 2008; Schmidlin et al. 2008). Recently, differential transcriptome profiling of *B. macrocarpa* identifies sets of deregulated genes including some previously described (e.g., expansin, PR-1) (Fan et al. 2015).

4.4.3 RNA3, p25 Protein, N-Gene Product, and p4.6 ORF

RNA3 is 1773 nt long and constitutes the major pathogenicity determinant associated with the rhizomania syndrome, because the presence and expression of the 25 kDa protein (p25) exacerbates root and foliar symptoms (Tamada et al. 1989; Jupin et al. 1992). Besides the role of an RNA sequence required for long-distance movement in *Beta* species (described above), two other ORFs reside on RNA3.

p4.6 ORF A 120 nt long ORF (ORF S) is present in the 3′ UTR of BNYVV RNA3, which could encode a putative 4.5 kDa peptide of 39 amino acids. Due to the noncoding nature of ncRNA3 (formerly called "RNA3sub") this ORF appears to be cryptic. This is corroborated by mutagenesis of the AUG codon or introduction of frame shift mutations within this ORF, without the production of any phenotypic effect (Lauber et al. 1998b, unpublished data).

N Gene Infection experiments conducted with *in vitro* transcripts of the first three BNYVV genomic components provoked the appearance of RNA3 deleted forms, where the p6.8 ORF appeared either in frame with the p25 ORF or, instead, as the first ORF on the deleted RNA3 species. Such expression of the p6.8 ORF product gave a necrotic phenotype on local lesions. Necrosis was reproduced even when the ORF was expressed by CaMV (Jupin et al. 1992). The 6.8 kDa product was named N due to the necrosis induced by its expression. If it exists, the translation mechanism of the N protein from full-length RNA3 is unknown yet. Indeed, mutagenesis experiments disrupting the p6.8 ORF in full-length RNA3 did not lead to phenotypic changes during the infection of local host plants. Experiments conducted with the N gene fused to eGFP reproduced the necrotic phenotype with the localization of the fluorescent fusion protein at the cortical ER at the early steps of the infection (Gilmer unpublished data).

p25 Protein The p25 protein plays a decisive role in the BNYVV life cycle. p25 is a multifunctional protein that has been shown to modulate symptoms and gene expression and behave as an Avr gene product in *Beta vulgaris*. Ectopic expression of the p25 protein in sugar beet leaves induces a yellowing of the infiltrated area (Peltier et al. 2011), whereas constitutive transgenic expression appears lethal in host plants but not in nonhost plants such as *A. thaliana* (Peltier et al. 2011) or *Nicotiana tabaccum* (Peltier and Gilmer unpublished data). p25 shares no homologies with other proteins except for the p26 protein that also has the motif $^{137}Fx_3FRGPGNx_2L^{154}$. The p25 protein was first localized in the cytoplasm and nucleus of infected cells by immunogold labeling and TEM (Haeberle and Stussi-Garaud 1995), but no mutants were analyzed in that study. Later, GFP-fusion constructs allowed characterization of the p25 nuclear localization signal (NLS, ^{57}**KR**IRF**R**^{62}). Deletion mutants allowed the identification of a nuclear export signal (NES, ^{169}**V**YM**V**CL**V**NT**V**^{178}) (see Fig. 5.6a). The presence of both import and export signals suggests the possible shuttling of p25 between the cytoplasm and nucleus of infected cells, independently of other viral factors. p25 is subjected to

posttranslational modification as suggested by its electrophoretic mobility (Niesbach-Klosgen et al. 1990; Klein et al. 2007). HA-tagged p25 immunodetection experiments performed after 2D gel electrophoresis revealed multiple isoforms with different isoelectric points (Gilmer unpublished data). Preliminary mass spectrometry experiments confirmed the presence of phosphorylated residues in both the N- and C-terminus of the protein. Due to the low number of lysine and arginine residues in the second half part of the protein, the exact position of the second phosphorylation remains uncertain. However, alanine and aspartic acid scanning mutagenesis performed on phosphorylation sites uncovered differences in foliar symptom expression and changes in p25 biochemical properties, such as multimerization and TA. Indeed, interaction studies performed using p25 expressed as yeast two-hybrid bait and prey fusion proteins revealed the existence of self-interactions, which required the entire protein, because deleted forms were not able to interact anymore (Klein et al. 2007). *B. vulgaris* cDNA library screening allowed the identification of putative cellular proteins interacting with the p25 protein (Thiel and Varrelmann 2009). One of these interactions involves an F-box protein with a Kelch domain, which has been further characterized, allowing the proposition of a model in which p25 interacts with the F-box protein to affect 26S proteasome activity by interfering with F-box function (Thiel et al. 2012). As stated in Chap. 5, the highly variable tetrad motif of p25 is linked to the ability of the virus to overcome resistance (see Fig. 5.6). A BNYVV strain carrying the protein tetrad motif (aa 67–70) introduced in a B-type viral background displayed differential symptoms on *T. expansa* host leaves (Klein et al. 2007), as well as when p25 was modified in the NLS/NES sequences. Interestingly, expression of p25 fused to the GAL4 DNA-binding domain or LexA protein in yeast two-hybrid reporter strain induced TA of the reporter gene. A domain involved in this TA was identified within the C-terminal part of the protein (between amino acid residues 103–160).

Hence, an NLS, a zinc-finger domain (aa 66–90), and a domain with TA capabilities are typical properties of transcription factors, indicating that p25 protein could be a transcriptional modulator of host genes. Experiments from E. Savenkov were validated in D. Gilmer's lab, confirming the TA functions of p25 (unpublished data). The ability for TA of plant genes and the Avr nature of p25 (Chiba et al. 2008, 2011) may explain on the one hand, the inability of researchers to obtain viable transgenic *Beta* species and, on the other hand, the phenotypic and metabolic changes observed in *A. thaliana* expressing the p25 viral protein (Peltier et al. 2011). Indeed, independent *A. thaliana* lines homozygous for a transgene expressing the p25 protein displayed a root-branching phenotype that persisted after backcrosses. These transgenic lines also contained higher levels of auxin and lower amounts of jasmonic acid derivatives as compared to wild-type plants. Transcriptomic profiling led to the identification of more than 3000 deregulated genes. Together with observations correlating an absence of p25 expression with an absence of rhizomania symptoms on *Beta* species (Tamada et al. 1989) and later with mutagenized artificial isolates (Koenig et al. 1991; Jupin et al. 1992; Tamada et al. 1999), we can associate p25 expression with the root proliferation phenotype that is linked to deregulation of gene expression.

One remaining question concerns the regulation of p25 expression. Because the expression of Avr protein induces a cell death response, p25 expression early in the viral life cycle would be deleterious to the completion of this cycle. Therefore, p25 protein synthesis needs to be tightly regulated and has to occur after viral RNA amplification. This delayed or conditional expression may explain the difficulty to detect p25 after in vitro translation of RNA3 transcripts. Possible expression mechanisms include the requirement of a viral effector to trans-activate RNA3 translation or the coupled transcription/translation of RNA3 during replication as exemplified by (Allo) leviviridae maturase protein synthesis (Priano et al. 1997). This later hypothesis does not exclude the requirement of a viral effector. The 450-nt-long 5′ UTR of RNA3 could participate in this regulation of p25 expression. This could explain why no p25 was detected in yeast carrying an RNA3 expression vector, but p25 was highly expressed when the RNA3 5′ UTR was shortened (Gilmer unpublished data).

4.5 Silencing Suppression and Movement of BNYVV

Besides the replication complex, another key player in the BNYVV life cycle is the VSR. BNYVV infection experiments performed in GFP-silenced *Nicotiana benthamiana* 16C plants restored GFP production mainly within vascular tissues (Chiba et al. 2013). The VSR ORF encodes a 14 kDa cysteine-rich protein called p14 (Chiba et al. 2013), resides at the 3′ proximal RNA2 extremity, and is translated from the third subgenomic RNA species of RNA2 and RNA2sub-c (Gilmer et al. 1992a). Mutagenesis experiments disrupting p14 expression highlighted its important role in viral accumulation and long-distance movement in a systemic host as well. Before the discovery of antiviral RNA silencing mechanisms, p14 was thought to regulate viral RNA accumulation and CP production (Hehn et al. 1995). BNYVV p14 is a thermolabile protein that forms dimers (Chiba et al. 2013). TEM performed on immunogold-labeled infected tissues revealed an accumulation of p14 in the cytoplasm and in the nucleolar compartments. Such localizations were confirmed using GFP-fusion constructs observed by confocal laser-scanning microscopy, both inside and outside of the viral context. Site-directed mutagenesis allowed the identification of a nucleolar localization signal (NoLS, $^{74}K_LK_CKK_{QN}K_{NHS}K_{HVQNGYL}RK_VR^{97}$), which was dependent on a functional zinc-finger domain, involving C^{68}, C^{71}, C^{105}, and C^{108} residues. Recent analyses showed the stabilization of GFP expression within patch test experiments outside of the viral context. This hallmark of a viral suppressor of RNA silencing helped confirm the VSR function of p14. However, the strength of p14 VSR activity appeared lower than that of the polerovirus (turnip yellows virus) P0 in this assay (Chiba et al. 2013). Attempts to decipher p14 activity within the RNA silencing cascade revealed its ability to reduce secondary small-interfering RNA (siRNA) production. Moreover, p14 VSR activity has been uncoupled from its nucleolar localization (ibid). Therefore, p14 could presumably act as a VSR in the cytoplasm, affecting transitivity of plant RNAi, which may explain its

role in long-distance movement of the virus, and its inhibition of siRNA amplification and siRNA-mediated cell signaling. Together with p14 action in long-distance movement, it is worthwhile to present yet unpublished results of allelic mutants of p14 that can be complemented by the presence of RNA3 unless the coremin motif is mutated. Such complementation is still under investigation and could be linked to an inhibition of the 5′-3′ exoribonuclease leading to aberrant RNA accumulation, leading to a saturation of the RNA silencing machinery. If this hypothesis is confirmed, this would link the systemic spread of BNYVV to its counter silencing functions.

The p31 protein encoded by RNA4 could represent another actor in the suppression of the antiviral RNA silencing response. Although p31 does not display a VSR activity in leaves, its expression in *N. benthamiana* roots enhances silencing suppression (Rahim et al. 2007). The mechanism of this effect is still unknown. Furthermore, there is no evidence for the existence of a similar pathway in roots of *Beta* species.

4.6 Future and Prospects: RNA-RNA Network in the Regulation of "a BNYVV Life"

While many efforts have centered on establishing relationships between the structure and the function of BNYVV-encoded proteins, the regulatory role of viral RNA structures has been neglected, with the notable exception of the 5′ and 3′ UTRs, which participate in viral RNA amplification. Stepping back to look at the initiation and progression of the viral life cycle within the host, taking into account known properties of cellular defense effectors, it seems evident that viral genome expression must be finely tuned.

Starting from the initial inoculation step from infested soil (Fig. 4.1, upper left panel), viruliferous resting spores germinate and a zoospore migrates to then encyst to the plant root cell. While the viral life cycle has been thought to occur in the planktonic form of the vector (Verchot-Lubicz et al. 2007), if one takes into consideration the obligate parasite form of *P. betae*, which requires a host cell for its energy renewal and reproduction, an active replication/encapsidation of the viral genome in the vector would be detrimental to viruliferous *P. betae* fitness versus the aviruliferous form. In such a situation, the viruliferous vector would burn ATP more rapidly than the aviruliferous one, leading to clearance of the BNYVV-containing vector. This is one of the reasons why I argue that there is no replication step inside the vector. The zoospore content is then transferred into the root cell cytoplasm, where the viral particles are released. Osmotic changes, ionic strength, or the presence of cellular factors dictate a partial uncoating of the rod-shaped virions, supposedly at both extremities. This uncovers both 5′-capped and 3′-polyadenylated UTR structures on all the viral particles. Taking into account the position of the initiation codons that serve for the translation of 5′ proximal ORFs, only RNA1-encoded

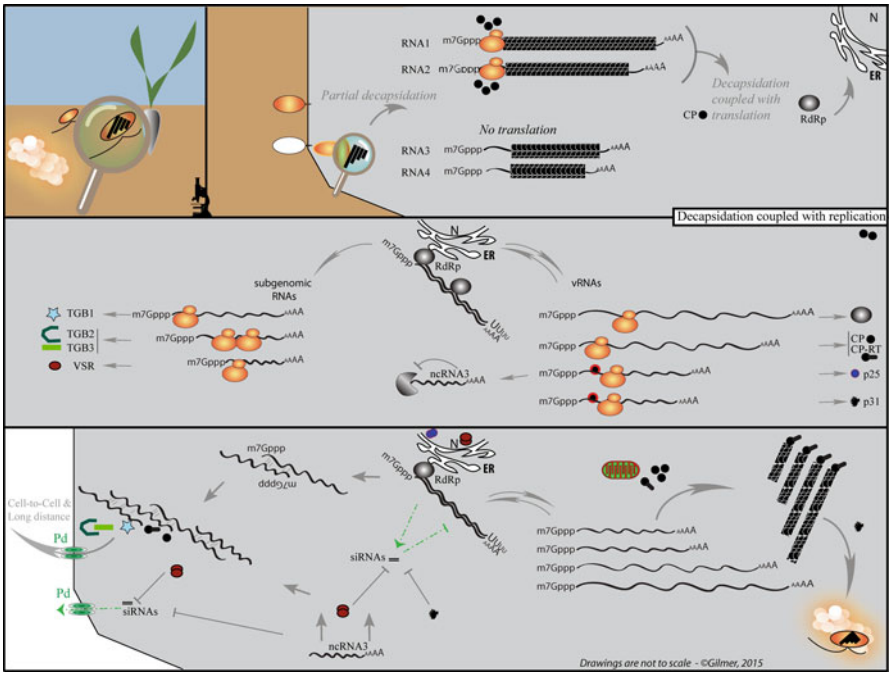

Fig. 4.1 Drawing of a putative BNYVV infection cycle. *Polymyxa betae* sporosores and zoospores are responsible for the delivery of viral particles (*black rods*) in the cytoplasm of root cells. After partial uncoating, stripping of CP subunits (*black dots*) of the RNA is coupled with ribosomal translation and thereafter with viral replication. Replication machinery amplifies genomic RNAs and ensures the production of subgenomic RNAs required for the synthesis of movement proteins (TGB1, TGB2, TGB3, and viral suppressor of RNA silencing). While replication is effective, small genomic RNAs become compatible for translation thanks to an unknown effector (*red-circled black dot*). Exoribonuclease stalls on ncRNA3 during RNA decay allowing inhibition of its cellular activities. Newly produced viral RNAs undergo on the one hand packaging for efficient loading into newly formed zoospores or sporosores. This step requires CP and CP-RT subunits and probably an association with mitochondria. On the other hand, nascent viral RNAs associate to form an RNA-RNA network able to maintain viral formula within the traveling complex essential for the long-distance movement within the plant vascular system. This complex exits the cell using existing plasmodesmata. Cap *m7Gppp*, polyadenylated sequence AAAA, *N* nucleus, *Pd* plasmodesmata, *VSR* viral suppressor of RNA silencing

p237 ($_{154}$AUG$_{156}$) and RNA2-encoded CP ($_{145}$AUG$_{147}$) are available for ribosomal initiation of translation, whereas RNA3-encoded p25 ($_{446}$AUG$_{448}$) and RNA4-encoded p31 ($_{380}$AUG$_{382}$) [RNA5-encoded p26 ($_{439}$AUG$_{441}$)] remain inaccessible. Partial uncoating of RNA1 and RNA2 would favor a coupled uncoating to assist translation of, on the one hand, the replicase component (RdRp) and, on the other hand, a few CP subunits (Fig. 4.1, upper right panel). Replicase association with the ER would form a replication complex that would then recognize 3′ UTRs and initiate synthesis of complementary viral RNAs, releasing the remaining CPs associated with viral matrices. In this situation, CP-RT synthesis is not believed to occur,

because the structural elements required for read-through remain masked by CP subunits; therefore, only virus-released and newly synthesized CP subunits are present in the cytoplasm. A similar model, named "striposome," has been described for TMV (Michael and Wilson 1984; Wilson 1984).

Why is RNA3 not translated immediately? As p25 (AVR) expression triggers plant defense responses leading to cell death, an immediate translation of the protein would destroy the replication-competent cell and thus eliminate the viral RNAs. This is corroborated by the low efficiency of RNA3 translation *in vitro* (Gilmer unpublished data). Hence, before the translation of small genomic RNAs, BNYVV RNA1 (RdRp) and RNA2 products (TGB proteins and VSR) take control of the cell fate to limit plant cell defense mechanisms. In such a situation, RNA3 and RNA4 will remain "silent" until a "riboswitch" event (Fig. 4.1, center panel, red-circled black dot) triggers the expression of these small genomic RNAs. Such a switch could consist of an RNA-RNA interaction regulated by nascent viral RNA production or an interaction of the 5′ UTR with a viral protein allowing p25 and p31 protein production. Viral RNA accumulation will then face cellular RNA degradation pathways, allowing the production of ncRNA3 thanks to a 5′–3′ exoribonuclease (Xrn) activity. Moreover, ncRNA3 accumulation has been shown to inhibit Xrn activity in vitro (Flobinus et al. in preparation). This inhibitory effect of ncRNA3 on a key player of the RNA degradation pathway is supposed to induce the accumulation of uncapped "aberrant" cellular RNA, known substrates of the silencing machinery. Silencing of *N. benthamiana* XRN4 was shown to enhance the systemic movement of TMV (Peng et al. 2011) and other viruses, such as a tombusvirus (tomato bushy stunt virus) (Jaag and Nagy 2009). A possible explanation for this effect could be the saturation of the silencing machinery by dsRNA and then siRNAs produced from uncapped and non-processed cellular mRNAs. This will produce a decoy effect that diverts the cellular Dicer-like proteins to reduce antiviral silencing defense. A coup de grâce will then be given by VSR action. However, the simultaneous but contravening effects caused by the saturation of the silencing machinery and inhibition of silencing factors by VSR action would need to be resolved. Gene expression levels and cellular and viral protein turnover could explain the requirement of both pathways for a fine-tuned regulation of the viral infection progression. Whatever the mechanisms, it appears that movement proteins together with both VSR function and ncRNA productions are required for the long-distance movement of the BNYVV genome.

Viral long-distance movement was thought to involve virions because deletion of the CP impairs systemic spread. However, given that mobile viral particles encounter a phenomenon similar to size exclusion chromatography in vascular conduits, small particles will be rapidly separated from larger ones, leading to a depleted viral formula in more distant cells. One solution for the maintenance of viral genome integrity would be a close interaction between viral genomic RNAs. Such RNA-RNA complexes could then be recognized by viral proteins (TGB, VSR, and presumably CP/CP-RT) to allow them to be transported cell to cell and long distance in the plant.

Preliminary experiments performed in collaboration with Dr. C. Ratti (Dall'Ara et al. unpublished data) support this hypothesis. These investigations have opened new questions about the plant response to systemic infection and should be extended to help us understand other multipartite plant viruses. Plants could prevent the systemic spread of viruses either by protein expression or simply by generating competing RNA sequences that interfere with viral RNA-RNA interactions. Understanding these phenomena is a new challenge for plant virologists.

Acknowledgments I would like to thank current and past members of my team, worldwide collaborators for the generous discussions and brainstorming, and Dr. Todd Blevins for his help in language editing. A special acknowledgment is reserved for my mentor Prof. Gérard Jonard.

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Chapter 5
Genetic Diversity of Beet Necrotic Yellow Vein Virus

Tetsuo Tamada, Hideki Kondo, and Sotaro Chiba

Abstract Beet necrotic yellow vein virus (BNYVV) is a member of the genus *Benyvirus* in the family *Benyviridae* with multipartite positive-sense single-stranded RNA genomes encapsidated in rigid rod-shaped particles. The members of *Benyvirus* including four species are similar to those of the family *Virgaviridae*, in respect of viral particle assembly, movement, and plasmodiophorid transmissibility. Recent studies revealed that ancestors and/or relatives of benyviruses may have infected a wide range of hosts such as plants, insects, algae, and fungi. For phylogenetic analyses of BNYVV genes, worldwide BNYVV isolates form four clades, A-I, A-II, A-III, and B, from which at least ten subgroup isolates (strains) have derived. These original BNYVV types and their progeny strains might have existed in East Asia, and each source had introduced infection to cultivated sugar beet plants and might have spread worldwide only in the last half century. Along with the growth of resistant varieties in rhizomania-infested areas since the 1980s, strong selection pressure has been imposed on the RNA3-encoded *p25* gene, and, consequently, resistance-breaking variants that have single amino acid changes in the p25 protein have been generated. RNA5-encoded *p26* gene is also associated with resistance breaking as well as symptom severity in sugar beet roots.

Keywords Sugar beet • Rhizomania • BNYVV • Benyvirus • *Virgaviridae* • Plasmodiophorids • Evolution • Nucleotide diversity • Resistance breaking

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E. Biancardi, T. Tamada (eds.), *Rhizomania*, DOI 10.1007/978-3-319-30678-0_5

For plant viruses and other organisms, variation is an intrinsic property. As organisms reproduce, individuals that differ genetically from their parents are generated by mutations, and their progeny may result in variants. In plant viruses, genomic variation caused by mutation is enhanced by recombination, reassortment, and acquisition of extra-genomic components. The distribution of genetic variants in populations of viruses is likely to contribute to their evolutional history and the resulting taxonomic relationships among them. In general, small genomes of RNA viruses are tightly packed with information and overlaps of coding and regulatory sequences and with different coding sequences. In particular, the few encoding proteins have different functions in the virus life cycle, imposing different selection pressures on the corresponding genes (Garcia-Arenal et al. 2001). Furthermore, recent nucleotide sequence databases for viral and cellular genomes and transcriptomes have led to the discovery of non-retrovirus RNA viral sequences integrated into the genomes of diverse eukaryotic organisms (Kondo et al. 2013). In this chapter, we address taxonomic positions and evolutional origins of beet necrotic yellow vein virus (BNYVV), BNYVV variation, geographical origins and migration, and virulence changes (i.e., host resistance breaking).

5.1 Benyviruses

5.1.1 Origin of Benyviruses

BNYVV is the type member of the genus *Benyvirus* belonging to the family *Benyviridae*. The virus has multipartite, positive-sense, single-stranded RNA [(+) ssRNA] genomes encapsidated in rigid rod-shaped particles (Gilmer and Ratti 2012; Gilmer et al. 2013). With rod-shaped particles, benyviruses are similar to viruses in the members of the family *Virgaviridae*, which include six genera: *Tobamovirus*, *Furovirus*, *Hordeivirus*, *Pecluvirus*, *Pomovirus*, and *Tobravirus*, with a proposed genus *Goravirus* (Adams et al. 2009; Gilmer and Ratti 2012; Sekine et al.2015) (Figs. 5.1 and 5.2). Two rod-shaped viruses, Nicotiana velutina mosaic virus (NVMV) (Randles and Rohde 1990) from a *Nicotiana* plant and Chara australis virus (CAV) (Gibbs et al. 2011) from a freshwater alga, are taxonomically unclassified, but they share some characteristics with benyviruses and virgaviruses (Fig. 5.1). Tobamoviruses (including tobacco mosaic virus) and CAV have a monopartite genome, whereas other virgaviruses and NVMV have multipartite RNA genomes (Fig. 5.1). Furo-, peclu-, and pomoviruses, like benyviruses, are transmitted by plasmodiophorid protists, and tobraviruses are transmitted by nematodes. Hordei- and pecluviruses (and probably goraviruses) are seed transmissible.

There are several discriminating properties between viruses of the family *Virgaviridae* and *Benyviridae*: phylogenetic relatedness, genome organization, and expression strategy (Tamada 1999; Adams et al. 2009) (Fig. 5.1). First, the respective replicase-associated proteins (methyltransferase [MT], helicase [HE], and

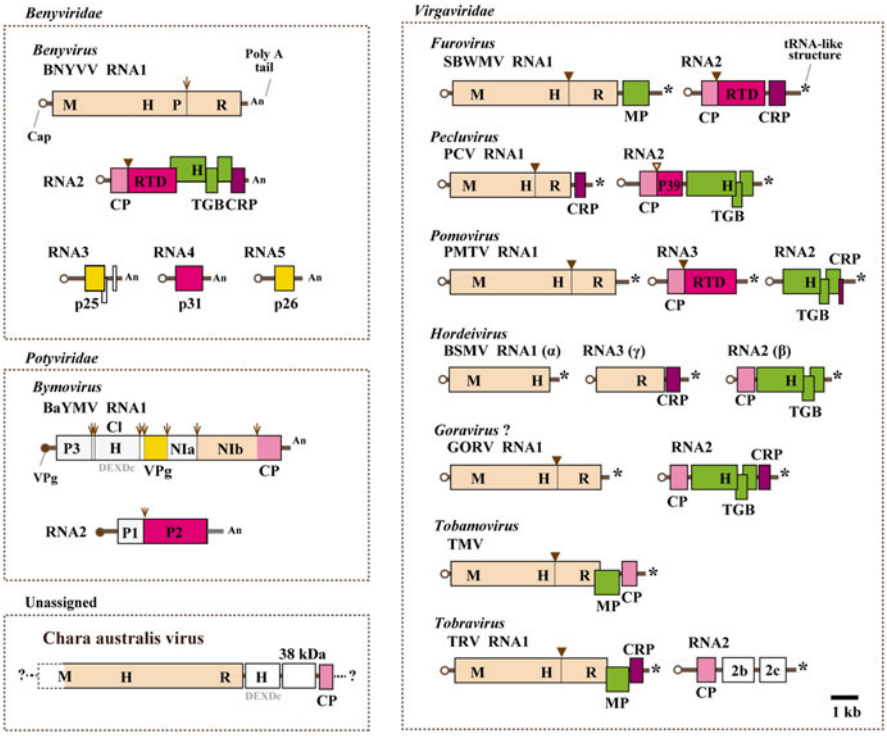

Fig. 5.1 The genome structure of plasmodiophorid-transmitted viruses and other related, rod-shaped viruses. Type members of the genus shown: *BNYVV* beet necrotic yellow vein virus, *BaYMV* barley yellow mosaic virus, *SBWMV* soil-borne wheat mosaic virus, *PCV* peanut clump virus, *PMTV* potato mop-top virus, *BSMV* barley stripe mosaic virus, *GORV* gentian ovary ring-spot virus, *TMV* tobacco mosaic virus, *TRV* tobacco rattle virus. Marks in domains are as follows: *M* methyltransferase domain, *H* helicase domain (including DEXDc domain), *R* RNA-dependent RNA polymerase domain, *MP* movement protein, *CP* coat protein, *RTD* readthrough domain, *CRP* cysteine-rich protein, *TGB* triple gene block proteins, *P* protease, *CI* cytoplasmic inclusion protein, *VPg* genome-linked protein, *NIa* nuclear inclusion protein a-protainase, *NIb* nuclear inclusion protein b (including RNA-dependent RNA polymerase), *P1* cysteine proteinase, *P2* putative vector transmission factor. The filled triangles, open triangle, and arrows indicate translation readthrough sites, leaky scanning site, and protease cleavage sites, respectively

RNA-dependent RNA polymerase [RdRp] domains) of benyviruses show higher similarity to those of the animal viruses of the family *Togaviridae* and *Hepeviridae* than to those of other rod-shaped plant viruses (Fig. 5.2). Second, benyvirus genomic RNAs have capped and polyadenylated structures at the 5′ and 3′ ends, respectively. Virgavirus genomic RNAs are similarly capped at the 5′ termini, but their 3′ ends have tRNA-like structures instead of the polyadenylated form (Fig. 5.1). Third, the replication-associated protein of benyviruses is encoded by a single open reading frame (ORF) producing a large polyprotein that is autocatalytically processed to give two species of products (Fig. 5.1). In contrast, virgaviruses

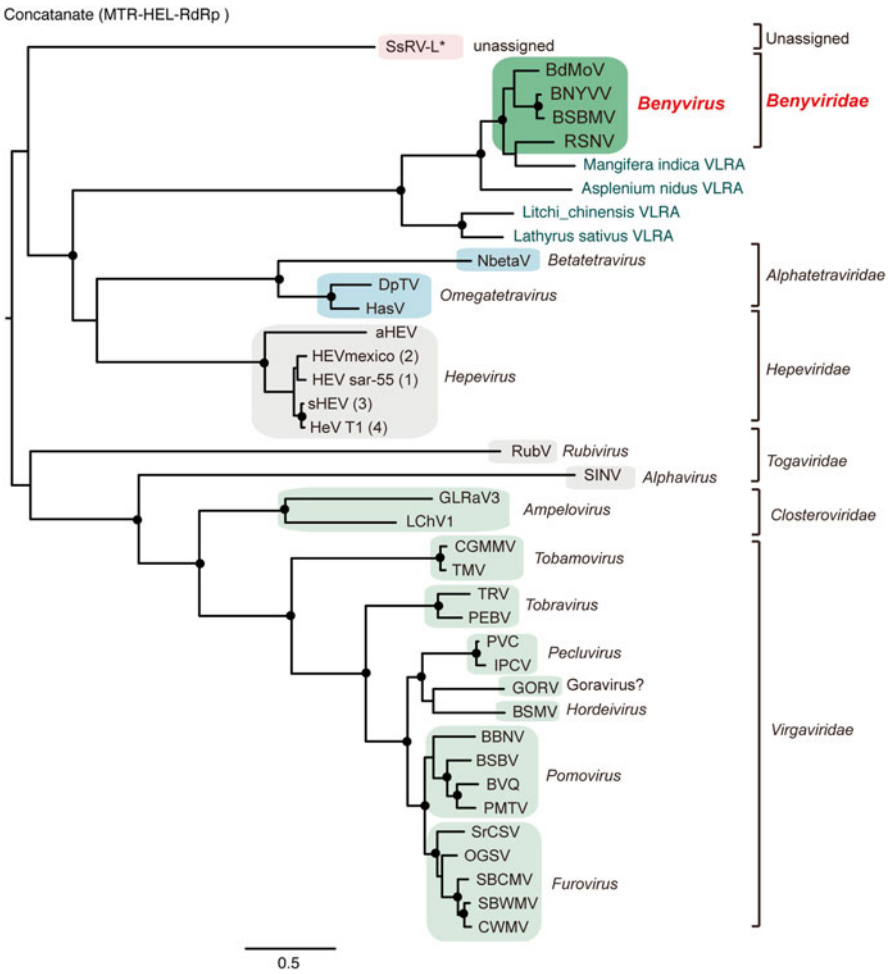

Fig. 5.2 Maximum likelihood (ML) phylogenetic tree based on the concatenated amino acid sequences of three domains (MT–Hel–RdRp) in the replicase proteins of benyviruses and other related RNA viruses. Closed circles on the nodes represent highly supported branches by aLRT analyses with the SH-like calculation (values greater than 0.9 are shown)

contain replicase-associated motifs on two ORFs, separated by a leaky stop codon (tobamo-, furo-, pecul-, pomo-, and tobraviruses) or by two respective genomic segments (hordeiviruses) (Fig. 5.1).

In contrast to such dissimilarity of the genomes, phylogenetic analyses of the coat protein (CP) show that benyviruses are included in a cluster of virgaviruses, in which they are more closely related to furo- and pomoviruses than to peclu-, hordei-, and tobamoviruses (Fig. 5.3). For movement proteins (triple gene block [TGB]), benyviruses are distantly related to peclu-, hordei-, and pomoviruses, but less distantly related to NVMV (Kondo et al. 2013). A remarkable common charac-

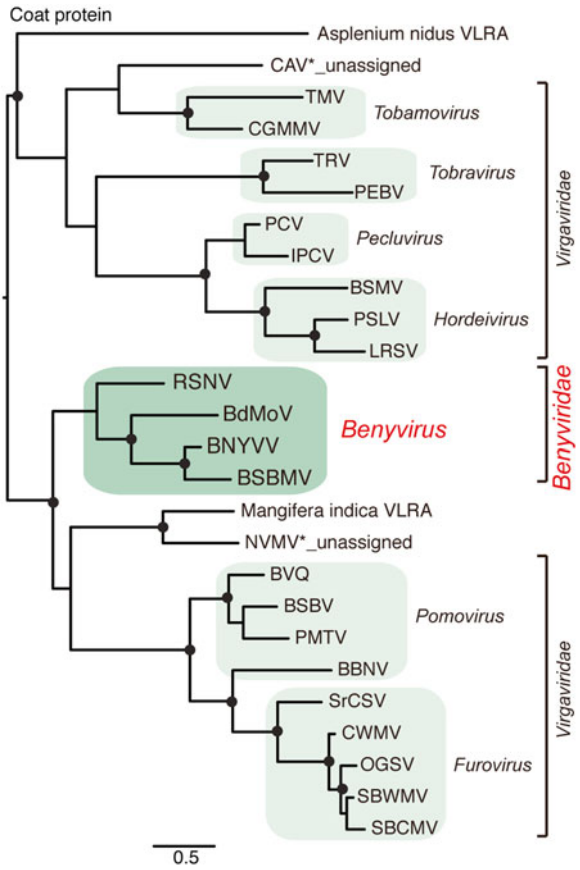

Fig. 5.3 Phylogenetic (ML) tree based on the coat proteins of benyviruses and selected virgaviruses. Closed circles on the nodes represent highly supported branches in an aLRT analysis with the SH-like calculation (values greater than 0.9 are shown)

teristic of beny-, furo-, peclu-, and pomoviruses is their transmissibility by plasmodiophorids (Tamada and Kondo 2013). These viruses have common elements: the CP-readthrough (CP-RT) domain and the analogue (p39 of pecluviruses) (Fig. 5.1). For BNYVV and potato mop-top virus (PMTV, pomovirus), the minor capsid proteins (CP-RT) are present at one extremity of the virus particles strongly implicated in vector transmission (Tamada and Kondo 2013).

Only three species (*Polymyxa graminis*, *P. betae*, and *Spongospora subterranea*) of the 41 species in the order Plasmodiophorida are recognized as plant virus vectors (Tamada and Kondo 2013). Some species of plasmodiophorids are known as parasites of aquatic angiosperms or brown algae. These organisms were reported to host several marine viruses with (+) ssRNA genomes, in which the virus is transmitted via water upon lysis of the host cell (Lang et al. 2009). Plasmodiophorids may

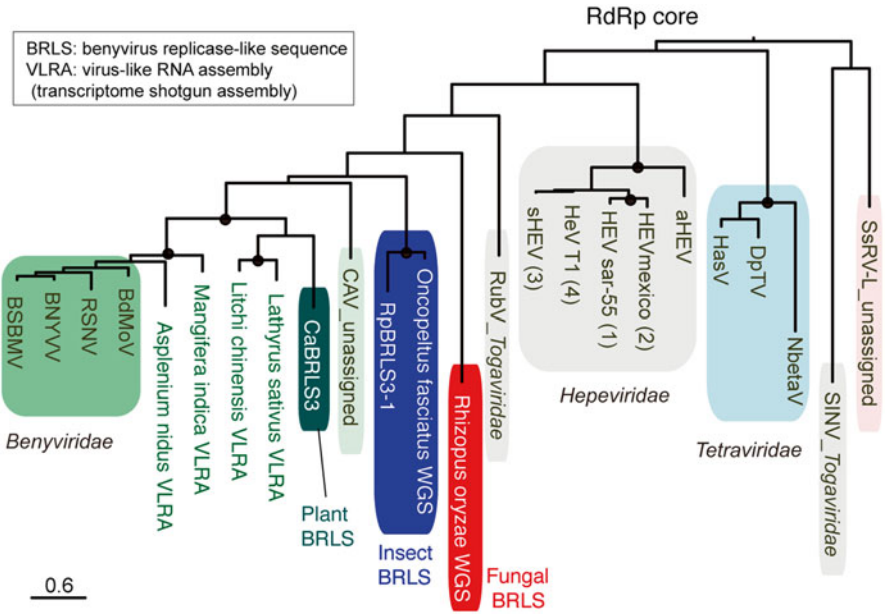

Fig. 5.4 Phylogenetic (ML) tree based on the RdRp core domain of benyviruses and their related sequences (BRLSs) together with other RNA viruses. Closed circles on the nodes represent branches from an aLRT analysis using the SH-like calculation (values greater than 0.9 are shown)

serve as vectors for entry through the thick cell walls of the host organism (Neuhauser et al. 2011). Although there are no relationships between such marine viruses and plasmodiophorid-transmitted plant viruses, the discovery of CAV from a freshwater green alga is worth noting because CAV has rod-shaped particles and its polymerase is most closely related to that of the benyviruses (Gibbs et al. 2011) (Figs. 5.1 and 5.4). Identification of a natural vector of CAV, possibly a plasmodiophorid, will provide important information.

On the other hand, Kondo et al. (2013) reported that benyvirus replicase-related sequences (BRLSs) are present in whole-genome shotgun assemblies of the chromosomes of the chickpea plant (*Cicer arietinum*) and a blood-sucking insect (*Rhodnius prolixus*), which might be fossil records of ancestral benyviruses in plant and insect genomes (Fig. 5.4). Similarly, the authors also found novel BRLSs in the genome of a milkweed bug, *Oncopeltus fasciatus*, and a zygomycete filamentous fungus, *Rhizopus oryzae* (H. Kondo, unpublished results). Furthermore, other benyvirus-like sequence fragments were found in the transcriptome shotgun assembly libraries of a few species of plants and a species of bark beetle. Most recently, several near-complete benyvirus-like sequence assemblies (VLRAs) in public databases were found from four plants, mango (*Mangifera indica*), a fern (*Asplenium nidus*), litchi (*Litchi chinensis*), and grass pea (*Lathyrus sativus*) (Morozov and Solovyev 2015) (Figs. 5.2 and 5.4). These data strongly suggest that ancient beny-

viruses and their relevant viruses had a much wider range of hosts including plants, insects, algae, and fungi.

5.1.2 *Members of the Genus* Benyvirus

The family *Benyviridae* contains one genus *Benyvirus* with four species: beet necrotic yellow vein virus (BNYVV), beet soil-borne mosaic virus (BSBMV), rice stripe necrosis virus (RSNV), and burdock mottle virus (BdMoV) (Gilmer et al. 2013). *Virus*, rice necrosis, burdock mottle virus. For phylogenic relationships among replicase proteins of the four benyviruses, BNYVV and BSBMV are most closely related to each other, and both are more closely related to BdMoV than to RSNV (Fig. 5.2). Beet soil-borne mosaic virus (BSBMV), burdock mottle virus (BdMoV) rice necrosis (RSNV). Benyvirus RNA1 and RNA2 encode housekeeping genes required for virus replication, assembly, cell-to-cell movement, and RNA silencing suppression (Tamada and Kondo 2013). BNYVV and BSBMV have additional small genome components such as RNA3 and RNA4, but no smaller segments have yet been reported for RSNV and BdMoV.

Beet necrotic yellow vein virus The virus has spread globally to all major areas where sugar beets (*Beta vulgaris* subsp. *vulgaris*) are grown since it was first found in Italy in the 1950s, but its origin is suggested to be in East Asia as described next section (Chiba et al. 2011). General features of BNYVV are described in Chap. 3.

Beet soil-borne mosiac virus The virus was first identified in Texas in 1988 as part of a complex of viruses associated with BNYVV (Rush and Heidel 1995). BSBMV is widely distributed only in the USA and has not been reported in other countries. Unlike BNYVV, the roots of BSBMV-infected sugar beets are generally asymptomatic. Systemic foliar symptoms commonly found on field-grown sugar beets include light leaf distortion, faint mottling, and light yellow vein banding (Heidel and Rush 1994; Rush and Heidel 1995). The predicted ORFs on BNYVV and BSBMV show 23–83 % amino acid identity and overall nucleotide sequences are 35–77 % identical (Lee et al. 2001). Like BNYVV, BSBMV has additional genomic components that encode homologous proteins with a moderate degree of identity (Heidel et al. 1997; Lee et al. 2001). Both viruses have a similar limited host range and are transmitted by the same vector *Polymyxa betae* (Rush 2003).

Rice stripe necrosis virus The virus was first observed in rice plants (*Oryza sativa*) on the Ivory Coast in Africa in 1977 (Louvel and Bidaux 1977) and recently reported in its neighboring countries, Burkina Faso, and Benin (Oludare et al. 2015; Sereme et al. 2014). RSNV has two genomic components RNA1 and RNA2 (Lozano and Morales 2009) and is transmitted by *Polymyxa graminis* (Morales et al. 1999). Infection may cause early seedling death and severe plant malformation; virus-infected rice plants develop a conspicuous chlorotic stripe, systemic necrosis, and stunting. In mature plants, panicles are malformed, causing a decrease in seed pro-

duction. Outside Africa, RSNV was found in the Department of Meta in the Eastern Plains of Colombia in 1991 (Morales et al. 1999). By 1994, the disease had spread to most of the rice-producing municipalities in the region. RSNV and its vector *P. graminis* were also detected in Ecuador, Panama, and Brazil. Although no severe outbreaks of RSNV have yet been reported in these countries, this virus disease is a considerable threat to rice production in South America (Lozano and Morales 2009).

Burdock mottle virus The virus was found in burdock plants (*Arctium lappa*) in Japan (Inouye 1973). Burdock, a domestic root vegetable crop in Japan, is a biennial plant belonging to the Asteraceae family. The virus usually causes mild chlorosis or mottling symptoms on the leaves of burdock plants. It is transmissible to several plant species by mechanical inoculation with sap, but its natural vector is unknown. BdMoV has two genomic components (RNA1 and RNA2). Kondo et al. (2013) found an AlkB-like domain, which is a member of the 2-OG-Fe(II) oxygenase superfamily, in the replicase protein encoded by BdMoV RNA1. Viral AlkB proteins and their homologues have been identified in a number of plant (+) ssRNA viruses in the family *Betaflexiviridae* and some viruses in the *Alfaflexiviridae* (order *Tymovirales*) (Martelli et al. 2007), but are not present in other benyviruses and rod-shaped viruses in the family *Virgaviridae* (Kondo et al. 2013). BdMoV is associated with characteristic viroplasm-like inclusions in the cytoplasm of virus-infected plant cells, but such inclusions are not found in other benyviruses (Kondo et al. 2013). BdMoV might thus have uniquely evolved from ancestral benyviruses, a scenario supported in part by its distant phylogenetic relationship with other members (Fig. 5.2).

