Akshay Kumar Chakravarthy
Venkatesan Selvanarayanan *Editors*

# Experimental Techniques in Host-Plant Resistance

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# Foreword

The last five decades have witnessed rapid progress in the development of crop varieties resistant to insect pests. Advances in instrumentation technology, biotechnology and molecular biology have allowed scientists to adopt newer approaches and devise more precise techniques for identifying plants containing resistance and/or tolerance to insect pests. Improved insights into the genetics and breeding of crop plants for resistance have further facilitated developments in host plant resistance studies. Current research efforts focused on durable pest resistance form the most important programmes in food crops, horticulture and forestry, where pest-resistant plants continue to be economically and ecologically superior to broad-spectrum insecticides. Identifying and utilizing pest-resistant varieties remain very cost-effective, often requiring less time than transgenic techniques. Continued growth in the human population and the associated need for increased food availability reinforce the pressing demand for efficient high-throughput plant germplasm screening schemes to identify and exploit sources of resistance.

The book *Experimental Techniques in Host-Plant Resistance* contains 30 chapters contributed by 60 authors, who addressed the needs for the development of more precise yet rapid methods of identifying and implementing insect crop plant resistance. They have attempted to collate information on standardized procedures used to determine resistance mechanisms; molecular marker-assisted selection of resistance; durability of insect-resistant plants in varying climatic regimes; and understanding the role of resistance in tritrophic interactions. I am hopeful the book will educate all researchers about the need to adopt refined methods to investigate and identify insect-resistant plants. The use of such improved techniques and technologies will greatly benefit the large-scale cultivation of insect-resistant crops across diverse landscapes and human communities.

Department of Entomology, Kansas State University, Manhattan, KS, USA
March 2019

C. Michael Smith

# Preface

The earliest land plants evolved around 450 million years ago from aquatic plants devoid of vascular systems. The diversification of flowering plants (angiosperms) during the Cretaceous period is associated with speciation in insects. Early insect herbivores were mandibulate, but the evolution of vascular plants led to the co-evolution of other forms of herbivory, such as leaf feeding, sap sucking, leaf mining, tissue boring, gall forming and nectar feeding. Plant defence against biotic stress is an adaptive evolution by plants to increase their fitness. Plants use a variety of strategies to defend against damage caused by herbivores. Plant defence mechanisms are either inbuilt or induced. Inbuilt mechanisms are always present within the plant, while induced defences are produced or mobilized to the site where a plant is injured. Induced defence mechanisms include morphological and physiological changes and production of secondary metabolites.

Host plant resistance is one of the eco-friendly methods of pest management. It protects the crop by making it less suitable or tolerant to the pest. While books on the theoretical aspects of host plant relationships/resistance are available, an exclusive book on the practical aspects is lacking. There is a wide gap between the theory and the experimental procedures required for conducting studies on host plant resistance for the postgraduate students and young researchers. A dire need for a book on practical aspects was strongly felt. Initially, a practical manual was prepared which eventually evolved into the present book. We hope this book, *Experimental Techniques in Host-Plant Resistance*, will be useful. The book provides information on major aspects of screening crop germplasms, sampling techniques, genetic and biochemical basis of HPR, behavioural studies on plant volatiles and some of the recent approaches in HPR. Besides the detailed procedures, the 'references', 'further reading' and 'illustrated examples' provide the additional material for the benefit of readers and workers alike. Illustrated examples provide at a glance, the tools and experimental setups for executing the techniques. The examples can also serve to

help evolve an idea or understand the principle based on which the techniques have been developed. It further broadens one's prospective to meet the desired objective or goal. We hope this will further kindle interest in researchers to develop improved techniques in host plant resistance.

Bangalore, Karnataka, India — Akshay Kumar Chakravarthy
Chidambaram, Tamil Nadu, India — Venkatesan Selvanarayanan
March 2019

# Acknowledgements

Host Plant Resistance is indeed a dynamic field, and researchers are continuously refining and updating techniques for studying different aspects of host plant resistance/relationships. It was a daunting task to collate documented information and concisely put it in a volume. The editors thank the contributors for their time and attention to write chapters on different aspects of host plant resistance. The chapters have been categorized under the following five parts: (I) Prerequisites for Host Plant Resistance Studies; (II) Instrumentation in Host Plant Resistance Studies; (III) Techniques in Host Plant Resistance Studies; (IV) Ecological and Climatographic Factors in Host Plant Resistance Studies; and (V) Genetics, Plant Breeding and Molecular Tools in Host Plant Resistance Studies. 60 contributors have written chapters for this book. The wide-ranging techniques can be applied across several species of insects with slight modifications or changes. Often, the screening germplasm results are not comparable directly, and we hope this book will go a long way in rendering the data comparable.

The editors are grateful to Mr. Subhash S., Dr. Doddabasappa B., Mr. Kumar K. P., Mr. Nitin K. S. and other postgraduate students and faculty from different universities in India. The editors are also thankful to the vice chancellors of the universities in India for encouragement. Select photos and line diagrams have also been taken from few books and other sources mentioned under "references"; we are thankful for the contribution. This has been done primarily for the benefit of a large number of undergraduate and postgraduate students and also for the research scholars who constitute a major share of the readership. The contribution from the selected books/publishers/authors/editors for this compilation is gratefully acknowledged by the editors. Colleagues from entomology across several universities and institutes in India and abroad have made valuable contributions to the book. We indeed are lucky to have the Foreword from a fine expert on host plant resistance, Dr. C. Michael Smith, Kansas State University, USA. We are highly indebted and

thankful to Dr. C. Michael Smith. We also take this opportunity to thank Dr. S. Lingappa and Dr. P. Narayanaswamy who inspired us and stimulated ideas on insect–plant relationships. The editors are grateful to them all. Finally, the editors are thankful to Springer India Private Ltd., New Delhi, for their deep interest and enthusiasm in publishing this book.

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**Dr. A. K. Chakravarthy** Head and Principal Scientist, Division of Entomology and Nematology, he is an author of many books and over 300 scientific papers and 50 chapters in Entomology and Natural history. His interest includes insects, birds, bats, rodents and mammals. With nearly four decades of experience in teaching, research and extension, Dr. Chakravarthy has been the investigator for over 35 research projects and has guided more than 25 students for post-graduation. A Ph. D. from Punjab Agriculture University and fellow from IARI, New Delhi, he is a member of several national and international scientific academia, an adviser, a panellist, a referee, reviewer and editor and is associated with publication of several national and international journals worldwide. A field-orientated, widely travelled biologist, he is actively working on novel approaches in integrated pest management, host plant Interaction, vertebrate pest management, biodiversity and environmental conservation issues. Recently, he has published books titled, *New Horizons in Insect Science: Towards Sustainable Pest Management*, *Economic and Ecological Significance of Arthropods in Diversified Ecosystems: Sustaining Regulatory Mechanisms* and *Arthropod Diversity and Conservation in the Tropics and Sub-tropics*, The Black spotted, Yellow Borer, *Conogethes punctiferalis* Guenee and Allied Species by Springers.

**Dr. V. Selvanarayanan** Professor of Entomology, Faculty of Agriculture, Annamalai University, he has 25 years of teaching and research experience. He was a gold medallist in post-graduation, and his doctoral research resulted in identification of new species of tomatoes resistant to insects. He was awarded with the Best Research Paper Award for 2006 and Distinguished Teacher Award for 2010 at Annamalai University. He has authored more than 80 research articles and 4 books. He is a fellow of Plant Protection Association of India and life member in many professional societies. He has spearheaded many externally funded research projects. He is passionate about travelling and has visited the UK, USA, UAE, China and other countries. He enjoys interacting with peers and delivers invited lectures in scientific meetings. As additional responsibilities, he served as Deputy Controller of Examinations and as Head, Department of Entomology at Annamalai University. He is on the editorial board of select scientific journals.

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# Part I
# Prerequisites for Host Plant Resistance Studies

# Introduction


**V. Selvanarayanan**

**Abstract** Since the seventeenth century, efforts have paved way for developing insect-resistant varieties. Resistant varieties are compatible with biological and cultural practices. Resistant varieties are especially of value in low-income group crops. Resistant varieties may enhance the efficacy of insecticides and environment quality. On the whole, resistant varieties form an ideal means of pest suppression.

**Keywords** Resistant Varieties · Role in pest management · History · Resistant variety · Ecology

## 1 Introduction

Plants acquired insect-resistant characters due to their co-evolution with insects. Since crop domestication was initiated by mankind, exploitation of desirable plant types was common. Intensive agriculture enabled more research impetus on exploiting crop varieties possessing insect tolerance or resistance. An insect-resistant crop cultivar yields more than a susceptible cultivar. Use of such insect-resistant/insect-tolerant cultivars evinces enhanced productivity without harming the benign environment. The genetic diversity of many crop plants offers ample scope for exploitation of resistant traits. The relationship between an insect and crop cultivar is influenced by the kind or mechanism of resistance, namely, antixenosis (non-preference), antibiosis and tolerance (Painter 1958). Resistance in a crop cultivar is exerted due to the presence of biophysical or biochemical factors resulting in any one of the above mechanisms of resistance. Cultivated varieties, wild lines, land races and other distantly related genera of crop plants are gathered and screened for resistance at the field and/or glasshouse conditions, and promising resistant sources are being exploited since early period, for large-scale cultivation or for future breeding programmes.

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## 2 Early Success

Since the eighteenth century, extensive attempts have paved way for developing insect-resistant crop varieties. In 1788, early maturing varieties of wheat were developed to escape from Hessian fly, *Mayetiola destructor* (Say), damage (Chapman 1826). This was followed by the identification of wheat cultivar "Underhill" in New York as resistant to Hessian fly (Havens 1792). Apple varieties "Winter Majetin" and "Northern Spy" were recommended as resistant to woolly aphid, *Eriosoma lanigerum* (Hausmann) (Lindley 1831). Later, the entire wine industry of France was devastated by incidence of phylloxera, *Daktulosphaira vitifoliae* (Fitch). To overcome this infestation, grafts of rootstocks of American grapes, *Vitis labrusca*, with the scions of French grapes, *Vitis vinifera*, were popularized which evinced revival of the vine industry (Smith 2005). With the advent of intensive agriculture, development and use of insect-tolerant/insect-resistant crop varieties gained prominence.

The International Rice Research Institute (IRRI), Manila, Philippines, developed improved varieties that possessed resistance to key insect pests and diseases. Rice variety IR36 was found resistant to brown planthopper, green leafhopper, stem borers, gall midge, blast, bacterial blight and tungro. This cultivar was planted in about 11 million ha in the world which enhanced the economic returns of rice growers and processors exponentially (Khush and Brar 1991).

## 3 Role in Pest Management

By reducing pest numbers, resistant varieties helped to shift the pest/predator ratios in favour of biological control (Heinrichs 1994). Varietal resistance is usually compatible with biological control but may also have an adverse effect on natural enemies. In field studies at IRRI, Philippines, the brown planthopper, *Nilaparvata lugens* (Stal), and spider *Lycosa pseudoannulata* (Bosenberg and Strand) ratio increased with the level of susceptibility from ASD7 and IR36, both highly resistant rice cultivars, to IR42 and Triveni, moderately resistant cultivars to IR8 and TN1, susceptible cultivars. The resistant plant may also enhance the predatory activity. Predation rate of the mirid bug, *Cyrtorhinus lividipennis* Reutger, when feeding on the first instar brown planthopper nymphs increased on the resistant cultivar, IR36, compared to the susceptible IR8.

Use of resistant varieties is most useful in crops of low value where yields fluctuate due to weather and other factors. Resistant varieties are of special significance for countries like India where holdings are small and farmers are not equipped to take up other methods of pest suppression. Physical and mechanical control methods are the oldest and offer several primitive ways of suppressing the pests.

For example, *Lablab niger* Medick creeper type is generally tolerant to pod borers compared to field-type *L. niger*. Collection and destruction of larvae would be easier on resistant/tolerant cultivars than susceptible cultivars (Chakravarthy 1978). Chakrabarthy et al. (1970) reported grape varieties, namely, Bangalore Blue and Bhokri, to be resistant to the defoliator, *Adoretus devanceli* (Blanch), than Bangalore Purple, Khalili and Anab-e-shahi grapes, and hence it was convenient to pick and destroy defoliator on resistant than susceptible grape varieties.

Host plant resistance may enhance the efficacy of insecticides. Evaluation of insecticides in rice indicated that they cause higher mortality of plant hoppers and leafhoppers feeding on resistant than on susceptible rice varieties (Heinrichs 1994). The mortality of brown planthopper reared on either moderately resistant ASD7 or a highly resistant cultivar "Sinna Sivappu" was higher than when feeding on a susceptible TN1 cultivar. The $LD_{50}$ of white-backed planthopper was 9.4 on the susceptible variety TN1 treated with ethylan but only 2.8 on moderately resistant N22.

Certain insects in addition to infesting crop plants also transmit pathogens causing diseases and thus serve as vectors. Host plant resistance is an effective means to manage both the disease and the vector. For instance, certain banana varieties showed resistance to bunchy top because of its less susceptibility to the banana aphid, *Pentalonia nigronervosa* Coq., the bunchy top vector (Verghese 2001; Hooks et al. 2009). Certain species of citrus like *Citrus nobilis*, *C. aurantifolia* and *C. rashmi* had significantly lower populations of the vector aphid (*Toxoptera* sp.), from among 22 species and 3 hybrids (IIHR 1984). Birch et al. (1992) showed the use of restriction fragment length polymorphism (RFLP) analysis, in distinguishing biotype of the virus vector aphid, *Amphorophora idaei* Borner, thus aiding in raspberry screening.

The effect of resistant host plants on pest population is specific, cumulative and persistent. It offers no environmental hazards and is eco-friendly. It is compatible with other pest management methods. However, the occurrence or selection of pest biotypes limits the effectiveness of resistant variety. Resistant factors may be incompatible with desired economic characters. In many crops, a variety resistant to one pest may be susceptible to another pest. Pod borer-resistant cultivars of *Lablab niger* are susceptible to the aphid, *Aphis craccivora* Koch (Chakravarthy 1992). Similarly, a variety resistant to insect pests may be susceptible to diseases as in the case of sesame wherein capsule borer-resistant varieties are susceptible to phyllody disease (VijaiAnandh and Selvanarayanan 2005).