5.2 Variation

5.2.1 Phylogenetic Characterization

Previously, BNYVV isolates were simply classified into two groups, A and B types, based on their *CP* genes (Kruse et al. 1994; Koenig et al. 1995; Saito et al. 1996). An additional group, P type, that contains an additional RNA segment (RNA5) was isolated from the Pithiviers area of France (Koenig et al. 1997), but it is closely related to the A type. A-type BNYVV is distributed worldwide, whereas the B-type virus has been found only in limited areas of Europe, although isolates with the B-type *CP* gene were also found in Japan (Miyanishi et al. 1999) and in China (Li et al. 2008). RNA5-containing viruses occur in small limited areas of Europe, but are widely distributed in China and in Japan (Koenig and Lennefors 2000; Li et al. 2008). These Japanese and Chinese isolates containing RNA5, called the Japanese type (J type), are distinguishable from the other three types of BNYVV.

In order to clarify this BNYVV variation, Schirmer et al. (2005) analyzed *CP* (RNA2), *p25* (RNA3), and *p26* (RNA5) of BNYVV isolates worldwide, collected mainly from Europe, and Chiba et al. (2011) later analyzed these genes plus *p31* (RNA4) from worldwide isolates of BNYVV, including many Asian isolates. These phylogenetic results can be summarized as follows:

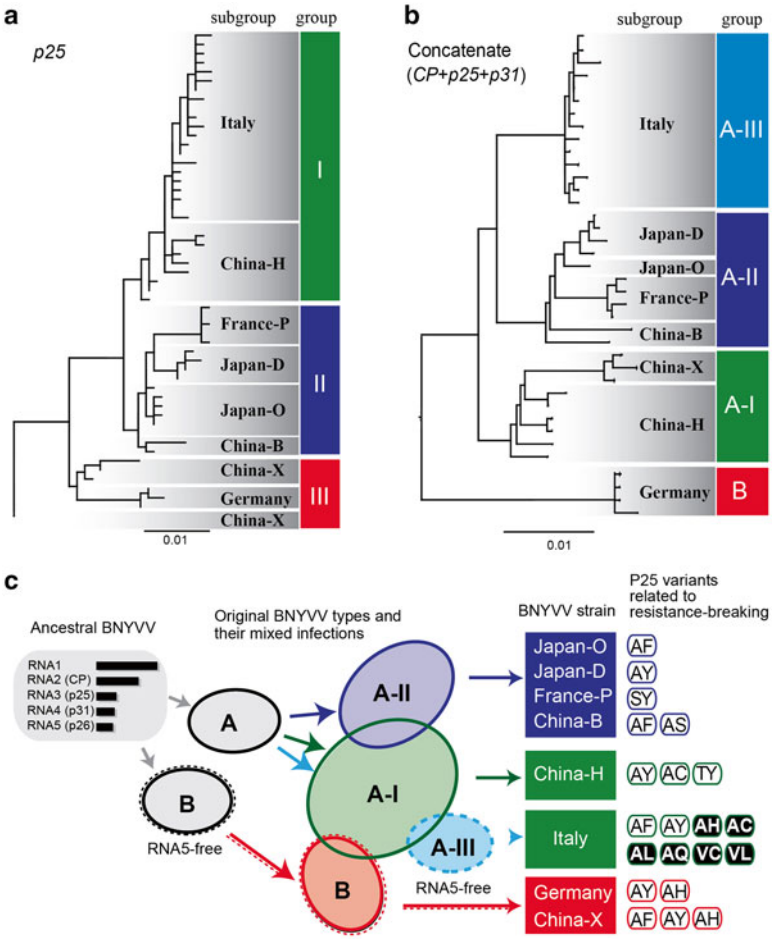

Fig. 5.5 Phylogenetic (ML) tree of BNYVV isolates based on the RNA3-*p25* gene (**a**) and the concatenated sequences of three genes (RNA2-*CP*+RNA3-*p25*+RNA4-*p31*) (**b**). (Redrawn and modified from Chiba et al. (2011)). For the concatenated tree, nine potential reassortant isolates containing anomalous sequences were removed. (**c**) Model of evolutional steps for BNYVV strains. Four original BNYVV types (A-I, A-II, A-III, and B) correspond to the groupings of the concatenated tree (**b**). Represented BNYVV strains were based on a subgroup of the p25 tree. *Solid- or dash-lined circles* indicate BNYVV populations with and without RNA5, respectively. Names of p25 variants are based on the p25 amino acid residues at positions 67 and 68, which are associated with resistance breaking (RB). *Black boxes* indicate *Rz1*-RB types; *white boxes* indicate non-RB types

- The *CP* sequences comprise two distinct groups, A- and B-type CP.
- The *p25* sequences have three main groups (p25-I, p25-II, and p25-III) that can be divided into several subgroups that each contains isolates from a single geographical region (Fig. 5.5a).

Table 5.1 Geographical distribution of BNYVV strains derived from different original types (From data of Chiba et al. (2011) and Zhuo et al. (2015))

Original type	Strain	CP	p25	p31	RNA5	Distribution
A-I	China-H	A or B	I	I	+	China, Japan, UK
A-I	China-Y	A or B	I	I	+	China
A-II	France-P (=P type)	A	II	II	+	France, Kazakhstan, UK, Iran
A-II	Japan-D	A	II	II	+	Japan
A-II	Japan-O	A	II	I or II	+	Japan
A-II	China-B	A or B	II	II	+	China, Germany
A-II	China-L	B	II	I	+	China
A-III	Italy (=European A type)	A	I	III	−	Europe, USA, Middle East
B	China-X	A	III	I or III	+	China
B	Germany (=B type)	B	III	IV	−	Germany, France, Belgium, Austria, Switzerland, Czech Republic, China

Groupings are based on the phylogenetic trees, in which *CP*, *p25*, and *p31* genes form two (A and B), three (I–III), and four (I–IV) groups, respectively
+ = RNA5 present
− = RNA5 absent

- The *p31* sequences cluster into four groups (p31-I, p31-II, p31-III, and p31-IV), which correspond, with a few exceptions, to the four clades of the phylogenetic tree constructed based on concatenated sequences (Fig. 5.5b).
- The *p26* sequence (RNA5) is present in most isolates, except those in the Italy and Germany subgroups, and fell into three groups (p26-I, p26-II, and p26-III), one of which contains the P-type isolates and another only two Japanese isolates.
- The concatenated analyses from joining the three essential sequences (*CP*, *p25*, and *p31*) while excluding possible reassortants showed that most BNYVV isolates form four distinct lineages (clades), named A-I, A-II, A-III, and B (Fig. 5.5b). Of the European strains, the A-type cluster was included in clade A-III and the P type in A-II.

On the basis of these phylogenetic analyses, Chiba et al. (2011) proposed the early presence of four types of BNYVV populations that contained different combinations of three to four BNYVV genes and probably mixtures of these types (Fig. 5.5c). If an ancestral BNYVV population contained five RNA components, RNA5-lacking variants could occur commonly during evolution or spread because RNA5 is not essential for BNYVV infectivity (at least in the known host plants). When the *CP*, *p25*, and *p31* gene sequences are considered, an original BNYVV population would have split into two types, A and B, and, subsequently, the A-type population would have separated into three subtypes (Fig. 5.5c): A-I and A-II subtypes, both containing RNA5 but differing in the *p25* and *p31* genes, and A-III subtype, which lacks RNA5 and also differs in *p25* and *p31* (Table 5.1). The B type also lacks

RNA5 and has different *p25* and *p31* gene sequences (Table 5.1). It is considered that the A-I-type source was present, at least partially, as mixed infections with A-II, A-III, and B types (Fig. 5.5c, Table 5.1).

5.2.2 Nucleotide Diversity

Estimates of the nucleotide diversity of virus genes give additional information about the variability and structure of BNYVV populations. Chiba et al. (2011) showed that the mean nucleotide diversity of all BNYVV isolates for the four sequenced genes (*CP*, *p25*, *p31*, and p*26*) was similarly low (0.016–0.025) and that the BNYVV within-group mean nucleotide diversity was even lower (0.002–0.018). These values suggest that BNYVV populations are genetically very stable or have diverged recently and that the mutation rate is similar among the four genes but the mutation frequency differs with the isolate groups (Chiba et al. 2011). Lower values (0.014–0.030) for the mean nucleotide diversity were also reported for Chinese BNYVV isolates (Zhuo et al. 2015).

Selection pressures also varied greatly depending on the gene and the geographical source of isolate (Chiba et al. 2011). For all isolates, the value of *dN*/*dS* (the ratio of nonsynonymous to synonymous substitutions) for the *CP* gene was 0.089, similar to that reported for the CPs of many other plant RNA viruses, indicating that they are strongly conserved (Garcia-Arenal et al. 2001). In contrast, the values of *dN*/*dS* for the other genes (0.778–0.327) are several times greater than that for the *CP* gene. Similar results were also obtained by Schirmer et al. (2005). Also, the *dN*/*dS* values for *p25* differ greatly in different *p25* groups (1.167–0.433), suggesting that, for each gene, different degrees of selection may operate in different geographical areas. In particular, the *p25* gene of the A-III-type strain (p25-I) is subject to strong positive selection. From this result, Chiba et al. (2011) speculated that an initial source of this Italy strain (European A-type strain) was introduced into Italy some years before the discovery of rhizomania in the 1950s and might have derived from a single genotype, with the resulting progeny variants spreading worldwide in sugar beet crops during the subsequent 30 years or so. Indeed, as described next, the existence of such a strong positive selection pressure on the *p25* gene may be associated with the cultivation of resistant sugar beet varieties.

5.3 Geographical Origins and Migration

5.3.1 Migration

Phylogenetic analysis of the *p25* gene sequences indicates that there are eight clusters of BNYVV isolates (China-B, China-H, China-X, France-F, Italy, Japan-D, Japan-O, and Germany) (Chiba et al. 2011) (Fig. 5.5a). In addition, two new clusters in the p25 tree were recently identified in China (China-L and China-Y) (Zhuo et al.

2015). The first eight clusters are also seen most clearly in the concatenate (*CP* + *p25* + *p31*) phylogenic tree (Fig. 5.5b), and these phylogenetic trends correlate best with the history of isolation and the geography and spread of the isolates. Table 5.1 shows the geographic distribution of BNYVV strains that consist of different sets of BNYVV genes. The first occurrence and possible migration process of each BNYVV strain are described below.

A-III Type The Italy strain (=European A type), first found in Italy in the 1950s, spread within Europe to the Middle East and to the USA over about 30 years (Koenig and Lennefors 2000; Schirmer et al. 2005; Koenig et al. 2008; Mehrvar et al. 2009; Chiba et al. 2011;). Thus, this strain is widely distributed throughout the world, but it is not found in Japan and China.

B Type The Germany strain (=B type) was first found in Germany and France in the early 1970s and later in Belgium, Austria, Switzerland, and the Czech Republic (Koenig and Lennefors 2000; Koenig et al. 2008; Schirmer et al. 2005). In another cluster, the China-X (Xinjiang) strain was found in Ningxia Huizu, Gansu, and Xinjiang Provinces.

A-II Type The France-P (Pithiviers) strain (=P type) was first found in small areas of France in the 1970s but then also in Kazakhstan (Koenig and Lennefors 2000), the UK (UK-MH isolate) (Ward et al. 2007), and Iran (IR-GR1 isolate) (Mehrvar et al. 2009). The Japan-O (Obihiro) strain was first found in the 1960s and widely distributed in central and eastern Hokkaido in Japan. Japan-D (Date) strain was isolated from southern areas of Hokkaido since the 1960s. The China-B (Baotou) strain (including Bao and Har4 isolates) was found in Neimenggu and Heilongjiang Provinces in China (Li et al. 2008). A German isolate OW1 (Koenig et al. 2008) also belongs to this China-B strain. A new strain, China-L, was identified in Gansu, Xinjiang, and Heilongjiang Provinces in China (Zhuo et al. 2015).

A-I Type The China-H (Hohhot) strain was found in Neimenggu, Ningxia Huizu, Xinjiang, and Heilongjiang Provinces in China. Japanese isolates belonging to this strain group, called Japan-T (Tsubetsu), were first found in small areas of eastern Hokkaido in the 1960s. The UK-FF isolate found in the UK (Ward et al. 2007) also belongs to the China-H strain. A new strain, China-Y, was identified in Neimenggu, Gansu, Xinjiang, and Heilongjiang Provinces in China (Zhuo et al. 2015).

5.3.2 Geographical Origins

As described already, East Asia holds the greatest diversity of BNYVV genomes in Chinese and Japanese isolates. Chiba et al. (2011) thus suggested that the ancestral BNYVV may have emerged from unknown native hosts in East or Central Asia

rather than in the Middle East or Europe. It is also thought that the four ancestral lineages of BNYVV and their derived progeny strains (Fig. 5.5c) probably existed in native hosts in East Asia long before the beginning of sugar beet cultivation. Each population of ancestral BNYVV might have passed through successive genetic bottlenecks in the course of transmission in different hosts at different places during different periods (Chiba et al. 2011). These events might have occurred relatively recently, perhaps only in the last half century.

Sugar beet was developed as a crop for sugar products in Europe in the early part of the nineteenth century, and then its cultivation spread to the USA and other temperate regions in the mid-nineteenth to twentieth century (Winner 1993). In Japan, sugar beet was first grown in Hokkaido in 1880 and regular cultivation started around 1920 (Winner 1993). At least three different sources of BNYVV must have been present in only a few fields in different districts of Hokkaido before the 1960s. BNYVV then became widespread in Hokkaido within about 10 years. Such rapid spread is thought to be due primarily to the return of BNYVV-infested waste soils from sugar beet factories to healthy fields and/or from transplanting methods using paper pots that contained virus-infested soils. In China, sugar beet was first experimentally grown in Heilongjiang in 1909 but then little grown until it became more generally cultivated as a new field crop in the 1960s. Since the first finding of BNYVV at Baotou in Neimenggu in 1978 (Gao et al. 1983), BNYVV has spread to many areas along the Hwang River (Yellow River; Neimenggu, Ningxia Huizu, and Gansu Provinces) and in two distant provinces, Xingjian and Heilongjiang, far from the Hwang River, in the 1980s. Thus, along with wider cultivation of sugar beets as a new crop, BNYVV might have spread from at least five sources in the respective sugar beet areas of China.

The natural host ranges of both BNYVV and its vector *P. betae* seem to be limited (Tamada and Baba 1973; Barr and Asher 1992; Hugo et al. 1996), but wild beet (*Beta vulgaris* subsp. *maritima*, a perennial species from the Mediterranean Coast) or related species may not be their native hosts (Chiba et al. 2011). In this respect, Al Musa and Mink (1981) detected BNYVV using *Gomphrena globosa* as a bait plant from soil collected from underneath cherry trees in an area in Washington State in the USA where sugar beet had never been cultivated. *G. globosa* (Amaranthaceae) is a common weed in subtropical and tropical America and widely used as a diagnostic indicator plant for viruses. Indeed, both BNYVV and *P. betae* can infect this plant, and virus-carrying resting spore clusters of *P. betae* have been observed in its roots (T. Tamada, unpublished data). Furthermore, BNYVV and *P. betae* were suggested to have much wider host ranges than previously reported, including monocotyledonous plants (Legrève et al. 2005; Mouhanna et al. 2008). Further discovery of natural hosts will provide a clue to the origin and evolution of BNYVV and related viruses.

5.4 Emergence of Resistance-Breaking Variants

5.4.1 Identification of Viral Genes Involved in Host Resistance

BNYVV infection is usually confined to the root system. The resistance of sugar beet varieties is due to the restriction of virus multiplication and translocation in the roots (Giunchedi et al. 1987, 1988; Poggi-Pollini and Giunchedi 1989; Paul et al. 1992; Scholten et al. 1994; Tuitert et al. 1994; Tamada et al. 1999), although the mechanism of resistance to BNYVV remains unknown. BNYVV resistance genes are derived from accessions of wild beet, *B. vulgaris* subsp. *maritima* (De Biaggi 1987; Lewellen et al. 1987; Whitney 1989; Geyl et al. 1995; Biancardi et al. 2002).

To find a lead on the resistance mechanism, Tamada et al. (1999) examined the effects of RNA3 (comparing wild-type *p25* to partially deleted *p25* mutants) on symptom development in susceptible (cv. Monomidori) and partially resistant (cv. Rizor) sugar beet varieties under laboratory and field conditions. BNYVV with wild-type RNA3 caused typical rhizomania root symptoms in the susceptible variety, but most plants of the resistant varieties did not develop symptoms and their roots had 10 to 20-fold lower concentrations of the virus than in susceptible plants. In contrast, BNYVV with mutant RNA3 caused no symptoms in either susceptible or resistant varieties, and their virus levels were similar. In Northern blots, wild-type RNA3 was not detectable in most of the taproots of a resistant variety, but the RNA3-deleted form was detectable, which suggests that replication of wild-type RNA3 was inhibited in roots of resistant plants. These results suggest that RNA3-encoded p25 is not only responsible for rhizomania development in susceptible varieties, but it may also be involved in the inhibition of virus translocation from rootlets to taproots in the partially resistant variety.

A similar phenomenon was observed in experiments based on the phenotypes that developed on rub-inoculated leaves of sugar beet (Tamada 2007; Chiba et al. 2008). When susceptible and partially resistant varieties were rub-inoculated with certain isolates of BNYVV, the resistance phenotype displayed a range of symptoms from no visible lesions to necrotic or grayish lesions at the inoculation site, and only very low levels of virus and viral RNA accumulated. The susceptible phenotype showed large, bright yellow lesions and developed high levels of virus. Based on the phenotypes produced after foliar rub-inoculation with BNYVV, differential lines MR0, MR1, and MR2 were selected from *B. vulgaris* subsp. *maritima* accessions (Tamada 2007). Chiba et al. (2008) found that the different responses of various BNYVV isolates (from experiments including site-directed mutagenesis of infectious cDNA clones) are due to amino acid residues at position 68 in the p25 protein; thus, BNYVV with phenylalanine (F) acts as a stronger elicitor than BNYVV with tyrosine (Y) or histidine (H). For example, MR1 plants were resistant to isolates with F, but not to isolates with Y or H. In MR2 plants, isolates with F usually induced no symptoms, but isolates with H induced larger necrotic symptoms in which the p25 elicitor seemed to be less active. In contrast, isolates with leucine (L) or cysteine (C) induced bright yellow susceptible phenotypes in MR1

and MR2 plants. All isolates tested caused the susceptible phenotype in MR0 plants. Possibly, differences in the nature of the amino acid residue at position 68 may affect the accessibility of host factors. Thus, Chiba et al. (2008) found that the p25 protein functions as an avirulence (Avr) factor in leaves of resistant *B. vulgaris* plants, that there is a virus strain–cultivar-specific resistance interaction, and that amino acid residues in p25 control this host-specific resistance. Sequence analyses of BNYVV isolates collected worldwide did indeed reveal sequence variations in the p25 protein, with two positions (67 and 68) recognized as an especially variable region (Schirmer et al. 2005; Chiba et al. 2011). A specific amino acid motif "tetrad" at positions 67–70 (Fig. 5.6a) was proposed (Schirmer et al. 2005; Klein et al. 2007). It is interesting to note that the resistance reaction in *p25–Beta* plant systems is more similar to typical *R*-gene-mediated resistance observed in fungal and bacterial effector–plant systems than in those of viral protein–plant systems. In general,

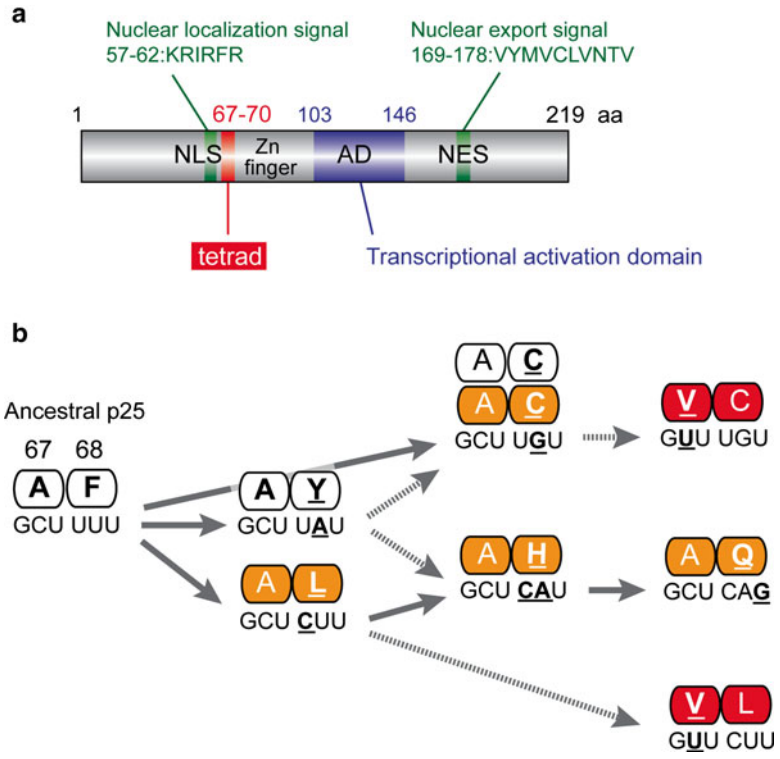

Fig. 5.6 (**a**) The characteristic features of the BNYVV p25 protein, indicating the positions of nuclear localization signal, tetrad amino acid motif, Zn finger domain, acidic transcriptional activation domain, and nuclear export signal. (**b**) Mutational steps of the p25 amino acids at positions 67 and 68 to confer the *Rz1* resistance-breaking (RB) type (Redrawn and modified from Chiba et al. (2011)). *Orange* and *red boxes* indicate amino acids associated with the mutant virulent and severely virulent types, respectively. *White boxes* indicate an Avr type. *Gray* and *dotted* arrows indicate transversion and transition substitutions, respectively

plants recognize essential viral proteins such as replicase and capsid proteins to trigger a resistant reaction, but the p25 pathogenic effector, which is otherwise dispensable for viral infection, is specifically targeted in *Beta* plants.

5.4.2 Emergence of Resistance-Breaking Virus and RNA3 Variation

In 2002, severe symptoms of rhizomania were observed in the *Rz1*-resistant sugar beet varieties planted in the Imperial Valley of California. Since it was first reported in North America in 1984 (Duffus et al. 1984), BNYVV has been detected in all major production regions of the USA (Rush et al. 2006). However, despite its broad distribution, the damage caused by this virus has significantly lessened during the last 15 years since the introduction of regionally adapted, *Rz1*-resistant varieties.

Liu et al. (2005) suggested that resistance-breaking (RB) variants of BNYVV had emerged in the Imperial Valley. Since then, RB variants have appeared in other sugar beet regions in the USA including Colorado, Idaho, Minnesota, Nebraska, and Oregon (Liu and Lewellen 2007). Acosta-Leal and Rush (2007) reported that changes to valine (V) from alanine (A) at position 67 and to aspartic acid (D) from glutamic acid (E) at position 135 in the p25 protein were strongly associated with the ability of the virus to overcome *Rz1* resistance in the Imperial Valley. However, Liu and Lewellen (2007) did not find any correlation between *p25* sequences of North American isolates and the viral titer in soil-inoculated *Rz1* plants in greenhouse experiments. The main variation was found at amino acid positions 67 and 68 in the p25 protein; i.e., in non-RB isolates, the two positions were always occupied by AC, but in RB isolates, AF, AL, SY, VC, VL, as well as AC were found.

As regards RB, Pferdmenges et al. (2009) found that two US isolates (with VL) from the Imperial Valley and Minnesota and a Spanish isolate (with VC) had the ability to overcome *Rz1* resistance in sugar beet grown in infested soils in the greenhouse, but an Italian (with AH) or German (with AY) isolate did not. These isolates belong to European A-type strains (=Italy strain) (Table 5.1). Pferdmenges and Varrelmann (2009) also showed that RB was not associated with virus concentration or level of viruliferous *P. betae* in the soils. Furthermore, Koenig et al. (2009b) demonstrated by reverse genetics using European isolates that a single U/C nucleotide substitution changing A to V (at position 67) in the p25 protein allowed an increase in virus accumulation in roots of mechanically inoculated, partially resistant sugar beet seedlings. Likewise, Acosta-Leal et al. (2010) showed that, for Minnesota isolates, wild-type p25 had an AC amino acid that was replaced by VC, whereas for California isolates, AL was replaced by VL; therefore, V at position 67 was apparently associated with RB in both cases. On the basis of all these results, a mutation with V at position 67 appears to be critical in overcoming *Rz1*-mediated resistance.

Furthermore, Chiba et al. (2011) assessed in detail the virulence of representative BNYVV isolates from Europe, the USA, and Asia using the *P. betae* vector to inoculate roots of susceptible and *Rz1*-resistant sugar beet varieties. Many isolates of the Italy strain (= European A type) were able to overcome *Rz1* resistance at various levels of resistance breaking, whereas isolates of the Germany strain (=B type) and France-P, Japan-O, and Japan-D strains (from all of which RNA5 is absent or was eliminated) could not overcome *Rz1*-mediated resistance. In particular, amino acid changes from F or Y to C, H, or L at position 68 in the p25 protein appeared to be associated with symptom induction in *Rz1*-resistant varieties, and, furthermore, an amino acid change from A to V at position 67 appeared to be associated with more severe symptoms.

To differentiate BNYVV isolates containing *p25* variants in relation to RB, Chiba et al. (2011) referred to the aforementioned variants as AF, AY, AC, AH, AL, AQ, VC, and VL, based on the amino acids at positions 67 and 68 (Fig. 5.5c). In Europe, AF and AY variants were infrequently detected, whereas AC, AH, and AL variants were prevalent (Schirmer et al. 2005; Chiba et al. 2011). In the USA, VL variants as well as AC, AH, and AL were prevalent (Liu and Lewellen 2007). Overall, Chiba et al. (2011) suggested that the relative RB ability of these *p25* variants can be estimated as AF < AY < AC = AL = AH < AQ < VC = VL. However, the AC and AH variants of the Italy strain were RB, whereas AC and AH variants in the Chinese isolates seemed to be non-RB, and amino acid differences in p25 between Chinese and Italy isolates suggest that other positions in p25 or a gene or genes other than *p25* may influence RB.

On the basis of results from the *p25* variants of the Italy strain, Chiba et al. (2011) presumed successive steps of mutations from wild type to RB type at two amino acid positions in the p25 protein (Fig. 5.6b). The ancestral amino acid F (wild type) at position 68 changed to Y and C by a transversion substitution (U<u>U</u>U → U<u>A</u>U and U<u>U</u>U → U<u>G</u>U, respectively). The reverse also occurs and may explain how Y or C was frequently present in p25 of Chinese and Japanese isolates (Li et al. 2008; Chiba et al. 2011; Zhuo et al. 2015). As an alternative, the first step is a change from F to L by a transition substitution (<u>U</u>UU → <u>C</u>UU). At the next step, although Y can change to C or H by a transition (U<u>A</u>U → U<u>G</u>U or <u>U</u>AU → <u>C</u>AU, respectively), the transversion from L to H (C<u>U</u>U → C<u>A</u>U) is probably more frequent. In a further step, the wild-type p25 amino acid A at position 67 also changes to V by a transition substitution (G<u>C</u>U → G<u>U</u>U). Note that the order of these mutational changes parallels the degree of RB ability discussed.

On the other hand, in 2004, a new strain with an unusual p25 tetrad, AYPR, was first isolated from rhizomania-resistant sugar beet varieties with severe symptoms in the Netherlands (Bornemann et al. 2015). Similar strains with AYPR were detected from 26 field soil samples in England in 2007. In greenhouse experiments, Bornemann et al. (2015) showed that this strain can accumulate at higher levels in young plants with *Rz1*-resistant genotype but not in *Rz1*+*Rz2*-resistant genotypes.

5.4.3 *Association of RNA5 with Resistance Breaking*

In Europe, BNYVV P type with RNA5 occurs only in a small region near Pithiviers in France (Koenig et al. 1997) and in two sites in the UK (Ward et al. 2007). However, most Asian BNYVV isolates contain RNA5 segments with sequence variation (Miyanishi et al. 1999; Schirmer et al. 2005; Chiba et al. 2011; Zhuo et al. 2015). Japanese isolates with RNA5 caused more severe symptoms in susceptible and partially resistant sugar beet plants (Tamada et al. 1996). Similarly, P-type BNYVV (France-P strain) moved more rapidly in plants than the A- or B-type BNYVV, and, in partially resistant varieties, symptoms in the taproots were much more severe than with the A- or B-type virus (Heijbroek et al. 1999). Pferdmenges et al. (2009) reported that the P-type strain, which has RNA5, and RB strains (US and Spanish isolates) had the ability to overcome *Rz1* resistance in sugar beet in the greenhouse. Similarly, Chiba et al. (2011) showed that the wild-type P-type isolate with RNA5 caused virulence in *Rz1* plants, but a laboratory P-type isolate that lacked RNA5 did not, suggesting that the RB ability of this P-type virus is due to the presence of RNA5. Thus, *p25* and *p26* genes may enable the virus to overcome *Rz1* resistance in a synergistic fashion, allowing the virus to replicate and spread more efficiently. The p26 and p25 proteins are remarkably similar (e-value = 4×10^{-10}, 22 % sequence identity) and probably have a common ancestor (Simon-Loriere and Holmes 2013). We suggest that the p26 protein acts as an alternative effector, which may be responsible for the survival of BNYVV in some resistant plant species including potential natural hosts.

Galein et al. (2013) reported interannual variation of BNYVV types and p25 tetrads in the Pithiviers area where rhizomania is severe and mixed infections of BNYVV P-, B-, and A-type strains are predicted. Before 2005, three CP types and five tetrads had been found: the B type with AYHR (54 %), the P type with SYHG (32 %), and the A type with AFHR (3 %), AHHG (3 %), and ALHG (6 %). In analyses of samples collected between 2008 and 2012, the major variants were the B type with AYHR (56 %) and P type with SYHG (32 %), and 18 minor tetrad variants were identified. In a single sugar beet root, presumptive indications of BNYVV reassortment were found in about 20 % of the samples. Several different tetrads associated with the CP of A or B type were found in RNA5-containing strains. Thus, the tetrad sequences were highly diverse in areas with mixed infections of the different BNYVV types (Meunier et al. 2005; Koenig et al. 2009a).

5.5 Conclusions

In the hypothesis we have described to explain the evolutionary history and route of spread of BNYVV, several subpopulations diverged from at least four original types of populations, the A and B lineages diverged early, and further divergences produced three A lineages (Fig. 5.5b). These original sources and their mixed

infections are presumed to have been present in East or Central Asia and adapted to indigenous host plants long before sugar beet was cultivated. Perhaps for the last half century, BNYVV sources with diverse origins from native hosts infected cultivated sugar beet plants in different areas during different periods (e.g., A-III-type virus in Italy, A-II type in France and Japan, and B type in Germany), and they have spread extensively or to a limited extent. Along with the introduction of BNYVV to new areas and with the successive cultivation of resistant varieties, new RB variant viruses have emerged via amino acid changes in pathogenicity-related viral genes (e.g., the *p25* gene).

As inoculum sources of virus-carrying *P. betae* increase, so does the possibility that new viruses or variant viruses will arise via mutations, recombination, or reassortants of viral genomes. Indeed, mixed infections of different strains or different species of viruses (e.g., BSBMV, beet soil-borne virus, beet virus Q) in sugar beet roots are now commonly observed.

Generally, it is thought that BNYVV variants probably coexist as quasi species in the field, as pointed out by Liu and Lewellen (2007), Acosta-Leal et al. (2008), and Chiba et al. (2011). Therefore, it is important to understand how new virus variants provide an advantage against regionally adapted resistant sugar beet varieties. In this regard, Bornemann and Varrelmann (2013) used deep sequencing to analyze the p25 tetrad variability of several BNYVV wild-type non-RB and RB strains of different geographical origins. In most cases, they found that the sugar beet genotype had a strong selective effect on the accumulation of different p25 tetrads and that RB tetrad mutations were selected with a loss of relative fitness, with the exception of certain strain.

Finally, interesting additional evidence concerning BNYVV evolution is the finding of BRLSs in both plant and insect genomes (Kondo et al. 2013), as recorded in the form of endogenous molecular fossils of viral genomes, despite benyviruses having a host range restricted to plants and plasmodiophorid vectors. These findings and the fact that benyviruses are most closely related to CAV isolated from algae suggest that ancestral and extant benyviruses may have infected a broad range of hosts (algae, fungi, plants, and insects). This evidence provides insights into the origin and evolution of the benyviruses and related viruses, and further work is expected.

Acknowledgments We thank Dr Beth E. Hazen for editing the chapter.

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Part III
The Vector

Chapter 6
The Plasmodiophorid Protist *Polymyxa betae*

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Abstract *Polymyxa betae* Keskin, the vector of beet necrotic yellow vein virus (BNYVV), is an obligate root parasite of sugar beet belonging to the family Plasmodiophoraceae and the class Phytomyxea. The genus *Polymyxa* includes two species *P. betae* and *P. graminis*, which are morphologically indistinguishable but separated by host range and can be distinguished by ribosomal DNA analysis. Plasmodiophorids have a complex life cycle consisting of two different phases: the resting spore stage, producing primary zoospores, and the sporangial stage, producing secondary zoospores. Infection of root cells by zoospores involves encystment on the cell surface followed by the direct injection of zoospore contents into the cell. The host range of *P. betae* is restricted to species of the family Chenopodiaceae and related plant species, whereas *P. graminis* infects graminaceous plants. Isolates of *P. graminis* are considerably diverse based on their ecological and biological characteristics, whereas *P. betae* has not shown the same degree of diversity, although there is some host-specific variation among *P. betae* isolates. Plasmodiophorid-transmitted viruses such as BNYVV are genetically very diverse, but these viruses have common elements that are involved in vector transmission. Serological and molecular techniques involving polymerase chain reaction (PCR) methods can be utilized for the detection and quantification of both *P. betae* and *P. graminis*.

Keywords Sugar beet • Rhizomania • *Polymyxa betae* • BNYVV • Plasmodiophorids • Virus transmission • Genetic diversity

Polymyxa betae Keskin was first described as an obligate root parasite of sugar beet (*Beta vulgaris* subsp. *vulgaris*) and other chenopodiaceous plants by Keskin (1964), and its association with symptoms of rhizomania disease in sugar beet was observed in Italy (Canova 1966; D'Ambra and Keskin 1966). Subsequently, it was

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E. Biancardi, T. Tamada (eds.), *Rhizomania*, DOI 10.1007/978-3-319-30678-0_6

established as the vector of beet necrotic yellow vein virus (BNYVV, benyvirus) that is the causal agent of rhizomania (Tamada and Baba 1973; Faccioli and Giunchedi 1974; Putz and Vuittenez 1974). *P. betae* is also a vector of three other viruses of sugar beet: beet soil-borne mosaic virus (BSBMV, benyvirus), beet soil-borne virus (BSBV, pomovirus), and beet virus Q (BVQ, pomovirus), some of which may be associated with rhizomania (Meunier et al. 2003; Rush 2003; McGrann et al. 2009a).

6.1 Taxonomy

Polymyxa betae is a species of the family Plasmodiophoracea. The members of this family were previously classified as fungi but are currently included in the protist supergroup *Rhizaria*, a sister group to the omnivorous vampyrellid amoebae, belonging to the class Phytomyxea (phytomyxids) (Neuhauser et al. 2014). Phytomyxea is a large group of obligate biotrophic, endobiotic parasites of plants, diatoms, brown algae, and oomycetes (Braselton 2001; Neuhauser et al. 2011). On the basis of 18S ribosomal DNA (rDNA) analyses, this class is divided into two distinct orders: the Plasmodiophorida, which comprises mainly parasites of green plants, and the Phagomyxida, which are parasites of diatoms and brown algae. All plasmodiophorid species are characterized by the following distinctive features (Box 6.1): cruciform nuclear division, zoospores with two anterior unequal whiplash flagella, multinucleate plasmodia, obligate intracellular parasitism, and environmentally resistant, long-lived resting spores. The production of zoospores and long-lived resting spores are of crucial importance in the epidemiology of the diseases caused by these organisms and the viruses they transmit.

In the family Plasmodiophoraceae, 41 species belonging to the following ten genera have so far been recognized: *Polymyxa*, *Spongospora*, *Plasmodiophora*, *Ligniera*, *Membranosorus*, *Octomyxa*, *Sorodiscus*, *Sorosphaerula* (basionym: *Sorosphaera*), *Tetramyxa*, *and Woronina* (Neuhauser et al. 2010, 2011). Members of the first three genera, *Polymyxa*, *Spongospora*, and *Plasmodiophora*, are of significant agronomic importance. *Plasmodiophora brassicae* causes the important clubroot disease of cabbage and other brassicaceous crops. *Spongospora subterranea* is an agent of powdery scab of potato and also a vector of potato mop-top virus (PMTV, pomovirus) that causes the tuber spraing disease on potato. Two species of *Polymyxa* serve as vectors of several important plant viruses (Kanyuka et al. 2003; Tamada and Kondo 2013).

Phylogenetic analyses of rDNA suggest that the genus *Polymyxa* is very closely related to *Ligniera* and *Sorosphaerula* (basionym: *Sorosphaera*), while it is more distantly related to *Spongospora* and *Plasmodiophora* (Ward and Adams 1998; Bulman et al. 2001). The *Polymyxa* genus includes two species, *P. betae* and *P. graminis*, which are morphologically indistinguishable but separated by host range (Barr 1979; Barr and Asher 1992; Braselton 2001). *P. graminis* multiplies primarily in grass and cereal species in the Gramineae, whereas *P. betae* infects species in the

Box 6.1: Terminology of Plasmodiophorids
Terminology for plasmodiophorids with a complex life cycle has become confused because of contributions from a variety of disciplines (Braselton 1995). The same author referred to Karling's proposal that is a standard set of terms with the use of common synonyms included within parentheses, viz., "resting spore" (cyst), "sporosorus" (cystosorus), "sporogenic" (cystogenous), and sporangial (sporangiogenous). Karling's opinion is that the use of the term "cyst" in another part of the life cycle for thick-walled, single-celled, resting structures was inconsistent and confusing, because zoospores encyst on the host prior to infection. By replacing the term "cyst" with "resting spore," for consistency, the term for the aggregation of resting spores is changed from cystosorus to sporosorus. For other recommended terms, sporogenic instead of cystogenous is used to refer to developmental stages that lead to resting spores, and sporangial instead of sporangiogenous describes the thin-walled sporangia (zoosporangia) that contain zoospores. Sporogenic and sporangial phases of plasmodiophorid life cycles are referred to as secondary and primary, respectively.

family Chenopodiaceae and some related plants. The two species of *Polymyxa* can be clearly distinguished by rDNA analysis (Ward et al. 1994; Ward and Adams 1998).