Considering the above concepts, development of resistant varieties of crops should be a recurrent, continuous and concerted attempt. Substantial success has been achieved in resistance breeding of crops to manage plant pests and diseases. Buddenhangen (1991, 1996) argued that the popularity of insecticides, and even insect-based integrated pest management, has reduced efforts in resistance breeding. Attempts on resistance breeding are generally focused on exploiting qualitative vertical resistance. This approach is convenient because high levels of resistance

can be achieved and because the method is compatible with breeding schemes used for enhancing crop performance through control of major genes. However, its gene-for-gene nature can sometimes lead to its breakdown of resistance due to selection pressure on the pest species. Classic examples of such resistance breakdown in insect pests include the Hessian fly, *Mayetiola destructor* Say, and green bug, *Schizaphis graminum* (Rondani), on wheat and the brown planthopper, *Nilaparvata lugens* Stal.; green leafhopper, *Nephotettix virescens* (Distant); and rice gall midge, *Orseolia oryzae* (Wood-Mason), on rice. While plant resistance breeding is often identified as a component of integrated pest management (IPM), its actual integration with other pest control methods in IPM systems has been limited. In most cases, breeding programmes have sought single technology solutions to pest problems (i.e. complete resistance), much like insecticides. This is often hard to achieve for some pests, e.g. stem borers in cereals, and while partial resistance can be obtained as in field bean, *L. niger*, to pod borer (Chakravarthy 1978, 1992). Partially resistant varieties, however, may have a value when viewed in an IPM context, where the contribution of natural enemies and other factors complements their effects (Thomas and Wage 1993).

Integrated pest management (IPM) approach can also contribute to the durability of plant resistance to pests. The action of mortality factors, such as natural enemies, can reduce pest populations and thereby delay the selection pressure to overcome the resistance factors. The role of natural enemies in maintaining durability in this manner has been demonstrated (Gould 1996).

In view of the above-said advantages, exploring, exploiting and employing host plant resistance will envisage eco-friendly pest management, for which realistic cooperation among entomologists, pathologists and plant breeders is needed. For developing insect-resistant cultivars, proper practical expertise and standard protocols are necessarily to be followed. Only such attempts will enable development of crop varieties with durable resistance (Figs. 1, 2, 3, 4, 5, 6, 7 and 8).

**Fig. 1** Hessian fly – *Mayetiola destructor*. (Source: www.bugwood.com)

**Fig. 2** Grape phylloxera – *Daktulosphaira vitifoliae*. (Source: BugGuide.Net)

**Fig. 3** Woolly aphid – *Eriosoma lanigerum*. (Source: barmac.com.au)

**Fig. 4** Camellia or black citrus aphid – *Toxoptera aurantii*. (Source: www.aphotofauna.com)

**Fig. 5** Banana aphid – *Pentalonia nigronervosa*. (Source: http://www.musarama.org)


**Fig. 6** IR 20 rice variety resistant to key pests. (Source: http://www.agritech.tnau.ac.in/)


**Fig. 7** IR 36 rice variety resistant to key pests. (Source: http://www.agritech.tnau.ac.in)


**Fig. 8** Brown planthopper –*Nilaparvata lugens*. (Source: http://www.pestnet.org)

**Acknowledgement** The author is thankful to the authorities of Annamalai University and the sources like http://www.pestnet.org, http://www.agritech.tnau.ac.in, *http://www.musarama.org*, www.bugwood.com, BugGuide.Net, www.aphotofauna.com and barmac.com.au for the photographs.

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# Germplasm Exploration and Collection


**A. K. Chakravarthy and V. Selvanarayanan**

**Abstract** A prerequisite for detecting sources of resistance to insect pests is to have diversified crop germplasm. Information on major sources for obtaining germplasm plays a crucial role in developing resistant crop varieties. This chapter indicates 220 sources of germplasm centres for more than 25 crop plants. Monoculture and intensive cultivation practices endanger the local germplasms, and care should be taken to conserve them. Wild species of the cultivated crops need also to be conserved. Local expertise, manpower and resources need to be best utilized for identifying resistant sources.

**Keywords** Germplasm collection · Utilization · Germplasm sources · Conservation

## 1 Introduction

Genetic resources of crop plants are diverse and offer ample scope for exploration and exploitation. Russian geneticist N.I. Vavilov emphasized that the success of crop improvement programmes depends on the strength of the germplasm gathered. Vavilov and his coworkers identified that China, India, Central Asia, Asia Minor, the Mediterranean, Ethiopia, Central America and western-central South America are the great centres of floral genetic diversity in the world (Maps 1 and 2).

Germplasm collections may consists of (1) improved modern cultivars under cultivation, (2) unimproved or purified cultivars no longer in cultivation, (3) breeding stocks developed by breeders but not released for cultivation, (4) land races, (5) weed races and (6) wild species (Panda and Khush 1991).

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## 2 Major Sources of Crop Germplasm

Extensive efforts of centres of excellence in the world have culminated in the maintenance of huge germplasm of crop plants. Early efforts of Russian geneticist Vavilov and his colleagues beginning in 1916 enabled the establishment of the world's first scientific gene bank at St. Petersburg, Russia, comprising more than 250,000 accessions. Since then, many crop-specific and general gene banks have been established. A major impetus to germplasm exploration, collection and preservation was given since the inception of the International Board for Plant Genetic Resources (IBPGR) in 1974 by the Consultative Group for International Agricultural Research (CGIAR). Later in 1991, it was rechristened as the International Plant Genetic Resources Institute (IPGRI). In 1994, it was given autonomous status as one of the centres of CGIAR. Subsequently, IPGRI took over the governance of International Network for Improvement of Banana and Plantain (INIBAP). During 2006, both organizations merged and christened as Bioversity International with headquarters in Rome, Italy. Since inception of IBPGR, genetic resources of all crops are maintained.

Besides Bioversity International, other international agricultural research centres such as CIAT (beans, cowpeas), Cali, Columbia; CIP (cassava, potato), Lima, Peru; CIMMYT (maize, wheat), Mexico City, Mexico; ICARDA (legumes), Aleppo, Syria; ICRISAT (millet, sorghum), Patancheru, India; IITA (cassava, cowpeas), Ibadan, Nigeria; and IRRI (rice), Los Baños, Philippines, maintain major germplasm holdings. In addition to such international institutes, national-level germplasm preservation centres are established, like the National Bureau of Plant Genetic Resources (NBPGR) in India or Plant Gene Resources of Canada (PGRC). Many sources of germplasm also exist in foreign national seed collections maintained by voluntary organizations and private seed companies.

The following is the list of major sources of germplasm of important crops (Batugal et al. 2005; Khan 2007; Smith 2005).

### *2.1 Banana/Plantain, Musa* spp.

Bioversity International Musa Germplasm Transit Centre, Belgium
Indonesian Tropical Fruit Research Institute, Solok, West Sumatra, Indonesia
Institut des Sciences Agronomiques du Rwanda (ISAR), Rubana, Rwanda
National Research Centre for Banana (ICAR), Tiruchirappalli, India

### *2.2 Barley,* Hordeum vulgare*; Oat,* Avena sativa*; and Rye,* Secale cereale

International Center for Agricultural Research in the Dry Areas (ICARDA), Syria

International Maize and Wheat Improvement Center (CIMMYT), Mexico
Waite Agricultural Research Institute Barley Collection, Adelaide, Australia
Ohara Institute for Agricultural Biology, Okayama University, Kurashiki, Japan
Agricultural Research Institute, Kromeriz, Czech Republic
Swedish Seed Association, Svalov, Sweden
USDA-ARS National Small Grains Collection, Aberdeen, Idaho, USA
Biotechnology and Biological Sciences Research Council (**BBSRC**) Cereal Collections, Germplasm Resources Unit, John Innes Centre, Norwich, UK

## *2.3 Bean,* **Phaseolus** *Species, and Cowpea,* **Vigna** *Species*

Centro Internacional de Agricultura Tropical (CIAT), Colombia
Instituto Nacional de Investigaciones Agricolas (INIA), Mexico
International Institute of Tropical Agriculture (IITA), Nigeria
University of Cambridge, UK
USDA-ARS Western Regional Plant Introduction Station, Pullman, Washington, USA
N.I. Vavilov Institute of Plant Industry, St. Petersburg, Russia
Indian Institute of Vegetable Research, Varanasi, India

## *2.4 Capsicum*

Asian Vegetable Research and Development Center (AVRDC), World Vegetable Centre, Tainan, Taiwan
Germplasm Bank of the Agricultural Experimental Station, La Consulta INTA, Argentina
International Center for Tropical Agriculture (CIAT), Cali, Colombia
Indian Institute of Horticultural Research, Bengaluru, India
Indian Institute of Vegetable Research, Varanasi, India

## *2.5 Castor*

AGES Linz - Austrian Agency for Health and Food Safety/Seed Collection, Austria
Agricultural Research Station Teleorman, Teleorman County, Romania
Biodiversity Conservation and Research Institute, Ethiopia
Centro Nacional de Pesquisa de Algodao (CNPA), Brazil
Comunidad de Madrid. Universidad Politécnica de Madrid. Escuela Técnica Superior de Ingenieros Agrónomos. Banco de Germoplasma, Spain
Indian Institute of Oilseeds Research, Hyderabad, India

EMBRAPA/CENARGEN, Brasilia, Brazil
Empresa Baiana de Desenvolvimento Agricola SA, Brazil
Faculty of Agriculture, University of Zagreb, Croatia
Genebank, Leibniz Institute of Plant Genetics and Crop Plant Research, Germany
Genetic Resources Institute, Azerbaijan
Gobierno de Aragón. Centro de Investigación y Tecnología Agroalimentaria. Recursos Forestales, Spain
Institute for Agrobotany, Hungary
Institute for Plant Genetic Resources 'K. Malkov', Bulgaria
Institute of Biodiversity Conservation (IBC), Ethiopia
Institute of Botany, Azerbaijan
Institute of Crop Germplasm Resources, Chinese Academy of Agricultural Sciences, China
Institute of Crop Science (CAAS), China
Institute of Field and Vegetable Crops, Maksima Gorkog, Serbia
Institute of Oil Crops Research, China
Institute of Oil Crops, Ukraine
Instituto Agronomico de Campinas, Brazil
Maize Research Institute, Zemun Polje, Serbia
Medicinal and Aromatic Plants Research Station Fundulea, Romania
Millennium Seed Bank Project, Seed Conservation Department, Royal Botanic Gardens, Kew, UK
N.I. Vavilov All-Russian Scientific Research, Institute of Plant Industry, Russia
National Bureau of Plant Genetic Resources (NBPGR), India
National Genebank of Kenya, Crop Plant Genetic Resources Centre, KARI, Kenya
Plant Breeding and Acclimatization Institute, Poland
Research Station of Medicinal Crops, Ukraine
USDA-ARS-PGRCU, Griffins, Georgia, USA

## *2.6 Cassava,* Manihot esculenta

Centro Internacional de Agricultura Tropical (CIAT), Colombia
Central Tuber Crops Research Institute, Thiruvananthapuram, India
International Institute of Tropical Agriculture (IITA), Nigeria
National Root Crops Research Institute, Umudike, Nigeria
National Crops Resources Research Institute, Kampala, Uganda

## *2.7 Chickpea,* Cicer arietinum*, and Lentil,* Lens culinaris

Ethiopian Gene Bank, Addis Ababa, Ethiopia

International Center for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria
International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India
Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), Gatersleben, Germany
Institute of Crop Germplasm Resources, Beijing, and People's Republic of China
Laboratorio del Germiplasmo, Bari, Italy
Nordic Gene Bank, Alnarp, Sweden
USDA-ARS Western Regional Plant Introduction Station, Pullman, Washington, USA
N. I. Vavilov Institute of Plant Industry, St. Petersburg, Russia
Australian temperate field crops collection, Victoria, Australia

## *2.8 Citrus*

Citrus Experimentation Station, University of California, Riverside, USA
National Citrus Germplasm Repository, Chongqing, Sichuan province, China
Regional citrus genetic resources repository, Huangyan, Zhejiang province, China
Regional citrus genetic resources repository, Guiling, Guangxi province, China
Regional citrus genetic resources repository, Changsha, Hunan province, China
Regional citrus genetic resources repository, Guangzhou, Guangdong province, China
National Research Centre for Citrus, Nagpur, India
Malaysia Botanical Garden
Fruit Tree Research Station, Tsukuba, Japan
Commonwealth Scientific and Industrial Research Organization (CSIRO), Adelaide, South Australia
The Citrus Germplasm Bank, Instituto Valenciano de Investigaciones Agrarias in Valencia, Spain

## *2.9 Coconut*

Experimental Station of Betume, Neópolis, Sergipe-SE (Brazilian Agricultural Research Corporation -EMBRAPA), Brazil
Oil Palm Research Institute, Kusi, Kade, Ghana
Centre de Coopération Internationale en Recherche Agronomique pour le Développement - Vanuatu Agricultural Research and Technical Centre (CIRADVARTC), Santo, Vanuatu
Cocoa and Coconut Institute (CCI), Stewart Research Station, Madang Province, Papua New Guinea

Ministry of Natural Resources Development, Kiribati
Taveuni Coconut Centre, Fiji
Coconut Industry Board (CIB), Kingston, Jamaica
National Agriculture Research Institute (NARI), Mon Repos, East Coast Demerara, Guyana
Centro de Investigacion Cientifica de Yucatan (CICY), Mexico
Coconut Research Institute, Chinese Academy of Tropical Agricultural Science (CRICATAS), Hainan Province, China
Coconut Genebank, Philippine Coconut Authority (PCA), Zamboanga, Philippines
Menumbok Coconut Station, Department of Agriculture (DOA), Menumbok, Sabah, Malaysia
Coconut Genebank, Dong Go Experiment Station, Vietnam
Chumphon Horticulture Research Centre (CHRC), Thailand
International Coconut Genebank, Sikijang, Pekanbaru, Riau Province, Indonesia
Indonesian Coconut and Other Palmae Research Institute (ICOPRI), Manado, Indonesia
Coconut Research Institute of Sri Lanka (CRISL), Bandirippuwa Estate, Lunuwila, Sri Lanka
Central Plantation Crops Research Institute (CPCRI), Indian Council of Agricultural Research (ICAR), Kasaragod, India

## *2.10 Cotton*

Empire Cotton Growing Corporation, Shambar, Sudan (former)
Institute of Genetics and Plant Experimental Biology, Tashkent, Uzbekistan
Presidencia Roque Saenz Pena, Argentina
National Center for Genetic Resources Preservation (NCGRP), Fort Collins, USA
Southern Plains Agricultural Research Center, USDA-ARS, College Station, Texas, USA
Central Institute of Cotton Research, Nagpur, India
Cotton Research and Development Unit, Ministry of Agriculture, Christ Church, Barbados

## *2.11 Maize*

Instituto Colombiana Agropecuario, Colombia
Instituto Nacional de Investigaciones Agricolas (INIA), Mexico
International Maize and Wheat Improvement Center (CIMMYT), Mexico
International Center for Insect Physiology and Ecology (ICIPE), Kenya
International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), India
Pioneer Hi-Bred International, Inc., Johnson City, Iowa, USA

Northrup, King & Co, Eden Prairie, Minnesota, USA
USDA-ARS North Central Regional Plant Introduction Station, Ames, Iowa, USA