6.2 Life Cycles of Plasmodiophorids

General features of the life cycle of plasmodiophorids (Braselton 1995; Kanyuka et al. 2003; Rochon et al. 2004; Tamada and Kondo 2013) are summarized as follows. The life cycle consists of two major phases (Fig. 6.1): the sporogenic phase that produces primary zoospores via the formation of resting spores and the sporangial phase that produces secondary zoospores. The zoospores are the only part of the life cycle outside the host and are the main manner of dissemination. Both types of zoospores are propelled by two whiplash flagella of unequal length. Each phase of the life cycle is initiated by the attachment of zoospores and penetration of epidermal or root hair cells. Zoospores enter a root cell by the encystment on its surface of a tubular structure (*Rohr*) that contains a dagger-like body (*Stachel*) (see Fig. 6.2b, c). The encysted zoospore produces an outgrowth (*adhesorium*), from which the zoospore cytoplasm including the Rohr and Stachel is passed, followed by injection of the zoospore contents into the host cell cytoplasm (see Fig. 6.2d). The zoospore contents enlarge within the host cell, and the young sporangial plasmodia develop several septa that divide into lobes, followed by several cycles of noncruciform mitotic nuclear divisions, and eventually develop into a multinucleate sporangial plasmodium, resulting in an aggregate of zoosporangia (sporangiosorus). Mature

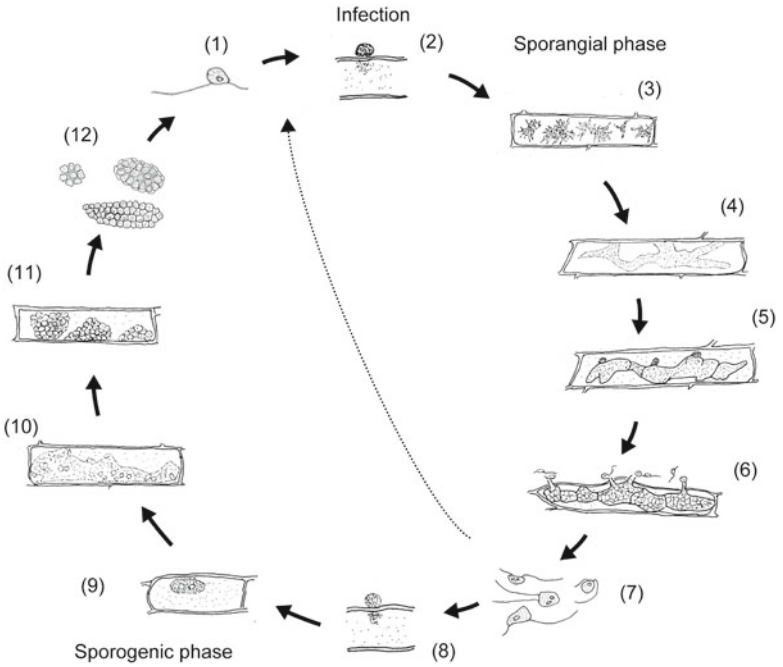

Fig. 6.1 Schematic diagram of the life cycle of *Polymyxa betae*, which consists of sporangial and sporogenic phases (Selected and redrawn from the figures of Keskin (1964)). Morphological characteristics of *P. betae* at each stage of the life cycle are shown as follows: *1* primary zoospores, *2* infection, *3* numerous branched plasmodia, *4* sporangial plasmodium, *5* constricted and septate sporangial thallus, *6* discharge of secondary zoospores from zoosporangium, *7* secondary zoospores, *8* infection, *9* small young sporogenic plasmodium, *10* larger sporogenic plasmodia, *11* cleavage of sporosorus plasmodium, *12* resting spore clusters

zoosporangia develop exit tubes that dissolve an opening in the host cell wall (see Fig. 6.3), from which secondary zoospores are discharged. The zoospores initiate another round of infection, producing either sporangial or sporogenic plasmodia. During the sporogenic phase, the nucleus undergoes cruciform division, followed by meiosis. The sporogenic plasmodium is then cleaved and the aggregates of unicellular resting spores are produced. Immature resting spores are tightly packed and angular shaped, but they become more rounded with a multilayered cell wall. Mature resting spores are usually grouped in clusters with characteristic morphology. Each resting spore releases a primary zoospore that initiates another round of infection via the production of a sporangial plasmodium (Fig. 6.1). Factors determining either the sporangial or sporogenic development phase for *Polymyxa* species

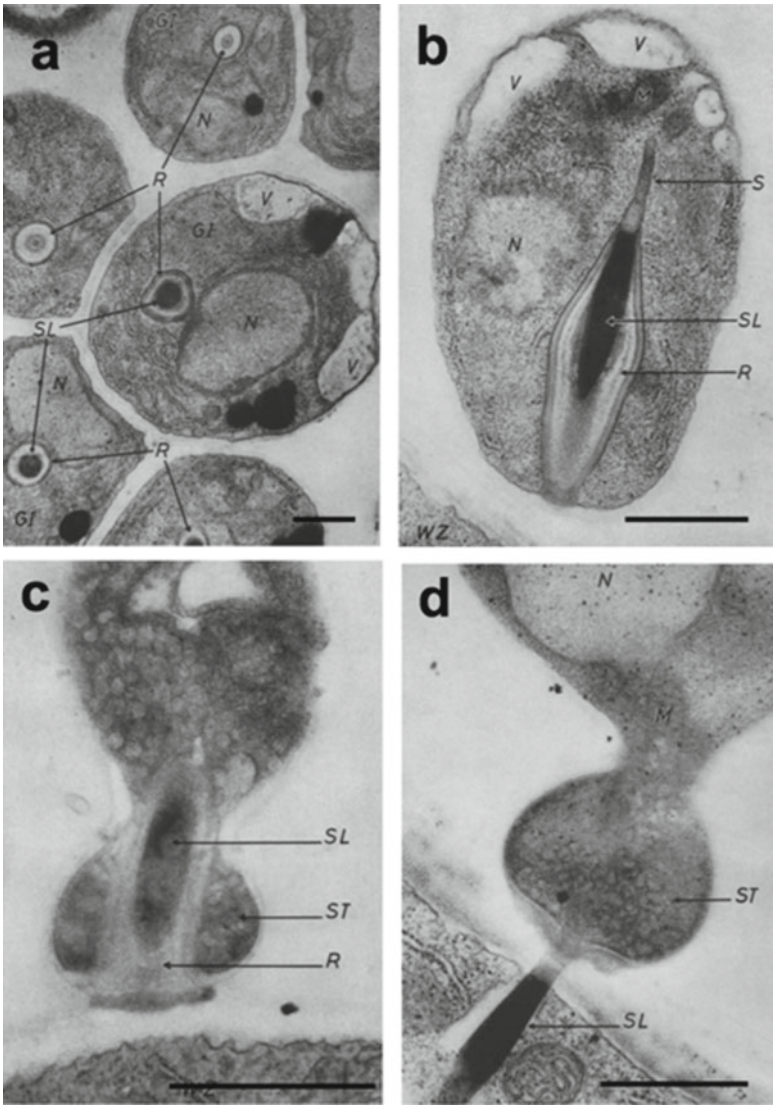

Fig. 6.2 Electron micrographs of the infection process by a zoospore of *P. betae* (Selected from figures of Keskin and Fuchs (1969)). (**a**) Mature zoospores, (**b**) a zoospore just attached to a host cell, (**c**) soon after a zoospore has attached to the host cell, (**d**) penetration of a zoospore into the host cell; inside the figures: *ER* endoplasmic reticulum, *GI* Golgi apparatus, *M* mitochondria, *N* nucleus, *R* Rohr, *S* Schlauch (tube), *SL* Stachel, *ST* stylet, *V* vacuole, *WZ* host cell, *Bar* 0.5 μm

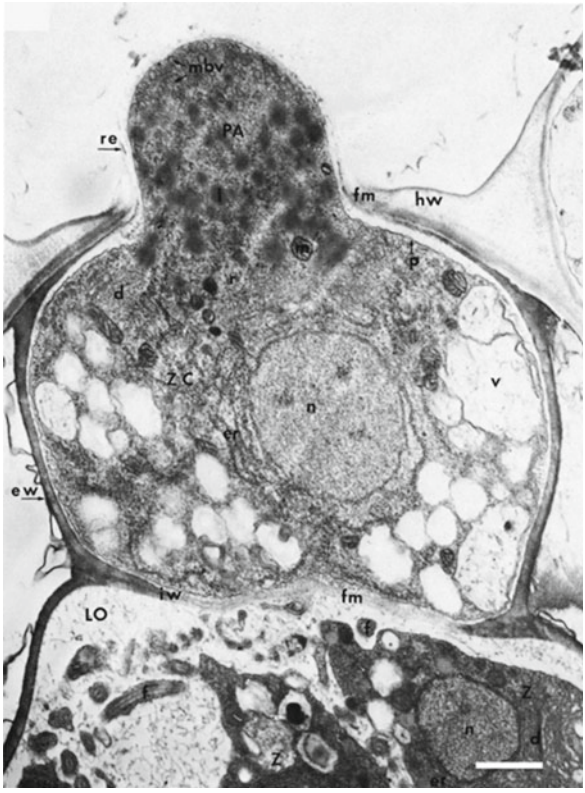

Fig. 6.3 Electron micrograph of zoospore exit-tube emission from a zoosporangium of *P. betae* (From D'Ambra and Mutto (1977), with permission of NRC Press through CCC). At the top of the papilla, there are many membrane-bound vesicles, some of which are fused. Behind this area, there are abundant lipid droplets. Mitochondria show prominent cristae, there is much endoplasmic reticulum, and many large vacuoles are present. The cross wall dividing the zoosporangium from the compartment supporting the papilla is softened and shows the presence of fibrillar material. *d* dictyosome, *er* endoplasmic reticulum, *ew* enveloping wall, *f* flagellum, *fm* fibrillar material, *hw* host wall, *iw* internal wall, *l* lipid droplet, *LO* zoosporangium lobe, *m* mitochondrion, *mbv* membrane-bound cytoplasmic vesicles, *n* nucleus, *p* plasmodesmata, *PA* papilla, *r* ribosome, *re* wall remnants, *v* vacuole, *Z* zoospore, *ZC* zoosporangial compartment adhering to the host wall. *Bar* = 1 μm

are unknown, and there is an apparent overlap of the presence of the two phases. At early developmental stages, sporangial and sporogenic plasmodia are morphologically very similar, but at later stages sporogenic plasmodia can be distinguished by plasmodiophorid-characteristic morphology.

6.3 Infection and Germination of *Polymyxa betae*

6.3.1 Morphology

The morphology of *Polymyxa betae* is described by Keskin (1964), Karling (1968), Barr (1979), and Abe and Ui (1986). The morphological feature of *P. betae* at each stage of the life cycle is schematically illustrated in Fig. 6.1. Resting spores are made up of separate cells held together by a binding material. They are globular, polyhedral, or slightly angular and 4–7 μm in diameter, and each cell produces a primary zoospore on germination. Primary zoospores occasionally fuse with each other while actively motile. Sporangial plasmodia are lobed, elongated, or irregular with small exit papillae, usually becoming elongated and enveloped by membranes at maturity. Zoosporangia are one to several in a cell. They are ovoid or subglobular and usually elongate with prominent bulges, developing one to several 5–8 μm long exit papillae. Secondary zoospores that swarm in sporangia before emerging are similar in shape and size to the primary zoospores with two unequal flagella, 4–5 μm and 16–20 μm long, respectively. Sporogenic plasmodia are similar to sporangial plasmodia, but usually smaller and denser in appearance, cleaving into segments which become resting spores. These unite to form resting spore clusters, each comprising 4–300 individual resting spores, several of which can develop in a root cell.

6.3.2 Infection and Liberation of Zoospores

The infection process and life cycle of *P. betae* has been described by Keskin (1964), Keskin and Fuchs (1969), and Barr and Asher (1996). In particular, Fuchs (1966) observed the behavior of zoospores on the epidermal cells and root hairs of sugar beet seedlings by using phase contrast microscopy and time-lapse motion pictures. These observations are described in detail below. Electron microscopy of the infection process is also represented in Fig. 6.2 (Keskin and Fuchs 1969). When the preformed zoospore settles on a host cell membrane, the short flagellum is fastened to the wall and soon disappears, but the long flagellum continues to move slowly until it disappears 5–10 min later. During the next 90–120 min, the spore becomes encircled by a membrane and, at the point of its attachment to the host cell, a dark area appears (Fig. 6.2b, c). Such a differentiated zoospore increases in relative size at the moment when the protoplast is injected into the host cell through a stylet-like connection (Fig. 6.2d), which occurs in less than 1 min. The newly generated thallus may fill much of its host cell and then starts to undergo incomplete compartmentation without wall formation. Dark zones appear irregularly over the ripening zoosporangia, and at these points a papilla grows out and the contents of the zoosporangium begin to differentiate. Subsequently, the papillae become tubular, about 5–8 μm long. The papilla bursts and the contents are extruded, remaining enclosed in a membrane attached to the open end of the tube. When the zoospores

leave the zoosporangium, they appear to creep actively one after the other from a small compartment at the base of the papillum. Development from infection until liberation of zoospores takes about 70–75 h in young host cells (Fuchs 1966).

D'Ambra and Mutto (1977) observed the process of exit-tube differentiation and liberation of zoospores from zoosporangia by electron microscopy (Fig. 6.3). Zoosporangia are divided into segment by cross walls, and the zoospore exit tube does not grow out from the zoosporangium directly. The exit tube arises from a compartment differentiated by the zoosporangium during its ripening, which is separated from it by a wall. This compartment, corresponding to the dark areas described by Fuchs (1966), is a single uninucleate cell whose content does not differentiate to form zoospores. At the beginning of exit-tube formation, the thallus wall in contact with the host cell wall becomes thinner and softened. At the same time, thallus cytoplasm shows membrane-bound vesicles, in which the endoplasmic reticulum increased and vacuoles enlarge (Fig. 6.3). Vesicles are concentrated in the area of exit-tube formation, suggesting that they are involved both in host and thallus wall lysis (Fig. 6.3). Indeed, enzymatic activity is indicated by thallus and host cell wall softening. Wall profiles and microfibril orientation are involved in mechanical forces at this point.

6.3.3 Distribution of Polymyxa betae *Within Root Tissue*

Barr and Asher (1996) observed that initial infection of *P. betae* occurred in the root epidermal cells 48 h after inoculation, and even after plasmodia developed there, infection was restricted to this outer layer of cells (Fig. 6.4a). Mature zoosporangia developed in 8 days at 20 °C, and at that time they were observed in the majority of epidermal cells but not within the root cortex. However, after 18 days all stages (plasmodia, zoosporangia, and resting spore clusters) in the life cycle were observed throughout the root cortex, but they were never seen in either the endodermis or the stele (Fig. 6.4b). It appears that *P. betae* is unable to penetrate the endodermal cells surrounding the vascular tissues (Barr and Asher 1996).

Such cortical invasion by a zoosporic protist suggests that some zoospores may encyst on the interior wall of infected cells and penetrate adjacent cells deeper within the root, in the same manner as occurs in the initial infection of epidermal cells (Aist and Williams 1971; Barr 1988; Barr and Asher 1996). Alternatively, there may be active movement of myxamoeboid plasmodia between cells, through holes that are created by the enzymatic digestion of cell wall components (Mithen and Magrath 1992). It was also observed that the plasmodia are spread passively by concurrent division with the host cells (Buczacki 1983). However, the wide distribution of zoosporangia within the cortex suggests that migrating zoospores are the most effective mechanism for internal spread.

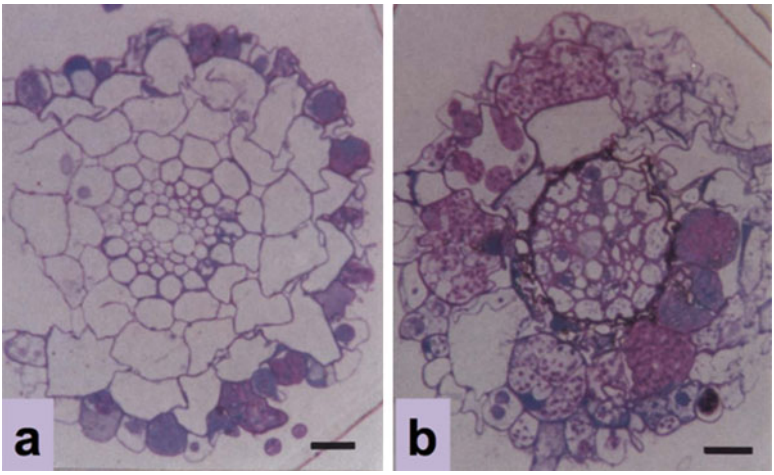

Fig. 6.4 Distribution of *P. betae* in cross sections of sugar beet seedling roots at 8 (**a**) and 18 (**b**) days after inoculation (Selected from figures of Barr and Asher (1996), with permission of Elsevier through CCC). Plasmodia, in various stages of development, are restricted to epidermal cells (**a**), and plasmodia and zoosporangia are present throughout the root cortex but not in the endodermis or stele (**b**). Semi-thick sections were stained with a methylene blue-azure II-basic fuchsin stain. *Pink* shows ground color, *blue* zoospore, and *dark blue* nuclei. *Bar* = 50 μm

6.3.4 Germination of Resting Spores

For a plasmodiophorid organism, high soil moisture and temperature are essential to enable the resting spores to geminate. For *P. betae*, a temperature around 25 °C is optimal for germination of resting spores and subsequent infection by zoospores (Abe 1987; Blunt et al. 1991). No infection was observed at 10 °C, and the minimum temperature for germination and infection appeared to be between 10 and 15 °C (Horak and Schlösser 1980; Abe 1987; Blunt et al. 1991). Abe (1987) also showed that germination of resting spores is affected by pH; for example, no germination was observed in aqueous solution below pH 5.3, although resting spores can survive under soil conditions below pH 5.0. Thus, the degree of germination of resting spores of *P. betae* depends on a combination of soil temperature, moisture, and pH (Abe 1987; Blunt et al. 1991).

It is generally assumed that resting spores of *P. betae* require root exudates as a stimulus for germination, a mechanism which would allow the plasmodiophorid to survive for long periods in the absence of the host plants (Schlösser 1988). The chemical nature of the stimulus is not known; however, microscopic observation showed that at least some resting spores germinate in distilled water alone and can be stimulated to germinate by preheating soils to 40 °C (Beemster and de Heij 1987). As with other fungal propagules surviving in soil, the complexity of the soil environment suggests a balance between exogenous stimuli required to overcome soil fungistasis and endogenous factors (Schlösser 1988; Asher 1993).

It is known that germination of resting spores is not synchronous. As regards the structure and the chemical composition of the spore wall, D'Ambra and Mutto (1977) and Langenberg and Giunchedi (1982) reported the presence of two distinct layers in a resting spore wall of *P. betae*, an outer electron-dense and an inner electron-transparent layer. By scanning electron microscopy, Ciafardini and Marotta (1988, 1989) showed that morphological modifications occur in the cell wall during the maturation process of sporosori and that the germination of the resting spores of *P. betae* takes place in particular fragile areas of the cell wall by cleavage. Furthermore, Ciafardini et al. (1995) investigated the architecture of the resting spore wall by cytochemical and enzymatic procedures along with electron microscopy. The mature cell wall is composed of five layers: the first two are intimately interconnected, the third is rather electron transparent and fibrous, the fourth is thin and rich in polysaccharide materials, and the fifth, innermost layer varied in appearance according to the stage of maturation. As a whole, the wall was resistant to enzymatic treatment with pronase, lipase, and phospholipase but was destroyed by lysozyme. There was evidence for a progressive modification of the upper area of the cell wall from a simpler to a more complex structure during the maturation of the spore and in relation to germination (Ciafardini et al. 1995).

6.4 Host Range and Genetic Diversity

Keskin (1964) first demonstrated that the host range of *Polymyxa betae* is restricted to sugar beet (*Beta vulgaris*) and other chenopodiaceous plant species (*Atriplex halimus*, *A. hortensis*, *A. undulata*, *Chenopodium album*, *C. bonus-henricus*, *C. murale*, *C. polyspermum*, *C. quinoa*, *C. rubrum*, *C. vulvaria*, *C. amaranticolor*, and *Spinacia oleracea*). Such a restricted host range is the critical basis for differentiating *Polymyxa betae* from *P. graminis*. In this first host range study, soils from Europe were used, while Barr (1979) reported that *P. betae* from Canada could be divided into two special forms: f. sp. *amaranthi* on *Amaranthus retroflexus* and f. sp. *betae* on Chenopodiaceae. The former infected only *A. retroflexus*, whereas the latter infected chenopodiaceous plants such as spinach, *C. album*, *C. capitatum*, *A. hortensis*, sugar beet, and garden beet. *P. betae* f. sp. *betae* included a European biotype on beet that grew on *C. album* and an Ontario biotype that grew on *C. album* but in only one case infected beet. Both the European and Ontario biotypes grew well on spinach, suggesting that these are locally adapted to specific hosts. Sugar beet is grown extensively in Europe, while in Ontario it had not been grown commercially since 1967.

In Japan, Abe and Ui (1986) confirmed the above results of Barr (1979) and further proposed a new forma specialis, i.e., *P. betae* f. sp. *portulacae* that infects only *Portulaca oleracea* and *P. grandiflora*. The authors also showed that host ranges of *P. betae* isolates from sugar beet and *C. album* are clearly different; for example, sugar beet isolates did not infect *C. album*, whereas *C. album* isolates did not infect sugar beet. Spinach, *C. murale*, *C. capitatum*, and *C. ficifolium* were common host

plants for both isolates. Thus, isolates from certain plant species may not infect plants from other families or even other plants within the same family. Furthermore, populations of *P. betae* from a single field soil were quite heterogeneous, with individual isolates showing significant variability in host specificity (Barr 1979; Abe and Ui 1986; Barr and Asher 1992).

Legrève et al. (2005) reported that the host range of *P. betae* is much wider than previously known and includes several families hitherto unsuspected. Altogether, 29 species belonging to the families Amaranthaceae, Asteraceae, Brassicaceae, Caryophyllaceae, Chenopodiaceae, Papaveraceae, Poaceae, and Urticaceae were identified (see Chap. 7). This host range study was conducted using sensitive detection methods such as polymerase chain reaction (PCR). It was notable that *P. betae* could infect monocotyledonous plants such as members of the Poaceae (Mouhanna et al. 2008).

Desoignies et al. (2011) reported that *P. betae* was detected in roots of *Arabidopsis thaliana* (Brassicaceae) using light microscopy and PCR. Infection severity was relatively low in this species compared with sugar beet, but all stages of the life cycle were present. The phenotype of *P. betae* in root cells of *A. thaliana* differed from that in sugar beet; for example, the spore-forming phase was more prevalent in comparison with the sporangial phase, and the sporosori contained a lower number of spores. Interestingly, the morphology of *P. betae* spores on *A. thaliana* is very close to that of *Plasmodiophora brassicae* resting spores produced on the same plant (Koch et al. 1991). This phenotype might be related to a specific interaction between the two plasmodiophorid species and *A. thaliana*. Moreover, Smith et al. (2013) have detected both *P. graminis* and *P. betae* sequences in roots of *A. thaliana*, indicating the possible infection of *Arabidopsis* plants by the two *Polymyxa* species.

On the other hand, there is also remarkable variation in the host specificity of *P. graminis* isolates from various origins. Susceptibility and multiplication rate in infected plants was found to differ among various isolates from distinct plants adapted to specific climatic regions and from distinct plants in the same country (Adams and Swaby 1988; Legrève et al. 1998).

Molecular diversity and phylogeny of isolates of *Polymyxa* species have been studied using restriction fragment length polymorphisms and sequences of rDNA (Ward et al. 1994; Ward and Adams 1998; Morales et al. 1999; Legrève et al. 2002; Smith et al. 2011). Analyzed rDNA sequence fragments contain partial ribosomal RNA (rRNA) genes; that is, some regions such as the 5.8S and 18S genes show relatively few differences between species, whereas other regions, such as the internal transcribed spacers (ITS) 1 and 2 regions between genes, are much more variable. Analysis of these and subsequent results (Smith et al. 2013; Neuhauser et al. 2014) show that the two *Polymyxa* species, *P. betae* and *P. graminis*, can be clearly distinguished and that there are several subgroups (ribotypes) of *P. graminis* (Fig. 6.5). In particular, Legrève et al. (2002) classified *P. graminis* ribotypes as different *formae speciales* that correlated with host range, temperature requirements, and geographical origin. For example, *P. graminis* isolates from temperate regions grew well at 17–20 °C, whereas *P. graminis* isolates from tropical climates were more aggressive

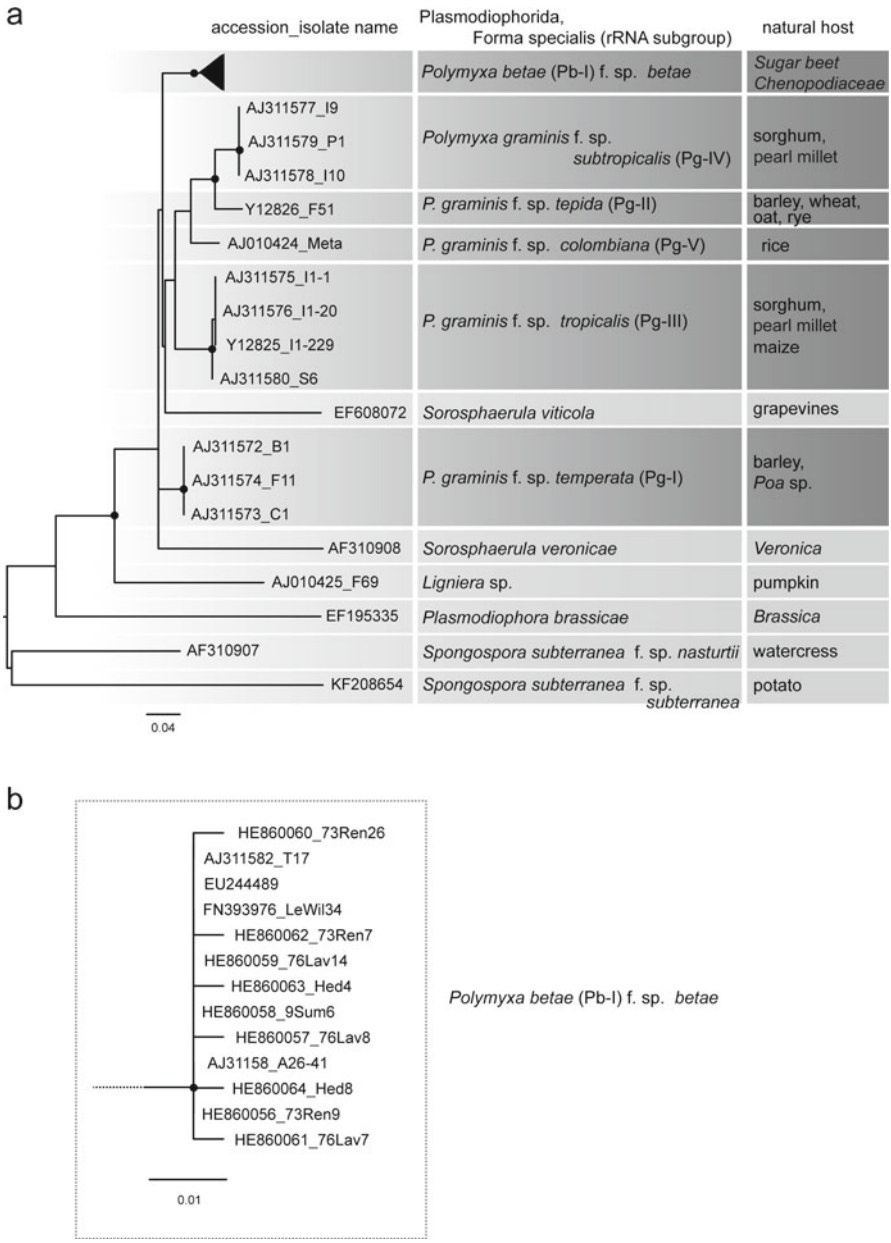

Fig. 6.5 Phylogenetic (ML) tree calculated from the rRNA 5.8S gene and two internal transcribed spacers (ITS1 and ITS2) of selected plasmodiophorids (**a**). (**b**) Clade of the collapsed branch (*black triangle*, the isolates of *Polymyxa betae*) indicated in (**a**) (Courtesy, Hideki Kondo). *Closed circles* on the nodes represent highly supported branches by aLRT analysis with the SH-like calculation (only values greater than 0.9 are shown)

and had a higher temperature optimum of 27–30 °C. On the basis of the ecological characteristics and rDNA analysis, five special forms were proposed: *P. graminis* f. sp. *temperata*, *P. graminis* f. sp. *tepida*, *P. graminis* f. sp. *subtropicalis*, *P. graminis* f. sp. *tropicalis*, and *P. graminis* f. sp. *colombiana* (Legrève et al. 2002) (Fig. 6.5).

6.5 Virus-Vector Relationship

Plasmodiophorid-transmitted viruses are known to include about 20 species belonging to five genera: *Furovirus*, *Pecluvirus*, and *Pomovirus* (family *Virgaviridae*), *Benyvirus* (family *Benyviridae*), and *Bymovirus* (family *Potyviridae*) (Adams et al. 2009; Tamada and Kondo 2013) (see Fig. 5.1). Out of 41 species of the family Plasmodiophoraceae, only three species, *Polymyxa graminis*, *P. betae*, and *Spongospora subterranea*, are recognized as virus vectors. Most of the viruses cause severe diseases in major crops such as rice, wheat, barley, oat, sugar beet, potato, and peanut. For example, *P. graminis* transmits at least 15 species of viruses (Kanyuka et al. 2003) including soil-borne wheat mosaic virus (SBWMV, furovirus), wheat spindle streak mosaic virus (WSSMV, bymovirus), wheat yellow mosaic virus (bymovirus), rice stripe necrosis virus (benyvirus), and peanut clump virus (pecluvirus).

Spongospora subterranea transmits potato mop-top virus (PMTV, pomovirus). *P. betae* is a vector of BNYVV and can also transmit three other viruses: beet soil-borne mosaic virus (BSBMV, benyvirus), beet soil-borne virus (BSBV, pomovirus), and beet virus Q (pomovirus), which are sometimes implicated in the rhizomania complex in sugar beet (McGrann et al. 2009a). All these viruses can be present in their plasmodiophorid vectors during all stages of the life cycle and can survive in soil within the long-lived resting spores.

As regards viral genes involved in vector transmission, the coat protein-readthrough (CP-RT) proteins of beny-, furo-, and pomoviruses, the p39 protein of pecluvirus, and the P2 protein of bymovirus (see Fig. 5.1) are considered to play an important role in the transmission process (Tamada and Kusume 1991; Tamada and Kondo 2013). In particular, elimination or mutation of the C-terminal portion of the RT domain and p39 or P2 proteins is correlated with loss of vector transmission. There is very little direct sequence similarity among these proteins, but they do have some structural similarity that might be involved in transmission (Dessens and Meyer 1996). The CP-RT proteins of BNYVV and PMTV are shown to be present in a small proportion of virions at one extremity. The RT domains and P2 proteins contain two putative transmembrane regions, suggesting that these transmembrane regions are involved in attachment to the zoosporangial plasmodesmata and may assist virus particles to move between the cytoplasms of the plant host and the vector (Adams et al. 2001). In the case of BNYVV and BSBMV, the RNA4-encoded protein is involved in efficient vector transmission (Tamada and Abe 1989; Rahim et al. 2007; D'Alonzo et al. 2012).

Mechanisms of virus acquisition by plasmodiophorids from host cells and virus entry into host cell cytoplasm are unknown. However, it is considered that these processes are taking place either when zoospores penetrate the host cells and transfer their contents into the host cell cytoplasm or at the sporogenic plasmodial stage (see Fig. 6.1), when there is only a thin membrane boundary separating the plasmodiophorid from the host cell cytoplasm (Kanyuka et al. 2003; Tamada and Kondo 2013). Acquired viruses are thought to be carried within rather than on the surface of the resting spores and zoospores. Viruses cannot be removed from zoospores by washing or inactivated by application of antiserum. Moreover, for BNYVV, resting spores remain viruliferous even after treatments with diluted NaOH and HCl (Abe and Tamada 1986). Viruslike particles have been observed within numerous vacuoles in young immature plasmodia or zoospores (Tamada 1975; Abe and Tamada 1986; Rysanek et al. 1992), but this has not been demonstrated for resting spores, because the impermeability of their multilayered wall renders their ultrastructure difficult to study (Kanyuka et al. 2003). However, in the case of SBWMV transmitted by *P. graminis*, viral RNA and movement protein (MP) but not CP were detected in resting spores, suggesting that the *Polymyxa* vector does not transmit intact SBWMV particles to host cells, but more a ribonucleoprotein complex consisting of MP and viral RNA (Driskel et al. 2004). In the case of WSSMV, however, CP was detected in resting spores. For BNYVV, viral proteins that are involved in virus replication and movement were detected within zoosporangia and resting spores of *P. betae* (Lubicz et al. 2007). These results suggest that viral translation and movement may occur within the vector. Thus, the mechanism of virus transmission by *Polymyxa* still remains a mystery, and further study is needed to determine whether the plasmodiophorid is actually a host for viruses or simply a vector for virus transmission.

6.6 Detection and Discrimination

6.6.1 Light Microscopy

The simple method to confirm the presence of *P. betae* in host plants such as sugar beet is by direct microscopic observation of roots from plants. Plant roots are carefully washed with tap water to remove soil and organic matter attached to the roots. Resting spore clusters can readily be seen in young lateral roots, but detection of plasmodia or zoosporangia may require staining with dyes such as cotton blue. To confirm the presence of *P. betae* in soil, *Polymyxa* can be baited by growing susceptible sugar beet plants in the tested soil under moisture and temperature conditions that are suitable for infection.

6.6.2 Serological Techniques

Mutasa-Göttgens et al. (2000) produced highly specific rabbit polyclonal antibodies for *P. betae* using a novel recombinant DNA approach. The authors identified and cloned a cDNA fragment from *P. betae* whose product, a glutathione S-transferase (GST), was expressed in zoospores, sporangia, and resting spores. Polyclonal antiserum produced from GST was found to react specifically with *P. betae* in sugar beet roots and with *P. graminis* in barley roots and to cross-react with *Plasmodiophora brassicae* in cabbage roots. However, no cross-reaction was detected with protein extracts from potato roots infected by *Spongospora subterranea*. In all cases, there was no interaction with proteins from host plants or from other microorganisms found in association with roots of sugar beet, barley, cabbage, and potato. Thus, as the antibodies showed little cross-reactivity to other root parasites of sugar beet, they may be useful in the detection and quantification of *P. betae* in plants and soil and in examining localization of *P. betae* in their hosts by microscopy. Kingsnorth et al. (2003a) further developed the enzyme-linked immunosorbent assay (ELISA) test for *P. betae* using the polyclonal antibody combined with a new monoclonal antibody recognizing the GST of *P. betae*. A close correlation was found between the numbers of *P. betae* zoospores in serially diluted suspensions and ELISA absorbance values. The ELISA test using monoclonal antiserum was shown to enhance the specificity of detection of *P. betae*.

6.6.3 Molecular Techniques

Mutasa et al. (1993) first reported the development of a specific DNA probe for the detection of *P. betae* in plant tissue. PCR primers for improved sensitivity and faster detection of the *P. betae* probe were identified (Mutasa et al. 1995), followed by the development of improved PCR primers that allowed amplification of *P. betae* DNA sequences in a single-tube nested reaction (Mutasa et al. 1996). This reaction system does not require an additional step, reducing the risk of contamination. It was possible to detect *P. betae* sequences in as little as 1 pg of total genomic DNA from infected roots, which is equivalent to a 10,000-fold increase in sensitivity compared to detection by Southern hybridization, as used previously (Mutasa et al. 1993). For rapid analysis of amplified products, primers can be modified to generate products that could be detected in a colorimetric assay with a commercially available kit. Kingsnorth et al. (2003b) subsequently developed a quantitative real-time PCR method for detection of the *P. betae* GST. This is the most sensitive and accurate method of *P. betae* detection, although positive signals are likely to be derived mainly from viable material, because the mRNA of GST is labile.

Although conventional PCR techniques were applied for discrimination and detection of *Polymyxa* species (Ward et al. 1994; Ward and Adams 1998), Ward et al. (2005) developed *P. graminis*-specific real-time PCR assays using primers that

were specific to *Polymyxa* species (*P. graminis* and *P. betae*) with a TaqMan probe (Applied Biosystems) that was specific for *P. graminis* only. It was also confirmed that real-time PCR with nonspecific DNA-binding dyes such as SYBR Green (Applied Biosystems) is suitable for detection of *P. betae* as well as *P. graminis*. Notably, the real-time PCR assay was shown to be highly sensitive, allowing detection of a single zoospore in the sample. It also appeared to be reliable in quantifying *Polymyxa*, whether as different numbers of zoospores or as different proportions of infected roots mixed with either healthy roots or uninfected soils. This assay, however, would not be applicable where both *P. graminis* and *P. betae* are present in the tested soils.

6.7 Conclusions

Polymyxa species are specialized obligate parasites which, though appearing to do little direct damage to their host plants, are highly effective vectors of pathogenic viruses. Their longevity of survival as thick-walled resting spores and rapid multiplication in roots in the form of zoosporangia and zoospores ensures effective survival and propagation of the virus. In addition, the unusual mechanism of plasmodiophorid infection, by direct injection of zoospore contents into the root cell, may aid virus transmission. Whether or not the vector acts as a host for virus multiplication within or outside the plant root requires further study.

The predominant specialization of *P. betae* on the Chenopodiaceae and *P. graminis* on the Gramineae, a differentiation supported by molecular data, may be due primarily to parasite- rather than host-mediated factors (McGrann et al. 2009b). Whole-genome sequencing of *Polymyxa* species could help to resolve this and contribute to our understanding of the mechanisms involved in parasitism and vectoring ability of these widely distributed and economically important organisms.

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Chapter 7
Ecology and Epidemiology

Tetsuo Tamada and Michael J.C. Asher

Abstract There are many factors affecting the development and spread of rhizomania, caused by beet necrotic yellow vein virus (BNYVV) that is transmitted by zoospores of the vector *Polymyxa betae*. Virus-carrying resting spores of *P. betae* retain infectivity for long periods in soil. The amount of virus-carrying *P. betae* (inoculum potential) in soil can be assessed by means of a bioassay using bait plants. The dynamics of disease development were demonstrated by introducing different initial inoculum levels into a noninfested field, and this showed that rapid increases of inoculum potential occurred under sugar beet cultivation during the first and following years. It also highlighted the danger of introducing even small amounts of contaminated soil. Both *P. betae* and BNYVV have limited natural host ranges, and common arable weeds may play only a minor role as natural field reservoirs of the virus. Resting spores of *P. betae* within soil particles are dispersed in several ways, such as by wind, water (e.g., flooding, irrigation), manure, animals, transport vehicles, farm machinery, and plant materials. BNYVV is not transmitted within seed or pollen, but its spread can result from soil-contaminated seeds. The germination of resting spores, the release of motile zoospores, and the production and release of secondary zoospores are all influenced by soil type, soil moisture, and temperature. In particular, zoospores of *P. betae* infect most rapidly and actively at relatively high temperature and moisture conditions in neutral or alkaline soils. Therefore, temperature in the spring and early summer has an important influence on disease severity. Temporal and spatial models to predict the rate of development and spread of rhizomania have been developed.

Keywords Sugar beet • Rhizomania • *Polymyxa* • BNYVV • Soil-borne disease • Disease spread • Environmental factors

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E. Biancardi, T. Tamada (eds.), *Rhizomania*, DOI 10.1007/978-3-319-30678-0_7

7.1 Inoculum

Rhizomania is caused by beet necrotic yellow vein virus (BNYVV, benyvirus) that is transmitted by zoospores of the vector *Polymyxa betae*. BNYVV survives in clusters of resting spores of *P. betae* in the soil. The virus-carrying resting spores remain infective for long periods in the field. Dried infected roots have been stored at room temperature for many years without apparent loss of infectivity, and contaminated air-dried soil retained its disease potential for more than 15 years (Abe and Tamada 1986). In several cases, severe yield reductions were observed when sugar beet was grown in fields that had had no inoculum sustaining crops during the previous 10–15 years (Schlösser 1988). After a break from sugar beet of more than 7 years, a considerable percentage of infected fields still had a high disease potential (Grunewald et al. 1983). The survival period of virus-carrying resting spores in the field is thought to be at least 7–10 years (Schlösser 1988).

7.1.1 Inoculum Assessment

Quantification of inoculum (i.e., virus-carrying resting spores of *P. betae*) in soil is very important for ecological and epidemiological studies and can also be valuable in forecasting and advising farmers on crop management. The presence of virus-carrying *P. betae* in soil is commonly assessed by means of a bioassay using sugar beet seedlings as bait plants.

Beemster and de Heij (1987) first described the possibility of obtaining quantitative data for the detection of both *P. betae* and BNYVV. After exposure to soil for 4 days in a Petri dish, the bait plants were transplanted into sterile sand and, after 5 weeks, *P. betae* and BNYVV infections were examined by light microscopy and enzyme-linked immunosorbent assay (ELISA), respectively. Assessment of the percentage of plants infected allowed an estimation of the level of infestation of the soil. Uchino et al. (1990) also estimated the infestation level of soil by ELISA value. Several, 1-month-old sugar beet seedlings were transplanted together into a polyethylene pot containing the sample soil (with the addition of 5 g calcium carbonate per about 100 g soil) and grown at 25 °C in the greenhouse. Bulk samples of sugar beet rootles were tested by ELISA. With heavily infested soil, positive results in a dilution series were obtained from samples containing as little as 10 % and 0.1 % of the original soil when incubated for 5 and 10 days, respectively. Results obtained from 3 weeks incubation showed a positive correlation between ELISA values and the level of infestation of the soil. Similar experiments were reported by Ciafardini (1991), who developed a quantitative analytical method for evaluating the concentrations of infective units of *P. betae* in soil, using the most probable number (MPN) technique and ELISA. Bait plants were grown in glass cylinders and incubated for 40 days at 20 °C. This method was suitable for the routine analysis of numerous soil samples and can be used to estimate the degree of infestation of soil by virus-carrying

P. betae. Tuitert (1990) also estimated the MPN of infective units of virus-carrying *P. betae* by assessing the numbers of infected plants in serial dilutions of soil. Optimal results were obtained by growing bait plants for 6 weeks at 23 °C with 16 h light and 15 °C with 8 h dark. MPNs determined after 6 weeks were 16-fold higher than after 3 weeks and did not increase further by 12 weeks (Tuitert and Bollen 1993).

Generally, a quantitative assessment of inoculum of *P. betae* and BNYVV can be achieved by using serial dilutions of the test soil. The dilution end point for severely infested soil samples was about 10^{-4}–10^{-5} so, for most samples to be assayed for BNYVV, a three- to tenfold dilution would be sufficient to estimate levels of infestation. With these procedures, inoculum potential rather than inoculum density is measured, and the results of the assay are presented as MPNs of infective units (Tuitert 1990; Adams and Welham 1995). However, the results of bioassays are influenced by various factors such as the pretreatment of soil, the volume of soil tested, the age of bait plants, the duration of the assay, and the environmental conditions.

In studies of the detection of artificially introduced resting spores of *P. betae* in a bioassay, Keskin et al. (1962) reported that only 1–2 % of single resting spore clusters (each containing 10–30 spores) gave rise to infection of seedling rootlets. Tuitert and Bollen (1993) also showed that only 1.7 % of resting spores in artificially infested sand were detected, corresponding to 50–100 resting spore clusters. Similarly, one infective unit of *P. betae* was shown to correspond to at least 50 clusters (Abe and Tamada 1986) and 100–150 clusters (Fujisawa and Sugimoto 1976). Such a low level of detection may be attributed to only a small proportion of resting spores germinating, because of partial or non-synchronous germination. Furthermore, only a small number of primary zoospores, even if released from resting spores, may succeed in contributing to detectable infection.

7.1.2 Inoculum Potential

Resting spores of *P. betae* usually accumulate in the upper 20 cm of the soil layer, but can also be found at a depth of 60 cm (Abe 1987; Heijbroek 1987). Uchino and Kanzawa (1991) studied the effect of inoculum present at different depths on disease infection and development. Experimental plots were created in a noninfested field by the addition of infested soil in a layer at different depths, ranging from the surface to 70 cm deep. The results showed that severe infection occurred where the layers were 0–20 cm, 0–40 cm, and 20–40 cm deep, whereas less severe infection but considerable yield loss was observed in the layer at 50–70 cm. This indicates that the presence of inoculum even below a depth of 50 cm (subsoil layer) may cause substantial damage to sugar beet.