## *2.12 Mango*

Regional Fruit Research Station, Vengurla, Maharashtra, India
Germplasm Centre, Bangladesh Agricultural University, Mymensingh, Bangladesh
Central Institute for Subtropical Horticulture (ICAR), Lucknow, India
Central Horticulture Experiment Station, Aiginia, Bhubaneswar, Odisha, India
*Instituto de Investigaciones de Citricos y otros Frutales*, Alquízar, Artemisa province, Cuba

## *2.13 Peanut*

International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India
Texas A&M AgriLife Research Center, Stephenville, Texas, USA
North Carolina State University, North Carolina, USA
EMBRAPA- National Center for Research on Genetic Resources and Biotechnology (CENARGEN), Brasilia, Brazil

## *2.14 Peas,* **Pisum**

Biotechnology and Biological Sciences Research Council (**BBSRC**) Pisum Collections, Germplasm Resources Unit, John Innes Centre, Norwich, UK

## *2.15 Potato*

Indian Institute of Potato Research, Shimla, India
Brunswick Genetic Resources Centre, Braunschweig, Germany
Central Columbia Collection, Tibaitata, Columbia
Center for Genetic Resources, Wageningen, Netherlands
Chilean Tuberous Solanum Collection, Valdivia, Chile
Collection of Tuberous Solanum of Argentina, Balcarce, Argentina
Commonwealth Potato Collection, Pentlandfield, Scotland
Gross Lusewitz Potato Species Collection, IPK, Gatersleben, Germany
International Potato Center (CIP), Lima, Peru

USDA-ARS United States Potato Genebank, Sturgeon Bay, WI USA
N. I. Vavilov Institute of Plant Industry, St. Petersburg, Russia
Fredericton Research and Development Centre, Fredericton, New Brunswick, Canada

## *2.16 Rice*

Bangladesh Rice Research Institute
Central Rice Research Institute, India
Centro Nacional de Recursos Geneticos e Biotecnologia, Brazil
Centre de Cooperation Internationale en Recherche Agronomique pour le Developpement, France
Institute of Crop Resources Research, Jiangsu Academy of Agricultural Sciences, China
International Center for Crop Germplasm Resources, Chinese Academy of Agricultural Sciences
Institute of Crop Research, Sichuan Academy of Agricultural Sciences, China
International Rice Research Institute (IRRI), Philippines
International Institute of Tropical Agriculture (IITA), Nigeria
Malaysian Agricultural Research and Development Institute, Malaysia
Indian Institute of Rice Research, Hyderabad, India
National Institute of Agrobiological Resources, Japan
Rice Division, National Board for Plant Genetic Resources, Bangkok, Thailand
University of Kyushu, Japan
USDA-ARS Genetic Stocks – *Oryza* Collection, Stuttgart, AR USA
USDA-ARS National Small Grains Collection, Aberdeen, ID USA
West African Rice Development Association, Buoake, Ivory Coast

## *2.17 Sesame,* **Sesamum** *Species*

Oil Crops Research Institute, Chinese Academy of Agricultural Sciences (CAAS)
National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India
All India Coordinated Research Project on Oilseeds (Sesame), Nagpur, India
Oilseeds Research Programme, National Cereals Research Institute, Bida, Niger State, Nigeria

## *2.18 Sorghum,* **Sorghum** *Species*

International Crops Research Institute for the Semi-Arid Tropics (ICRISAT),

Hyderabad, India
Northrup King & Co., Eden Prairie, MN, USA
Texas A&M Sorghum Germplasm Collection, Mayaguez, Puerto Rico
USDA-ARS Plant Genetic Resources Conservation Unit, Griffin, GA USA

## *2.19 Soybean,* Glycine max

Asian Vegetable Research and Development Center (AVRDC), Tainan, Taiwan
Australian National Soybean Collection, Canberra
USDA-ARS Soybean Germplasm Collection, Urbana, IL USA
Soybean Germplasm Collection, University of Illinois, Urbana, IL USA

## *2.20 Spices (Black Pepper, Cardamom)*

Indian Institute of Spices Research, Kozhikode, India

## *2.21 Sugarcane,* Saccharum *Species*

Indian National Sugarcane Germplasm Collection, Coimbatore, India
USDA-ARS National Germplasm Repository, Miami, Florida, USA

## *2.22 Sunflower*

The North Central Regional Plant Introduction Station, Ames, Iowa, USA
Indian Institute of Oilseeds Research, Hyderabad, India

## *2.23 Sweet Potato,* Ipomoea batatas

Central Tuber Crops Research Institute, Thiruvananthapuram, India
International Potato Center (CIP), Lima, Peru
USDA-ARS Plant Genetic Resources Conservation Unit, Griffin, GA USA

## 2.24 *Tomato,* Lycopersicon *Species*

Tomato Genetics Resource Centre, Department of Plant Sciences, University of California, Davis, USA

Experimental Garden and Gene Bank, Radboud University, Nijmegen, The Netherlands

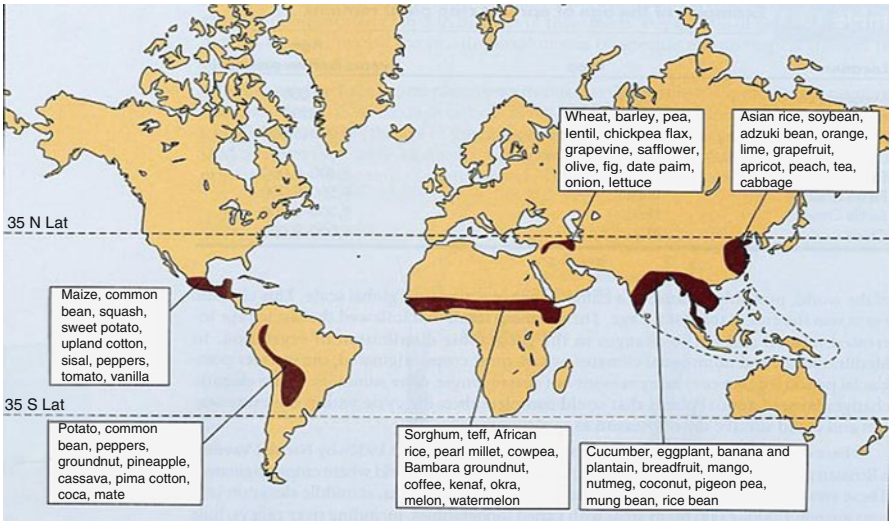
AVRDC Vegetable Genetic Resources Information System (AVRGIS), World Vegetable Centre, Taiwan

Germplasm Bank of the Agricultural Experimental Station La Consulta INTA, Argentina

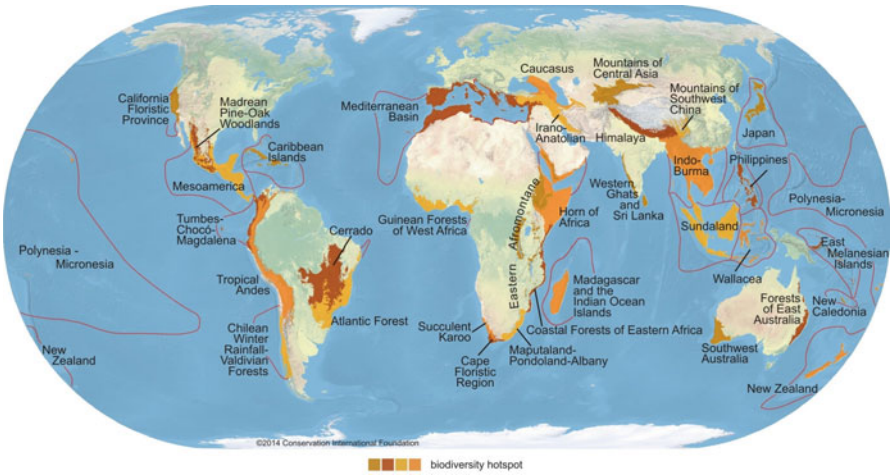
Indian Institute of Vegetable Research, Varanasi, India

## 2.25 *Wheat,* Triticum aestivum

Australian Wheat Collection, Tamworth, New South Wales, Australia




**Map 1** World map showing the centres of origin for important crops distributed in the tropics. (Source: Biosciences for Farming in Africa 2012)




**Map 2** Biodiversity hotspots in the world. (Source: Conservation International Foundation 2014)

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# Comparison of Germplasm Evaluation Parameters


**Jayalakshmi Narayan Hegde and Sandeep Singh**

**Abstract** Workers often screen crop germplasms based on one or more than one parameters. It is necessary that appropriate parameters are compared for efficacy and efficiency before the screening technique is standardized. An appropriate parameter will facilitate in locating a promising cultivar. Described in this chapter are four case studies involving insects with chewing and sucking mouthparts.

**Keywords** Germplasm · Evaluation parameters · Efficacy · Efficiency

## 1 Introduction

Screening plant germplasm is prerequisite before in-depth investigations are initiated. Selection of appropriate parameters for screening crop germplasms to insect damage is basic and important in the development of insect-resistant varieties under field conditions or polyhouse (Ramalho 1977).

**Example** Comparison of germplasm evaluation parameters in *Lablab niger* Medick to pod borer attack. The % pods and seeds damaged is used to screen cultivars to pod borer attack. Grow local bean cultivar in an area of 250 $m^2$ for the study (Chakravarthy and Lingappa 1984).

- The field is divided into quadrats of 50 $m^2$ each.
- Twenty-five inflorescences per quadrat are selected randomly at bloom and labelled.

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- Weekly observations on:

  (a) % flower and bud damage.
  (b) Number of eggs on bud and flower.
  (c) % pod damage.
  (d) % seed damage.
  (e) Numbers of eggs on pod are recorded.

  Data were taken up to pod maturity in all labelled inflorescences.

- The number of seeds per pod was always four, although seeds in very few pods were five. However, for calculating the potential weight of a seed, the number of seeds per pod was taken as four.
- On this basis, the potential weight of a seed was calculated at 0.26 g.
- Correlation coefficients were calculated between and among ten parameters, and later some of them were compared with seed weight loss (Table 1).
- The relative accuracy of parameters was judged based on the extent of positive correlation the parameter showed with seed weight loss.
- For relative efficacy, the time required for recording observations on each parameter was also recorded (Table 2).

**Table 1** The correlation coefficients between the combinations of four parameters selected for evaluation of *Lablab niger* germplasm to borer damage

| Pairs of parameters | Correlation coefficient |
|---|---|
| Number of eggs on bud and flower and on pod | |
| % flower and bud damage and % pod damage | |
| % flower and bud damage and % seed damage | |
| % flower and bud damage and number of eggs laid on pod | |
| Number of eggs on bud and flower and % pod damage | |
| Number of eggs on bud and flower and % seed damage | |
| % pod damage and % seed damage | |
| % flower and bud damage and number of eggs laid on bud and flower | |
| % pod damage and eggs on pod | |
| Eggs on pod and % seed damage | |

**Table 2** Association between the five parameters (1–5) and seed weight loss (g) to pod borers in *Lablab niger*

| Parameter | Correlation coefficient |
|---|---|
| % flower and bud damage | |
| Number of eggs laid on bud and flower | |
| % pod damage | |
| % seed damage | |
| Number of eggs on pod | |

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# Determining Relative Abundance and Distribution Patterns of Insect Pests


**T. Prabhulinga and A. D. N. T. Kumar**

**Abstract** Abundance of pests on a crop is an important criterion for timing the screening of germplasms. Screening should be aimed against more than one key pest. This will facilitate developing resistant/tolerant variety against multiple species. Determining relative abundance also indicates the distribution patterns of the target insect species on the plant. Relative abundance also aids in developing sampling plans for the key pests.

**Keywords** Relative abundance · Sampling · Distribution patterns · Timing of screening

## 1 Introduction

The relative number of a species in a habitat is an important factor in ecology especially in the applied sense. Measures of abundance, which are estimated by counting the number of individuals in a specified area, are used to reflect population level and well-being. Thus, abundance of insects has a pivotal role to play in many ecological contexts, including the limitation of species ranges and geographical distribution patterns of species. Relative species abundances are measured for a trophic level. Species occupying the same trophic level will potentially or actually compete for the same resources.

A sample of the relative abundance of pod borers of field bean (*Lablab niger*) is enumerated below.

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- *Lablab niger* plants of erect (*L. niger* var. *lignosus*) and creeping (*L. niger* var. *typicus*) types were used to determine the relative abundance and distribution patterns of the lab lab pod borer, *Adisura atkinsoni* Moore, the dominant species.
- Numbers of life stages of pod borers were made to estimate their relative abundance.
- The pattern of oviposition at weekly or 10-day intervals was also analysed.
- 'Local' (erect type) and 'EC- 36417' (creeping type, trailed on 2.5 m bamboo poles), of *Lablab niger* that are highly preferred for oviposition (Chakravarthy 1978, 1983), cultivars were chosen to study the pattern of oviposition by *A. atkinsoni* moths along the plant vertical axis.
- The plants' heights at which the borer moths laid eggs were recorded.
- The vertical distribution of *A. atkinsoni* eggs was also observed under laboratory conditions in Bengaluru.
- Field collected blooms of 'local' cultivar inserted in flasks (500 ml) containing water were placed in oviposition cages (1 $m^3$).
- Four pairs of *A. atkinsoni* moths were introduced into five cages (1 $m^3$) made of wood and wire mesh.
- Fresh blooms and 10% honey solution were changed every day for 9 days in the cage.
- Five *L. niger* fields in the study area were visited to record the spatial distribution of *A. atkinsoni* eggs and larvae.
- Fields were divided into a varying number of quadrats of 1$m^2$ each, and the recorded numbers of eggs per 100 blooms in each quadrat were maintained.
- Spatial distribution pattern of less than 10-day-old pods was simultaneously recorded in two (B and C) fields to see if egg distribution of *A. atkinsoni* closely followed the distribution of such pods on the plants.
- Pods of *Lablab niger var.* 'EC-36417' and 'local' that were collected from 100 blooms per quadrat from the two fields were split open to record the number and stage of larvae present inside the pods.
- All spatial distribution patterns were based on the sample mean and the variance. A test for departure from randomness based on the variance ($S^2$) to mean ($\bar{X}$) ratio was calculated as follows:

$$I - S^2\bar{X} = \frac{\left(X_i - \bar{X}\right)^2}{\bar{X}(\eta - 1)}$$

where $X_i$ is the number of eggs of larvae in the *i*th units in a sample. Values of $I$ greater or smaller than one indicated over- and under-dispersion, respectively. The exponent $K$ of the negative binomial distribution was estimated from samples following Southwood (1978).

- As per the method, a value of $K > 8$ indicated that the distribution is approaching a Poisson distribution; and the smaller the value of $K$, the greater the extent of aggregation. Mean size of the clump ($A.$) was calculated using Arbous and

Kerrich's (1951) formula, by which if $A. = <2$, the aggregation would seem to be due to environmental impact.