The question arises as to how much inoculum is required for disease development and to what extent does inoculum potential build up in the following years under sugar beet cultivation. Tuitert and Hofmeester (1994) explored this by

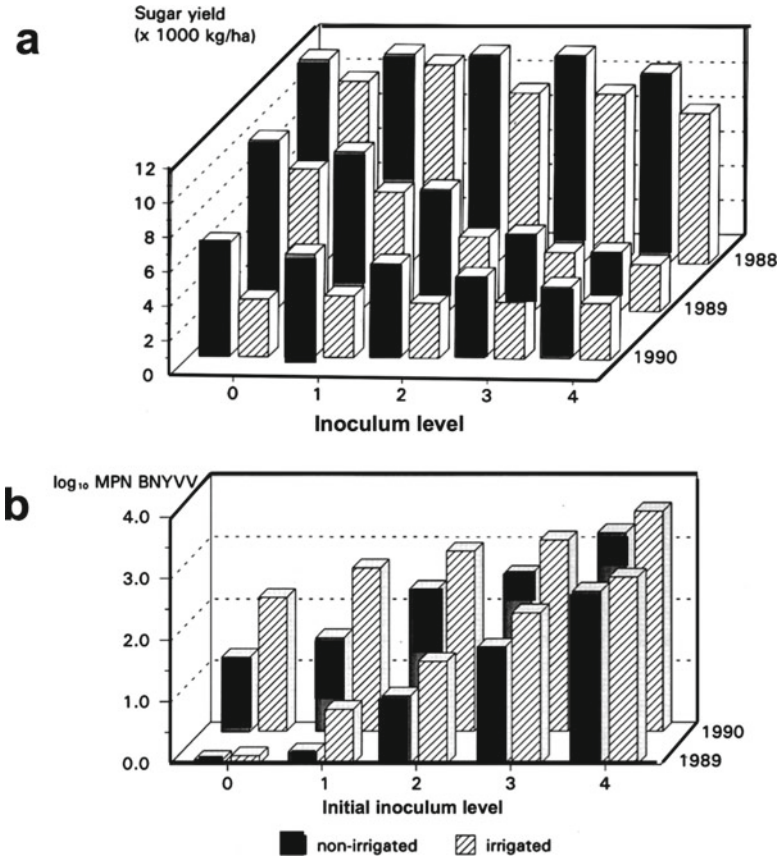

Fig. 7.1 Epidemiology of BNYVV in sugar beet at different inoculum levels in three successive crops in the presence or absence of irrigation. Approximate amounts of BNYVV-infested soil introduced in 1988 were 0, 0.5, 5, 50, and 500 kg ha^{-1}, which correspond to inoculum levels 0–4. (**a**) Sugar yield of three successive sugar beet crops (From Tuitert and Hofmeester 1994); (**b**) The inoculum potential of BNYVV in soil in May in 1989 and 1990 (From Tuitert and Hofmeester 1992). Inoculum potential given as arithmetic means of $\log_{10}$ (MNP+1); MPN is the most probable number of infective units per 100 g soil

applying different inoculum levels (in the form of mixtures in different proportion of infested and noninfested soils) to plots in a field that had never previously grown sugar beet. Sugar beet was then cultivated, with or without irrigation, for three successive years (Fig. 7.1). In the first year, root symptoms were not observed, but at harvest BNYVV-infected plants were detected by ELISA in low numbers at all inoculum levels. Root weight at harvest was not affected, but sugar content decreased with increasing inoculum level, leading to a reduction in sugar yield of 10 % at the highest inoculum level. In the next year that sugar beet was grown in the same field plots, symptoms of BNYVV-infected plants were more severe in plots with higher

inoculum levels. Root weight and sugar content decreased progressively with increasing inoculum level, resulting in sugar yield reductions of 11–66 % compared to the noninfested plots (Fig. 7.1a). In the third year, the whole field was heavily infected. Thus, it was shown that introduction of even very small amounts of infested soil to a field can cause a yield reduction in the first year and more severe reductions in the following years (Fig. 7.1a).

Tuitert and Hofmeester (1992) also examined the dynamics of inoculum of BNYVV and *P. betae* during the first 2 years following infestation in the same field (Fig. 7.1b). Cultivation of the first sugar beet crop resulted in a 10,000-fold multiplication of inoculum of virus-carrying *P. betae*, whereas in the second crop, the maximum increase was about 70-fold. This rapid increase of inoculum highlighted the danger of introducing small amounts of infested soil and, at a time when effective control measures (e.g., resistant varieties) were unavailable, emphasized the importance of measures to reduce the spread of infested soil (Tuitert and Hofmeester 1992).

7.1.3 Weeds as Alternative Hosts

The observed longevity of inoculum survival in the field gives rise to doubts as to whether weeds play a significant role as alternate hosts perpetuating virus-carrying *P. betae*. Earlier studies on alternative hosts of *P. betae* showed that its host range was limited to a few members of the families Amaranthaceae, Asteraceae, Caryophyllaceae, Chenopodiaceae, Portulacaceae, and Solanaceae (Keskin 1964; Barr 1979; Abe and Ui 1986; Barr and Asher 1992). Recently, *P. betae* and BNYVV have been reported to have much wider host ranges. For example, Legrève et al. (2005) showed that both virus and vector could be detected by reverse transcriptase polymerase chain reaction (RT-PCR) in 29 of 64 plant species grown in infested soil. They included Amaranthaceae (*Gomphrena globosa*), Asteraceae (*Chrysanthemum segetum*), Brassicaceae (*Capsella bursa-pastoris* and *Thlaspi arvense*), Caryophyllaceae (*Silene alba*, *Silene noctiflora*, *Stellaria media*, and *Stellaria graminea*), Chenopodiaceae (*Atriplex halimus*, *Atriplex hortensis*, *Atriplex undulata*, *Chenopodium album*, *Chenopodium bonus-henricus*, *Chenopodium murale*, *Chenopodium polyspermum*, *Chenopodium quinoa*, *Chenopodium rubrum*, *Chenopodium vulvaria*, *Chenopodium amaranticolor*, *Beta vulgaris*, and *Spinacia oleracea*), Papaveraceae (*Papaver rhoeas*), Poaceae (*Hordeum vulgare*, *Poa pratensis*, *Apera spica-venti*, *Digitaria sanguinalis*, and *Elymus repens*), and Urticaceae (*Urtica urens*). Back transmission to sugar beet was shown in several species, but not in all species listed above. Also, *P. betae* was detected by RT-PCR in five other species: Apiaceae (*Aethusa cynapium*), Asteraceae (*Achillea millefolium*, and *Anthemis arvensis*), Poaceae (*Lolium perenne*), and Solanaceae (*Solanum nigrum*). In addition to the species described above, Hugo et al. (1996) reported that *P. betae* infected *Amaranthus retroflexus* and *Amaranthus caudatus-viridis* (Amaranthaceae),

Silene vulgaris (Caryophyllaceae), and *Papaver argemone* (Papaveraceae). Furthermore, Mouhanna et al. (2008) reported that several other weed species seemed to be alternative hosts for BNYVV and *P. betae* (German isolate). These included the monocotyledonous plants *Alopecurus myosuroides*, *Lolium multiflorum*, *Sorghum vulgare*, and *Sorghum halepense* and dicotyledonous plants *Calystegia sepium*, *Capsella bursa-pastoris*, *Centaurea cyanus*, *Convolvulus arvensis*, *Galinsoga parviflora*, *Matricaria inodora*, and *Stellaria media*. However, there is only very limited virus multiplication in many weeds that were recognized as positive, and common arable weeds probably play only a minor role in the epidemiology of BNYVV, even if they are hosts. In addition, most weed species are largely controlled by herbicides in arable rotations involving sugar beet.

On the other hand, populations of *P. betae* from a single field soil have been shown to be quite heterogeneous, with individual isolates showing significant variability in host specificity (Abe and Ui 1986; Barr 1979). For instance, isolates of *P. betae* from *Amaranthus retroflexus* and *Portulaca oleracea* infect species only within the Amaranthaceae and Portulacaceae, respectively. Furthermore, Abe and Tamada (1986) found that the ability of the vector to transmit BNYVV differs among isolates of *P. betae*; e.g., the virus was maintained only in resting spores obtained from sugar beet, spinach, *Chenopodium murale*, and *Chenopodium capitatum*. Indeed, the inoculum potential (virus-carrying *P. betae*) of severely infested soils, in which *Chenopodium album*, *Amaranthus retroflexus*, or *Portulaca oleracea* had been grown, was greatly reduced, compared to the inoculum potential of soil after growing sugar beet or Swiss chard, which was significantly increased (Abe and Tamada 1984). Moreover, when virus-carrying isolates of *P. betae* were grown in the roots of *Chenopodium ficifolium*, which is susceptible to BNYVV, although the virus was detected in the roots, the released zoospores did not transmit the virus (Abe and Tamada 1986). Possibly, *P. betae* can transmit BNYVV to this plant species, but may not acquire the virus from it. Together, these results further suggest that arable weeds, even if infected by BNYVV and *P. betae*, do not always play a role as natural field reservoirs of the virus.

7.1.4 Inoculum Potential After Growing Resistant Varieties

Genetic resistance is the most promising way to control rhizomania, and a large number of virus-resistant varieties have been grown in infested areas, although they are not resistant to the vector. It is presumed that acquisition of virus by the vector is reduced in virus-resistant plants and, consequently, virus-resistant varieties delay the buildup of virus inoculum in the field compared to susceptible varieties. Such a reduction of inoculum potential by resistant varieties could have important epidemiological implications. Indeed, Tuitert et al. (1994) showed that the population of resting spores of *P. betae* in partially resistant plants with a low virus content in the roots was less viruliferous than that in susceptible plants with a high virus content, suggesting that the growth of a resistant variety can be expected to delay the buildup

of virus inoculum in soil. In the field, the inoculum potential of BNYVV in soil was lower after growing a partially resistant variety than after growing a susceptible variety.

It might be expected that a much higher level of virus resistance in the host plant would have a correspondingly greater effect on reducing virus inoculum in field soil. In areas where virus-resistant varieties will continue to be grown in infested soils, it is important that the inoculum level in the soil does not increase. In addition, resistance to the vector might contribute to a further delay in the buildup of inoculum or a gradual elimination of inoculum of BNYVV. Such a reduction in the buildup of virus inoculum will contribute to the durability of disease resistance.

7.2 Dispersal

There are two different ways in which virus-carrying *P. betae* can be dispersed through or with soil. One is active dispersal by motile zoospores moving autonomously through soil, and the other is passive dispersal of resting spores without expending energy. Although zoospores can be passively displaced by water movement over the soil surface or through soil pores, resting spores can be dispersed by movement of contaminated soil in several ways, such as wind, water (flooding, irrigation), manure, animals (birds, farm animals), transport vehicles, farm machinery, and plant materials (Hillmann 1984; Heijbroek 1987, 1988; Schlösser 1988; Asher 1993). Dispersal of resting spores or adhering soil is described as follows:

Wind Dispersal of virus-carrying resting spores of *P. betae* with soil particles by wind plays an important role in disease spread. Windblow of soils from infested fields results in a largely unavoidable spread from field to field. Such aerial dispersal of resting spores has not been experimentally investigated, but the movement of the much larger cysts of the beet cyst nematode (*Heterodera schachtii*) in windblown soil has been reported (Schlösser 1988), indicating that wind dispersal could play a significant part in their transmission. Indeed, the widespread distribution of rhizomania in the inner regions of China is thought to be due to the strong wind, the so-called yellow dust that prevails during the spring. Wind dispersal has also been implicated in the widespread dissemination of soil contaminated with viruliferous resting spores of *Polymyxa graminis* which, coupled with frequent wheat growing, may account for the extensive distribution of soil-borne wheat mosaic virus (furovirus) and wheat spindle streak mosaic virus (bymovirus) in parts of the United States and Canada, respectively (Harrison 1977).

Water Resting spores of *P. betae* can be dispersed by erosion due to flooding or by watering in irrigated agriculture. In the Netherlands, the first occurrence of rhizomania was often observed along the edges of fields adjacent to ditches, following the deposition of contaminated mud and water from ditch-clearing activities. Indeed, Heijbroek (1987) showed that contaminated drainage water from infested sugar beet fields was used for irrigating or spraying crops, or infiltrated directly by

the deliberate raising of the water table during the growing season to supply the crop. In California, much of the local spread has been attributed to the use of furrow irrigation, with surplus water being run from field to field (Asher 1993).

Manure It has been shown that virus-carrying resting spores of *P. betae* are able to survive through the intestine of sheep fed on sugar beet root fragments infected with rhizomania (Hillmann 1984; Heijbroek 1988). Thus, the use of organic manure could carry the risk of spreading the disease from fields where animals are fed with sugar beet tails from beets grown on contaminated soil. Tops and leaves carry less risk, because no resting spores are present within these plant parts, unless they are contaminated with infested soil (Heijbroek 1988).

Factory waste The soil that accumulates from infected roots during the harvesting period usually has a very high inoculum potential. In particular, factory waste from the preparation of harvested sugar beet for processing is of potential significance in the spread of the disease (Schlösser 1988; Asher 1993). In Japan, where sugar beet seedlings are raised in paper pots and transplanted into the field, the use of contaminated waste soil in the pots resulted in a substantial increase of the sugar beet areas becoming infested (Ui 1973). Similarly, in France, the practice of returning the considerable amounts of waste soil to agricultural land contributed greatly to the spread of the disease (Richard-Molard 1985; Cariolle 1987). In the past, such material was returned directly to farms for soil improvement. Furthermore, the washing water used to remove the remaining soil from harvested roots is usually collected in ponds or lagoons, where the soil is allowed to sediment over a period of several months or years. These ponds were in the past distributed to farm land for soil improvement or were used directly for the production of crops (Asher 1993).

Agricultural implements It is clear that very small quantities of soil adhering to farm vehicles and machinery can readily disseminate the disease. The characteristic elongated or cross-shaped patterns of disease distribution are often observed in a recently infested field, corresponding to the direction of movement of machinery (Richard-Molard 1985; Cariolle 1987; Heijbroek 1988). In particular, there is a major risk of disseminating the disease by soil tillage or mechanical weed control within a field and by sugar beet or potato harvesting machines and transport vehicles throughout a whole area, because much of this work is done by contractors or cooperatives (Heijbroek 1988).

Plant materials Spread of virus-carrying resting spores of *P. betae* can be caused by movement of soil particles adhering to plant material such as seed potatoes, bulbs, and various rootstocks that have been grown in rhizomania-infested areas. In Japan, severe outbreaks of rhizomania in 1969 and 1970 were due to the introduction of sugar beet seedlings raised in nursery beds containing infested soil (Ui 1973).

Seed BNYVV is not transmitted by seed or pollen. Hesse et al. (1984) failed to detect any transmission of BNYVV in seeds from sugar beet plants grown in infested fields, if they were processed properly. However, if this is not done, the

spread of virus-carrying resting spores can result from soil contamination of seeds which have been produced in infested fields. This was demonstrated by Heijbroek (1988), who also confirmed the absence of direct transmission of BNYVV within seeds, even from systemically infected seed-bearing plants. In general, seeds for the commercial root crop are mechanically treated (processed) to remove the outer layers of the pericarp, along with any soil that might be attached (Heijbroek 1988). However, in the past, seed used for breeding purposes was often not processed and might well has contributed to the long-distance dispersal of the disease through exchanges between countries and between continents.

7.3 Factors Affecting Infection and Disease Development

The occurrence of the disease, its severity, and the extent to which it spreads within the growing crop are all dependent on the activity of virus-carrying zoospores of *P. betae*. Indeed, the germination of resting spores, the release of motile zoospores, and the production and release of secondary zoospores are influenced greatly by soil type, soil moisture, and soil temperature.

7.3.1 Soil Type and pH

Rhizomania occurs in any soil from a heavy clay to a light sand (Hamdorf and Lesemann 1979; Grunewald et al. 1983; Hillmann 1984). However, the incidence of the disease may be especially pronounced in badly drained or poorly structured soils. Thus, well-drained soils may reduce infection, whereas heavy soils with poor structure and compaction layers, which cause poor drainage, may enhance infection (Rush 2003). Higher levels of infection were observed on sandy soils than on black fen peat soils (Webb et al. 2000) though this may largely be due to differences in soil temperature (see below). Indeed, the effect of soil texture and structure on root infection is not always straightforward, because these soil characters can greatly influence soil moisture and temperature (Rush 2003).

On the other hand, soil pH is a major factor affecting root infection with virus-carrying *P. betae*, with neutral or alkaline soils having more severe infection. In Japan (Hokkaido), the incidence and severity of rhizomania in sugar beet fields are closely associated with soil pH, regardless of soil type. The native soils in Hokkaido are usually acidic, ranging from pH 4.5 to 7.5, and disease development was especially pronounced in soils having pH values above 6.5. Examples of the variation observed in two fields are shown in the schematic diagram of Fig. 7.2; disease incidence was closely related to soil pH, and the higher the soil pH, the more severe the disease. Abe et al. (1985) also showed that values of soil pH in several fields in the Chitose area fluctuated with changes in exchangeable calcium content, calcium saturation rate, and/or NO_3-N concentration in the soils (Fig. 7.3).

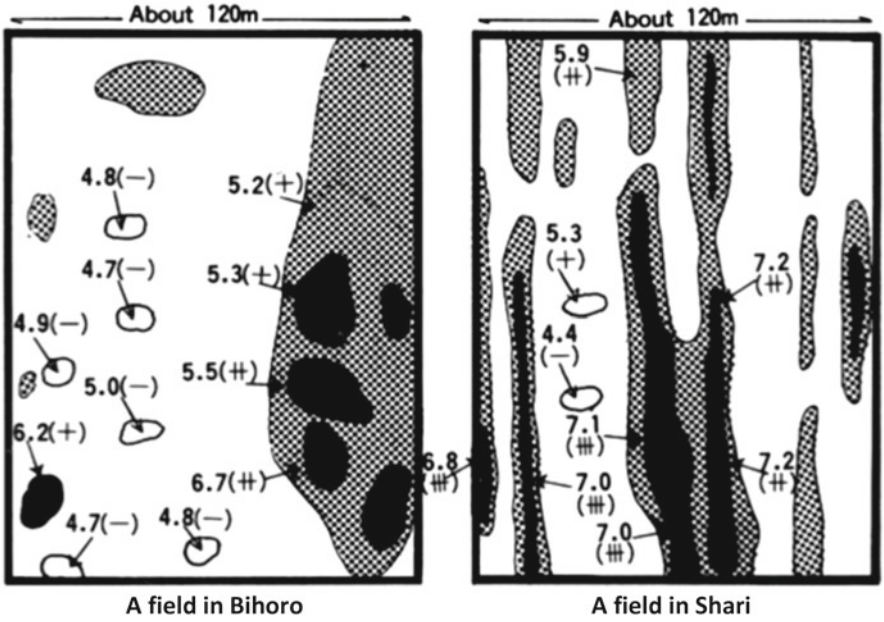

Fig. 7.2 Schematic diagram of the distribution of rhizomania incidence in two fields in Hokkaido (Japan), showing the relationship between disease severity, soil pH, and *P. betae* infection in different patches in a field (From Abe 1987). *Black* areas, heavily diseased; *shaded* areas, slightly diseased; *white* areas, healthy. *Figures* indicate soil pH. *Polymyxa betae* infection is shown in *parenthesis*. – not detected; + ~ +++ increasing degrees of infection

As regards the effect of soil pH on *P. betae* infection, Abe (1987) showed experimentally that zoospores of *P. betae* infect most rapidly and severely at pH 6.0 to 7.0, while the activity of zoospores decreases greatly below pH 6.0 (Fig. 7.4). For example, most of the zoospores at pH 6.0 to 7.0 were able to retain their infectivity for 10 h, but below pH 5.5 their infectivity was lost dramatically within a few hours (Fig. 7.4). However, resting spores can survive at lower values than pH 5.0, although their germination is inhibited and the activity of zoospores is also reduced (Abe 1987). Therefore, if the soil pH of contaminated fields is increased by liming, the disease potential can be restored. The application of superphosphate, acidic peat powder, or sulfur dust to reduce soil pH to 5.5 was shown to effectively reduce disease development (Miyawaki et al. 1983; Abe 1987), although decreasing soil pH has adverse effects on crop development (Asher 1993), and lime is normally applied to prevent this.

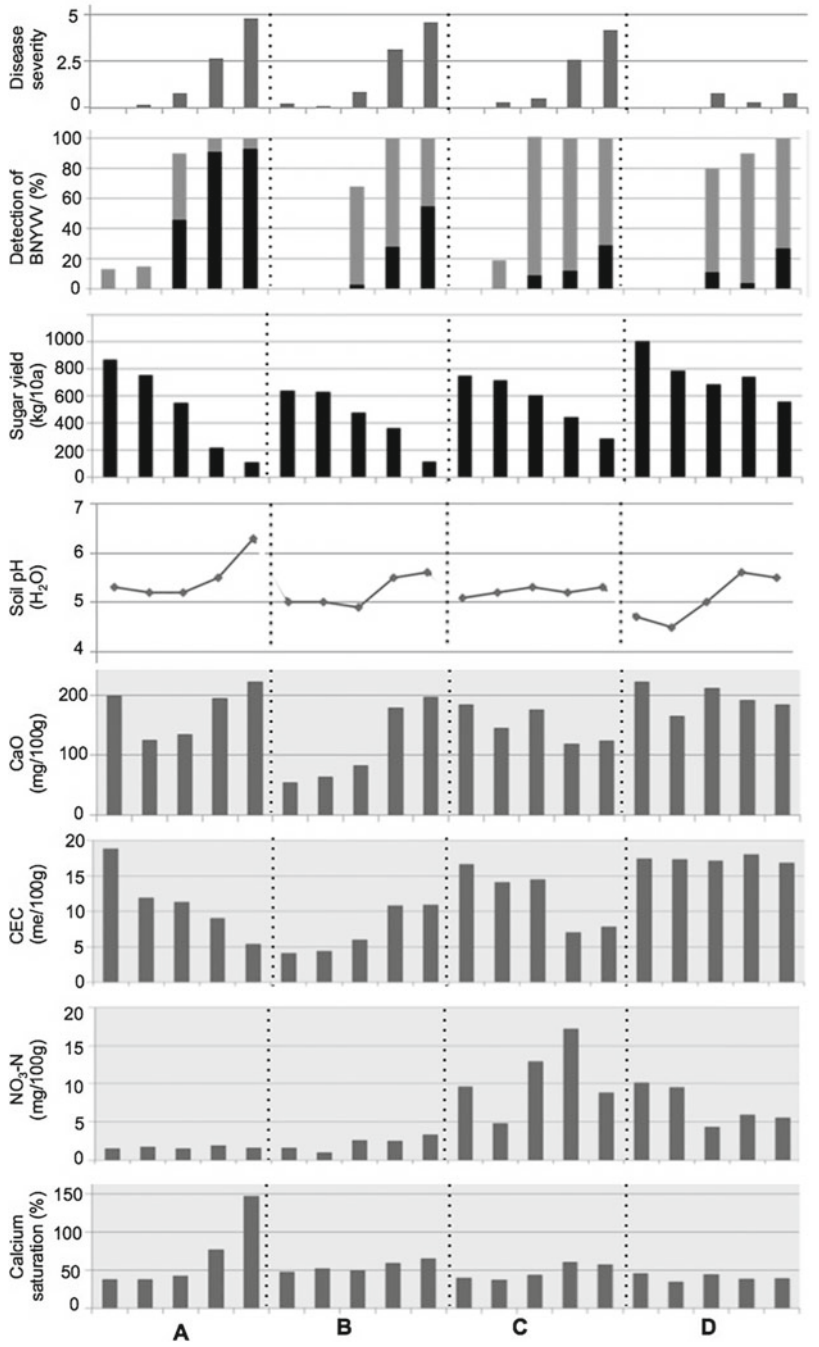

Fig. 7.3 A case example for disease incidence and chemical characteristics of soils in sugar beet fields in Hokkaido (Chitose area) (Represented by means of diagram from data of Abe et al. (1985)). Distribution of diseased patches in field: (**a**) widely, (**b**) widely and zonally, (**c** and **d**) limited. In each field, five sites showing different severity (including no symptom) were selected, in which about 30 plants per site were examined for disease severity, BNYVV detection, and yield parameters. Soils from around sampling sites were analyzed for chemical characteristics. The disease severity was estimated by five grades (1–5) of yellowing and stunting. BNYVV detection was done by ELISA from side roots (about 5–10 mm in size) (*black bar*) and fine rootlets (*grey bar*)

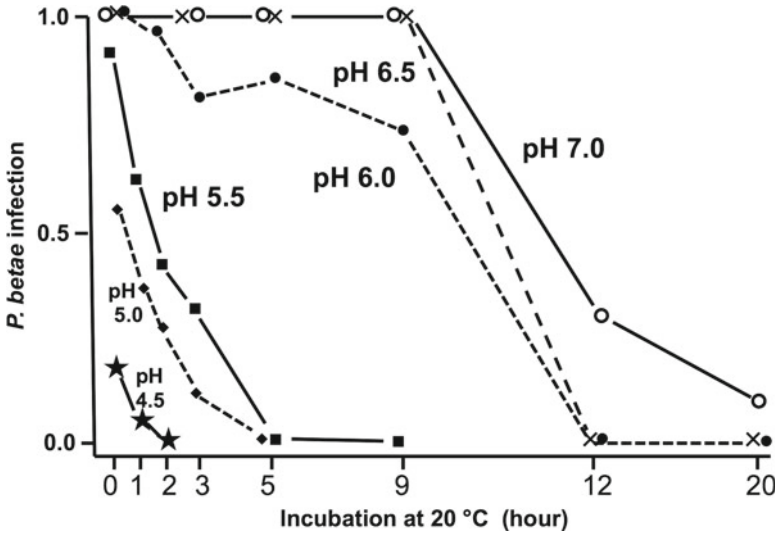

Fig. 7.4 Effect of pH on the activity (survival) of zoospores of *P. betae* (Modified from Abe 1987). Zoospore suspensions were incubated in different pH solutions adjusted with 1 % sulfuric acid or 0.5 % citric acid at 20 °C for different times and were inoculated to roots of sugar beet. To exclude effects on *P. betae* infection, solutions were adjusted to pH 7.0 immediately before inoculation. Zoospore activity was assessed by the degree of *P. betae* infection in roots

7.3.2 Moisture

Soil moisture is a critical factor affecting root infection by *P. betae*. Water is required for the resting spores to germinate and for the zoospores to swim through the soil pores to the roots. Soil moisture requirement is different in different soil types, and soil matric potential may directly influence the movement of the zoospores; e.g., it was shown that a limiting soil matric potential of greater than – 40 kPa is required (Gerik et al. 1990; de Heij 1991). *Polymyxa* zoospores range from 3 to 6 μm in size and the whiplash flagellum extends an additional 15 μm. At the nonconducive matric potential of – 40 kPa, the largest pore filled with water has a diameter of 7.4 μm, whereas at the conducive matric potential of – 20 kPa, the largest pore filled with water has a diameter of 14.7 μm (Cadle-Davidson et al. 2003). Hence *Polymyxa* zoospores can move freely in water-filled pores at – 20 kPa, but may encounter barriers at – 40 kPa (Cadle-Davidson et al. 2003).

As the amount of soil moisture is increased, the activity of *P. betae* zoospores is increased, raising the level and extent of infection. In practice, brief periods of rain at intervals during the spring and early summer months may be sufficient to stimulate zoospore release in the upper layers of the soil (de Heij 1991). However, poor soil structure, inadequate drainage, frequent heavy rainfall, and the use of irrigation (particularly excessive irrigation) have been found to result in much higher levels of infection in some areas or countries (Asher 1993). Tuitert and Hofmeester (1992)

showed experimentally that inoculum potentials increased more in irrigated plots than in nonirrigated plots after 1 year (Fig. 7.1b).

7.3.3 Temperature

Soil temperature is an important factor affecting *P. betae* infection and disease development and severity. A temperature exceeding about 15 °C was reported to be essential for infection of sugar beet seedlings by *P. betae* (Horak and Schlösser 1981; Abe 1987). Under controlled conditions, Blunt et al. (1991) showed that the optimum soil temperature for infection is about 25 °C; the time between sowing and the first detectable infection was shortest and the subsequent rate of infection most rapid at this temperature (Fig. 7.5). No infection was observed over 80 days at 10 °C, and the minimum temperature for germination of resting spores and infection appeared to be between 10 and 15 °C, confirming previous field observations (Horak and Schlösser 1981). In Japan, Abe (1987) reported that a soil temperature of about 23 °C stimulated infection of roots by *P. betae*, resulting in severe rhizomania outbreaks and a greater reduction in sugar yield than a temperature of 10 or 15 °C.

Blunt et al. (1991) showed that at 30 °C, primary infection was rapid (first observed only 5 days after sowing) but that subsequent development was sporadic and limited (Fig. 7.5). At this temperature, resting spores were found in less than 8 % of lateral roots during the 28 days of the experiment, and their density was always considerably less than on plants grown at 15 °C. Interestingly, infection under these conditions may induce the production of resting spores rather than further generations of zoospores. On the other hand, Legrève et al. (1998) reported that an isolate of *P. betae* from Turkey induced a high level of infection at 27–30 °C as well as at 19–26 °C, compared to a Belgian strain which had an optimum temperature of 19–26 °C. This suggests that there may be differences in temperature requirements among *P. betae* strains, as demonstrated with *P. graminis* strains from tropical regions, which have a higher temperature optimum of 27–30 °C (Legrève et al. 2002).

In Britain, soil temperature in the spring and early summer was shown to be of particular importance (Payne and Asher 1990), in which higher soil temperatures at sowing promoted early infection and more rapid development of *P. betae* (Blunt et al. 1991). This would suggest that rhizomania should be more widespread and infection more severe in countries with warm temperatures in the spring and early summer, when the roots are most susceptible and virus infection is established at an early growth stage in the plant (Blunt et al. 1992). This is generally the case in regions or countries having a Mediterranean or continental-type climate, where soil temperature may be as high as 20 °C at the time of sowing. By contrast, in the cooler sugar beet growing regions of the world, such as the United Kingdom, Eire, and the Scandinavian countries, the disease has developed more slowly and been less severe (Asher 1993).

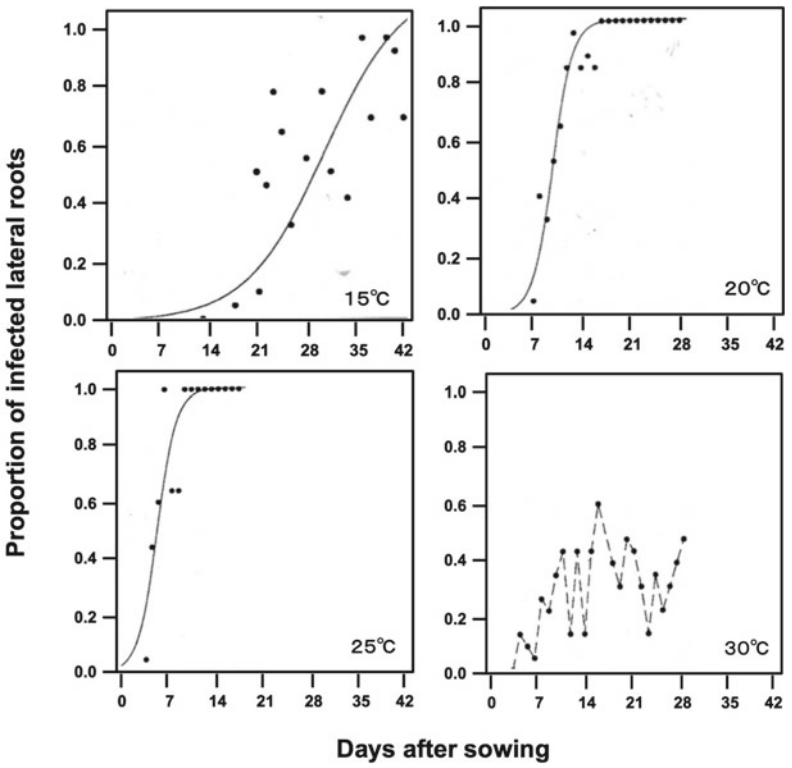

Fig. 7.5 Observed data, together with estimated logistic curves, for the progress of infection of *P. betae* on lateral roots of sugar beet seedlings at four temperatures (From Blunt et al. (1991), with permission of John Wiley and Sons through CCC)

7.4 Modelling Infection and Spread

In order to analyze the dynamics of *P. betae* infection, Webb et al. (1999) developed a model for the buildup of *P. betae* that integrates the dynamics of root growth, infection, and inoculum within seasons. The model incorporates two sources of infection: (1) primary zoospores from resting spores present in the soil at the start of the season and (2) secondary zoospores produced from sporangial plasmodia in infected roots during the cropping season. The model also consists of two components: one for the dynamics of the host in relation to infection by *P. betae* and other for the dynamics of primary and secondary inoculum.

Subsequently, Webb et al. (2000) extended the model to incorporate the effect of temperature on four key parameters: germination of resting spores, rate of primary infection, and the latent period between infection and the production of secondary zoospores and resting spores. The results showed that for UK temperature conditions, the effect of sowing date on infection was greater than that of year-to-year variations. The variation in inoculum buildup predicted when temperature data from

a range of soil types were used in the model agreed with field observations, where higher levels of infection were observed on sandy soils than on black fen peat soils.

A modification of these temporal models, describing inoculum amplification during the growing season, was combined with spatial models to predict the spread of rhizomania within and between fields on a farm and ultimately between farms within a region (Stacey et al. 2004). In highly mechanized production systems, most of the primary spread of the disease is likely to be on agricultural machinery, especially harvesters which are often shared on several farms (Richard-Molard 1985; Heijbroek 1988). When applied to the United Kingdom, the model accurately simulated both the number and distribution of farms that had developed rhizomania in annual surveys carried out over the 13 years since its first detection in 1987, prior to the availability of resistant varieties. By adjusting model parameters while running simulations, the effect of various control strategies could be examined. This highlighted the difficulty of containing the spread of such a soil-borne disease, e.g., by ceasing production on affected fields and implementing hygiene measures. Because of the long delay between the first contamination of a field with small amounts of infested soil and the appearance of symptoms (9 or more years in a 3 year sugar beet rotation), secondary spread can continue undetected during this period.

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Part IV
Control and Resistance Breeding

Chapter 8
Control of the Disease

Claudio Ratti and Enrico Biancardi

Abstract This chapter describes the preventative systems adopted for limiting the spread and the damage caused by rhizomania. However, the area affected by the disease is still expanding, notwithstanding the quarantine measures tried in several countries. Since the first observations, the new disease has appeared unusually dangerous for the beet crop, mainly due to the easy spread and the severe effects on sugar yield. When the etiology of rhizomania was discovered, the development of genetic resistances appeared among the few options available against the disease. Because the genetic control is incomplete and it is currently being overcome by new strains of the virus in some areas, new systems of agronomic and biological control are under evaluation for potential integration with the genetic resistances and have shown some evidence of antagonistic ability in depressing the development of *Polymyxa betae*. A genetically modified strain of *Pseudomonas fluorescens* was employed for evaluating the possible effect of this organism on lowering the spread of rhizomania. Using some species belonging to the genus *Trichoderma*, positive results were obtained in the glasshouse.

Keywords Sugar beet • Rhizomania • Quarantine measures • Crop rotation • Biological control

8.1 Disease Effects and Damages

In sugar beets affected by rhizomania, all growth parameters, without exception, are influenced by the disease. The leaf area index (LAI), leaf and root dry matter, leaf and root growth rate, etc., can be twofold higher in a resistant variety than in susceptible ones (Rezaei et al. 2014). In the case of later infections, roots display only a slight proliferation of rootlets, but, if individually analyzed, the diseased beets show remarkably reduced sugar content and processing quality (Box 8.1). The lower value of the

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E. Biancardi, T. Tamada (eds.), *Rhizomania*, DOI 10.1007/978-3-319-30678-0_8

Box 8.1: Processing Quality

Gross sugar yield is the most important trait for growers, and it depends on the weight of topped roots (excluding soil, leaves, tops, etc.) and the sugar content, i.e., the percentage w/w of sucrose present in the roots. In addition to the gross sugar yield, the processing quality must be considered, indicating roughly the percent of white sugar, which can be extracted in the factory from a given amount of beets. With increasing quality, the white sugar yield approaches the gross sugar yield. The inheritance of the character "sugar yield" is quantitative and strongly affected by the environment (Powers et al. 1963). A nonadditive variance is prevalent in controlling the trait "root production" (Campbell 2002), while for the "sugar content" the variance is mainly additive (Smith et al. 1973). There is a high negative correlation between sugar yield and root yield. In other words, if the root weight is increased by selection, the sugar content lowers and vice versa. Processing quality includes a number of chemical and physical traits of the harvested beets (Oltmann et al. 1984). Many of these characteristics are under genetic control, but the effect of cultural practices, harvest, storage methods, environment, etc., is normally greater (Harvey and Dutton 1993). Among the soluble impurities (nonsugars), sodium, potassium, alpha-amino nitrogen compounds, reducing sugars, etc., have been taken into account in breeding programs due to their negative effects on sugar extraction and crystallization (Last and Draycott 1977; Smith et al. 1977). The root concentration of these nonsugars can be reduced with mass selection (Powers et al. 1963; Smith et al. 1973; Coe 1987; Smith and Martin 1989; Campbell and Fugate 2013). Breeding for further improvements is problematic due to interactions among nonsugars, sucrose concentration, and root weight (Campbell and Fugate 2013). Gains in processing quality also are possible with an appropriate fertilizer and water management.

Some anatomical characteristics of the roots are associated with processing quality. Selection of smooth-root hybrids (with reduced or without the two vertical grooves) lowers the amount of adhering soil carried to the factory. This is desirable as the soil remaining on the roots after washing causes damage, especially during the slicing and diffusion phases (Theurer 1993). The amount of tare soil increases with the clay content of the soil with its moisture during harvest. Smooth-root varieties with improved root shape, i.e., reduced depth of the lateral grooves and reduced crown dimension, were developed through mass selection (Mesken and Dieleman 1988; Saunders et al. 1999).

processing quality is mainly in response to the higher concentration of sodium and reducing sugars (glucose and fructose) in diseased beets. On the other hand, alpha-amino nitrogen always is lower than in healthy (rhizomania-free) beets (McGinnis 1982; Bürcky 1987; Hofmeester and Tuitert 1989; Hoffmann and Märländer 2005). Root and sugar yields are severely depressed as well (Wisler et al. 1999).

Fig. 8.1 Rhizomania-diseased field used for mass selection. The *yellowing row* on the left is the susceptible check sown for verifying the uniform distribution of the disease across the field (San Pietro in Casale, Italy, 1986)

According to Lewellen et al. (2003), the occurrence of systemic infections of rhizomania is not frequent and only develops in the presence of early and severe disease conditions. In this case, leaves display yellowing (Fig. 8.1), but to lesser degree the chlorotic spots (Fig. 2.4) and some physiological, biochemical, and ultrastructural modifications associated with rhizomania (Hamdorf et al. 1977; Sallè et al. 1986). In case of early infections, the taproot shows:

- Typical proliferation of fibrous rootlets taking a beard-like appearance
- Constriction resulting in a "wine glass" shape
- Browning of vascular rings
- Development of perpendicular secondary roots, etc.

It is possible to detect the symptoms of rhizomania in sugar factories by means of the routinely analyzed parameters, even from beets harvested in supposedly healthy fields (Box 8.2) (Pollach 1984; Barocka 1985; Pollach et al. 1991). The RS index (named "Rizomania–Signal" in German) discriminates the diseased truckloads of beets from the healthy ones. The countless formulae in use are annually adjusted according to the climatic conditions of the campaign and usually are computed using the sum of the concentrations of potassium and sodium divided by the concentration of alpha-amino nitrogen. This ratio, also called the alkalinity coefficient (AK), is very sensitive according to Wieninger and Kubadinow (1971),

Box 8.2: Beet Processing
Sugar beet is harvested 5–9 months after sowing. Before lifting, beets are mechanically defoliated and topped because of the low sugar content of leaves, petioles, and crowns (Fig. 1.2). Additionally, these parts incorporate high concentrations of impurities, which reduce the sugar extraction rate. Roots, carefully separated from the soil, are transported to the factory as soon as possible in order to avoid sugar losses of a different origin. Leaves and crown remain on the field or rarely are used as cattle feed. After washing and cleaning, roots are sliced in cossettes, from which sugar is extracted by diffusion in hot water. The resulting "raw juice" is purified by means of lime and carbon dioxide. After filtration, the "thin juice" is concentrated by evaporation until the density becomes greater than 60 %. At this point, the crystallization of sugar can take place in this "thick juice" under vacuum and high temperature. Finally, sugar crystals are separated by centrifugation from the molasses, a thick brown syrup still containing about 45 % sugar (McGinnis 1982). Molasses is used for animal feed and for production of alcohols, glutamate, betaine, yeasts, vitamins, etc. The full extraction of residual sugar from the molasses is possible, but not economically viable. The pulp, i.e., the insoluble part of the exhausted cossettes, composed mainly by hemicellulose, is sold after drying as animal feed.

because the smallest presence of rhizomania immediately increases the sodium content while decreasing the alpha-amino nitrogen concentration (Hofmeester and Tuitert 1989). The processing of diseased beets is more expensive also due to the increased concentration of reducing sugars and the low extraction rate. In order to compensate, the factory must modify or lengthen some extraction procedures when there are not enough healthy roots to dilute the disease ones (Box 8.3) (Pollach 1984). Of course, the grower is penalized in his payment for delivering diseased beets.

The damage from rhizomania is compounded by other components and parameters negatively influencing the sugar extraction (Rosso et al. 1989). The deteriorated values from the processing are on all of the following: reduced sugar content, increased alkalinity index, lower purity of thick juice and dry matter (°Bx), higher pH, and increased content of glucose, fructose, malic acid, oxalic acid, citric acid, phosphoric acid, chlorides, sulfides, etc. Only the lower concentration of the nitrogen compounds (amino acids, nitrates, nitrites, etc.) does not reduce the efficiency of the sugar extraction (Rosso et al. 1989). In field trials organized by the same authors, the white sugar yield of the resistant and susceptible varieties was 10.5 and 5.0 t/ha, respectively (Fig. 8.2).

In Fig. 8.3 the distance (A) represents the still incomplete effects of the rhizomania resistances available today, while the economic damage of the disease is given by the difference between the yields of normal varieties in healthy and diseased field

Box 8.3: Processing of Diseased Beets

The difficulties of processing rhizomania diseased roots begin at harvest, with initial losses due to the fanginess and breaking of roots, as well as the incorrect topping of the crop caused by the death of beets and the related poor stand uniformity. Much soil attached to the rootlets, and many rotten roots are carried to the factory. The routine analyses made for grower payment on every load also consider the amount of tare, which is the quantity of substances other than the healthy taproots, such as soil, stones, leaves, rotten parts, bolted beets, etc. Of course, the weight of the tare has consequences on the commercial value of beets. As described above, every storage system should be avoided in the case of moderate to severe rhizomania infection. The cutting and diffusion phases of processing may be difficult due to the abnormal physical properties of the diseased root tissues. Other problems are caused by the high concentration of nonsugars and reducing sugars caused by the disease, which produce acidification and development of color in both the thin and thick juices. In the case of severe rhizomania infections, which also could lead theoretically to a negative amount of extractable sugar, the roots may be directly eliminated and destroyed. From this perspective, it would be more convenient to leave the severely diseased beets in the field. The decision about what to do is not easy because of the variability of symptoms in the individual roots and in the different parts of the field. Undoubtedly, the harvest of the diseased beets would significantly reduce the inoculum of viruliferous *P. betae* in the soil, but this improvement becomes useless because of the long-lasting vitality of the cystosori.

Box 8.4: Solarization

Using clear or black plastic sheets, it is possible to heat the underlying soil to temperatures sufficient to eliminate or reduce the inoculum of some soilborne pathogens, including *P. betae* and the vectored BNYVV. The soil covering is more effective on fungi than on bacteria, and, in general, its action is proportional to the mean temperature and to the length of the treatment. Higher temperatures are reached under clear plastic sheets, but the development of weeds is not hampered (Halloin 1993). Therefore, in most cases, the black plastic sheet is preferable even with the 3–5 °C lower temperature. Because solarization can greatly modify the biological activity of the soil, at least in the first 10–20 cm layer, the treatment should be applied very carefully. In fact, some pathogens, initially reduced by the treatment, can multiply quickly soon after the plastic is removed, thanks to the disappearance of antagonists (Katan and DeVay 1991). The method is quite expensive and therefore is rarely used in field crops today.

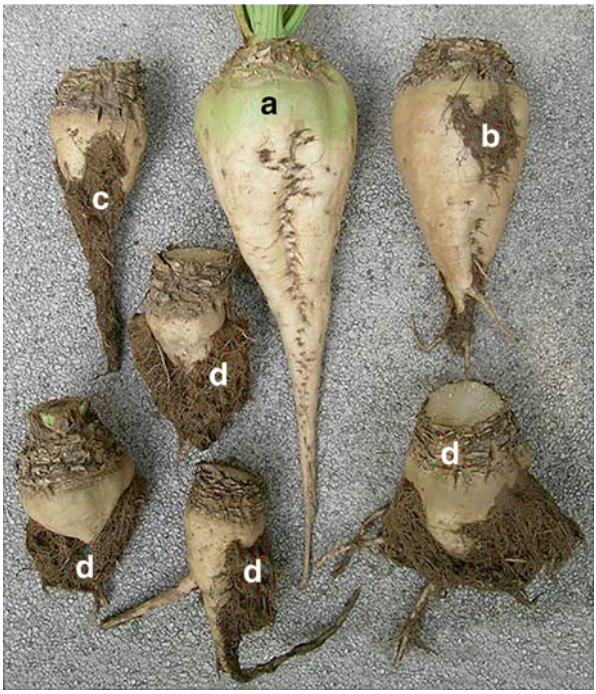

Fig. 8.2 Resistant and susceptible beets grown in different conditions of rhizomania attack (Courtesy, Ghedini 2001). (**a**) Susceptible variety in healthy soil, (**b**) monogenic resistant variety in diseased soil, (**c**) multigenic resistant variety in diseased soil, (**d**) susceptible beets in severely diseased field

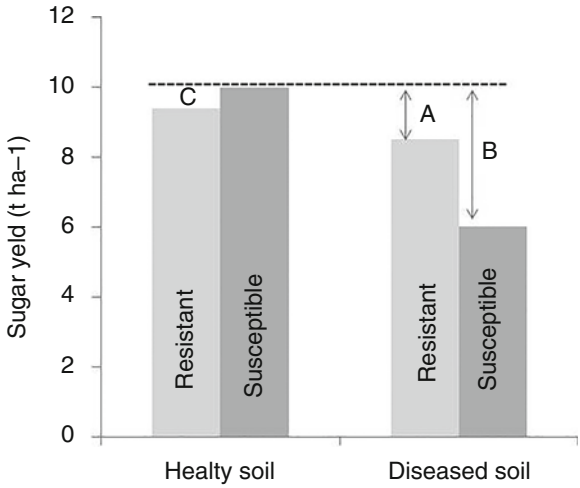

Fig. 8.3 Beets of resistant and susceptible varieties in healthy and diseased conditions (see Sect. 8.1)

(B). The value is reduced if resistant varieties are employed. In other words, the losses due to rhizomania are around 50 % or more in the case of susceptible varieties. The difference (C) represents the gap between resistant and susceptible varieties under healthy conditions. Today, the yield of both types of varieties is roughly the same in healthy soil (Wisler et al. 1999).

Rhizomania also develops on sugar beet grown as a winter crop. Because it is sown near the beginning of autumn or even later, the unfavorable climate and soil

conditions during and after emergence limit the inoculation by BNYVV. Favorable conditions of temperature and soil moisture usually begin some months later, when the roots are well developed and the inoculation by viruliferous *P. betae* is hindered. Also, even in the presence of high levels of inoculum, the shape of the diseased roots is quite normal. The only visible symptom is some limited development of rootlets on secondary roots, which often are undetected because they remain incorporated in the soil during harvest. More evident are the typical effects on sugar content and sodium concentrations on the analyses performed before processing. Because the normal spring-sown crop, at least in Europe, often coexists with the winter one, rhizomania in southern districts was discovered first in the former, where both the inoculum conditions and the damage are much more serious. Breeding of resistant winter varieties began around 1980, and, in 1994, the first ones were released (Zavanella, personal communication 2015).

8.2 Agronomic Control

The control of soil-borne diseases always is more difficult than managing those diseases which damage the aboveground parts of the plant. In fact, the soil becomes a sort of effective protective shield for the pathogens, and their symptoms frequently are evident only when the disease is in too advanced a stage to be managed. Moreover, soil-borne diseases often are present together with other pathogens, such as the mixture of species belonging to the genera *Fusarium*, *Rhizoctonia*, and *Aphanomyces*, which cause seedling damping-off. Over the last few decades, rhizomania has entered as a component of these combinations of diseases (Harveson and Rush 2002).

Due to the easy diffusion of the pathogenic agents, the identification of the diseased and rhizomania-free fields helps the farmer decide what to do under the threat or in the presence of rhizomania. He must take into account that fields never cultivated with sugar beet may be infected by the virus (Al Musa and Mink 1981). Remote sensing techniques may help in rapidly localizing the spread of the disease. In fact, the betacyanin and carotenoid contents in the leaves influence the reflectance of the canopy, and they are more related to the presence of rhizomania than to the nitrogen availability in soil (Steddom et al. 2003). Also, the 2–3 °C higher leaf temperature of the diseased beets, due to diminished transpiration and water uptake (Keller et al. 1989), may be utilized in remote sensing surveys (Ahrens 1987a). More often, a field inspection is performed, in which beet or soil samples are analyzed according to the local provisions of extension and quarantine services.

If the soil is judged healthy after ELISA analyses, the factors favoring the first BNYVV inoculation should be avoided. The more current recommendations of prophylaxis are to avoid:

- The movement of infected soil through farming equipment, workers, cattle, etc.
- The planting of *P. betae* host plants throughout the entire crop rotation (Hess et al. 1982)

- The return to the farm of the tare soil that was transported to the factory with the diseased beets (Dunning et al. 1984; Schäufele and Büttner 2002)
- Using water coming from infected fields for irrigation
- Excessive water use during irrigation (Sharifi et al. 2012)
- Water and wind erosion of soil in diseased fields by planting cover crops and using appropriate agronomic practices

Additionally, the sowing of resistant varieties in supposedly healthy fields helps in maintaining the inoculum at non-damaging levels. A good and regular stand helps in maintaining cooler soil temperatures, which may reduce the multiplication rate of *P. betae* (Neher and Gallian 2014). Transplanting developed beet seedlings in paper pots delays the inoculation of the rootlets for some weeks and enables a quite normal growth of beets (Richard-Molard 1984). The gain in white sugar was estimated by Schäufele (1983) at about 15 %, but even with this advantage, the practice is too expensive. Early sowing helps to avoid seed germination and seedling growth happening right when soil temperature and moisture favor the cystosori inoculation of beets (Blunt et al. 1991). Therefore, later-drilled crops infected during the earlier stage of growth increase the damage (Asher 1988; Hofmeester and Tuitert 1989). However, soil temperatures above 16 °C are quite rare during the planting period, at least in temperate-cold cultivation areas (Ahrens 1987b). At 11 °C, the inoculation of the zoospores into the rootlets still seems possible (Casarini-Camangi and Canova 1987a), but no infection has been observed below 10 °C (Webb et al. 2000). Evidently, natural selection has adapted *P. betae* to the local conditions. The optimum for zoospore movement and for virus transmission is around 25 °C (Horak and Schlösser 1980).

The prevention measures mentioned and the use of resistant varieties also are very useful when the field is believed healthy. In a 10-year survey made in Germany by Schäufele (1989a), he found a good correlation between rhizomania intensity and either the daily temperatures or cumulate rainfall from May to August. *P. betae* inoculum seems limited in soils with a pH less than 7.0 and with the calcium content below 2,000 ppm (Grünewald et al. 1983; Legrève et al. 1998). The inoculum of *P. betae*/BNYVV is in correlation with increasing soil pH induced by lime and exchangeable magnesium (Kutluk-Yilmaz et al. 2010). Attempts at reducing soil pH were tried using sublimed sulfur by Sugawara et al. (1973, 1978) with erratic results on decreasing the disease inoculum.

The BNYVV, which is well protected inside the thick-walled resting spores of *P. betae*, can pass unharmed through the intestines of sheep with only small percentage of reduction in number. Therefore, the spread of the viruliferous vector is possible in this way, especially when the sheep also is fed with other sugar factory residues (Heijbroek 1988). The same happens with pigs and cattle. Moreover, the resting spores survive for a long time in manure, still widely used as an organic fertilizer (Hillmann 1984; Schäufele 1989b). Due to the high temperature necessary in sugar extraction, alcohol distillation, drying processes, etc., the diffusion through coproducts such as fresh or dried exhausted cossettes, molasses, and vinasses, when used for animal feeding, seems quite impossible. The biomass collected in the fac-

tories after the roots are cleaned (tails, crowns, leaves, weeds, etc.) is sometimes fermented for biogas production. In this case, the infectivity of cystosori inside the feedstock material disappears within a week at temperature above 38 °C (Friedrich et al. 2010). Similar effects on *P. betae* were observed in composting materials under the combined effect of temperature, moisture, aerobic or anaerobic conditions, etc. The German rules for composting (i.e., 2 weeks >55 °C or 1 week >65 °C) allow for the elimination of the entire *P. betae* resting spores (Van Rijn and Termorshuizen 2007).

Tuitert (1993) verified that the *P. betae* viruliferous zoospores can move in the soil only in a limited range of 0.05 m and that the very first field infection involves usually just a few, isolated plants called "blinkers" (Neher and Gallian 2014). The distribution in patches is typical of the first phase of rhizomania diffusion (Truscott and Gilligan 2001) and often occurs in parts of the field more prone to waterlogging. The early symptoms are difficult to recognize because the yellowing patches of the diseased plants are mixed with other types of leaf discolorations and not clearly evident. The subsequent soil movement, caused by plowing, sowing, and other operations, lengthens the infected patches in direction of the rows (Schäufele 1989a; Tuitert 1993). Obviously, the speed with which the disease diffuses under field conditions depends not only on the dynamics of the soil but also on the reproduction rate of the inoculum (Webb et al. 2000). This means that with no-tillage practices the spread of the disease could be reduced. Of course, these practices are not recommended for sugar beet.

Harveson et al. (1996) found that the horizontal spread of the viruliferous zoospores occurs more with soil movement during tillage or harvest than through furrow irrigation. Consequently, the diffusion of rhizomania appears not as rapid as might be expected. After the first soil infection, according to Stacey et al. (2004), a damaging level of inoculum requires at least two or three sugar beet crops before some aboveground symptoms are expressed. In other words, the infection of healthy soil should become detectable on the leaves around 8–10 years after the first real contamination of the soil (Schäufele and Büttner 2002). This delay is in contradiction with the prompt inoculation response obtained in the greenhouse-controlled conditions. The different behavior can be explained by the perfect dilution of the cystosori obtained in the soil mixture used in the greenhouse, while under field conditions the diffusion of *P. betae* follows the usually limited displacement of the soil (Tuitert 1993). When the disease becomes evident on the leaves (Fig. 8.1), the spread across the field appears much more rapid, as was observed by Schäufele et al. (1995). The authors referred to an initially limited and restricted field infection, which spreads in 6 years throughout the field. Dealing with these issues, it must be remembered that there is an enormous variability of local situations.

Crop rotation is not effective against rhizomania, but by increasing its duration and sowing resistant varieties, it should be at least possible to slow the accumulation of viruliferous *P. betae* cystosori in the soil and, more important, to delay the development of resistance-breaking strains of BNYVV (Rush et al. 2006; Stevens et al. 2006). Additionally, if a variety were available that would leave fewer viruliferous

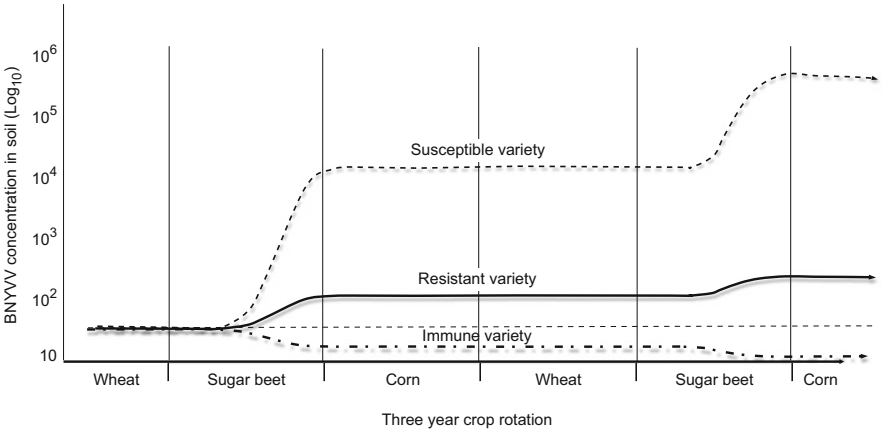

Fig. 8.4 Simulated trend of BNYVV soil inoculum in 3-year crop rotation, according to the resistance of sugar beet varieties. The logarithmic scale on the left is merely indicative

Fig. 8.5 Holly experimental hybrids separated into rhizomania-susceptible and rhizomania-resistant roots grown in diseased soil (Courtesy, Lewellen 1984)

cystosori in the soil after harvest than were present at the time of sowing, one could imagine that after some number of cycles of sugar beet crop, it would be possible to sanitize the infected fields. This timespan should be proportional to the ability of the variety to reduce the multiplication of the viruliferous cystosori, i.e., to the degree of rhizomania resistance of the variety grown (Figs. 8.4 and 8.5).

The use of resistant varieties (Rz1 and/or Rz2), when compared with susceptible ones, reduces the concentration of virus inoculum up to 6,000 times (Gilmer et al.

2007). For this reason, as mentioned above, sowing resistant varieties, even in healthy soils, should help maintain the BNYVV inoculum at low and not damaging levels. Gerik and Duffus (1988) found differences in the BNYVV vectoring ability of *P. betae* depending on its origin. The first infection of healthy soils by viruliferous *P. betae* is enhanced by the presence of non-viruliferous *P. betae* strains, because the noninfected zoospores can be infected easily by the virus soon after their injection in the sugar beet rootlets. We should remember that the BNYVV is present in only 5–20 % of the *P. betae* zoospores (Tuitert et al. 1994). Hence, if the remaining percentage of zoospores are easily infected, this would explain how the soil content of BNYVV might increase up to 10,000 times when susceptible sugar beet is grown (see Sect. 2.1). According to Tuitert and Hofmeester (1992), after the second one the increase was about 70-fold (see Sect. 7.1.2).

Irrigation should be avoided, if possible, during the seedling stage, above all when the soil temperature favors the movement and inoculation of the *P. betae* cystosori (Koch 1982; Hofmeester and Tuitert 1989). These authors also found a reduction of sugar content in the irrigated plots, but this would be a normal reaction to the weight increase (sugar content is measured as percent of fresh weight) of irrigated beets when compared to the nonirrigated crop. Similar negative effects were ascertained by Casarini-Camangi (1987b). In the presence of soil-borne fungal and/or viral diseases, the number of irrigations could be reduced in order to limit the damage, taking into account that under-irrigation is damaging as well (Harveson and Rush 2002).

In several countries, policies of containment (mainly of an agronomic nature) were adopted to limit the spread of the disease (Putz and Richard-Molard 1983). An example of a quarantine directive is given at http://wwwirishstatutebook.ie/1990/en/si/0210.html. In the UK, the disease was under statutory control for some years (Anon 2003). The beets were destroyed in the diseased fields, and further sugar beet cultivation was prohibited in the site (Dunning et al. 1984; Asher 1999). The control on imported vegetables was strengthened, and transplanting materials had to be certified as coming from rhizomania-free areas (Asher and Dewar 2001). Unfortunately, the quarantine measures have proven unable to limit the spread of rhizomania, mainly because of the large amount of infected soil moved by machinery during the harvest and the transfer of beets to the sugar factory, particularly when these operations are performed using off-farm equipment. To be fair, only a few grams of soil are enough for disease transmission, and that can happen through the dust raised by the machines and blown away. The failure of the limiting measures was caused by either the difficulty to assess the real effect on the disease in reasonable timespan or the sometimes long asymptomatic phase (Stacey et al. 2004; McGrann et al. 2009).

Due to the frequent occurrence of multiple soil-borne diseases caused by viruses and/or fungi, the sowing of mixtures of varieties with different genetic traits and degrees of single resistances has been attempted (Harveson and Rush 2002). In some cases, e.g., for foliar diseases of winter wheat, the seed mixture yields better than the sum of each variety grown alone (Mundt et al. 1995). In sugar beet, some

positive but erratic results were achieved for foliar diseases, such as curly top and cercospora leaf spot (Finkner 1976).

8.3 Chemical and Thermal Control

The soil fumigation with methyl bromide or dichloropropane with dichloropropene (D–D) resulted in effectively reducing the number of cystosori and consequently the inoculum of BNYVV. Bongiovanni (1965) used a mixture 1:1 w/w D–D and obtained an increase in sugar yield of about 140 % more than the untreated check. Martin and Whitney (1990) reported a fivefold increase in sugar yield on a rhizomania diseased field after fumigation. On the other hand, Bradley and Khan (2006) did not find any increase of sugar yield after fumigation in organic soils. This treatment was necessary against sugar beet cyst and root-knot nematodes (*Heterodera schachtii* and *Meloidogyne* spp.), because, at the time, no other management options were available and lengthened crop rotations were not always suitable (Gerik and Hanson 2011). Due to the excessive costs and potential harm to applicators and the environment, fumigation has almost disappeared. Fortunately with sugar beet, growers have been able for the most part to discontinue the practice of fumigation because of the recent release of varieties resistant to cyst nematode or the inclusion of nematode trap crops in rotations. As mentioned above, the effect of fumigation is temporary, because large quantities of diseased sugar beet tails, lateral roots, and small hair roots remain in the soil after harvest, ensuring an enriched inoculum for the next sugar beet crop (Asher et al. 2002; Sugawara et al. 1978).

Burketova et al. (1996) reported the effect of salicylic acid in improving sugar yield of susceptible varieties grown in diseased soil. No positive result was observed on resistant varieties. Concentration of sodium chloride ranging from 2 to 8,000 ppm in soil is negatively correlated with the disease severity, virus concentration, and percent of diseased roots (Iskander et al. 2014). At the highest level of salt concentration (6–8,000 ppm), the presence of cystosori in the roots seemed to decrease together with the inoculum and the infective ability of the viruliferous zoospores. It should be recognized that high concentrations of sodium (more than 8,000 ppm) are increasingly harmful for beets as well (Kaffka and Hembree 2004). Any other agrochemical applied to the soil seems until now unfitted for reducing the inoculum of rhizomania.

Solarization is a field practice which utilizes the solar radiation for heating soil with the aim of reducing the inoculum of soil-borne diseases. The increased temperature is achieved by covering the soil with clear or black plastic sheets with different compositions, sizes, and characteristics. The achievable temperature depends mainly to the latitude. Lewellen and Wrona (1997), using solarization and resistant varieties, obtained quite satisfactory results in the Imperial Valley, California (around 31° latitude). Under these conditions, the sugar yield of solarized plots was similar to that obtained with fumigation using methyl bromide/chloropicrin. Also in this case, however, the cost of the treatment did not justify the yield gain. High soil

temperature can be obtained with injections of steam. This system is suited especially for pasteurization of limited amounts of soil for greenhouse experiments.

8.4 Biological Control

Because of the lack of effective chemical protection against rhizomania and the foreseen limited durability of the current traits of resistance, alternative management methods have been sought, such as the use of antagonistic organisms, which might be able to reduce the inoculum of *P. betae* or to interfere with its pathogenic action. In the case of soil-borne fungal diseases damaging other crops, some species belonging to the genera *Trichoderma*, *Pseudomonas*, *Talaromyces*, etc., have shown some evidence of antagonistic ability in depressing the development of *P. betae* (Resca et al. 2001; Aksoy and Kutluk-Yilmaz 2008; Naraghi et al. 2014). A genetically modified strain of *Pseudomonas fluorescens* was employed for evaluating the possible effect of this organism on lowering the spread of rhizomania. Positive results were obtained in the glasshouse for sugar beet, but, according to McGrann et al. (2009), these methods seemed to be less effective under field conditions, mainly due the wide soil variability (Resca et al. 2001). Currently, this antagonism works well in field condition only against other viruses affecting crops like tobacco, papaya, lemon, etc. (Shen et al. 2014).

Other biologic and hence sustainable measures against the virus vector have been proposed by using some species of *Streptomyces* for seed and seedling treatments or to be incorporated in the seed pellet (Galein 2013). The lipopeptides released by *Bacillus amyloliquefaciens* are able to significantly reduce the systemic diffusion of *P. betae* inside the roots (Desoignies et al. 2013). The earthworm (*Lumbricus terrestris*) seems capable of reducing the *P. betae* inoculum after the passage of soil through its digestive system (Akca et al. 2014). If these biological control organisms also would work in an open field, they could support the main control of rhizomania given by the genetic resistances.

Greenhouse experiments were done to examine the antagonistic action, or cross protection, seen using beet soil-borne mosaic virus (BSBMV), which seemed to interfere with the BNYVV replication (Mahmood and Rush 1999). Cross protection is a mechanism that occurs when a plant infected by one virus (protecting) is then secured by the infection of a second virus (challenging). This phenomenon usually occurs between two strains of the same virus but sometimes among different viruses. Therefore, it was hypothesized an interference potentially useful for reducing the virulence of BNYVV. Beet soil-borne mosaic virus, first reported in California and Texas, by Liu and Duffus (1988), is very similar to BNYVV (Wisler et al. 1994), but does not cause damages to the roots of host plant. In the USA, BNYVV and BSBMV are often present in the same field, sometimes in the same plant. When sugar beet is inoculated with BNYVV, the negative effects are greater than if the inoculation were performed with both viruses. The lowering of the damage clearly is due to some kind of antagonism (Workneh et al. 2003), and, therefore, interactions such as

cross protection have been investigated. A high degree of reciprocal cross protection between BNYVV and BSBMV was seen in greenhouse experiments on *Beta vulgaris* seedlings inoculated with the protecting virus on roots and with the challenging virus on leaves delivered from the sap of *C. quinoa*-infected leaves (Mahmood and Rush, 1999). The degree of cross protection was increased by longer inoculation intervals between the first and the second inoculum. Moreover, RNA of both viruses was detected in doubly infected plants, but capsid protein of the BNYVV was undetected by serological tests, suggesting that BSBMV CP is involved in some mechanisms able to avoid superinfection in cross-protection tests.

However, experiments performed with soils naturally infested with *P. betae* zoospores carrying BNYVV and BSBMV seem to demonstrate that BNYVV is able to suppress BSBMV in mixed infections (Wisler et al. 2003). When BSBMV was present in mixed infections with BNYVV, its level was strongly reduced, even when the BNYVV titer was very low, particularly in rhizomania-resistant varieties. Furthermore, the *Rz1* allele of rhizomania resistance does not provide resistance to BSBMV. The significant reduction of BSBMV in the presence of BNYVV may be due to several factors such as competition for host infection sites by viruliferous *P. betae*; BNYVV-infected zoospores could be more aggressive, or one virus may have a competitive advantage once inside the cell (Wisler et al. 2003). In order to lower the damage caused by BNYVV, perhaps this useful interference could be improved by selecting more competitive strains of BSBMV.

However, these studies of BNYVV/BSBMV interactions have been conducted under different experimental conditions that have to be considered (Rush 2003). Sugar beet plants vortexed in a liquid inoculum become entirely infected, in contrast to infection through *P. betae* that usually remains localized into the roots and rarely goes systemic. With the vortex method, the first virus becomes established and interferes with subsequent infection of a second virus. Whereas, in natural infection through *P. betae* zoospores, the virus with the highest concentration usually colonizes the majority of the roots and will predominate. Moreover, in such experiments the initial inoculum density should be determined, and soil temperature must be manipulated in order to obtain repeatable results. In fact, BSBMV usually predominates at temperatures <20 °C, whereas BNYVV at temperatures >25 °C (Rush 2003). The same antagonism between BNYVV and beet oak-leaf virus (BOLV) was verified by Liu and Lewellen (2008).

Since the discovery of rhizomania, many attempts have been made to contain the disease with soil fungicides (D'Ambra et al. 1972) or fumigant treatments (Bongiovanni 1965; Alghisi and D'Ambra 1970; Martin and Whitney, 1990), but these means were ineffective or not economical or ecologically adequate. In addition, prolonged rotation cycles for sugar beet crops are not effective in controlling the disease because, as mentioned above, the vector's viruliferous resting spores remain viable in the soil for decades (Webb et al. 2014). Thus, once infected by BNYVV, the field could be considered almost permanently infected (Asher 1993).

In conclusion, the only effective way to reduce the incidence of the disease is through genetic resistances, first identified in a few Italian sugar beet genotypes (see Chap. 9). However, these beet genotypes also are subject to virus infection, although

they exhibit only moderate rootlet proliferation. Their reaction to the disease is characterized by the appearance of a barrier of suberized cells, which completely surrounds and walls off the affected cortical tissues (Poggi-Pollini and Giunchedi 1989).

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Chapter 9
Genetic Resistances

Leonard W. Panella and Enrico Biancardi

Abstract Soon after the first appearance of the disease, the presence of some traits of resistance to rhizomania was recognized in Italian varieties. In the mid-1980s, the breeding research led to the release of monogenic resistance, which reduced drastically the damage caused by beet necrotic yellow vein virus (BNYVV). At least two origins of the currently employed traits (Rz1 and Rz2) were identified in *Beta maritima* collected in the Po River Delta, Italy, and at Kalundborg Fjord, Denmark. Both traits are located on chromosome III and spaced far enough apart to be considered different loci. The crosses display an additive action, useful for increasing sugar yield even in the presence of Rz1 resistance-breaking strains of BNYVV. Some differences were detected in the mechanisms limiting the effects of the BNYVV, because the beets carrying the resistance Rz2 show reduced virus replication and more restricted cell-to-cell movement than Rz1. But the subject still is controversial. In the future, resistance to the vector, *P. betae*, could complement the effects of BNYVV resistances, if difficulties in the transfer of the trait would be overcome.

Keywords Sugar beet • Rhizomania • Genetic resistances • Rz1 • Rz2 • Genetic resources

Genetic resistance often has been recognized as the only viable mean for limiting soil-borne diseases. In sugar beet, it is only for beet necrotic yellow vein virus (BNYVV), i.e., rhizomania, that a set of fairly different and effective, single-gene resistances is currently available, while for other soil-borne diseases of sugar beet, multigenic traits with low heritability have been found. Varieties endowed with multiple resistances to different soil-borne diseases would be very useful, but so far nothing similar to the single-gene resistance to rhizomania exists (Harveson and Rush 2002).

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E. Biancardi, T. Tamada (eds.), *Rhizomania*, DOI 10.1007/978-3-319-30678-0_9

9.1 Sources of Resistance

The presence of varieties carrying some trait of resistance and a sufficient degree of genetic variability, originally were detected through differences within trials in fields naturally infected by the then unknown syndrome. The discovery of the disease outside Italy and the recognition of the unusually high yield losses captured the interest of research institutes, seed companies, growers' associations, and the beet sugar industry in Europe, Japan, and the USA. In relatively few years, thanks to international synergies and collaborations, the damage caused by the disease was almost completely eliminated, allowing the survival of the crop and the related industry. The probability of finding new sources of resistance, with the qualities demanded through the registration procedure, is quite low in commercial sugar beet germplasm. This primarily is due to the selection methods employed for breeding the currently used monogerm hybrid varieties and by their narrow genetic variability (Francis and Luterbacher 2003; Pavli et al. 2011; Litwiniec et al. 2015).

Seed companies prefer searching for new traits in germplasm adapted for sugar production, which has already been endowed with satisfactory levels of sugar yield, processing quality, and morphological traits. The search among other taxa of the genus *Beta* is more difficult and time consuming, but it should become more fruitful in the future. Hopefully, by means of molecular biology, the introgression of resistance genes into the cultivated germplasm will become easier than in the past (Francis and Luterbacher 2003). The transfer of monogenic and dominant genes of resistances into germplasm that already is regionally adapted is the easiest way (Meulemans et al. 2003).

9.2 Resistances to BNYVV

The first available type of rhizomania resistance with moderate effect on yield parameters was named "Alba type," because it was identified in varieties released by the Alba seed company, Padua, Italy (Biancardi et al. 2002) (Fig. 9.1). These genotypes surely were derived from cercospora leaf spot (CLS)-resistant lines, which, in turn, were obtained from crosses with *Beta maritima*, commonly named sea beet (Box 9.1). After 1970, Alba carried out mass selection on mother beets, cultivated in fields under 2-year rotations that gradually became severely infected by BNYVV, but which was not realized until later (Usai, personal communication). It is likely that for some years an efficient selection for rhizomania resistance was performed unconsciously in these fields. The association between Alba-type resistance and Munerati's germplasm is further confirmed because the seed company, which was founded in 1933, worked mainly with Rovigo materials, especially before the Second World War. Similar segregation patterns were found in some of Munerati's original families. Moreover, the multigerm variety Alba P was sold and utilized as a CLS-resistant variety. As is well known, the only currently available

Fig. 9.1 *Beta maritima* on the bank of Po di Levante River, "very near the Adriatic Coast." This photograph was found in 2015 on the original glass plate with only the year 1909 given. It most likely was taken in the June of 1909 by Munerati. The date and landscape correspond to the harvest of sea beet seed used for selection of resistance to cercospora leaf spot. Due to the similar lineage, it is likely that the depicted sea beet also has been the ancestor of the resistances to rhizomania employed worldwide in the last 30 years. (A print of this photo has been previously used by Biancardi et al. (2012) and was believed to date from 1951)

Box 9.1: A Fruitful Collaboration

In 1925, Coons was directed by the Agricultural Research Service (ARS) of the USDA to look for germplasm containing resistance to diseases (Coons 1936). He decided to look in the center of origin of the wild relatives of sugar beet, the North Atlantic Coast of Europe and within the European countries bordering the Mediterranean Sea. By the time Coons made his first trip to Europe in 1925, Munerati's work with resistance to cercospora leaf spot from *B. maritima* was well underway (Biancardi et al. 2012). But many of the commercial seed companies were reluctant to work with the germplasm because, as Coons commented on his first trip, "These plants, however, as seen by one of us in 1925, had not been freed from certain undesirable characteristics derived from the *B. maritima* parent-notably, the tendency to be multicrowned and to have sprangled roots, especially lateral roots emerging from the taproot at about a 90-degree angle" (Coons et al. 1955). However in 1935 when Coons returned again to Europe to collect germplasm, he commented, "Munerati had greatly improved his breeding stocks and furnished his American colleagues his family 'R 581' which, although not fully comparable with sugar beets in root or crown conformation, was externally resistant to Cercospora leaf spot and high in sucrose" (Coons et al. 1955).

(continued)

Box 9.1 (continued)

Seed of the Munerati's R 581 and other lines from his *Beta maritima* material such as "Mezzano" and "Cesena" were sent to USDA, university, and sugar beet industry researchers throughout the USA and incorporated into disease resistance germplasm. Despite some undesirable traits from the *Beta maritima* parents, as mentioned above, the USDA-ARS public plant breeders crossed it into many of their lines. It found its way into the programs at Salinas, California; Logan, Utah; Fort Collins, Colorado; East Lansing, Michigan; and Beltsville, Maryland (Panella and Lewellen 2005). Realizing that the collection of seed was dying in Beltsville, it was moved to Salinas, CA, where McFarlane worked to restore it, while incorporating disease resistance genes from *Beta maritima* (Panella and Lewellen 2007). It was also where a young scientist, Lewellen, began working with *Beta maritima* and, ultimately, the Rovigo Sugar Beet Research Station. Once Lewellen got his program established, he looked to the USDA-ARS gene bank, but also to Biancardi at Rovigo, for sources of resistance to important sugar beet disease. Until their retirement, Lewellen and Biancardi worked closely together on many projects. They, along with De Biaggi and Erichsen, were responsible for breeding the first varieties with resistance to rhizomania, with Rizor in Europe and the "Holly gene" in the USA. There were visits between the USA and Italy, as well as meetings at international congresses, but most importantly exchanges of breeding materials. Although they may be best known for their rhizomania research, they have worked together on cercospora leaf spot resistance, sugar beet cyst nematode resistance, and resistance to other diseases (Biancardi et al. 2012).

This collaboration continued when a new sugar beet researcher, Panella, joined the USDA-ARS sugar beet breeding program at Fort Collins, Colorado, in 1992. This was when rhizomania was being found in Colorado, and it was natural for him to begin to work with Lewellen and Biancardi, looking to *Beta maritima* for disease resistance genes to rhizomania and other diseases (Biancardi et al. 2012). Traveling to Italy to work with Biancardi on the *Beta maritima* book, Panella became acquainted with another young Italian sugar beet researcher, who had continued the sugar beet research from Rovigo at the University in Padua (Stevanato and Panella 2013). Together with Stevanato and Panella, ARS researchers, McGrath and Hanson (in East Lansing, Michigan), and Richardson (in Salinas, California), the longtime collaboration between USDA-ARS and the Italian sugar beet program born in Rovigo continues.

source of CLS resistance was the Rovigo germplasm (Skaracis and Biancardi 2000). Until 1980, Alba P was sold as multigerm variety and therefore potentially free to be used as a diploid pollinator by other seed companies. The first published use of mass selection on diseased soil began in 1966 at San Bonifacio and Albaredo d'Adige, Verona, Italy, and led to the identification of some diploid lines with the

required traits (Gentili and Poggi 1986). Through colchicine treatment, the Maribo seed company, Holeby, Denmark, obtained tetraploid pollinators and the triploid variety, "Ritmo," showing the features of quantitative resistance, both to rhizomania and cercospora leaf spot (Biancardi et al. 2002).

After the first observations on the spreading of the still unidentified syndrome (Donà dalle Rose 1954), the Sugar Beet Research Station at Rovigo went back to work on rhizomania resistance in 1976. Planting in April, in opportune soil temperature and moisture conditions, part of the institute's germplasm was evaluated in a naturally infected field at San Pietro in Casale, Bologna. In order to obtain uniform infection, a viruliferous *P. betae* inoculum was manually distributed after sowing and before a 26 mm rain (Ciafardini 1991). Individual selection was carried out in February on beets that had survived the winter, because their higher sugar content worked as a sort of antifreeze protection inside the roots. In some cases, it was possible to improve the selection based on the disease index (DI) (Table 11.1) or by means of Brix (°Bx) or refractometer degree of the root sap. The DI in Table 11.1 does not include symptoms on the leaves because they are not always correlated to the disease, because, among other things, they can be confused with nutrient deficiencies (Pavli 2010). The collection of brei samples necessary for more precise analyses was impossible due to the small size of the surviving roots. In these instances the use of the °Bx measurement was helpful in making selections. The storage and reproduction of the selected mother roots were critical, mainly because of the danger of the development of rot. Particular care and reduced water supply were adopted for the storage and for field transplanting of the roots for seed production.

Only one diploid, multigerm family, coded Ro 236, showed a relatively high percentage of survival. Root and leaf morphology were similar to the check variety, Alba P; the same was observed for the degree of CLS resistance (Biancardi et al. 2002). The rest of the entries, especially monogerm, O-Type, CMS lines, and tetraploid families, were almost completely destroyed. That the resistance was the multigenic form in the varieties Alba P and Ro 236 was suggested by the behavior of their F_2 progeny. This quantitative resistance was similar to that found in some lines of sea beet collected in the Po Delta of Italy (Figs. 2.3 and 9.1) (Biancardi and De Biaggi 1979; Lewellen and Biancardi 1990). Analyzing the improvements obtained in 3–4 cycles of backcross and recurrent selection on different genotypes carrying the Alba resistance, the heritability of the trait appeared relatively high (Biancardi, unpublished). But progress became more difficult in advanced cycles of selection. Histological analyses carried out on root tissue displayed a clear delay in the BNYVV diffusion through the xylem bundles (Giunchedi and Poggi-Pollini 1988). These observations suggested that the reduced symptoms and the better production under diseased condition were caused by an active reaction of the plant against the diffusion of pathogen (Lewellen and Biancardi 1990). This multigenic resistance probably was incorporated into the varieties Mezzano NP, Buszczynski CLR, GW 304, GW 359, GW 671, Monodoro, Dora, Ritmo (3×), Lena, Sanamono, and Bushel and in the varieties later released using the pollinators Ro 401 and Ro 412 (Biancardi et al. 2002) (Fig. 9.2). In field tests conducted in Germany in 1982 on rhizomania diseased fields, the varieties Dora and Lena produced 63 % and 85 % more white sugar, respectively, than the susceptible check (Bolz and Koch 1983).

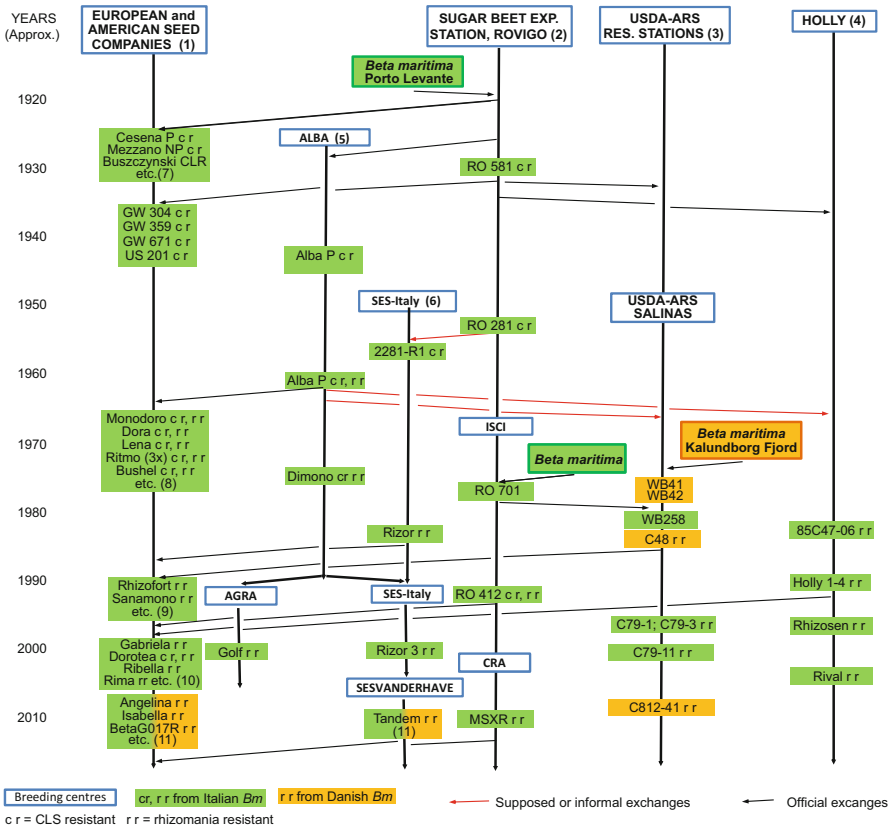

Fig. 9.2 Lineage of cercospora leaf spot and rhizomania resistances on the basis of published references or probable exchanges among the European and American research centers. Chronology in some cases is approximate due to graphic needs. It includes (**1**) Buszczynski, Synovie (PL); Centro Seme, Mezzano (I); Centro Produzione Seme, Cesena (I); KWS, Einbeck (D); Hilleshøg, Landskrona (S); Maribo, Holeby (DK); Lion Seeds, Maldon (UK); Great Western (now Western Sugar Coop); Denver (CO); American Crystal Sugar Company, Moorhead (MN); (**2**) Regia Stazione Sperimentale di Bieticoltura, Rovigo (I), named ISCI in 1968 and CRA in 2002; (**3**) USDA stations and other breeding activities at Beltsville (MD), Fort Collins (CO), Salt Lake City (UT), Salinas (CA), Riverside (CA), Waseca (MN), East Lansing (MI); (**4**) Holly Sugar, Colorado Springs (CO); (**5**) Alba Immobiliare, Ponte San Nicolò (I), joined with Agra; (**6**) SES-Italy, Massa Lombarda (I); (**7**) Commercial multigerm varieties endowed with CLS resistance; (**8**) monogerm varieties endowed with multigenic rhizomania resistance "Alba"; (**9**) varieties endowed with "Rizor" monogenic resistance similar to "Holly"; (**10**) varieties endowed with "Holly" monogenic resistance similar to "Rizor"; and (**11**) varieties with both monogenic resistances "Rz1" (Rizor and Holly) and "Rz2" (WB 42)

In 1979, the SES-Italy seed company, Massa Lombarda, Italy, began a research program aimed at discovering some source of resistance to rhizomania, possibly ready for a rapid development, and then releasing it quickly. It began with the field evaluation of the diploid germplasm belonging to the company (De Biaggi 1987).

Fig. 9.3 Field trial organized in diseased field where the first monogenic resistance to rhizomania has been detected. It is evident the quite normal green color of the leaves in the more resistant entry (highlighted plot) (Villa Serraglio, Italy, 1980)

Apart from the standard check varieties, the nursery included diploid pollinators and CMS lines with their maintainers (O-Type). The entries were sown in the spring of 1980 in rhizomania-infected fields located at San Martino in Argine, Bologna, and Villa Serraglio, Ravenna (Fig. 9.3). The fields revealed a severe and quite uniform infection, which allowed a satisfactory selection of the best individuals within the best entries. The beets, belonging to five multigerm families including 2,281, were selected on the basis of:

- Plant development and an intense green color of the leaves
- Regularly shaped roots, without internal or external symptoms of the disease
- Root weight

The remaining genotypes were almost totally destroyed by rhizomania. The 10–20 mother beets selected in each family, representing around 5 % of the original population, were overwintered (De Biaggi 1987).

In February 1981, the beets of each family were crossed with two F_1 male-sterile, high-yielding seed bearers (referred to as females in the text). In the year after, field tests of the ten hybrids were conducted at a site in France, where a more intense and uniform rhizomania infestation had been previously located. In July, the excellent performance of hybrids obtained with the pollinator 2,281 was confirmed by the French trials (Table 9.1). The remaining hybrids did not exceed the yield of Alba P and Domino (the hybrid monogerm version of Alba P) used as check.