- If in the majority of the samples $K$ is smaller than $X$, the statistic $U$ could be arrived at following Anscombe (1950).

$$U = S^2 - \bar{X}\left(1 + \frac{\bar{X}}{K}\right)$$

- A positive value of $U$ indicated that the distribution is skewed more than the negative binomial distribution and a negative value less skewed than the negative binomial distribution.

## 2 Distribution Patterns, Sample Size and Sampling

The above three parameters determine the success and accuracy of experiment on population. Before conducting an experiment, the distribution pattern of insect pest needs to be determined. We have to standardize the sample size too. The selection of reliable sampling method is also crucial.

As an example, three varieties of cotton belonging to *Gossypium arboreum* and *G. hirsutum* group were raised in the field following randomized complete block design using recommended package of practices of the University of Agricultural Sciences, Bengaluru. Distribution pattern of the spotted pod borer, *Earias* spp., could be studied as under.

## 3 The Steps Are as Follows

- Spatial distribution of eggs of the borer was determined by counting in a linear fashion, eggs on top two-thirds of the plant in five rows.
- Distribution pattern of egg was based on the mean to $S^2$ ratio, and $X^2$ test was used to confirm the distribution.
- Larval spatial patterns were realized by visual counts, both in damaged fruiting parts and those that remain undamaged.
- Larvae were counted in the five rows selected (ten plants per row per variety per sowing date) one after another.
- Vertical distribution was determined by dividing plant canopy vertically into three levels, viz. top (0–20 cm), mid (21–40 cm) and bottom (41–50 cm), and counting *Earias* eggs and larvae at each level.
- Data was subjected to one-way analysis of variance to get variations in mean between levels of plant height along vertical axis.

- To determine whether insects' preference for a particular height is density-dependent, the insect counts were pooled and their relative distribution among the levels and density classes found.

## 4 Sample Size

- Sample unit sizes of 5, 6, 7, 8, 9 and 10 cotton plants of each variety were compared with a unit size of 25 plants for sampling *Earias* spp. larvae. So, a sample unit size of 25 plants (about 5% of the plant population) was treated as 'large sample'.
- Each sample size is tabulated exhibiting number of units (plants), mean $(\bar{X})$, standard deviation (SD) and standard error (SE) of a number of *Earias* larvae per plant.
- Precision of a sample size was based on SE of *x*. Sample size having the least SE relative to 'large sample' was chosen as the most precise sample and is derived as:

$$n = \left( \frac{t \propto \frac{}{2} \times S}{C\bar{X}} \right)$$

where *t*/2 is the standard deviate corresponding to the desired probability level a, *C* is constant proportion of the *x* based on half width of the $(1 - \alpha)$ confidence interval and *S* is the standard deviation.

The standard deviation (*S*) is given by: $x_i^2$

$$\mathrm{SD} = \sqrt{\frac{X_i^2 - 1/n(\sum X_i)^2}{n-1}}$$

where *n* is the number of units in the sample, standard error (SE) is given by (*S*/*n*) and *S* is standard deviation.

## 5 Number of Larvae in Damaged Fruiting Structures

- The reproductive parts, viz. buds, squares, flowers and bolls, are randomly harvested from plants to find extent of borer infestation.
- Infested fruiting structures are debracted and dissected to count the number of *Earias* larvae.
- Correlation analyses run between percentage borer infestation and larval counts to find if damaged fruiting structures sampled provided an estimate of the larval population of the *Earias* spp. (Table 1 and Fig. 1).

**Table 1** Spatial distribution of *Earias* spp. eggs and larvae on four cotton varieties

| Variety | Sowing date | Mean per plant | Variance | Distribution | | Dispersion index | $X^2$ (Chi Square) |
|---|---|---|---|---|---|---|---|
| | | | | Uniform | Clump | | |
| | | | | | | | |
| | | | | | | | |

*Note*: Mean of 50 plants per variety per sowing date. + = $mean > S^2$, – = $Mean < S^2$. Values greater or smaller than 1 indicate over- and under-dispersion, respectively

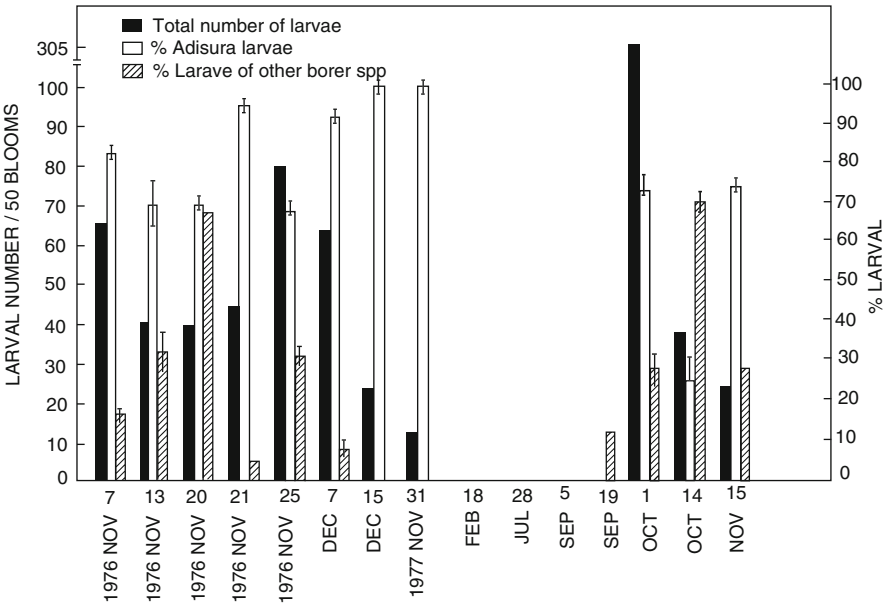


**Fig. 1** Relative abundance of pod borers on *Lablab niger*. (Source: Chakravarthy A.K 1983)

$K = \left(\frac{\bar{X}}{S^{2-\bar{X}}}\right)$, $\lambda = \left(\frac{\bar{X}}{2K}\right) \times V$, where $V$ is a function with a $X^2$ (chi square) distribution with 2 K df as per Arbous and Kerrich (1951), and when $K = 8$, the distribution is Poisson. When $\lambda = 2$, the distribution is due to environmental effect and not due to the inherent property of the insect population (Southwood 1978).

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# Mass Culturing of Phytophagous Insects for Host Plant Resistance Studies


**K. Kumar and G. Dhakshinamoorthy**

**Abstract** Studies on host plant relationships/resistance necessitate availability of insects in large numbers. Different orders of insects may require different mass culture procedures. Contamination by microorganisms and low yield of quality insects are often the impediments encountered while mass culturing. Procedures for six chewing and sucking key pests have been outlined in this chapter.

**Keywords** Mass rearing · Insect pests · Aseptic condition · Host plant relationships

## 1 Introduction

Studies on host plant resistance necessitate population of pests throughout the study period. One cannot depend on field population of test insects as it may not be homogenous. Hence, for host plant resistance studies, it is mandatory to collect and mass culture the test insects under laboratory conditions. Standard procedures and protocols have been developed over the years for key insect pests infesting cultivated crops. Mass culturing procedures for few select insects have been dealt in this chapter.