Table 9.1 Field trials illustrating the performance of Rizor compared with other varieties

Locality (year) rhizomania	Variety (seed company)	Roots (t/ha)	Sugar content (%)	Sugar yield (t/ha)
Pithiviers – France (1982) severe	2281 Rizor (SES)	41.90	14.22	5.95
	Alba P (Alba)[a]	22.60	11.77	2.65
	Domino (Alba)[a]	20.90	10.08	2.32
	LSD (P = 0.05)	*9.60*	*2.01*	*2.33*
S. Martino – Italy (1983) moderate	Rizor (SES)	52.39	41.11	7.39
	Monodoro (Hilleshøg)[a]	37.69	14.94	5.63
	Ritmo (Maribo)[a]	14.11	13.46	5.53
	Monofort[b] (VDH)	22.90	13.43	3.07
	LSD (P = 0.05)	*14.30*	*0.96*	*1.49*

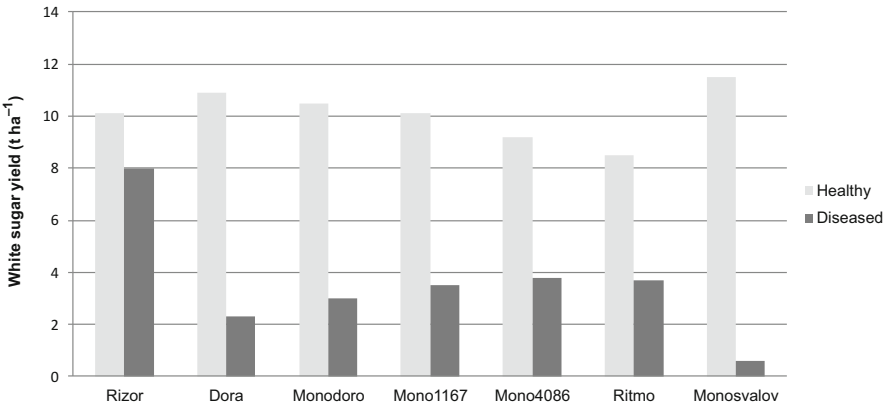

Fig. 9.4 Field trial conducted at Erstein, France, in diseased and healthy soils

The next year, the best hybrids were sown in France in two similar field trials, both conducted in infested and in rhizomania-free condition (Fig. 9.4). In addition, another two similar tests were sown in Italy (De Biaggi 1987). These trials confirmed that the resistance factor identified in the family 2281-R1 (used later in the hybrid "Rizor") offered a real possibility for significantly reducing the damage caused by rhizomania (De Biaggi 1987; De Biaggi et al. 2003). The virus concentration in the root tissues in susceptible check varieties and Rizor was 2,300 and 135 ng/g, respectively (De Biaggi 1987).

The French trials confirmed the higher level of resistance displayed by the monogenic factor carried by the first version of Rizor, when compared with the varieties of the "type Alba" (Bongiovanni 1984). However, the first available version of Rizor had some negative traits:

- A slight tendency toward bolting
- Unsatisfactory processing quality
- Sugar yield that was 10 % less than the susceptible varieties in rhizomania-free fields

But by using traditional breeding methods, Rizor was improved rapidly. In 1985, it was widely sown in France. At the same time, several experimental versions of Rizor were included in field trials in diseased districts all over the world. Until 1987, the breeding program for Rizor was carried out using normal family selection methods, integrated with artificial infection of individual plants followed by ELISA analyses. Later, the selected lines were multiplied using in vitro techniques (De Biaggi 1987; De Biaggi et al. 2003).

The origin of the resistance shown by the variety Rizor (i.e., the multigerm, diploid family 2281-R1) was not entirely clear. But recently, through the analysis of molecular variance (AMOVA) and use of principal component analysis of SNP markers on the original genotypes (which had been stored at low temperature), it was established that the Rizor and Holly resistances are not discernable as separate genetic sources (Stevanato et al. 2015). With this evidence, it was recognized that the pollinator SES 2281-R1 almost certainly originated from the Ro 281 family or some similar germplasm, which had been bred in public and private research stations and then bought, likely from Holly Sugar, currently located at Sheridan WI, through normal germplasm exchanges.

The resistances, Rizor and Holly, were soon recognized as monogenic and dominant because the rhizomania-resistant F_1 hybrid varieties were produced from rhizomania-susceptible females. In some genetic backgrounds, the segregation of *Rz1* was disturbed by minor genes (De Biaggi 1987). At these times, the presence of susceptible (*rzrz*) plants in the hybrid was around 10 %. This percentage was not negligible, especially from the sugar production point of view, and was difficult to reduce. This task became easier with the availability and the use of molecular markers.

In the first Rizor releases, it was observed that the viruliferous zoospores of *P. betae* inoculated the rootlets of susceptible genotypes in the same way as in the Rizor pollinator and in the Rizor hybrid (Giunchedi et al. 1985). The zoospores only moved easily through the xylem tissues in the roots of susceptible genotypes, whereas the movement in the genotypes carrying the Rizor resistance appeared slightly reduced. At that time, it was not possible to establish the physiological mechanism, which reduced spreading of the BNYVV (De Biaggi et al. 1986; Giunchedi et al. 1987; Giunchedi and Poggi-Pollini 1988). Further research ascertained that in Rizor, the zoospores multiply normally in the rootlets, but their migration toward the taproot is reduced or delayed by the development of a sort of barrier

of suberized cells, which interferes with the diffusion of the viruliferous zoospore from the affected areas (Asher and Kerr 1996; Marciano et al. 1977; Poggi-Pollini and Giunchedi 1989). Moreover, it was observed that the reproduction of *P. betae* in the roots of Rizor seemed to be hindered because the zoosporangia rarely were visible. This should lead to a slower reproduction of the plasmodiophoromycete and, consequently, to a reduced soil inoculum level after harvest, when compared with the susceptible varieties (Merdinoglu et al. 1987) (Fig. 8.4). Accordingly, the resistant plant seemed to react actively against the diffusion and the reproduction of the BNYVV. It was unclear if the diffusion of the virus inside the root happened cell to cell or by means of the vascular bundles (Geyl et al. 1995). At least in the initial observations, the resistance to rhizomania did not seem to act through limited inoculation of the viruliferous zoospores of *P. betae*. Another quality of Rizor, at least in its first releases, was the suitable CLS resistance, similar to Ritmo, confirmed by the similar weight of leaves and crowns in the late harvests (Bongiovanni 1986).

In the summer of 1983, Erichsen observed very poor growth and a diffused yellowing of leaves in a variety trial at Tracy (California) conducted by the Holly Sugar. Only the beets of some three-way experimental hybrids, such as 85C47-06 (Table 9.2), which had been produced by crossing different pollinators with the same CMS female, were normal (Lewellen et al. 1987). After ELISA analyses, it became clear that the field was uniformly infested with rhizomania (Duffus et al. 1984; Duffus and Ruppel 1993). Notwithstanding the presence of at least 30 % susceptible beets in the F_1, the sugar yield of the Holly hybrid was better than some European resistant checks. In this case too, the resistance factor segregated partially according to the action of a single dominant gene, demonstrated by a chi-square test on the backcrosses. The next year, similar results were obtained at Salinas by Lewellen et al. (1987), and the single, dominant gene was named *Rz* (Lewellen 1988) (Table 9.2).

The O-Type and CMS pair of lines carrying the Holly resistance was sold in Europe in 1986, and the first variety endowed with the "Holly" trait was "Gabriela" (KWS, Einbeck, Germany) released in 1990. In the initial reproductions of hybrids bearing the Holly trait, the seed quality was fairly low. These problems were rapidly resolved because the allele *Rz* proved to be easily handled and the negative qualities (low germination ability, cold susceptibility, etc.) were not linked with the resistance (Wisler et al. 1999).

Attempts to trace the source of the Holly resistant gene have not been successful. It has been speculated that it was partially derived from sea beet, perhaps from the Italian CLS-resistant accessions incorporated around 1935 into the germplasm of the USDA-ARS stations and the Great Western Sugar Company and other American seed companies (Lewellen and Biancardi 1990) (Fig. 9.2).

The mechanism of resistance for the Holly gene, coded *Rz1* by Scholten et al. (1999), appeared to be related to a reduction in BNYVV replication. Wisler et al. (1999) observed that the allele *Rz1* was incompletely dominant with various degrees of penetrance, as later confirmed by Pelsy and Merdinoglu (1996). In F_2 segregating, heterozygous plants, *Rz1rz1*, Giorio et al. (1997) observed a 1:2:1 ratio, which was thought to be caused by some kind of codominance. The *Rz1* types did not perform with the same intensity in the backcrossed genotypes; resistance was

Table 9.2 Field trials organized at Salinas in 1985 and 1986

Locality (year) rhizomania	Variety (seed company)	Roots (t/ha)	Sugar content (%)	Sugar yield (t/ha)	Disease index[c]
Salinas CA, USA (1985) moderate	84C39 – 031[a] (Holly)	60.80	12.90	7.82	2.67
	Rizor (SES)	40.10	13.80	5.48	3.08
	Monodoro (Hilleshøg)	37.50	11.90	4.50	3.29
	Monohikari (Mitsui – Seedex)	25.50	12.20	3.20	3.38
	HH37[b] (Holly)	24.10	9.90	2.41	3.60
	USH 11[b] (USDA)	20.10	9.00	1.80	3.66
	LSD (P = 0.05)	*8.30*	*1.10*	*1.08*	*0.54*
Salinas CA, USA (1986) severe	85C47 – 06[a] (Holly)	39.80	14.60	5.88	2.98
	Rizor (SES)	25.80	14.30	3.72	3.58
	Monodoro (Hilleshøg)	19.90	13.30	2.68	4.38
	Monohikari (Mitsui – Seedex)	22.80	13.40	3.06	4.45
	HH37[b] (Holly)	19.90	12.40	2.46	4.54
	USH 11[b] (USDA)	15.40	11.60	1.79	4.45
	LSD (P = 0.05)	*5.30*	*1.00*	*0.77*	*0.40*

See also Table 11.1
From Biancardi et al. (2002), modified
[a]Monogenic-resistant varieties
[b]Susceptible check
[c]Disease index (0 = no symptoms, 9 = dead)

dependent on both their genetic background and the presence of modifying genes (Rush et al. 2006). The effect of the genetic background that accompanies the resistance gene cannot be disregarded because it may modify significantly the expression of the trait (Meulemans et al. 2003).

Because recent fingerprinting analyses confirmed that the resistances of Rizor and Rz1 are almost identical, it is very probable that the Holly 1-4 line carrying the *Rz1* gene and the resistant family, SES 2281, were derived from the same common parent (Stevanato et al. 2015). The differences between the Holly line and the SES family evidently are due to diverse genetic backgrounds (De Biaggi, unpublished). It appeared possible to accumulate additive traits, which increase the effects of *Rz1*. The supposed modifying genes seemed to be the similar to those working in the Rizor resistance. Additionally, both alleles showed incomplete dominance, which means higher production in the homozygous state than in the heterozygous (Meulemans et al. 2003). In resistant genotypes, the virus was localized in the epidermis, cortex parenchyma, endodermis, and interstitial parenchyma but rarely in the vascular tissues (Scholten et al. 1994).

The combination of multiple types of resistance may be advantageous to provide higher levels of protection against BNYVV (Lewellen and Biancardi 1990). The effect of the Alba multigenic resistance on sugar yield in diseased fields is less than the monogenic or near monogenic sources like Rizor and Holly, and the hybrids between Alba and Rizor did not perform better than the parents. The expected het-

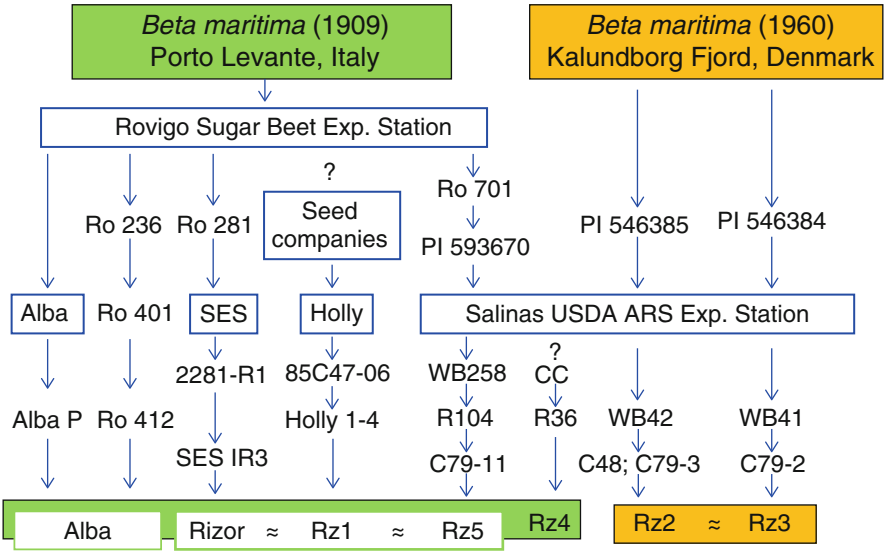

Fig. 9.5 Sources, year of sampling (between parentheses), and partial derivation of the currently employed resistances to rhizomania (Alba, Rizor, Holly, Rz2) or those resistances still under evaluation (Rz3, Rz4, and Rz5). *CC* Composite cross, ≈ similar Breeding centres

erosis effect also was not evident in hybrids between Rizor and Holly sources. Therefore, their equivalence began to be hypothesized. According to Barzen et al. (1992), the resistances of Rizor and Holly likely are due to the same major gene with incomplete dominance, located in chromosome III and interacting both with minor (or modifying) genes and the presence of different genetic backgrounds (Meulemans et al. 2003). Scholten et al. (1999) analyzed segregation in F_2 and backcross generations of a cross between the Salinas line R104 and Holly 1-4 (Rz1) and placed both resistance loci in the identical position on chromosome III. Because the line R104 is derived from the *B. maritima* accession Ro 701, collected in the Po Delta in 1978 (Biancardi et al. 2002), the common lineage of Rizor and Holly sources is confirmed (Stevanato et al. 2015) (Fig. 9.5).

The variety Rizor became available for farmers in 1985 (De Biaggi 1987), that is, 5 years before the marketing of the Holly Rz1 resistance, which was sold to the European seed companies in the form of a CMS and its corresponding O-Type. According to Dürr et al. (2000), some negative traits of the first Rz1 and the derived European varieties were evident, especially during emergence, mainly caused by the limited nitrogen uptake of the seedlings (Rush et al. 2006).

Notwithstanding the intense screening carried out in public and private breeding centers, no other traits of resistance were found in the cultivated sugar beet germ-

plasm. Consequently, attention turned to wild beets and in particular to *B. maritima* (van Geyt et al. 1990). The transfer of the monogenic resistance trait from *B. maritima* to sugar beet genotypes is relatively easy and usually is performed by means of backcrosses or recurrent selection, aided by the use of molecular markers, which have improved greatly the rate of success (Geyl et al. 1995). The accessions collected in different parts of the world and stored in gene banks were carefully checked. The wild germplasms of USDA-ARS collection were analyzed in the field and greenhouse at the USDA-ARS Research Station in Salinas, California. Differing degrees of rhizomania resistance were found in 17 entries (Whitney 1989). After crossing with susceptible beets, the segregating generations suggested the monogenic and dominant nature of the resistance, which appeared quite simply inherited. The most promising accessions were WB 41 and WB 42 (PI 546385), corresponding to *B. maritima* populations collected in 1960 at Kalundborg Fjord, Denmark, by Lund (Amiri et al. 2003; Doney and Whitney 1990; Lewellen 1991, 1997). In some accessions, including WB 42, high levels of resistance to rhizomania, CLS, root maggot, and *Erwinia carotovora* were detected (Doney and Whitney 1990). Using recurrent selection, the resistance trait of WB 42 was transferred into the high-yielding pollinator C37 (Lewellen et al. 1985) also carrying resistance to curly top, erwinia root rot, beet western yellows virus (BWYV), and beet yellows virus (BYW). The resulting line, C79-3 (Lewellen 1997), endowed with WB 42 trait, displayed a higher level of resistance to rhizomania than Rz1 (Scholten et al. 1999). The gene, coded *Rz2* by Scholten et al. (1999), was localized on chromosome III at a genetic distance of 20–35 cM from *Rz1*. The Rz2 resistance seems to be based on a single, dominant, major gene displaying distorted segregation as observed both in Rizor and Rz1 sources. It was predicted that, because the genes carrying the resistance Rz1 and Rz2 were at different loci, they would provide some heterotic effects after crossing (Amiri et al. 2003).

In accession WB 41 collected very close to the WB 42 site, another resistant gene was discovered (Figs. 9.2 and 9.5). The gene was named *Rz3* and the locus conferring resistance was localized on the same chromosome III, very near (5 cM) to *Rz*2. It is believed that the WB 41 and WB 42 resistances are induced by the same gene, perhaps belonging to the same allelic series and interacting with different modifying factors (Grimmer et al. 2007). In relation to WB 42, Scholten et al. (1997) put forward the hypotheses of either one or two major genes with distinct segregation or of two complementary major genes both necessary for expression of the resistance. The genomic region of 800 kb including *Rz2* is currently being analyzed in search of new candidate genes for resistance (Capistrano et al. 2014).

After the discoveries of Rz2 and Rz3, other presumed new sources of resistance were isolated by means of QTL analyses in accession R36 derived from composite crosses obtained at Salinas, and in WB 258 (formerly coded Ro 701, R104, and PI 546426), derived from *B. maritima* harvested in July 1978 in the Po Delta by De Biaggi and Biancardi (Biancardi et al. 2002; Lewellen 1991), likely near the same site where Munerati collected the seed in 1908 (Fig. 9.1). These hypothesized new resistances, termed Rz4 by Gidner et al. (2005) and Rz5 by Grimmer et al. (2008), respectively, showed evidence of distorted segregation and mapped very near to

Rz1, likely representing another case of an allelic series (Grimmer et al. 2007). All the listed resistances were released in 1997, included in the Salinas germplasm series C79-1 to C79-11, developed after backcrossing with the common recurrent parent C37 (Lewellen et al. 1985; Lewellen 1995a, 1997) (Figs. 9.2 and 9.5). Two populations developed from a combination of all of the germplasms in this series will be released as FC1740 and FC1741 (Panella, personal communication). As regards further types of resistances, Pelsy and Merdinoglu (1996) mentioned some Turkish sugar beet breeding lines and *B. maritima* ecotypes, but further developments of these materials are not known.

Another approach has been applied to search for new traits of resistance (Doney and Whitney 1990). The individually selected *B. maritima* mother roots of different populations were pooled and open pollinated. In the derived heterogeneous population, chance, but potentially useful, combinations of major and minor genes of rhizomania resistance can be found. From similar composite crosses, some new sources of rhizomania resistance were obtained by Lewellen (1995b) and Lewellen and Whitney (1993). Using the approach described above, one of the germplasm developed led to the creation and release of C79-8, where the Rz4 resistance was discovered (Gidner et al. 2005). With the same system, the lines C39R and C47R were identified, which bore quantitative traits of resistance, allowing the same level of production under rhizomania conditions as did Rizor or Rz1 (Lewellen 1995c). Both lines reduced the disease symptoms, but not the virus concentration in the roots (Rush et al. 2006). Potentially useful traits of resistance were located in *Beta corolliflora*, *Beta intermedia*, and *Beta lomatogona* (Paul et al. 1993).

9.3 Multiple Resistances

The level of resistance of Rizor ≈ Holly (Rz1) improves in crosses with *Rz2* (De Temmerman et al. 2009; Meulemans et al. 2003). The differences among the above-mentioned monogenic resistances, as well as the heterosis, are evident only in the case of Rz1 x Rz2 crosses (Amiri et al. 2003). First in 2002, some commercial varieties carrying the double resistance were introduced in the USA and in France, where they displayed better sugar yield both in the presence of the BNYVV-P strain and in other resistance-breaking BNYVV strains (De Temmerman et al. 2009; Rush et al. 2006; Smith et al. 2010). To the best of our knowledge, these results indicate that the accessions of *B. maritima* collected in Italy and more recently in Denmark are the only sources of resistances to rhizomania commercially deployed today (Pavli et al. 2011) (Figs. 9.2 and 9.5).

Heijbroek et al. (1999) tried to find an interaction between varieties carrying different sources of resistance and the three pathotypes of BNYVV (A, B, and P), known at that time. Because no significant differences were detected in field trials, the experiments were continued in the glasshouse, using substrate with the same concentration of the diverse BNYVV pathotypes. Pathotype B was less damaging for all the measured parameters, while pathotype P confirmed its already known

high level of virulence, likely due to it moving more rapidly inside the roots than either the A or B type (Heijbroek et al. 1999). Significant differences also were evident among the response of resistant varieties, but not among the pathotypes.

Resistance-breaking strains of BNYVV appeared around the year 2000 on varieties with the Rz1 resistance. The evolution of these strains after about 30 years of continuous employment of Rz1 almost was expected. The same happens for every disease, when the crop is protected for a long time by the same chemical or a single-gene resistance (Van Der Plank 1975). In the Imperial Valley of California, similar loss of resistance by Rz1 was observed during the 2002 campaign, evidently due to mutations in the virus. The new strain was coded IV-BNYVV. Satisfactory degrees of resistance were identified in the C79-9 germplasm released by Lewellen (1997), coming from the *B. maritima* accession coded WB 151 (PI 546397), collected in Denmark together with WB 41 and WB 42. Additional resistance-breaking strains observed in other parts of the USA (Minnesota, North Dakota) are similar but not identical (Acosta-Leal et al. 2009, 2010; Liu and Lewellen 2007). It is yet to be ascertained whether the resistance-breaking episodes occur independently or, simply, are due to contamination, i.e., through movement of infected soil (Bornemann et al. 2015). The first option appears more likely and indicates that the local genotype x environment interactions can modify the frequencies and the molecular background of the virus mutations. This means that similar strains may appear everywhere after a given period of virus multiplication, if the local conditions favor the disease agents.

The genomic composition of *P. betae* displays variability, dependent on geographical adaptation as well. Therefore, the vector could have a role in the resistance-breaking occurrences (Pferdmenges 2007). According to the same author, the resistance-breaking episodes were unconnected with the *P. betae* concentration in soil (Pferdmenges and Varrelmann 2009). Soon after, proof of these occurrences in soils infected by BNYVV type A was discovered in varieties endowed with Rz2 and also in the more recent varieties with the double-resistant Rz1 + Rz2 (Hleibieh et al. 2007; Scholten and Lange 2000).

9.4 Resistance to *Polymyxa betae*

Beet necrotic yellow vein virus (BNYVV), the causal agent of rhizomania, is vectored by the plasmodiophorid, *P. betae* (reviewed by Rush 2003). *Polymyxa betae* is ubiquitous in every beet-growing country and can carry several more or less harmful soilborne viral diseases (Lennefors et al. 2008; Rush 2003). BNYVV is not always present inside the cystosori, e.g., only 45 % of the cystosori tested in Californian soil samples were infected (Gerik and Duffus 1988). Normally, the plasmodia alone seem asymptomatic for the crop (Desoignies et al. 2014; Hleibieh et al. 2007; Scholten and Lange 2000) or cause minimal damage (Rush et al. 2006). But it has been reported in greenhouse tests that a viruliferous *P. betae* induced a significant depression in emergence and seedling growth (Liu and Lewellen 2008;

Wisler et al. 2003). Similar damage in field conditions was described by Davarani et al. (2013) but only in warm soil, which also was observed also by Blunt et al. (1991). The opinions are quite controversial, likely because the behavior of the plasmodiophorid depends not only on genotype x environment interactions but also on the behavior of host plant (Pferdmenges 2007). According to Abe and Tamada (1986), isolates of *P. betae* coming from plants other than sugar beet are unable to transmit the BNYVV.

Methods complementing the genetic resistance or alternative mechanisms for limiting the effects of BNYVV have been investigated. A primary target should be delaying or hindering the multiplication of *P. betae* by means of genetic mechanisms to prevent or reduce either the entry of the vector into the rootlets or the multiplication of the virus (Mesbah et al. 1997). In fact, the plasmodiophorid, as was shown by Lubicz et al. (2007), can assume the function of host, because the virus reproduces inside it as well. Several approaches to find resistance to *P. betae* have been tried without concrete results. No useful source of resistance to the plasmodiophorid has been discovered in screening sugar beet genotypes; on the contrary, a higher number of cystosori were found in BNYVV-resistant varieties than in the susceptible ones (Paul et al. 1993). In some entries belonging to the sections *Procumbentes* (now genus *Patellifolia*) and *Corollinae*, it has been observed that the zoospores penetrated the root of the resistant wild beets normally, but further diffusion was rarely observed (Barr et al. 1995) or further development of cystosori (Paul et al. 1993). Through monosomic addition lines, it was demonstrated that the genes inducing this sort of behavior are located on chromosomes IV and VIII of the host plant. Notwithstanding the dominance of the monogenic resistance (Barr et al. 1995; Paul et al. 1993), attempts to transfer the traits into cultivated genotypes were unsuccessful, mainly due to the cross-incompatibility among sugar beet and the species included in sections *Procumbentes* and *Corollinae* (e.g., *Patellaris*) (Box 1.5). The same attempts were undertaken using genotypes of *B. maritima* (Doney and Whitney 1990). Here, the traits of resistance to *P. betae* were "surprisingly common" and appeared to be quantitatively inherited (Asher et al. 2009; Asher and Barr 1990; Luterbacher et al. 2004, 2005). The resistance loci to the vector were located on chromosomes IV and IX and were termed *Pb1* and *Pb2*, respectively. In backcrosses with sugar beet germplasm, by screening with recombinant antibody, it was possible to transfer the resistance traits. BNYVV alone moves very slowly inside the root, being transferred more easily cell to cell by *P. betae* (Prillwitz and Schlösser 1993). Therefore, the degree of aggressiveness of the plasmodiophorid seems to have effects in spreading the virus inside the beet and in the related damage (Gerik and Duffus 1988). The same authors found significant differences of behavior in US isolates of *P. betae*. The multiple resistances, both to virus and to vector, could be a powerful means for further reduction of the damages caused by rhizomania due to the very different but complementary mechanisms.

9.5 Resistant Varieties

Owing to:

- The very limited or ineffective effects of crop rotation and other agronomic measures after soil infection
- The present and future difficulty of finding effective means of chemical control
- The encouraging, but still scarcely working, biological control systems for reducing the spread and the damage of rhizomania

The only affordable and relatively inexpensive means of management is given by genetic resistance (Pavli et al. 2011). The statement is justified by the availability of several already employed, effective, different types of resistance (Hull 1994). Other new traits are possible candidates to replace the currently used resistance sources in case of a possible reduction in efficacy. Using molecular biology-assisted techniques, the wild beet genomes (including almost all the species of the genera *Beta* and *Patellifolia*) could become further sources of suitable traits, stimulating future development of sustainable agriculture (Martin and Sauerborn 2013). Additionally, nothing is more environmental friendly than genetic resistance.

Another promising area of research is represented by the transgenic resistances (see. Chap. 10). Some transgenic varieties have shown quite normal sugar production under very diseased field conditions (Lennefors 2006). These effective resistances, near to immunity, are ready to be released and may be an important mean to reduce the effects of rhizomania, also under the condition of very aggressive virulence (Hleibieh et al. 2007; Mannerlöf et al. 1996; Pavli et al. 2011).

The rhizomania resistance traits in *B. maritima* are very unlikely to have originated by natural selection in presence of the disease factors. Bartsch and Brand (1998) did not find the presence of either *P. betae* or BNYVV in soils and roots of *B. maritima* at six sites along the coast of the North Adriatic Sea, including the Po River Delta, where the Rz1 resistance is found. This was mostly due to the lack of *P. betae*, which is decreased in the soil by the high salt content where *B. maritima* normally grows. The same lack of rhizomania and vector likely occurs in the Danish soils, where the sea beets that coded WB 41 and WB 42 were collected (Driessen 2003). Therefore, if rhizomania resistance in *B. maritima* has originated and developed by natural selection in diseased soils, as hypothesized above, this must have happened elsewhere or in some other manner.

Sugar yield of the first versions of the monogenic-resistant varieties was about 10 % lower than the susceptible ones in healthy soils (Whitney 1989). But this weakness gradually has been overcome. Today, the sugar yield of resistant and susceptible varieties is almost the same in rhizomania-free conditions. These varieties don't display significant yield variation either in absence or presence of rhizomania, excluding the cases of severe infections (Graf 1984) and resistance-breaking BNYVV (Fig. 9.5).

In conclusion, the dynamics of the host-pathogen relationship in rhizomania depend on numerous factors and reciprocal interactions, which still are quite

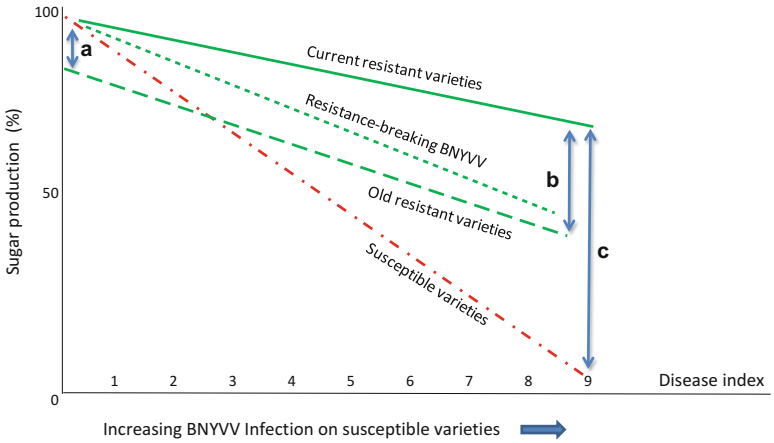

Fig. 9.6 Sugar yield varied with increasing infection. After release, resistant varieties were yielding less than the susceptible ones in healthy soil (*a*). Over time, this gap has been completely erased. The resistant varieties (old or new) are not immune to rhizomania and yield decreases with increasing infection but not nearly as much as yield decreases in susceptible varieties. The breeding progresses in the last 30 years and the effects of rhizomania resistances on sugar yield are represented by the *double arrows* (*b*) and (*c*), respectively

unknown or not predictable due to their extreme variability. The future development of disease management will require, as in the past, a continuing increase in multidisciplinary research projects (Fig. 9.6).

9.6 Mechanisms of Resistance

Some analytical techniques, such as immunogold-silver labeling, electron microscopy etc., have been used to detect the location of the BNYVV inside root tissues, with the aim of explaining the mechanisms, which allow the resistant beets to limit the damage caused by rhizomania. Because BNYVV can multiply both inside the *P. betae* and in the root cell (Geyl et al. 1995), it is not easy to establish the stage(s) of the viral pathogenesis as influenced by the genetic host resistance. According to Fraser (1990), the reaction of resistant beets against vectored viruses similar to BNYVV works mainly by limiting:

- The transmission of the virus
- Its multiplication inside the root
- Its movement inside the root
- Its pathogenicity

The major portion of research papers reported no difference between the BNYVV concentration in the rootlets of resistant and susceptible beets under similar inoculum condition. This means that the resistance does not reduce the entry of virulifer-

ous cystosori. But in the case of Rz2, a lower concentration of BNYVV was detected (Scholten et al. 1994). The inhibition of BNYVV multiplication seems to behave in a way that influences the reduction of the damage. In fact, the epidermal cell of the resistant rootlets infected by cystosori contains more BNYVV than in susceptible genotypes (Giunchedi and Poggi-Pollini 1988).

The reduced mobility of the virus has been recognized as the major effect of genetic resistance. The short-distance movement happens cell to cell and has been detected by means of virus antigen, which located virus in cells neighboring the cell containing the viruliferous cystosori (Hull 1989). The possibility of long-distance movement, i.e., through the vascular bundles, is still controversial (Scholten et al. 1994). In some cases the BNYVV was detected neither in the bundles of resistant nor of susceptible beets. In other cases, the xylem vessels of susceptible genotypes were infected by the virus, as seen by the inoculum concentration (Scholten et al. 1994). In resistant beets, the vascular bundles appeared smaller than in the susceptible ones, likely limiting in this way the movement of the virus. It was hypothesized that there was development of suberin barriers in the cell, which hinders the movement of the virus from lateral rootlets to the taproot (Poggi-Pollini and Giunchedi 1989). The ability of the BNYVV to spread in the roots depends also on the beet's age. In fact, if the infection happens after the seedling stage, the virus spreads in the rootlets, but not in the taproot, also in susceptible genotypes (Hull 1989), thus explaining the minor damage of the disease if the crop is sown early. Similar differences were detected when comparing the currently deployed resistances, which display quite diverse mechanisms in limiting the effects of the BNYVV. For example, the beets carrying the resistance Rz2 show minor virus replication and more restricted cell-to-cell movement than Rz1. In order to explain this behavior, Scholten et al. (1994) hypothesized the presence of different genetic systems or mechanisms of action in Rz1 and Rz2, which was later demonstrated.

9.7 Germplasm Conservation in the Service of Plant Breeding

Over the past 60 years, we slowly have come to realize that the crop wild relatives of sugar beet, especially sea beet, have become a crucial genetic resource in the breeding of sugar beet and other cultivated beet crops. During this time, we have seen a tremendous increase in our knowledge of the life history of this critical resource (Biancardi et al. 2012). But it is only in the last 30 years that we have acknowledged that the wild germplasm was vanishing (Doney et al. 1995; Pignone 1989) and that without this resource, we might not have the genetic means to improve the sugar beet crop (De Bock 1986; Doney and Whitney 1990; Doney 1993; Lewellen and Skoyen 1991; van Geyt et al. 1990). We have begun to understand that an effective conservation strategy must be grounded on a thorough understanding of the taxonomy, genetic diversity, and distribution of the crop wild relatives (Frese 2010).

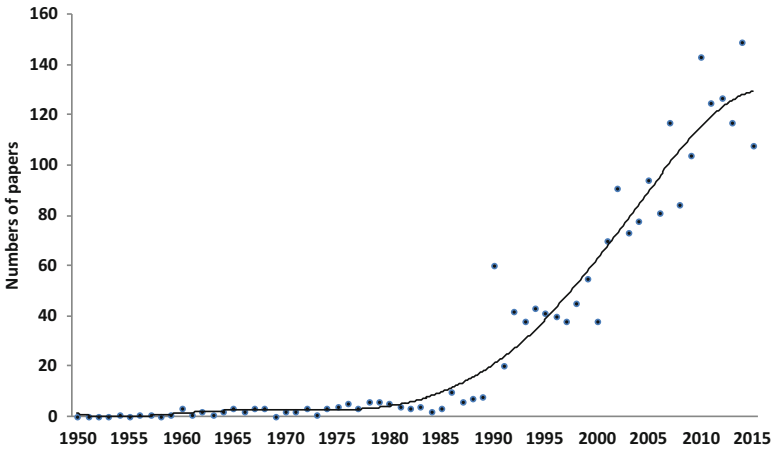

Fig. 9.7 Number of journal articles regarding rhizomania published per year. Until 1963, the papers were written only in Italian. An article in German regarding *Polymyxa betae* was edited in 1964, though not yet linked with rhizomania. To the best of our knowledge, the first papers on the disease in German or in English were published in 1967 and 1971 respectively (Summarized from different sources)

Some of our sources of resistance to rhizomania are the result of plant collections by the pioneers in this field. Coons of the USDA-ARS was a major supporter of using the wild *Beta* species, especially sea beet as a source of genetic diversity to improve cultivated beet in the USA. He made collection trips in 1925 and 1935 (Coons 1936) and again in 1951 and 1971 (Coons 1975), long before anyone in the USA was aware of rhizomania, yet some of the accessions he collected provided the genes for resistance to rhizomania. Similarly, Munerati may have transferred rhizomania resistance unintentionally from sea beet to cultivated beets as he worked on resistance to Cercospora *Cercospora* leaf spot.

Although there was a reluctance to use sea beet germplasm because of some of the undesirable traits, by the mid-1980s, commercial breeding programs had begun to reconsider (Frese et al. 2001). In Europe, Bosemark (1989) created the framework for plant breeders to introgress effectively the germplasm of crop wild relatives into elite breeding populations. This activity was mirrored in North America by the development of the Sugar Beet Crop Advisory Committee to work with the curator of the USDA-ARS *Beta* collection to provide evaluation data for plant breeders interested in crop improvement, especially for improved disease resistance (Doney 1998; Janick 1989; Panella and Lewellen 2007). Frese (1990) used many of these ideas to develop a strategy to enhance the genetic foundation of the sugar beet gene pool. Together these researchers founded the World *Beta* Network under the IBPGR to improve international collaboration among researchers and gene bank curators of *Beta* germplasm collections worldwide (Bosemark 1989). Today this effort of conserving genetic resources is a collaborative effort of the international community.

We know that species evolution is arrested in an ex situ collection, which provides only a snapshot of part of the existing genetic diversity at that place in time and space. There can be no additional adaptation to the changing environment, only adaptation to the changing gene bank seed reproduction process. Consequently, we have seen increasing awareness in conservation of beet wild relatives in situ and sea beet in particular (Biancardi et al. 2012; Frese 2010; Frese and Germeier 2009; Jarvis et al. 2015; Van Dijk 1998). It is up to all of us, researchers, plant breeders, and beet processors, to preserve these resources for those who will need them in the future (Fig. 9.7).

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Chapter 10
Engineering Transgenic Rhizomania Resistance

Ourania I. Pavli and George N. Skaracis

Abstract The only practical means to ensure viability and profitability of the sugar beet crop is to provide efficient protection against rhizomania, caused by beet necrotic yellow vein virus (BNYVV), through the use of varieties, specifically bred as resistant to the disease. Although breeding ingenuity has to date achieved successful control of the disease throughout the world, resistant varieties may still suffer significant losses. At the same time, evolutionary changes in the pathogen continuously pose new challenges and require adjustments in relevant breeding programs if they were to keep providing a durable crop protection through the use of better resisting varieties. Given the fact that acquiring resistance from the repertoire of the crops' gene pool is delimited by the scarcity of natural genetic sources of resistance to BNYVV, transgenesis offers the possibility to broaden the options for rhizomania resistance. Initial attempts to generate transgenic rhizomania resistance were based on the pathogen-derived resistance (PDR) concept. Recent understanding of the aspects underlying the antiviral pathways of RNA silencing however, has placed the focus of interest on generating rhizomania resistance based on the exploitation of the discovered innate defense mechanism. Alternative resistance strategies involved the employment of genes originating from nonviral sources. This chapter reviews the latest advances in breeding for rhizomania resistance in transgenic sugar beet plants.

Keywords Sugar beet • Rhizomania • Beet necrotic yellow vein virus • Resistance breeding • Transgenic rhizomania resistance

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E. Biancardi, T. Tamada (eds.), *Rhizomania*, DOI 10.1007/978-3-319-30678-0_10

10.1 Introduction

Over the years, the introgression of natural genetic sources of rhizomania resistance, through conventional breeding approaches including the marker-assisted backcross breeding, has been the most substantial means to ensure a viable sugar beet production in disease incidence areas (Biancardi et al. 2002). Although breeding efforts toward developing varieties specifically bred as resistant to the disease have so far been met with considerable success, resistant varieties may still suffer significant losses compared to their potential sugar yield under disease-free conditions (Johansson 1985; Casarini 1999). Such losses are further strengthened by the known high plasticity of viral genomes (Roossinck 1997) which poses serious threats to durability of plant virus resistance (Garcia-Arenal and Mc Donald 2003) as well as the fact that plant RNA viruses, such as the rhizomania-causing beet necrotic yellow vein virus (BNYVV), in the field exist as swarms of point mutants with different pathogenic properties. Relevant are the reports on changes in field and molecular BNYVV epidemiology, manifested by the emergence of highly pathogenic virus strains which are capable of breaking the currently employed Rz-based resistances (Schirmer et al. 2005; Liu and Lewellen 2007; Acosta-Leal and Rush 2007; Acosta-Leal et al. 2008, 2010a, b; Chiba et al. 2008, 2011; Koenig et al. 2009; Pferdmenges et al. 2009; Pferdmenges and Varrelmann 2009; Pavli et al. 2011b).

In view of the fact that the ongoing battle between constitutive defense and counter defense is in play to determine the disease outcome of the infection, there is an obvious need to search for additional resistance sources, capable of withstanding the fast evolution of BNYVV under conditions of high pathogen pressure. Given the scarcity of natural genetic sources of resistance to BNYVV, however, the possibility of acquiring resistance from the repertoire of the crops' gene pool is severely limited. In this regard, the prospect of generating transgenic virus-resistant plants has been explored as a means to broaden the options for rhizomania resistance.

Transgenic approaches employed to date include the transgenic expression of (a) viral genes and sequences, based on the pathogen-derived resistance (PDR) concept as proposed by Sanford and Johnston (1985); (b) virus-derived double-stranded RNA (dsRNA) molecules, as a means to trigger the RNA silencing-mediated resistance, generally regarded as the most successful variant of PDR; and (c) antiviral genes, originating from nonviral resistance sources. This chapter reviews the current state of the art on the use of transgenic approaches to combat rhizomania disease of sugar beet.

10.2 Pathogen-Derived Resistance

The concept of PDR, first perceived by Sanford and Johnston (1985), relies on the exploitation of genes originating from the pathogen as a means to obtain resistance in a variety of host-parasite systems. It was suggested that the deliberate expression

of such genes in an altered form, at varying levels or at different plants' development stages, could interfere with pathogen replication leading to a specific host resistance. Following the initial demonstration that the expression of a viral coat protein (CP) confers resistance ranging from immunity to delay and attenuation of symptoms (Abel et al. 1986; Beachy et al. 1990), the PDR approach was efficiently extended to a wide range of plant pathogens, a significant part of which pertains to plant viruses. Among possible targets for PDR-mediated virus resistance, the most broadly exploited virus genes were those coding for (a) coat protein, (b) replication-associated proteins, and (c) movement proteins (MPs) (Abel et al. 1986; Baulcombe 1996; Prins and Goldbach 1996; Prins et al. 2008). Contrariwise to the original notion, however, resistance levels often did not correlate with protein expression levels, thus highlighting the operation of alternative mechanisms acting at the RNA level. In this line, it was demonstrated that in many cases resistance relied on the expression of transgenic RNA and more importantly, such RNA-mediated resistance proved more promising in conferring high-level resistance or even immunity to virus infection (Prins et al. 2008). To this end, much emphasis has been recently placed on the expression of viral RNA sequences triggering a resident resistance mechanism, now known as RNA silencing. A separate section is devoted to this type of resistance.

The perspective of engineering PDR-based resistance against rhizomania disease of sugar beet has been pursued by means of expressing genes coding for the CP. Despite the proven ability to obtain transgenic virus resistance through the expression of the viral replicase and MP genes, these approaches have not been employed in developing resistance against rhizomania.

First attempts to achieve PDR-mediated resistance against BNYVV were based on the expression of the *CP* gene in sugar beet suspension cells (Kallerhoff et al. 1990). More specifically, *Agrobacterium tumefaciens*-mediated transformation was used to develop suspension cells expressing the viral CP followed by an in-frame region that encodes a read-through translation product, involved in virus assembly. The transformed protoplasts were subsequently challenge-inoculated with BNYVV, whereas evaluation of resistance was performed on the basis of estimating virus titers in transformed and untransformed protoplasts. Though amenable to infection, protoplasts deriving from *CP*-transformed sugar beet suspension cells presented significantly lower virus multiplication rates in comparison to protoplasts from non-transformed cells.

Accordingly, Ehlers et al. (1991) developed a protocol for the generation of transgenic hairy roots expressing the BNYVV-derived *CP* gene through *Agrobacterium rhizogenes*-mediated transformation. Although, transgene integration and expression was verified, both at the transcript and protein level, a CP-mediated protection could not be demonstrated, due to inability to infect the sugar beet hairy roots. In this regard, the CP-based rhizomania resistance at the whole plant level was first reported by Mannerlöf et al. (1996). The study involved the generation of two constructs carrying the coding region of the *CP*, their difference residing on the length of their untranslated leader sequences, and their subsequent use for *A. tumefaciens*-mediated transformation. Progenies obtained after two

cycles of selfing were challenged-inoculated with BNYVV and assessed for resistance, both in greenhouse and field conditions. Although accumulation of the viral protein could not be detected, virus multiplication was reduced in *CP*-expressing plants both in greenhouse and field experiments. Later studies, however, provide evidence that such a discrepancy between translatable levels and reduced virus accumulation may be explained on the basis of other mechanisms operating at the transcript level (for a review see Prins et al. 2008).

Alternatively to the expression of the *CP* gene, another PDR approach readily employed for the achievement of transgenic virus resistance relies on the expression of either functional or truncated versions of virus MPs as a means to interfere with virus cell-to-cell movement. In this line, the finding that overproduction of BNYVV-p15 relative to p13 results in inhibition of "triple gene block" (TGB)-based cell-to-cell movement (Bleykasten-Grosshans et al. 1997) provides evidence that the transgenic expression of p15 in sufficient amounts may be explored for the purposes of achieving resistance to the rhizomania-causing BNYVV.

10.3 RNA Silencing-Mediated Resistance

RNA silencing, referred to as RNA interference (RNAi) in animals and gene quelling in fungi, is a conserved regulatory mechanism occurring in a wide range of eukaryotic organisms, acting in a sequence-specific manner to control gene expression. RNA silencing induces mRNA degradation or translation inhibition at the posttranscriptional level, named posttranscriptional gene silencing (PTGS) in plants, or epigenetic modification at the transcriptional level, depended on RNA-directed DNA methylation (RdDM). PTGS was first observed when transgenic petunia plants with additional copies of an endogenous gene, encoding the chalcone synthase (CHS), became completely pigmentless due to a dramatic decrease in expression level of the respective genes (Napoli et al. 1990; van der Krol et al. 1990). Relevant findings were at the same period provided by Lindbo et al. (1993) who produced transgenic plants, expressing the CP of tobacco etch virus (TEV), that upon virus inoculation developed symptoms of systemic infection to later switch back to a healthy state. A notable advance in the knowledge concerning gene silencing, however, stemmed from the model proposed by the same authors as a way to explain the recovery phenotype observed. The central concept of the model was that the transgene-induced RNA degradation reflects a universal mechanism, based on the recognition and subsequent breakdown of all RNAs sharing a high sequence homology with the inserted transgene. In recent years, the research field of RNA silencing has been amazingly progressed, thus filling in our understanding of the pathway's sequel which is nowadays considered as a well-elucidated process (Baulcombe 2004; Meister and Tuschl 2004; Hammond 2005; Eamens et al. 2008; Agius et al. 2012). The triggering element of all existing RNA silencing pathways is a dsRNA molecule, capable of recruiting necessary components to carry out sequential phases of sequence-specific degradation (Fire et al. 1998; Hammond

et al. 2001). Following recognition, dsRNA is processed by Dicer-like (DCL) proteins into small interfering RNAs (siRNAs) of 21–24 nucleotides in length, which are loaded to members of the Argonaute (AGO) family to form an RNA-induced silencing complex (RISC). RISC then uses the siRNAs as guides for recognition of homologous RNAs to direct RNA degradation, translational repression, or DNA methylation of homologous target genes.

The gradual identification of biological pathways underlying RNA silencing has revealed that apart from the regulatory role in central developmental processes, RNA silencing also functions as a natural antiviral defense mechanism, a process named virus-induced gene silencing (VIGS) (Baulcombe 1999; Ding and Voinnet 2007; Ding 2010; Agius et al. 2012). In this regard, research has placed the focus of interest on the exploitation of the mechanism into more practical applications. In plants, RNA silencing, now considered as the most successful variant of PDR, has been primarily employed as an antiviral strategy against a wide range of viruses (Voinnet 2001, 2008; Waterhouse et al. 2001; Vazquez et al. 2002; Goldbach et al. 2003; Tenllado et al. 2004). Toward this direction, the mechanism is usually triggered by the introgression of a hairpin molecule, capable of duplex RNA formation, an arrangement known to act as a strong silencing inducer (Hamilton et al. 1998; Waterhouse et al. 1998; Chuang and Meyerowitz 2000; Johansen and Carrington 2001).

Aiming at building up RNA silencing-mediated rhizomania resistance, diverse targeting approaches have been so far developed. In all cases, transgenic plants were designed to trigger the accumulation of viral transgene-derived siRNAs that subsequently target the homologous BNYVV genome for degradation. A first attempt to artificially engineer rhizomania resistance via the RNA silencing defense pathway involved the production of transgenic *Nicotiana benthamiana* plants, expressing either the CP-encoding gene or its adjacent in-frame open reading frame (ORF), referred to as CP-readthrough domain (RTD) (Andika et al. 2005). Upon foliar rub-inoculation, only the RTD-expressing plants displayed highly resistant or recovery phenotypes. Analyses of transgene mRNA and transgene-derived siRNAs, accumulated prior and post infection, pointed that high-level resistance operates at a transgene-induced RNA silencing level, whereas the recovery phenotype was triggered by virus-induced silencing of the transgene. Inoculation tests using viruliferous zoospores of *Polymyxa betae*, however, revealed that roots of resistant plants, though amenable to infection, presented low multiplication rates as a consequence of transgene-induced RNA silencing. It is of interest that in resistant plants, levels of mRNA were higher in roots than in leaves, while transgene-derived siRNAs were lower in roots than in leaves. Similar findings of lower accumulation of siRNAs in the roots were obtained both in non-transgenic plants and transgenic plants showing a recovery phenotype. Overall results, support the conclusion that in both transgene- and virus-induced RNA silencing, the silencing activity in roots is lower than in leaves. Transgenes employed in this study however were transcribed as single-stranded RNA (ssRNA), a configuration known to function as a weak silencing inducer, therefore leading to a reduced activity of transgene-induced RNA silencing.

A first demonstration of the efficacy of RNA silencing in conferring rhizomania resistance in the sugar beet crop however was provided by Lennefors et al. (2006). The study involved the transgenic expression of a BNYVV replicase-derived inverted repeat, as a means to trigger the dsRNA-induced accumulation of siRNAs. Upon BNYVV challenge inoculation, using the *P. betae*-mediated natural infection process, transgenic plants displayed high-level resistance against various strains of BNYVV. At the same time, resistant plants were characterized by low levels of transgene mRNA and accordingly high accumulation of siRNAs, which are indicative of the operation of an RNA silencing-based resistance. More importantly, such resistance was equal or even higher as compared to the conventionally bred resistant plants, both under greenhouse and field conditions. Later studies have further revealed that the observed resistance was not compromised upon infection of transgenic plants with heterologous viruses such as beet soil-borne virus beet soil-borne virus (BSBS), beet virus Q (BVQ), beet mild yellowing virus (BMYV), and beet yellows virus (BYV) (Lennefors et al. 2008).

In the same research line, three hairpin constructs, carrying differing in size fragments of a highly conserved region from the BNYVV replicase gene, were assessed for their ability to confer BNYVV resistance in sugar beet hairy roots, developed through an *A. rhizogenes* shortcut approach (Pavli et al. 2010). Upon BNYVV inoculation, the composite seedlings showed a significant delay in symptom development as compared to the wild-type ones. At the same time, the transgenic root system of these seedlings was virus-free or presented marginally positive values while the non-transformed aerial parts of the same plants proved infected. These findings point to the conclusion that the expression of BNYVV replicase-derived dsRNA leads to resistant hairy roots, presumably as a result of an RNA silencing mechanism. Among the Ri T-DNA-transformed hairy roots tested, the ones endowed with the transgene of 459 bp manifested higher levels of resistance, probably owing to a better stability of the transgene.

Recently the potential of various hairpin constructs, carrying sequences of the 5′-untranslated region (5′-UTR) of RNA 2 or the flanking sequence encoding the p21 CP, to confer RNA silencing resistance against rhizomania has been explored (Zare et al. 2015). At first, the ability of constructs to confer resistance was assessed by means of transiently transforming leaves of both *Chenopodium quinoa* and sugar beet plants and evaluating resistance on the basis of symptom development and virus titers. As the constructs carrying two copies of the 5′-UTR with or without the p21-encoding sequence (constructs IHP-P and IHP-U, respectively) proved more efficient in inducing BNYVV resistance in both *C. quinoa* and sugar beet, the abovementioned structures were employed for stable transformation of sugar beet. Upon inoculation, both types of transgenic plants presented high-level resistance that was in fact comparable or higher than the conventionally bred resistant variety. However, the IHP-P-attributed resistance proved more efficient in conferring rhizomania resistance, most probably due to effects of transgene stability caused by differences in length of hairpin structures.

10.4 Resistance Based on Genes of Nonviral Origin

In parallel to the multitude of the extensively explored PDR-based strategies, a series of alternative approaches using genetic sources of nonviral origin have been further elaborated for the purposes of engineering transgenic virus resistance. Such strategies include the transgenic expression of antibodies, against a conserved domain in a key viral protein/enzyme, but also the expression of genes which act as elicitors of hypersensitive response (HR) and immune responses. In this line, attempts to achieve rhizomania resistance through the employment of antiviral genes from other than the BNYVV sources were based on the expression of antibodies and bacteria-derived harpin proteins.

The concept that the antibody-based recognition of pathogens and subsequent disruption of essential virus functions may be extended from mammals into plants gave rise to a series of research initiatives aiming at the generation of transgenic virus resistance. In sugar beet, such plantibody approach was pursued through the *in vitro* expression of single-chain antibody fragments (scFv), directed against the CP and the nonstructural protein p25, in *N. benthamiana* plants (Fecker et al. 1997). However, successful expression was mainly possible when scFvs were targeted to the endoplasmic reticulum (ER) and not to the cytosol, which is generally considered as their normal site of function. Transgenic plants were subsequently challenged-inoculated, both mechanically and through the use of the transmitting vector *P. betae*. Although confined in the ER, the CP-specific scFvs resulted in the inhibition of early infection and the development of milder symptoms at later stages of infection. The plantibody-based approach however, has not been further explored for the purposes of conferring protection against BNYVV.

In view of the well-established properties of harpins in inducing defense responses upon external application, transient or constitutive expression in plants, Pavli et al. (2011a) explored the potential of achieving rhizomania resistance through the expression of the harpin Z_{Psph} protein from *Pseudomonas syringae* pv. *phaseolicola. N. benthamiana* plants were constructed to express the HrpZ protein as an N-terminal fusion to the PR1 signal peptide (SP/HrpZ$_{Psph}$), to direct harpin accumulation to the plant apoplast. Upon challenge with the virus, the SP/hrpZ$_{Psph}$-expressing plants showed high-level resistance, as evidenced by either a complete absence of disease symptoms or a considerable delay in symptom development. Such phenotypic features were further accompanied by a significant reduction in virus multiplication, resulting in plants that were either virus-free or contained very low virus titer. As the resistance was systematically accompanied by visible necrosis, localized exclusively at the virus-inoculated area of resistant plants, it was speculated that such resistance may be attributed to a primed state of SP/hrpZ$_{Psph}$-expressing plants. Authors suggested that the necrosis might be attributed to an augmentation or synergistic effects of defense responses elicited by the extracellularly targeted harpin and virus infection. In order to assess whether the harpin-mediated resistance may be extended to the natural host of BNYVV, SP/HrpZ$_{Psph}$ has been further expressed into transgenic sugar beet hairy roots by using an *A. rhizogenes*-based root transformation approach. In accordance with the results

obtained from *N. benthamiana* plants, sugar beet hairy roots showed high-level resistance to BNYVV, manifested by the absence of disease symptoms as well as no or very low virus titer. It is of interest that the transgenic expression of the non-secreted protein version ($HrpZ_{Psph}$), both in *N. benthamiana* and sugar beet hairy roots, resulted in plants that upon infection with BNYVV presented similar features with the non-transgenic control plants. All together, these findings provide strong evidence that the resistant phenotype is clearly correlated with the harpin targeted for secretion to the plant cell exterior.

Following the demonstration that the expression of $SP/HrpZ_{Psph}$ confers enhanced rhizomania resistance both in a model plant and sugar beet (Pavli et al. 2011a), the same research group explored the possibility to further improve the level and durability of the $SP/HrpZ_{Psph}$-based resistance by simultaneously employing two entirely different defense mechanisms (Pavli et al. 2012). To this end, *N. benthamiana* plants that express the $SP/HrpZ_{Psph}$ protein as well as a conserved region originating from the BNYVV replicase gene, arranged as inverted repeat, were produced. Upon BNYVV-challenge inoculation, transgenic plants were either highly resistant or even immune to infection. More specifically, the majority of double-transgenic plants were completely symptomless, whereas the remaining plants showed mild disease symptoms. At the same time, the great majority of these plants were negative to infection, while the rest presented very low virus titer. It is worth noting that the performance of transgenic plants co-expressing the two transgenes was in all cases superior in comparison with the ones carrying a single transgene, presumably due to additive transgene effects. Such findings demonstrated, for a first time, that the simultaneous integration of two genetically distinct defense mechanisms may provide a novel tool to achieve high level and stable resistance against rhizomania.

10.5 Concluding Remarks

Despite societal concerns, primarily but not only within the EU about the use of transgenic crops in agriculture, there is no doubt that relevant approaches have proven capable of generating high-level, durable, and safe resistance against virus diseases in the field. Along this line, several studies have demonstrated the ability of transgenesis to enrich the breeder' arsenal in facing the problem of sugar beet rhizomania disease, a problem with everlasting significance, especially in view of the evolutionary changes in a pathogen continuously posing new challenges. Apart from ensuring the economic viability of the crop, transgenic resistance should ideally prevent or considerably delay a counteracting response of virus adaptation, leading to fitness gain and probability of resistance breakdown. To this end, the ever-increasing in-depth understanding of the molecular biology underlying virus infections allows for the design of transgenic resistance strategies presenting a substantially better perspective. The important issue to be addressed is the justified choice of the engineering approaches, mainly in terms of efficacy, durability, safety and, most importantly, eventual relaxation of public concerns.

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Chapter 11
Breeding Methods

Marco De Biaggi and Enrico Biancardi

Abstract Different breeding approaches are employed for identifying the traits of rhizomania resistance. The first screenings were made by means of visual evaluation and chemical analyses of individual beets or plot samples grown in rhizomania diseased field. Advanced selections are usually performed in glasshouse, where more uniform inoculum and growing conditions are possible. Traditional systems are still today employed for reproduction of selected beets. Molecular analyses on the resistance loci allow the accurate selection of the plantlets bearing the trait after different kinds of crosses. Until now, all the genetic resistances used in commercial varieties were identified in *Beta maritima*. The removal of undesirable traits of the wild parent is among the more difficult steps. Toward the end of the breeding process, the improved genotypes are hybridized with CMS seed-bearers after evaluation of their combining ability. The experimental hybrids are sown in multiyear trials under rhizomania attack for the control of the agronomic value before the registration process.

Keywords Sugar beet • Rhizomania • Breeding • Combining ability • Field trials • Seed production

Different approaches are employed for isolating traits of rhizomania resistances in never-screened sugar beet genotypes. The target is more difficult than other crops due to both the narrow genetic variability of the currently employed germplasm and the biennial cycle of sugar beet (Biancardi et al. 2005). The more common system consists in finding the traits of resistance in old commercial germplasm and to

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E. Biancardi, T. Tamada (eds.), *Rhizomania*, DOI 10.1007/978-3-319-30678-0_11

introgress them into more fitted genotypes (Panella and Lewellen 2007). In order to increase the genetic variability, the beets carrying some trait of resistance are pooled and freely intercrossed. The open-pollinated population is screened as usual, and the selected beets are then reproduced following the normal breeding procedures (Doney and Whitney 1990).

11.1 Screening for Resistance

The preliminary screening for resistance to diseases is based on the evaluation of host plants in field conditions and in the presence of the specific pathogen. This first step is performed for ascertaining not only the presence of resistance traits in a large number of genotypes but also their genetic variability (Graf 1987; De Biaggi 1987; Petersen 1994). The evaluation of the rhizomania symptoms on fully developed roots (Fig. 8.2) is made according to specific scales, as the disease index reported in Table 11.1. In case of systemic infection, the ranking becomes reasonably significant also with observation on leaves (Fig. 11.1).

The visual root evaluations are well correlated with the degrees of resistance, but the correlation improves if supported by ELISA tests. In weekly analyses, the ELISA values show significant variations likely due to soil temperature, namely, the factor more directly influencing the multiplication rate of BNYVV and *Polymyxa betae* inside the root tissues (Gerik et al. 1990; Wisler et al. 2003). In some conditions, the ELISA test becomes quite unaffordable owing to interferences with other soil-borne viruses and/or by uneven distribution of BNYVV in the plant tissues. In this occurrence, DAS- and TAS-ELISA (double- and triple-antibody sandwich enzyme-linked immunosorbent assay) work slightly better (Wisler et al. 1999). The level of resistance in field condition is established also through the usual analyses of

Table 11.1 Scale used at Rovigo for ranking the rhizomania resistance through observations of the roots

Disease index		
0	Highly resistant	No visual symptoms
1	Very resistant	Nearly normal taproot, c
2	Resistant	1 < intermediate symptoms < 3
3	Slightly resistant	Taproot slightly to moderately constricted, moderate bearding, slight browning in taproot sections, and leaf yellowing
4	Slightly susceptible	3 < intermediate symptoms < 5
5	Susceptible	Taproot wineglass shaped, secondary root bearded, browning in taproot sections
6	Fairly susceptible	5 < intermediate symptoms < 7
7	Moderately susceptible	Severe bearding and stunting, tail almost rotten
8	Very susceptible	7 < intermediate symptoms < 9
9	Highly susceptible	Beet totally rotten

Fig. 11.1 Sugar beet showing the typical rhizomania symptoms both on root and leaves

sugar content, root yield, and quality performed on single beets or on plot samples (Giunchedi et al. 1985, 1987; Wisler et al. 1999) (see Chap. 12).

The systems for reproducing the selected beets are applied according to the targets to be achieved, the genotypes under selection, and the traits of resistance to be transferred or improved. The traditional methods include:

- Progeny tests
- Inbreeding of single beets
- Hybridization with CMS lines in order to quantify the combining ability
- Testcrosses

Cycles of backcrosses are needed for adapting new resistant genotypes to the local requirements and for the removal of negative traits coming, for example, from *Beta maritima*. Recurrent selection schemes are sometime employed for improving the specific and general combining ability (Lewellen and Biancardi 1990). The efficacy of these methods has been considerably improved by the DNA analyses, as explained in Chap. 12. For a complete description of the traditional sugar beet breeding systems, the readers are addressed to the following publications: Coons (1936), Knapp (1958), McFarlane (1971), Baroka (1985), Hecker et al. (1985), Bosemark (1993), Campbell (2002), and Biancardi et al. (2005).

The negative correlation between BNYVV content in the roots and degree of resistance is confirmed, among other things, by the dosage (number of alleles) and by the frequency (ratio of *Rz* to *rz* alleles) of the dominant *Rz* in 3× hybrids. In this case, the BNYVV titer increases in the respective genotypes as follows: *Rzrz*<*Rzrzrz*<*rzrzrz* (Wisler et al. 1999). It means that one dose of *Rz* in 2× heterozygote form (*Rzrz*) is enough to induce better resistance than in *Rzrzrz* 3× genotypes. This is among the reasons which lead to preferring the diploid (2×) level rather the triploid (3×) in breeding for rhizomania resistance (Zivic et al. 2011; Meulemans et al. 2003). The difficulty in genic management and evaluation of tetraploid germplasm is also a reason to limit at the 2× level the breeding activity for rhizomania resistance. The same difficulty happens more widely in reproducing the resistant 4× families used as pollinator of 3× hybrid varieties (Bosemark 1993). In other words, the 2× varieties can be improved more rapidly than the 3× ones, which means, among other things, better costs-benefits ratio.

Through field evaluations, it is possible not only to establish the degree of genetic variability among and/or inside a great number of accessions but also to select directly the beets with the desired levels of yield and quality. Symptoms of rhizomania are scored during the growing season and at the time of the normal harvest, when the yield and quality data are usually at the hit of their significance. The soil and inoculum uniformity in the field (nursery) plays an important role in order to avoid the selection of plants grown in locally low infected soil and consequently with low BNYVV content even if the plant is susceptible (Francis and Luterbacher 2003). Attention is paid also at the uniformity of cultural practices and at the regular beet stand.

As regards the soil uniformity, it may be taken into account that the vertical variability is often high notwithstanding the uniform surface (see Chap. 12). Two or more replications and the presence of some susceptible and resistant checks should be foreseen to avoid problems due to unpredicted variability in growing conditions. It is needed that the natural or artificially induced infection of the roots happens at the opportune time and temperature, when the host plants are at the stage of their maximum susceptibility. Of course, the presence of other pathogens should be possibly avoided. In conclusion, the only variable on the nursery or field trials should be the genotypes to be screened (Campbell et al. 2008).

After the first screening, advanced selections are usually made in glasshouse, where the uniformity of the inoculated soil mixture, temperature, soil moisture, etc. are completely under control. Besides, other diseases interfering with rhizomania are easily eliminated. Therefore, the level of resistance and the BNYVV titer are ascertained more precisely than in field conditions and with less sampling problems. The main weakness of the greenhouse evaluations is given by the limited volume and depth of soil available for the plantlets, which require to be analyzed much earlier than in field conditions, where the beets can be sampled when they are fully developed and ready for harvest and sugar extraction, i.e., when the most important economical traits can be exactly evaluated.

It was demonstrated that the positive correlation between the virus concentration and the ELISA value is valid also if the plants are analyzed in early stages of growth

(Geyl et al. 1995). In other words, the difference in virus content between resistant and susceptible beets becomes detectable at about 20–30 days after emergence (Casarini-Camangi 1987; Bürcky and Büttner 1991). Büttner et al. (1995) found a strong positive correlation between the fresh weight of the lateral rootlets and the BNYVV content 24 days after sowing. Giunchedi et al. (1987) evidenced good correlations among the ELISA value in roots grown in controlled conditions, the visual evaluation of the symptoms, and the sugar yield in field trials. These results enabled the wide employment of greenhouse procedures in every phase of selection (Paul et al. 1992; Scholten and Lange 2000). If the plants analyzed in early stages do not survive the root sampling, they are multiplied *in vitro* in order to obtain more rapidly the amounts of seed necessary for further selections and/or field trials (Keller and Lüttge 1991; Giorio et al. 1997).

For ensuring the reproducibility of the results in glasshouse, the experiment conditions and, above all, the virus/vector concentration in soil mixtures, should be similar (Francis and Luterbacher 2003). Different procedures, including the "most probable number" method, are employed to estimate the concentration of *P. betae* cystosori in the mixtures used in greenhouse by means of increasing of soil dilutions (Tuitert 1990; Ciafardini and Marotta 1989; Pelsy and Merdinoglu 1996).

The substrate where the genotype under evaluation is sown or transplanted is the natural infected soil (Grimmer et al. 2008) or its mixtures with river sand, peat, and other materials. Different ratios of soil/sand (v/v) have been used: from 1:1 (Wisler et al. 2003) to 1:9 (Scholten et al. 1994). In order to avoid the unwanted presence of other diseases, soil and mixtures are adequately sterilized. Usually, 12 h at 105 °C is enough if the temperature is uniformly distributed (Scholten et al. 1994). For ensuring an even infection, frequently used is a nutrient liquid solution where the roots of diseased beets have been previously submerged for the zoospore extraction. The healthy seedlings put in contact with the liquid suspension of zoospores became infected within 30 min (Peters and Godfrey-Veltman 1989). In this way, the infection is regularly distributed around the root, whereas using diseased soil or mixtures, the inoculation of the primary zoospores is much more uneven due to their limited mobility (Tuitert 1993). Similar well-distributed infection is possible using water-saturated soil mixtures (Pferdmenges et al. 2009). A useful method for producing *P. betae* zoospores to be employed in glasshouse and laboratory experiments is described by Paul et al. (1993).

As regards the nondestructive methods applied on developed beets allowing the direct reproduction of the selected plants, Fujisawa and Sugimoto (1979) proposed the evaluation based on the virus rub-inoculation of the leaves of bait plants. The foliar reactions developing after around 2 weeks are in good correlation to the variety behavior in infected field. Horak and Schlösser (1980), for the same target, used root homogenate of beets to be selected according to the symptoms of BNYVV multiplication on the leaves of *Chenopodium amaranticolor*, *Chenopodium quinoa*, *Tetragonia expansa*, etc. (OEPP/EPPO 2006). The test works quite well also for sea beet accessions (Fujisawa and Sugimoto 1979; Grassi et al. 1988, 1989).

By the visual evaluation of the leaves wilting caused by the reduced water uptake in diseased beets, it has been obtained good correlation levels with the BNYVV titer

in the roots (Keller and Lüttge 1991). At the same time, the diseased leaves increase their temperature, changing also the concentration in P, K, and Ca. Putz and Richard-Molard (1983) found differences in leaf thickness between more and less resistant genotypes. The above parameters may be quite easily measured and used as rapid methods of early selection, even if the analyses of the root tissues appear to be more reliable (Ahrens 1987; Scholten and Lange 2000). Another similar and rapid method of early selection is possible measuring the net-CO_2 uptake in the leaves of the same order of emission (Keller and Lüttge 1991). All these methods have gradually lost their importance with the introduction of molecular analyses in breeding systems (see Chap. 12).

11.2 Breeding for Resistant Varieties

The more widespread method employed for production of commercial varieties was usually based on the hybridization of pollinators carrying the homozygous allele of rhizomania resistance (*RzRz*) with susceptible (*rzrz*) F1 CMS seed-bearers (Fig. 11.2 and 11.3) (Box 9.1). The opposite, i.e., cross of resistant (*Rz1Rz1*) seed-bearer

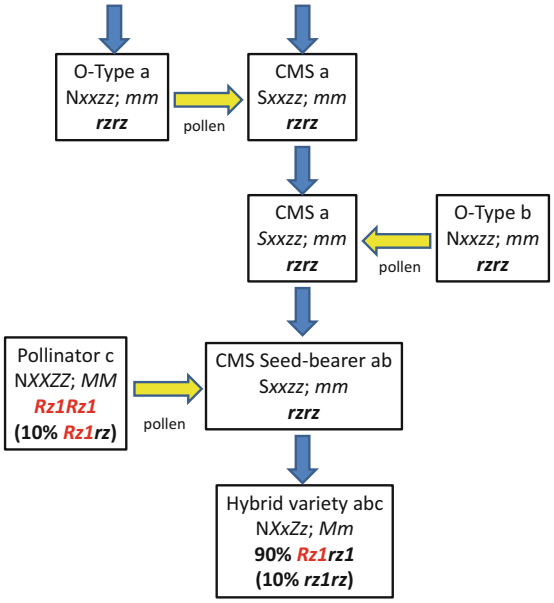

Fig. 11.2 Production of three-way rhizomania resistant variety. The CMS and monogerm (mm) seed-bearer (a) is reproduced by O-Type (b) not used for the reproduction of the CMS itself. The rhizomania susceptible CMS (ab), crossed with the resistant pollinator (c) produces the resistant (*Rzrz*) hybrid variety. If the pollinating beets (c) are not completely homozygous (*RzRz*), a proportional percent of susceptible (*rzrz*) beets is produced in the variety (between parentheses)

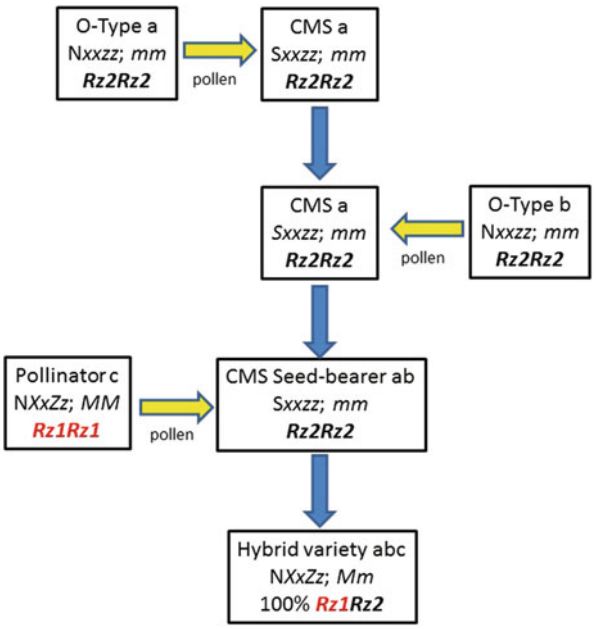

Fig. 11.3 Production of a 3 way hybrid by means of both homozygote parents carrying two different *Rz* genes

and susceptible pollinator (*rz1rz1*), is possible, but sugar yield of the hybrids is not satisfactory for unknown reasons. The heterozygous (*Rzrz*) condition of some beets in the resistant male parent gives rise to corresponding amount of susceptible beets (*rzrz*) in the hybrid variety, which reduces proportionally the yield performances. The problem has been recently eliminated, analyzing, by means of markers and specific SNP (see Chap. 12), all the stecklings used as pollinators in hybrid seed reproduction (Box 11.1).

The 100 % of resistant beets in the variety is obtained if the resistance allele is homozygous at least in one parent being heterozygous in the other. In presence of heterozygous beets in the parents (between parentheses), a proportional amount of susceptible beet can be foreseen in the hybrid seed.

In situation of severe rhizomania attack, the *RzRz* genotypes are more resistant than *Rzrz* ones due to the incomplete dominance of *Rz*. Consequently, the presence of homozygous resistant genes is advisable both on pollinator and on CMS seed-bearer (Wisler et al. 1999; Rush and Merz 2003) (Fig. 11.2 and 11.3) (Box 8.3). Some of recently released varieties have been obtained crossing both parents homozygous for one (i.e., *Rz1Rz1 or Rz2Rz2*) or two genes of resistance (*Rz1Rz2*), as Tandem (De Temmerman et al. 2009), Angelina (Rush et al. 2006), and Isabella (http://www.kws-uk.com/aw/KWS/united_kingdom/Company/Submenu-1-Topic-1/Sugar-Beet-Press-Archive/~ewxk/NEW-BEET-VARIETY-OFFERS-ALTERNATIVE/). The

Box 11.1: Stecklings and Mother Beets
Mass or individual selection in sugar beet is performed on mother beets, which are completely developed plants sown and harvested as the commercial crop. The defoliated and cleaned roots, after morphological selection, are individually sampled, analyzed, and selected accordingly. The best mother beets are stored and reproduced in spring. The steckling (or planchons) are beets planted usually in August in nursery and transplanted after overwintering in seed production fields. Normally, the stecklings are employed only for seed production, since any type of traditional selection can be made, with the exception of the flowering and seed production behavior (monogermity, pollen production, male sterility, seed shape, etc.) (Biancardi et al. 2005). The selection in early stage of development became usual for rhizomania resistance based on molecular traits, which do not change during the growing cycle. In this case, the analyzed plantlets to be reproduced are grown up to the steckling stage and overwintered in the same way.

double resistance (Fig. 11.2 and 11.3) is useful not only for increasing sugar yield but also for avoiding or delaying, through additive effects, the breakdown of resistance due to evolution of BNYVV (Liu et al. 2005; Pferdmenges 2007).

More difficult is to employ the sources of resistance identified in *Beta maritima* and moreover in other species of the genus *Beta*. In the former case, the trait of interest must be separated from the negative background of the wild beets, which usually behave (Fig. 11.4):

- Annual cycle
- Low root weight
- Low processing quality
- Low sugar content
- Multiple crowns
- Root fanginess and woodiness

This step requires a number of backcrosses, and it is greatly simplified if the resistance trait is monogenic and dominant. The transfer of the resistance found in the North Atlantic *B. maritima* accession, such as WB41 and WB42, which supplied the Rz2 and Rz3 resistances, respectively, is relatively easier than the Mediterranean sources due to the prevailing biannual trait (Hautekèete et al. 2001) and the minor fanginess of the roots (see Sect. 9.2). The evolution of molecular markers technique fastens the isolation and the transfer of the resistance traits (see Sect. 8.2).

Production of rhizomania-resistant seed does not differ to the procedures followed for the normal varieties, as the postharvest seed processing and pelleting operations (Box 9.1 and 11.2). The maximum level of attention must be paid in selecting the field for the nursery, which can be absolutely rhizomania-free. Apart from the lower seed yield of the rhizomania diseased beets, it must be taken into account that 1 ha of nursery is enough for transplanting 10 ha of seed crop. If the

Fig 11.4 Breeding lines obtained crossing *Beta maritima and* showing the tpical fanzines taken by the wild parent

Box 11.2: Seed Production

Essentially two systems are employed for production of sugar beet hybrid varieties. Using the direct system, the parents are sown in the same field from which the commercial seed will be harvested. Beets are spaced at greater distances than in the sugar crop and, having to survive the winter, they are less protected from the frost. Seeds are planted at 0.06–0.14 m intervals within rows that are 0.6–0.75 m apart. A row of multigerm pollinators is sown every three or four rows of monogerm CMS seed-bearers (Kockelmann et al. 2010. In the indirect system, beets first are planted in a nursery. At the appropriate time, usually after vernalization, the small roots (stecklings) are transplanted into seed production fields located elsewhere (Bornschauer et al. 1993). The ideal stand in the nursery is between 1.0 and 1.2 M plants per hectare. The rows are drilled from 0.20 to 0.25 m apart depending on seed traits, soil, harvesting system, climate, etc. The nursery normally is planted in August, and the stecklings are harvested in February or March. Pollinators and CMS usually are transplanted into distinct rows: every 0.4–0.5 m in rows 0.7–0.8 m apart, for a target population density of about 36,000 stecklings per hectare. The ratio of pollinators/seed-bearers is usually 1:4, which can also become 1:2 in case of poor pollen production and release (De Biaggi, unpublished). The rows of pollinator are eliminated at the end of June, and the harvest of the seed-bearers begins when most of the seed has turned a light tobacco color

(continued)

Box 11.2 (continued)
and starts to come away easily. Earlier harvests will not lead to great losses, but the seed is partially unripe and there is the risk of poor germination. The loss of seeds increases as the time to the harvest increases after this stage. The stalks are laid out in windrows for some days until seed moisture is 10–15 %. Rain during this period is damaging because it promotes the development of fungal parasites on the seeds and always results in lowered germination. Threshing machines are equipped for reducing the seed losses. Where the climate does not allow the drying in the field, stalks are transported to the factory to be processed as soon as possible.

soil of the nursery is diseased, more effective and rapid system for rhizomania spreading does not exist. This serious risk is reduced, when possible, by the direct sowing of seed production fields (Box 9.1).

As in the normal (susceptible) varieties, the germination quality of seed plays an important role in obtaining a rapid emergence and a regular stand and is negatively influenced by any stress suffered by the seed crop. Drought and the presence of pathogens around the glomerulus are the most dangerous factors. The introduction of drip irrigation, with one dripper for each seed-bearer beet, improves greatly not only the yield of seed but also the germination ability, avoiding both the damages of low water supply and reducing, at the same time, the development of diseases since the leaves and seed stalks are not moistened, as it happens during the sprinkler irrigation (Kockelmann et al. 2010).

11.3 Field Trials and Release of Resistant Varieties

The evaluation of performance in field conditions is one of the more important steps of breeding new varieties. Field trials are necessary to determine, in addition to the diseases resistances, the agronomic value of the selected genotypes during and at the end of the breeding procedures. The evolution of symptoms and the productive effect of rhizomania are easily scored and statistically processed (Simmonds 1987). In advanced stages of selection, a more comprehensive set of traits, which includes processing quality, are evaluated in replicated plots in the presence of three to five best performing commercial varieties endowed with different degrees and types of resistance. The set of resistances is useful also to detect the possible development of BNYVV resistance-breaking strains. The same varieties and checks are usually sown also in healthy soil for the yield control of the experimental hybrids in absence of rhizomania. In fact, the behavior of varieties in healthy conditions is important since frequently the disease does not develop as foreseen before sowing. It must be

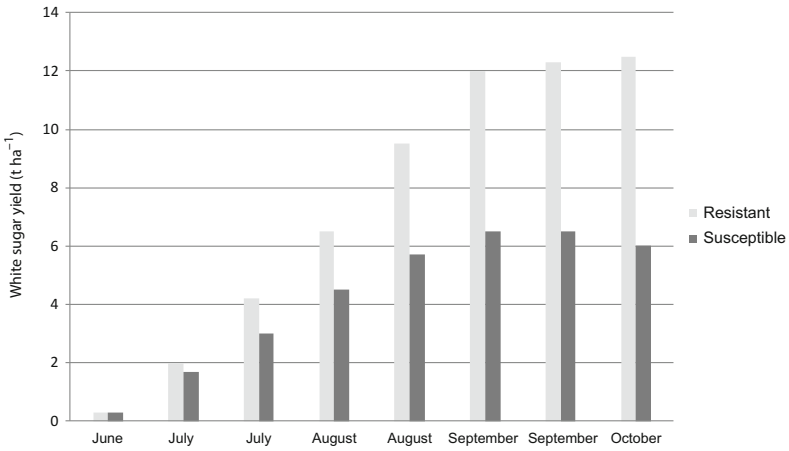

Fig 11.5 Sugar yield of resistant and susceptible varieties in diseased field trials harvested every 2 weeks. Mean of four trials organized by ISCI in 2008 with five susceptible and five resistant varieties

taken into account that the use of resistant varieties is recommended also in healthy soil, in order to delay the always possible infection (see Sect. 8.2).

The most critical data in the field trials is the areal white (or extractable) sugar yield of the new hybrids compared with the locally more diffused varieties. Before the release, the stability of sugar production must be also carefully evaluated, being this trait required for good performances in different locations and years. In a variety of trials for evaluation of rhizomania resistance, the soil should be uniform and representative of the area. As in the nursery, other pathogenic viruses may be possibly avoided, because the response of the varieties becomes more clear when rhizomania acts alone and not in association. Randomized block designs with four replications provide sufficient precision if the trials are repeated for at least 2–3 years in several locations, as usually performed by the registration procedures (Simmonds 1987). In this case, it is not necessary to obtain the highest possible precision in the single trials, increasing, for example, the number of replications. The number of harvests depends on the objectives of the experiment. Wisler et al. (1999) found that the ELISA absorbance value, which is proportional to the BNYVV titer in the root, goes down in the second part of the campaign, thus reducing the differences among varieties (Fig. 11.5). This is likely due to the effect of lowering soil temperature on the reproduction of *P. betae* inside the roots, which in turn influences the BNYVV titer. In the main part of growing conditions, the late harvests are normally the more diseased and the differences among the entries more significant. The single harvest is more fitted to the plot-harvesting machinery employed today. The field trials are usually organized on three rows per plot, 5–10 m long and without border rows (Biancardi et al. 2005).

The yield and quality data are processed with specific software, which supplies means, statistical differences, and interactions among entries, locations, and years.

The yield stability is evaluated by assessing the interaction varieties × localities × years that is often related to a wide genetic base of both parents of the variety (Perkins and Jinks 1968). As mentioned above, the yield stability is a very valuable quality for farmers and can be quantified only in presence of GxE interaction, which needs to be evaluated in an adequate range of environmental conditions in different locations and years. The stable varieties usually perform better by means of localities and years. In fact, this parameter is one of the more reliable criteria for the recommendation of sugar beet varieties.

By means of field trials, it is possible to know the breeding progresses obtained during the years, as it was made, for example, in Germany. Sowing in the same trial the seed of varieties used in the years from 1983 to 1998, the increasing performances of rhizomania-resistant varieties compared with the susceptible checks was demonstrated. It was evident the continuous increment of white sugar yield of the resistant varieties also in healthy conditions (Büttner and Mangold 1998). Increasing the number of trials, the real annual incidence of the disease can be better evaluated. In the result of multiyear trials, it is also possible to look at some too rapid yield improvement of some varieties, evidently impossible by means of breeding alone.

For more detailed information regarding the sugar beet trials, the following literature is available:

- Plot samples – Beiss and von Müller (1974)
- Processing and storage of samples – Wauters 2002; Burba et al. 1975; Oltmann et al. 1984
- Chemical analyses and data processing – Oltmann et al. (1984)
- Experimental designs – Chocran and Cox (1957); Clarke (1980)
- Statistical analysis – Petersen (1994); Yndgaard et al. (2002); Kristensen and Hill (2002)
- Software – Schwarzbach (1984); Utz (1991); Kristensen and Hill (2002)

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Chapter 12
Assisted Selection

Piergiorgio Stevanato, Enrico Biancardi, and Peyman Norouzi

Abstract Early attempts at the industrial processing of sugar beet required frequent and rapid analysis of sucrose. Some instruments used in the factory (polarimeter, refractometer, etc.) were soon applied for analyzing the sugar content of individual beets subjected to mass and progeny selections. The analytical support increased exponentially when the processing of quality beets became necessary to improve the sugar extraction rates. After the discovery of rhizomania, the analyses were used widely for assessing the effect of the disease on the host plant and hence for breeding purposes. Around the mid-1980s, some enzymatic techniques as ELISA were applied to directly detect both beet necrotic yellow vein virus and, to a lesser extent, *Polymyxa betae*. More recently, molecular markers and related techniques have found wide application in breeding, making it possible to establish the presence of resistance traits in genotypes under selection. Other kinds of analyses are needed in order to quantify the presence of rhizomania in field conditions and in soil mixtures for greenhouse experiments.

Keywords Sugar beet • Rhizomania • Assisted selection • ELISA test • Genome analyses

12.1 Soil Sampling

Soils are characterized by a very high variability both on the surface and even more in a vertical direction. In cultivated soils, plowing inverts and mixes the surface layer, which becomes relatively homogeneous. The mixing effect is slower in a horizontal direction due to the more limited soil displacement. Since the root system

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E. Biancardi, T. Tamada (eds.), *Rhizomania*, DOI 10.1007/978-3-319-30678-0_12

of some crops, including sugar beet, may develop down to 3 m, the sampling to establish the amount of fertilizers needed by the crop should at least approach that depth (Stevanato et al. 2010).

The sampling points for soil analyses involving only the plowed layer are distributed randomly, located following apposite grids, or taken walking in a "W" shape across the field. Their number per surface unit is directly proportional to the variability of the parameters to be analyzed. Regarding rhizomania, the variability is high especially in the first phases of infection, when limited parts of the field are diseased (see Sect. 8.2). The top soil layer is usually sampled down to 0.15 or 0.30 m, i.e., around half of the plowed depth (Hofmeester and Tuitert 1989; Kutluk-Yilmaz et al. 2010; Galein 2013). After sugar beet harvesting and before plowing, the concentration of viruliferous *Polymyxa betae* cystosori is normally higher in the top layer (0–0.25 m). After plowing and the inversion of the soil, the opposite occurs (Biancardi, unpublished). Hence, the inoculum should result as less than it really is if quantified by means of insufficiently deep samples (see Sect. 7.1). In fact, the inoculum in the non-sampled layer (0.25–0.50 m) depends mainly on the number and frequency of past sugar beet crops:

- Is not negligible due to the long-lasting survival of the resting spores
- Can be very different from the layer above
- Can infect the roots also when the resting spores are present at 0.50–0.70 m (Uchino and Kanzawa 1991)

Only a variable percentage (5–20 %) of zoospores are infected by the virus (Tuitert 1990). The analyses of *P. betae* in soil can thus supply incorrect data on the BNYVV concentration, since, among other things, some tests are unable to distinguish if the resting spores are dead or alive.

Using quantitative DAS-ELISA, optical density values of *P. betae* in soils with and without virus are not significantly different. It appears that the *P. betae* content in lateral hairy rootlets is not the only significant factor for the vector to successfully transmit BNYVV. Microscopic observation of cystosori in infected roots was used for preliminary studies.

The single soil cores are analyzed separately when a map of the disease can be drawn and when uniform infection is sought for nurseries or field trials. More frequently, for routine control of the disease levels, the cores are carefully mixed after drying, in order to obtain single samples or a few subsamples per field. In some limited situations, remote sensing and precision farming techniques (see Sect. 8.3) are successfully applied for control of the spread of the disease on large area (Steddom et al. 2003).

It is impossible to test the presence and concentration of BNYVV directly on the soil. The virus inoculum is thus determined by means of baiting plants, such as susceptible sugar beet varieties, *Beta maritima*, *Chenopodium quinoa*, *Chenopodium amaranticolor*, etc., grown in a greenhouse on mixtures containing variable proportions of diseased soil (see Chap. 2).

12.2 Root and Leaf Sampling

BNYVV becomes systemic under moderate-severe attack and/or in particular climate situations. This is in disagreement with different field experiences, which report that some symptoms, like canopy yellowing and vein necrosis, can become evident 6–7 weeks after emergence in field conditions, also under low infection (Biancardi, unpublished). This divergence is likely due to the temperature interacting with the causal agents during the early crop development. In dry and warm growing areas, vein necrosis is observed very rarely but canopy yellowing is normal.

The viruliferous zoospores of *P. betae*, once penetrated in the rootlets, develop zoosporangia and new zoospores, which spread in the root tissues (see Chap. 2). Their concentration changes in the different parts of the taproot, where the chemical composition, including the sucrose concentration, is also very variable. These not negligible differences in root composition could influence the concentration, multiplication, and movement of the disease agents. The BNYVV dynamics inside the beet are still not completely understood, especially with regards to both the differing behavior induced by the degrees of resistance and the variable relationship BNYVV/*P. betae*.

Quite uniform inoculation of the rootlets is possible by means of liquid zoospore suspensions. In this way, the viruliferous *P. betae* zoospores are spread more regularly around the taproot. Using solid infected soil mixtures, inoculation of the rootlets is less uniform due to the limited mobility of the zoospores. The further diffusion of BNYVV depends on a number of variables that result in its uneven distribution across the taproot. Consequently, the samples taken on rootlets, before the spread of BNYVV in the taproot, are less affected by error than samples taken later or in other parts of the root.

The presence of rhizomania is detectable, as mentioned above, by more or less intense yellowing of the leaves. However, the yellowing, as other foliar symptoms (circular chlorotic spots, vein necrosis, elongated petioles, etc.), also depends on climate factors, lack of nitrogen and some microelements, other diseases, etc., becoming quite erratic over the years and sites (see Sect. 8.1). Root sampling is preferred since the rhizomania causal agents are less subjected to other variables. Some selection methods based on the leaves' reaction after BNYVV inoculation are briefly described in Sect. 11.1. Due to the different morphology of beet leaves, the tests should be done on leaves of similar order of emission.

12.3 Sampling for Molecular Analyses

For molecular analysis of BNYVV, fresh lateral roots of plants are prepared and RNA extraction done according to Acosta-Leal et al. (2008); DNA is extracted from fresh or frozen roots.

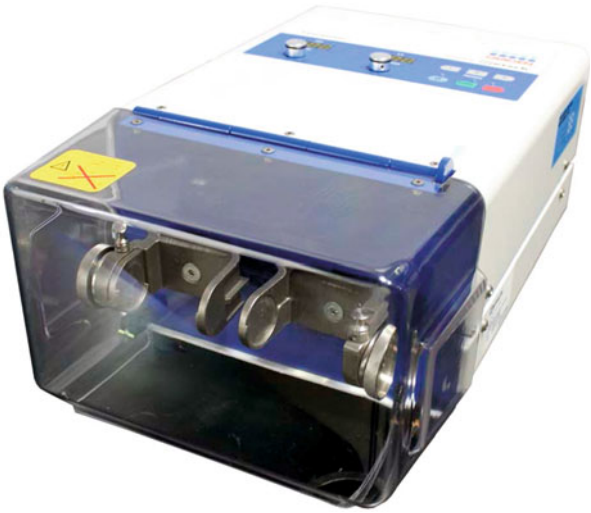

Fig. 12.1 High-throughput QIAGEN TissueLyser for microtiter-based DNA extraction (QIAGEN, Germany)

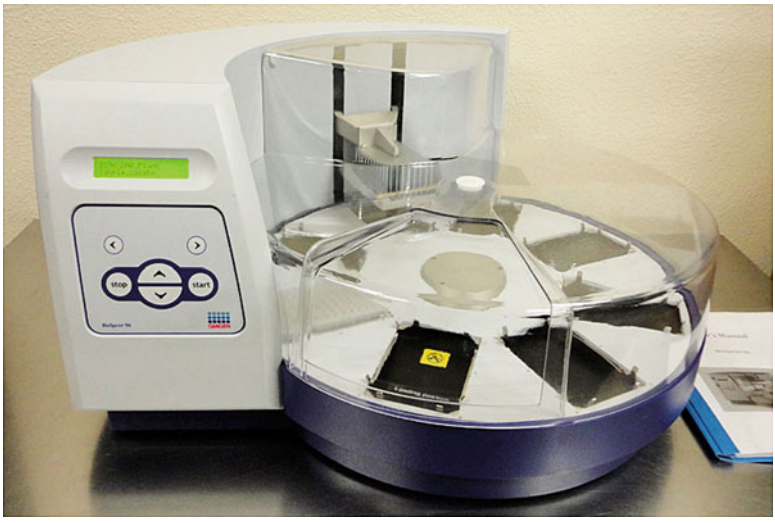

Fig. 12.2 BioSprint 96 instrument for automated DNA or RNA extraction (QIAGEN, Germany)

The roots are frozen in liquid nitrogen and ground to a fine powder in lysis buffer [400 mM Tris-HCl (pH 8.5), 60 mM EDTA (pH 8.5), 150 mM NaCl, 1 % SDS]. All centrifugation steps are conducted at 11,000 rpm for 5 min at 4 °C. Stevanato et al. (2014a, b) reported the use of a homogenizer and a workstation for automated DNA isolation (Figs. 12.1 and 12.2).

12.4 Conventional Chemical Analyses

Prior to the introduction of the ELISA techniques, resistance to rhizomania was evaluated with visual ranking (Table 11.1), frequently assisted by analyses of sucrose and processing quality. Since the drop in sugar content is one the main effects of the disease, the polarimeter analyses are useful especially in the presence of low infection on beets ready to be harvested. The sodium content is also very sensitive and directly correlated to the disease severity (see Sect. 8.1).

The analyses are routinely performed on representative (60–100 kg) plot samples for both yield and quality evaluations in field trials and also on beets delivered to the sugar factory. In the case of individual selection by means of mother beets, a sample of at least 15–20 g can reproduce not only the composition of the entire root, but also ensure the survival of the selected beets. Due to the abovementioned uneven distribution of both virus and fungus, the sampling procedures must be performed carefully, since the precision of the analyses depends closely on these procedures.

12.5 BNYVV Concentration

12.5.1 ELISA Test

The enzyme-linked immunosorbent assay (ELISA) is widely adopted to detect the presence of BNYVV in sugar beet. ELISA uses enzymes to highlight fluorescence emitted by antibody molecules associated to the antigen. The enzyme is covalently attached to an antibody molecule, creating an immunological tool with high specificity and sensitivity.

The general procedure for ELISA is based on adding the antigen (in liquid phase) to the wells of a plate where it adheres to the walls. A primary antibody binds specifically to the antigen. An enzyme-linked secondary antibody is added that reacts with a chromogen, producing a color change to quantitatively or qualitatively detect the antigen. The "reading," usually based on detection of intensity of transmitted light by spectrophotometry, involves quantitation of transmission of some specific wavelength of light through the liquid. The sensitivity of detection depends on amplification of the signal during the analytic reactions (Bhattacharya 2013). Various types of ELISAs have been employed with modifications to the basic steps. For instance:

- Indirect ELISA
- Sandwich ELISA
- Competitive ELISA
- Multiple and portable ELISA

The most used ELISA protocol for BNYVV detection in sugar beet is sandwich ELISA, DAS (double-antibody sandwich), and TAS (triple-antibody sandwich).

The sandwich ELISA quantifies antigens between two layers of antibodies (capture and detection antibody). The antigen to be measured must contain at least two antigenic epitopes capable of binding to an antibody, since at least two antibodies act in the sandwich.

Detection of BNYVV in sugar beet roots is relatively simple and is usually based on the use of monoclonal antibodies. Monoclonal antibodies recognize a single epitope that allows fine detection and quantification of small differences in antigen. Several suitable antibodies have been produced (Koenig et al. 1984; Grassi et al. 1988; Torrance et al. 1988) and detection kits are now available from commercial companies. One advantage of using a purified specific antibody to capture an antigen is that it eliminates the need to purify the antigen from a mixture of other antigens, thus simplifying the assay and increasing its specificity and sensitivity.

12.5.2 TAS-ELISA

According to Henry et al. (1992), this test uses ELISA microtiter plates and includes negative sugar beet controls, homogenizer buffer control, and positive control (either known positive sugar beet material or positive leaf material from BNYVV inoculated onto *Chenopodium quinoa*). A solution of antibodies specific to BNYVV is placed on microtiter plates. The antibodies bind to the surface of the plate due to the effect of electrostatic attraction. Each sample (usually a rootlet extract) is placed in a well on the plate. If the antigen, i.e., BNYVV, is present, it binds to the antibody and stays trapped in the plate. A second layer of specific BNYVV monoclonal antibody is then added to the plate. If the virus has been trapped in one of the wells, it will also bond to this antibody. An alkaline phosphate enzyme conjugates at appropriate dilution in antibody buffer to each well. If the antigen is in contact with the enzyme, it is transformed and emits a visible signal in a spectrophotometer.

The ELISA test is negative if the absorbance of the sample is less than three times that of the healthy control or positive if equal to or greater than three times that value (EPPO 2006). The absorbance value is significantly positively correlated with rhizomania disease index score and negatively correlated with individual root weight, plot root weight, and sugar yield. This information is useful both in resistance breeding and for sugar beet growers and processors (choice of varieties, inoculum production, crop rotations, detection of diseased beets, etc.) (Wisler et al. 1999).

Some commercial kits for BNYVV detection use the double-antibody sandwich (DAS) ELISA. This method is based on adding a conjugate monoclonal antibody and then leaving the following step of adding the conjugate. A complete set of DAS-ELISA, used for detection of BNYVV, contains all reagents, controls, microtiter plates (F-96), and substrate buffer necessary for testing 480 or 960 samples with a working volume of 200 µl/test/well. All buffers as well as equipment for sample preparation and disposables are also available. The best samples for testing are root-

lets (fibrous roots) grown after rainfall or irrigation. The samples are homogenized 1:20 (w/v) in extraction buffer (Bioreba 2014).

ELISA testing is the normal way of screening many samples for BNYVV and can be used as the sole screening test provided the antisera is of high specificity. The test is inexpensive, fast, and can detect at low virus concentrations. If an additional confirmatory test is required, it should be preferably based on different biological principles (EPPO 2006). ELISA is therefore the preferred assay for routine virus detection, although RT-PCR has increasingly been used for detection and identification of viruses due to its higher level of sensitivity (Yardimci and Çulal-Kılıç 2011).

12.5.3 Polymerase Chain Reaction

The second test for detecting the presence of BNYVV is the polymerase chain reaction (PCR), which consists of an amplification of the RNA of the virus. The PCR is a molecular biology method that is used to copy a known DNA sequence (i.e., a gene) a great many times. The result of this reaction is that if the virus is present initially in the analyzed sample, a given DNA sequence belonging to it will be copied between a million to a billion times.

The PCR polymerase chain reaction is based on the activity of thermostable polymerase. A polymerase will synthesize a complementary sequence of bases to any single strand of DNA providing it has a double-stranded starting point. This is very useful because you can choose which gene you wish the polymerase to amplify in a mixed DNA sample by adding small pieces of DNA complimentary to your gene of interest. These small pieces of DNA are known as primers because they prime the DNA sample ready for the polymerase to bind and begin copying the gene of interest. During PCR, temperature variations are used to control the polymerase activity and binding of the primers.

The PCR polymerase chain reaction consists of the following steps:

- The plant DNA is extracted from a sample of roots or leaves.
- Denaturation: To start the reaction, the temperature is raised to 95 °C. At this temperature, all double-stranded DNA is “melted” into single strands.
- Annealing: The temperature is then lowered to ~60 °C. This temperature allows the binding of the primers to complementary regions of denatured DNA strands.
- Extension: The enzyme DNA polymerase is added to assemble a complete copy of each of the two strands of DNA by the initiators. The optimal temperature for the polymerase to operate is 72 °C.

This process is repeated a great many times generating millions of copies of the DNA fragment to be identified.

The virus responsible for rhizomania is made up of RNA and not DNA. In this case, the PCR must be preceded by a viral RNA retranscription into viral DNA. This is known as reverse transcription polymerase chain reaction (RT-PCR) (Meunier et al. 2003). The synthesis of DNA from an RNA template via reverse transcription

produces complementary DNA (cDNA). Reverse transcriptases (RTs) use an RNA template and a short primer complementary to the 3′ end of the RNA to direct the synthesis of the first-strand cDNA, which can be used directly as a template for the PCR. This combination of (RT-PCR) allows the detection of low-abundance RNAs in a sample, and production of the corresponding cDNA, thereby facilitating the cloning of low-copy genes. After amplification it is necessary to analyze the amplified DNA by the electrophoresis method.

12.5.4 Electrophoresis

Electrophoresis is used for separating molecules according to their size and electrical charge. An electric current is passed through a medium that contains the mixture of molecules. Each kind of molecule travels through the medium at a different rate, depending on its electrical charge and molecular size. Separation of the molecules occurs based on these differences. After amplifying the gene, it is possible to run the amplified DNA out on an agarose gel and stain it with a dye that makes it visible. Electrophoresis is used to determine whether the DNA virus sequence in question has been copied during the PCR. This consists of placing all the DNA molecules produced by the PCR in a gel and forcing them to migrate through an electric field.

Nucleic acid molecules are size separated with the aid of an electric field where negatively charged molecules migrate toward the anode (positive) pole. The migration flow is determined solely by the molecular weight as low-weight molecules migrate faster than heavier ones. They separate according to their size: the shortest molecules will travel further than the longest ones. The brighter the visible band, the more copies of your target have been created.

12.5.5 Real-Time PCR

Real-time PCR is identical to a standard PCR, except that the progress of the reaction is monitored by a detector during each PCR cycle (Fig. 12.3). Each real-time PCR technique uses some kind of fluorescent marker that binds to DNA. The number of gene copies during the reaction increases and corresponds to increasing fluorescence. Quantification is done by measuring the increase in fluorescence during the exponential phase of PCR. Data collected in this phase of the reaction yield quantitative information on the starting quantity of the amplification. The results thus obtained are much more accurate than the traditional PCR. Fluorescent reporters used in real-time PCR include double-stranded DNA (dsDNA) – binding dyes or dye molecules attached to PCR primers or probes that hybridize with the PCR product during amplification. The fluorescence generated during PCR can be detected by various chemical reactions. There are two alternatives:

Fig. 12.3 QuantStudio™ 12K Flex Real-Time PCR system (Life Technologies, USA)

- Non-specific detection. Fluorescent colorants that bind to DNA, such as: SYBR Green I, BEBO, BOXTO, EvaGreen, etc.
- Specific detection. Based on the hybridization of specific probes, such as: TaqMan, Molecular Beacons (Scorpion primers, QZyme, LUX primers), LightUp probe, hybridization probes

The change in fluorescence over the course of the reaction is measured by an instrument that combines thermal cycling with fluorescent dye scanning capability. By plotting fluorescence against the cycle number, the real-time PCR instrument generates an amplification plot that represents the accumulation of product over the duration of the entire PCR reaction.

The advantages of real-time PCR include:

- Ability to monitor the progress of the PCR reaction as it occurs in real time
- Ability to precisely measure the amount of amplicon at each cycle, which allows highly accurate quantification of the amount of starting material in samples
- Amplification and detection occur in a single tube, eliminating post-PCR manipulations

The steps of the real-time PCR correspond to those of a regular PCR. There are three main phases that make up each cycle in a PCR reaction in real time: denaturation, annealing, and extension. The reactions are generally performed in 40 cycles. Rhizomania is caused, as mentioned above, by an RNA virus, therefore, with real-time PCR you need to pass through reverse transcription from RNA into cDNA by the action of the enzyme reverse transcriptase (RT).

There are two methods for the production of cDNA:

- The first is called quantitative two-step PCR reverse transcriptase. Usually, this cDNA synthesis reaction uses random primers used to give a fair representation of all targets in real-time PCR applications. About 10 % of the cDNA product is then transferred to a separate tube for the PCR reaction in real time.
- The second method is one-step qRT-PCR (quantitative RT-PCR). This combines the synthesis of the first-strand cDNA and PCR reaction in real time in the same tube. So it simplifies installation and reduces the chance of contamination. Gene-specific primers (GSP) are required. This is because using oligo (dT) or random primers will generate nonspecific products in the one-step procedure and reduce the amount of product of interest.

12.5.6 TaqMan

Harju et al. (2005) used TaqMan for the detection of BNYVV. The TaqMan is very specific, because it combines specificity of primers and specificity of the TaqMan probe – typically does not detect nonspecific PCR products. The increased sensitivity by using the TaqMan is potentially useful, given the uneven distribution of BNYVV within infected sugar beet roots (Kaufmann et al. 1992). The most significant advantages of real-time over conventional PCR are time and labor savings, made by eliminating the need for post-PCR gel electrophoresis. In a direct comparison between the conventional PCR and TaqMan RT-PCR in BNYVV RNA detection, it has been shown that the TaqMan assay was 10,000 times more sensitive than the conventional analysis, as it detects up to a dilution of 1:100,000, compared with conventional BNYVV analysis that detects only at a dilution of 1:10 (Harju et al. 2005). In contrast, qPCR is more expensive, due to the cost of dual-labeled oligonucleotide. The costs involved in the development of qPCR can be reduced by increasing the number of samples. By adopting multiplex TaqMan assays, the detection of two (or more) different target sequences in the same reaction reduces the cost of reagents. The development of reliable multiplex TaqMan assays has been demonstrated (Mumford et al. 2000) and can easily be reached after some restricted optimization.

12.6 *Polymyxa betae* Concentration

The presence of *P. betae* zoospores and cystosori within the plant is usually examined through microscopy or with DNA-based tests. DNA-based tests are able to detect the presence of the pathogen but not to quantify the amounts of *P. betae* or if it is alive or not. An accurate real-time PCR method for detecting DNA and mRNA was developed to identify *P. betae* using the glutathione-S-transferase protein

expressed at all fungal stages of development (Kingsnorth et al. 2003). This protein has also been used to develop a triple-antibody sandwich ELISA (TAS-ELISA) (Kingsnorth et al. 2003) useful to easily detect and quantify the BNYVV content in roots, using specific antisera.

12.6.1 Microscopy

Soil samples are taken from fields where sugar beet is grown, including those with rhizomania occurrence in the past but with no problems concerning sugar beet growing. Altogether, about 6 kg of soil is taken on several sites from each field. The soil samples are then dried at room temperature in a laboratory and sieved through 2 mm screens. Sugar beet baiting plants (var. Regina) are sown in pots with soil samples mixed in equal parts with autoclaved compost to facilitate root removal of baiting plants at harvest. The plants are grown under controlled conditions with a 16-h photoperiod at 20 °C (night) and 23 °C (day). The pots are watered directly as needed. After 6 weeks, roots of baiting plants are harvested, washed, and examined by optical microscope for the presence of *P. betae*.

12.6.2 Immunogold-Silver

Traditional methods to detect and quantify the vector and virus in soil are based on baiting plant bioassays using soil dilutions to estimate the most probable numbers (MPN) of infective propagules (Tuitert 1990). These methods are expensive and time-consuming, taking more than 8 weeks to complete for a single soil sample.

12.6.3 Scanning Electron Microscopy

Zoospores of *P. betae* transmitted BNYVV from naturally infected sugar beet seedlings to aseptically grown sugar beet or spinach (*Spinacia oleracea*) seedlings in the absence of other fungi. Virus was positively identified by local lesion assay, electron microscopy, and leaf-dip serology. This established *P. betae* as the vector of BNYVV, although virus was not detected in ultrathin sections of more than 220 zoospores (Giunchedi and Langenberg 1982).

Sporosori of *P. betae* were made with the scanning electron microscope on sugar beet roots. Sporosori were extracted from the host cells 15, 20, and 30 days after inoculation, purified, and observed with the scanning electron microscope. The morphology of sporosori differs, depending on their stage of maturation (Ciafardini and Marotta 1988).

12.6.4 Fluorescence Resonance Energy Transfer

Detection and relative quantification of *P. betae* content is done and confirmed in the same roots of baiting plants using a specific nanobiosensor kit for *P. betae*. The nanobiosensor method is based on fluorescence resonance energy transfer (FRET) using antibody-attached quantum dots and GST-conjugated rhodamine according to the kit producer's instructions (Safarpour et al. 2012). The test is conducted by first placing 250 µl of Tris-HCl buffer in each well. Ten microliters of the rhodamine-antigen solution is then added. This is followed by an addition of 10 µl of the QD-labeled antibody solution. The baseline data are then recorded by a microplate reader (Tecan, Austria). The microplate reader is operated as follows: the excitation wavelength is set at 350 nm (the excitation wavelength of CdTe QDs) and the emission of the quencher (rhodamine) is located at 580 nm. At the detection stage, the suspicious roots (0.1–0.5 mm thick) are mashed in Tris-HCl buffer (1 g plant material/500 µl buffer). Twenty microliters of the prepared extract is then added to each well and the second round of data is obtained. If no or negligible baseline shift (negative) is observed, the sample is free of *P. betae* but a significant baseline downward shift (positive) will reveal that the sample contains the pathogenic agents. The baseline shift measured for an immunodominant membrane protein (IMP) as the negative control (X ± 3SD) is used in differentiating healthy and infected samples.

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Chapter 13
Perspective

J. Mitchell McGrath

Abstract The few existing sources of rhizomania resistance are vulnerable to resistance-breaking strains of the beet necrotic yellow vein virus. Since rhizomania will likely continue as a threat to sustainable sugar beet production, and cultural controls to mitigate disease damage are currently ineffective, additional sources of genetic resistance must be developed. Evidence suggests that existing rhizomania resistance genes are members of the canonical NB-LRR resistance gene family in plants; thus, the search of additional variants in and among readily crossable *Beta* species with sugar beet is likely to be beneficial. Developing novel sources of resistance will best be accomplished armed with greater detail and knowledge of the pathogen system, including genetics of the host, the virus, and the fungal vector. Aspects of current knowledge and technology that will help in deducing future "targets of opportunity" for developing additional as well as durable resistances suggest that there are numerous strategies which provide optimism for reducing the impact of rhizomania on sugar beet.

Keywords Sugar beet • Rhizomania • *Rz*-mediated resistance • R-genes • *Polymyxa betae*

Rhizomania is perhaps the greatest current biotic concern to beet production worldwide, having demonstrated its ability to spread to new regions and overcome currently deployed genetic host resistances (Pavli et al. 2011). Fortunately, resistance-breaking virus strains appear to be spreading slowly. This provides a chance to understand the host–virus–vector interaction in sufficient detail to develop additional durable sources of rhizomania resistance and breed them into new varieties. However, this is not a trivial task since the current rhizomania resistances are, in part, the result of serendipity recognized by keen eyes, and details of the host–virus–vector interaction are just beginning to be described. This chapter is meant to suggest some short- and long-term work that will assist in ameliorating the effects of rhizomania on sugar beet production. It is unlikely that this is an exhaustive list

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E. Biancardi, T. Tamada (eds.), *Rhizomania*, DOI 10.1007/978-3-319-30678-0_13

since each new detail will suggest alternatives in meeting the challenge of this nearly ubiquitous beet disease. What specific strategies will be effective in developing new rhizomania resistances will depend in large part on understanding the biology of the host–pathogen interaction in sufficient detail to recognize approaches that may yield progress, with the end goal being complete immunity to the virus. There are many opportunities, and as our knowledge increases, additional opportunities will present themselves.

To summarize briefly, rhizomania results from a tripartite interaction between a susceptible beet host (*Beta vulgaris*), a plasmodiophorid soil-borne vector (*Polymyxa betae*), and a four- or five-component virus (beet necrotic yellow vein virus, BNYVV) (reviewed in McGrann et al. 2009). Interruption of this interaction at any point during the infection and disease process will prevent rhizomania or will otherwise reduce the impact of infection. The only control measure to date has been to reduce the impact of infection through the use of resistance genetics and breeding. Sugar beet seed companies have deployed the *Rz*1 and *Rz*2 rhizomania resistance genes, with *Rz*1 present in almost all their varieties and *Rz*2 is generally found in combination with *Rz*1. The valid concern is that these genes will be overcome over time. Unfortunately, there are no present cultural controls available due to the persistence of the vector in the agro-ecosystem, its ease of virus acquisition, and the virus's persistence in the vector. Thus, the only foreseeable control will continue to be genetic resistance, and the available means to accomplish this are to prevent the virulent interaction from occurring, to mitigate the damaging effects of the interaction once it has occurred, or both. Fortunately, an improving understanding of host plant resistance and immunity, a better understanding of fungal pathogen biology, and insight into how plant viruses co-opt host metabolism to influence disease physiology all assist in contemplating "targets of opportunity" for durable rhizomania resistance in sugar beet (Fig. 13.1).

There are two commercially deployed genetic loci that confer rhizomania resistance in sugar beet, *Rz*1 and *Rz*2. There appear to be three variants described at the *Rz*1 locus (*Rz*1, *Rz*4, and *Rz*5) and two at *Rz*2 (*Rz*2 and *Rz*3) (see McGrann et al. 2009 for review). Both loci map to linkage group 3 of sugar beet and are separated by an estimated genetic distance of 5–20 cM or more. More precise allelism tests would be useful to finely locate these loci and determine the full complement and effect of available alleles. Recently, a patent has been filed for *Rz*3, which presents the best current information about the structure of a sugar beet *Rz* resistance gene (Törjèk et al. 2014). As surmised from previous co-localization of rhizomania resistance with R-genes (Hunger et al. 2003), the available evidence strongly suggests that *Rz* resistance genes are indeed of the NB-LRR type (nucleotide-binding site, leucine-rich repeat) (De Ronde et al. 2014). In plants, such genes tend to be located in tandemly arrayed clusters, which may facilitate generation of novel alleles through mis-pairing and recombination of adjacent tandem copies during meiosis (David et al. 2009). Pairing and recombination between clusters is also likely to generate novel variants. Thus, it is plausible that additional *Rz* resistance variants are waiting to be identified in the primary *Beta vulgaris* germplasm pool as well as the secondary germplasm pool of related wild species (McGrath et al. 2011), and

Fig. 13.1 This image of *Beta maritima* (sea beet) was taken in June 2011 by Panella and Biancardi on the Po Delta, levy about 500 m from Porto Levante (Italy). The plant is developing and producing seed in extreme conditions, only on few grams of salty debris among the stones. Moreover, it is growing within a few meters of the Adriatic Sea, which bathes the plant with salty water during frequent storms. No other plant thrives in a similar location. From sea beets collected in 1909 along this shoreline (see Fig. 9.1), within 250 m from where this image was taken, resistances to cercospora leaf spot and rhizomania (of the Alba, Rizor, and Holly types) were extracted and bred into sugar beets. These resistances form the backbone of genetic resources available for limiting damage to these widespread and destructive diseases. Undoubtedly, sea beet will be an important source for other genetic resistances in the future. This picture is also a metaphor of the sugar beet crop, which since the beginning was always the underdog to sugar cane as a copious and less expansive producer of sucrose. Unlike sugar cane and severe diseases, the sugar beet crop has become an important source of income to growers in temperate regions throughout the world

that new variants will continue to arise and be discovered at some (perhaps low) frequency within the cultivated materials as well as in related species (Litwiniec et al. 2015).

Our limited understanding of resistance alleles suggests that the first task in developing additional rhizomania resistance is to understand variation at existing *Rz* resistance loci. It is likely this will be accomplished in the near future. Because NB-LRR loci are generally complex, containing numerous copies at a locus, and each of these copies could have a potentially unique immune system role, it is unlikely that the full picture of R-gene diversity in beets can be gleaned from current genome assemblies (Meyers et al. 2002; Monosi et al. 2004). A problem with current beet genome assemblies is that they are derived from short-read sequencing reads such that assembly of genome regions rich in repeat sequences is biased in favor of a single element. Unless other technologies are used to complement short

reads (as in the case of *Rz*3, see Törjèk et al. 2014), locus location can be assigned, but copy number at that locus remains ambiguous. More recent long-read sequencing technologies are better suited to discriminating between highly similar copies of repeated loci. These technologies are beginning to be applied in sugar beet, and the R-locus structures of at least a few beet genomes will help to reveal rates of recombinational mutation and assess the specific nucleotide positions that confer *Rz* resistance. This information may be used directly to modify R-genes with the most effective nucleotide combinations and/or gene copy numbers for higher levels of *Rz*-mediated resistance.

The biology of NB-LRR mediated resistance is fascinating and only beginning to be appreciated (reviewed in Caplan et al. 2008). Basically, the NB-LRR system functions as a gatekeeper for response to non-self recognition. An NB-LRR member gene product does not have to directly interact with a pathogen molecule (a so called pathogen effector): it is possible for the pathogen effector to interact with another host factor that then activates the NB-LRR system through the N-terminal domain of the R-protein (Caplan et al. 2008). This initiates downstream responses that may include production of reactive oxygen species (ROS), accumulation of salicylic acid, transduction of signals mediated by MAPK (mitogen-activated protein kinase) pathways, and transcriptional reprogramming, among other processes (Nicaise 2014). NB-LRR proteins can form higher order complexes and even carry transcription factor domains, thus they would seem to be evolutionarily plastic and able to generate novel alleles with selective advantages for survival outcomes in response to pathogen attack (Sarris et al. 2015). Exquisite specificity can be achieved by this system, and it is not surprising that the NB-LRR apparatus is tuned to recognize pathogen effectors from many different pathogens. The specificity appears to come at the price of increased complexity that includes numerous R-genes and a diversity of their interactions and downstream responses. This specificity of a disease–resistance interaction network opens up possibilities for resistance to fail with a small systemic perturbation, leading to a disease outcome (gene-for-gene systems would fall into this category).

What this means for rhizomania resistance research is that the five described *Rz* alleles at both loci need to be characterized for commonalities of structure that would allow deduction and prediction of function. It is possible, but less likely, that the known *Rz* alleles are direct pathogen recognition molecules. Therefore, knowledge of the conserved structure of *Rz* proteins, specifically the amino acid residues that characterize *Rz* resistance proteins as distinguished from susceptible alleles, is needed to prove which of the myriad pathogen effectors and host-interacting proteins may be responsible for molecular recognition events. It is possible that the pathogen effector is not even of viral origin, since until only recently, virus resistance mechanisms have been included in generalized models of plant–pathogen resistance (Mandadi and Scholthof 2013).

One of the interesting findings regarding sugar beet NB-LRR genes is that the TIR domain has not been detected (Tian et al. 2004). NB-LRR proteins are often grouped into two major classes whether a TIR (Toll/interleukin-1 receptor) or a CC (coiled-coil) domain is found at the N-terminal of the protein (Eitas and Dangl

2010). The TIR domain is thought to have a role in the hypersensitive response, leading to localized cell death and thus restriction of the pathogen's spread through the plant (de Ronde et al. 2014). The status of sugar beet N-terminal domains of R-genes has not been reported exhaustively. However, it is tempting to speculate that TIR-based immunity engineered into a sugar beet NB-LRR protein could have a role in creating novel disease resistances, perhaps including rhizomania.

Review of downstream immunity responses is beyond the scope of this perspective and the reader is referred to the increasing literature related to plant immunity in general. Relatively few detailed plant–pathogen interactions have been described to date, and it is not known whether these insights apply to the sugar beet–*P. betae*–BNYVV patho-system. It seems likely that some of the processes are shared between sugar beet and other model and crop systems, and that these could be exploited to enhance rhizomania resistance (Nicaise 2014). However, it cannot be overemphasized that these characterizations need to be done for sugar beet, not only for the rhizomania system but also for each of the major sugar beet pathogens worldwide (Biancardi et al. 2005). With the advent of sugar beet genome sequences (e.g., Dohm et al. 2014), it is anticipated that this task will become more precise and informative with respect to the specific genes involved and thus allow greater precision in choosing targets of opportunity for sugar beet crop protection.

Some progress has been made in characterizing the rhizomania interaction with sugar beet. Using near isogenic lines with and without *Rz* resistance genes, Larson et al. (2008) and Webb et al. (2015) were able to identify changes in the proteome in response to challenge with BNYVV versus non-inoculated plants. The proteins detected likely represent stable downstream targets and responses of the *Rz*-mediated immunity system and encompass those described in other plant host–pathogen interactions. These data reinforce that the sugar beet–rhizomania system is not fundamentally deviant relative to other plant pathogenic processes. However, uncertainty as to the putative identities of gene products deduced by homology comparisons with other species, the relative diversity of biological processes detected, and temporal variation in protein abundance across time points assayed make it difficult to draw strong conclusions regarding mechanisms of rhizomania resistance. The idea that rhizomania interactions with sugar beet are not fundamentally different than other plant immunity responses is further supported through demonstration that the BNYVV RNA3 P25 pathogenicity component interacts specifically with beet, *Arabidopsis*, and *Nicotiana* F-box proteins involved in targeting proteins for degradation, supporting the notion that P25 is involved in the suppression of the beet's innate immune response (Peltier et al. 2011; Thiel et al. 2012; Litwiniec et al. 2014).

An important part of the plant antiviral immune system is destruction of invading viral RNAs such that replication, transcription, and/or translation are prevented; thus, viral titers and gene products do not accumulate to damaging concentrations. RNA silencing mechanisms exist to recognize host from nonhost RNA, and viruses exploit suppression of this mechanism to replicate. Suppression of silencing can be overcome in some cases, such as when virus genes are engineered to express in a plant host and thereby provide a prophylactic copy of a viral gene that presumably

"primes" the immune RNA silencing machinery to high activity prior to infection. Beets expressing BNYVV coat protein or replicase genes do indeed confer a measure of rhizomania resistance when so configured (Mannerlöf et al. 1996; Pavli et al. 2010). Extra measures of resistance could be anticipated by combining multiple triggers of the beet's innate immunity, much like stacking of *Rz* resistance alleles in current varieties. It is logical to think that such resistance would be more difficult for the virus to overcome and thus be more durable. Pavli and colleagues (2012) developed an interesting approach toward durable rhizomania resistance. In this case, *Nicotiana benthamiana* was used as a surrogate transformable species. The transformed plant received a harpin gene and a BNYVV replicase gene, where the harpin gene acts as a bacterial effector gene involved in pathogen recognition, presumably acting prior to activation of the NB-LRR component of immunity. The replicase gene presumably functions in the RNA silencing immune pathway. As more components of the immunity system are identified and assessed in sugar beet, it is conceivable that effective components could be stacked in combination, which ideally would confer rhizomania resistance indefinitely. Transgenic approaches may be a viable option if the high expense of developing such varieties can be justified. In this respect, newer genetic engineering technologies such as targeted gene replacement, perhaps using a CRISPR/Cas9 or similar technology (Belhaj et al. 2015), will reduce the cost of developing new germplasm but may not address the high cost of obtaining regulatory approval of such varieties.

Resistance to BNYVV as the causal agent of rhizomania is the most direct target of intervention, however other targets are also available. Resistance to the plasmodiophorid soil-borne vector *P. betae* is another attractive option that would confer indirect rhizomania resistance via precluding virus infection. This strategy may be easier to implement as a durable resistance approach versus continually screening germplasm for resistance to newly virulent strains of BNYVV that avoid existing Rz-mediated resistance. Three lines of evidence suggest that immunity to could be achieved:

- *Polymyxa betae* has a very restricted host range in nature, affecting only a few species in the genus *Beta*. The only other *Polymyxa* species described affects wheat (and perhaps beet as well), suggesting that *P. beta*–plant interactions are an exception and that the obligate biotrophic nature of this interaction is highly specific and perhaps easily disrupted (Neuhauser et al. 2010).
- Closely related *Beta* species appear immune to *P. betae* infection, again suggesting a highly specific interaction that has perhaps only recently been exploited by BNYVV to vector rhizomania (Tamada and Kondo 2013). Genomics applied to these related species may help to uncover a genetic basis of this immunity, and, by using traditional breeding methods, perhaps this immunity could be transferred to sugar beet.
- Resistance to *P. betae* may be found within the primary germplasm pool of *Beta vulgaris*. Asher and colleagues described two loci from *B. vulgaris* subsp. *maritima* that confer resistance to *P. betae* opening up the possibility that resistance may be readily incorporated into cultivated materials (Asher et al. 2009).

In summary, the dynamics and mechanics of the plant immune system are only beginning to be unraveled. Insights gained need to be confirmed in sugar beet (for each of sugar beet's pathogens) and extended to the rhizomania infection process. Opportunities exist to apply these insights to specific interactions with the BNYVV as well as to interactions with the vector of rhizomania. It is expected that the beet immune system will follow similar paradigms as in other angiosperms, including the panoply of effector- and plant-mediated pathogen responses. However, the recognition of each pathogen is likely a highly specific interaction. Thus, modulation of the host response to either the virus or the vector requires an understanding of the specific molecules and mechanisms involved in triggering sugar beet defenses. Further, downstream responses may be subject to environmental influences as well as the degree and strength of the interference of the host response by the pathogen's infection processes. Such conditional responses may be difficult to ascertain, and in this respect, model systems may offer an efficient opportunity to examine the molecular interactions that facilitate the development of disease, particularly rhizomania.

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Appendix

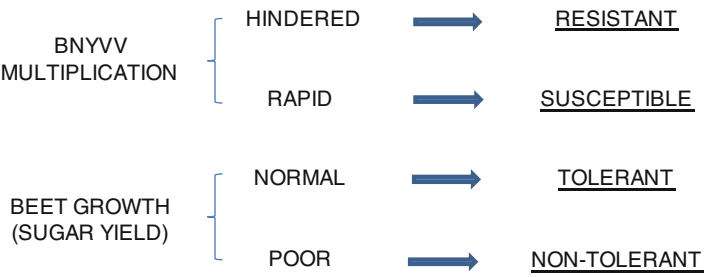

Fig. A1 Terms generally used (*right*, *underlined*) to define the kind and intensity of reactions employed by sugar beet to limit the effects of rhizomania on yield

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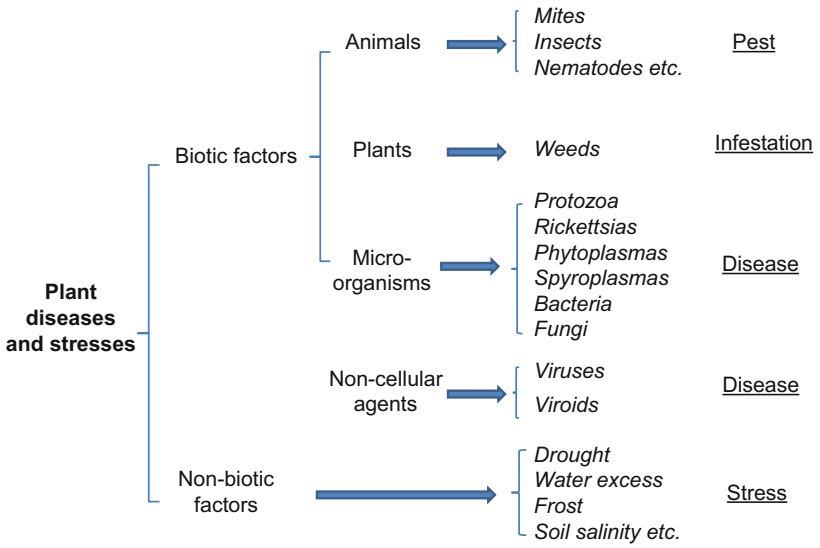

Fig. A2 Terms used to define the pathologies affecting the sugar beet crop (*right*, *underlined*) according to their origin

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