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## 2 Tobacco Caterpillar, *Spodoptera litura* (Muthukumaran and Selvanarayanan 2008)

### 2.1 Materials Required

1. Plastic buckets of 10–12 lit capacity
2. Muslin cloth
3. Conical flasks (250 ml; 500 ml)
4. Plastic basins of 30 cm diameter
5. Sterile sand
6. Black cloth
7. Adult emergence cage (0.3 $m^3$)
8. Formalin (analytical grade)
9. Sodium hypochlorite
10. Sugar or honey solution
11. Fresh Nerium leaves and castor leaves

### 2.2 Procedure

- Adult moths are collected from light traps and allowed in the mating and oviposition cage (plastic bucket) at the rate of five pairs per cage. Sugar/honey solution (10%) is provided in a cotton swab for adult feeding. The bucket is covered with black cloth.
- *Nerium* leaves are provided for egg laying. The petioles of the leaves are immersed in water kept in a small conical flask (250 ml). Egg masses laid on the *Nerium* leaves are collected every day.
- The adult moths are examined after egg laying for possible pathogen infection. If they contain pathogens, the egg masses collected from them are destroyed. If no infection is found, rearing process is continued.
- Subsequently, the egg masses are disinfected in 10% formalin for 30 min and then washed thoroughly in running water to remove formalin completely. The eggs are stored in a vial after drying under a fan for 30 min.
- The eggs may hatch in 3–4 days. The emerging larvae are transferred onto fresh castor leaves in the plastic bucket (15 lit capacity) and are covered with a cloth. The first and second instar larvae are allowed to feed in groups of 15–20 individuals.
- From third instar onwards, the larvae are reared at 25 larvae per bucket with a cloth cover.
- Adequate castor leaves are provided for feeding. After fourth instar, the larvae are reared at 15–20 larvae per bucket with a cloth cover. The full-grown larvae may

reach prepupal stage in 12–14 days. The prepupae are collected, allowed on moist sand in a basin and covered with a cloth. The pupae are collected in 2 days and are kept in an emergence cage. Healthy adults upon emergence are collected and allowed in mating cages (buckets) having black cloth covering, and adult feed (sugar honey solution) is provided. For NPV production on Spodoptera Arivudainambi and Selvanarayanan (2000) procedure may be adopted.

## 3 Tomato Fruit Worm, *Helicoverpa armigera* (Arivudainambi and Selvanarayanan 2000)

### *3.1 Materials Required*

1. Rearing container for larvae
2. Artificial diet
3. Oviposition chambers/cages
4. Cardboard
5. Muslin cloth
6. Plastic clips
7. Plastic buckets
8. Petri dishes (0.1–0.3 dia)
9. Test tubes (20 cm length; 3 cm dia)
10. Glass jars (750 ml; 1 litre capacity)
11. Sucrose solution 10%
12. Vitamin mixture
13. Rubber septa
14. Plastic containers (0.1 $m^3$, rectangular)
15. Mud pots (0.2 m dia)
16. Glass vials (screw cap)
17. Sodium hypochlorite
18. Sterile water
19. Saw dust
20. Adult emergence cage (0.3 $m^3$)

### *3.2 Artificial Diet*

Different artificial diets like chickpea-based diet and wheat germ diet have been used for rearing *H. armigera* under laboratory conditions. Chickpea-based diet is the best for *Helicoverpa* rearing (Armes et al. 1992).

## 3.3 Larval Rearing Containers

As cannibalism is the major problem in the mass culture of *H. armigera*, multicellular larval rearing tray or individual diet bottles should be used. The major disadvantage in using multicellular tray is the easy dissemination of disease, and individual bottles are difficult to handle like cleaning. So plastic containers of suitable size can be used.

## 3.4 Oviposition Chambers

Several kinds of oviposition chambers have been used for mass rearing *H. armigera* in laboratory conditions. For example, transparent plastic jars, earthen pots covered with cotton cloth, etc. are used. The major disadvantage of mud pot is the prolonged darkness that leads to unfavourable condition for the adult moth. The rearing procedure briefly is as follows:

- Larvae of *H. armigera* are gathered from host plants like tomato, cotton, millets, etc.
- The collected larvae are reared in artificial medium in seclusion in small glass vials.
- The larvae are allowed to feed continuously without any disturbance and periodically observed for pupation.
- An adult emergence cage can be fabricated as detailed below, with a modification of the oviposition cage described by Kumar and Ballal (1990). The adult emergence cage is made up of a cylindrical iron frame (40 cm high and 25 cm dia) and is fitted with a circular cardboard at 25 cm height from the base of the frame. The frame is encircled with muslin cloth using plastic clips and inserted in a plastic bucket (35 cm dia and 30 cm height) filled with $3/4^{th}$ of water. Petri dishes (6 inches dia) containing pupae are kept over the cardboard at the centre of the frame. Eight strips of cloth (15 cm $\times$ 5 cm) are hung from the top of the frame to enable the emerging moths to alight upon stretching their wings and cuticle hardening.
- Newly emerged adults are collected in 10 cm $\times$ 3 cm tubes and allowed in glass jars (14.5 cm height and 10.5 cm dia) having cloth covers at 5 pairs per jar for mating and oviposition. Little amount of 10% sucrose solution enriched with commercial vitamin mixture is provided optimally as adult diet in small rubber septa inside the mating cage. The top of the mating jar is covered with moist muslin cloth, and also strips of cloth are hung vertically inside, which serve as oviposition substrate.

- Moisten the cloth using wet sponge at periodical intervals. The egg cloths are replaced daily as well as dead or dying insects are removed. Relatively higher humidity (80–85%) and lower temperature (25 ± 2 °C) need to be maintained for both adults and eggs. The egg cloths are collected from the 2nd day after mating and preserved in small plastic containers (7 cm dia × 8 cm height).
- The egg cloths are kept in the plastic containers under 3/4 moist sand on plastic trays to increase the humidity and to lower the temperature.
- Once the neonate larvae start emerging, the egg cloths are taken out of the plastic containers and spread over the canopy of young chickpea plants (10 days old), grown already in plastic trays or mud pots.
- The young larvae are allowed to feed on chickpea up to 2nd instar and then transferred to artificial diet in small glass vials (5 cm) under seclusion, as the larvae are cannibalistic.
- Healthy pupae are gently taken out of pupation cell in the diet and surface sterilized with 1.8% solution of sodium hypochlorite followed by two rinses of sterile water.
- Then the pupae are air-dried in shade, spread over a layer of sterilized saw dust in 150 mm diameter petri dishes at 50 pupae per petri dish and are kept inside the adult emergence cage.

## 4 Diamondback Moth (DBM), *Plutella xylostella*

### *4.1 Materials Required*

- Disposable paper cups
- Mustard seeds
- Rearing cages
- Petri plates

### *4.2 The Rearing Procedure Is as Follows*

– Seedlings of mustard are used as rearing material. Mustard seeds are soaked in water for 15–24 h and treated with disinfectants like carbendazim for 30 min. 3–5 g of seeds are transferred to a small disposable cups (6.2 cm dia and 7.0 cm height).The seeds sprouted after 1 day are ready for oviposition of adults or feeding by larvae from 3rd day onwards.

– Larvae are collected from the field and allowed for feeding on mustard seedlings.
– After pupation, pupa is pooled and kept inside the rearing cage (45 × 30 cm).
– 300–500 pupae are kept in a petri plate in the cage.
– After adult emergence, the moths are fed with vitamin-enriched 10% sugar solution provided inside the cage.
– A cup containing 3-day-old mustard seedlings is kept in the cage containing 300–500 adults for oviposition. After 24 h, the seedling cup is taken out and a fresh set is kept. About 1000–1500 eggs having yellow tinges are obtained on the mustard seedling in a day. The seedlings that are deposited with eggs are exposed to natural light for 2–3 h.
– Later the larvae are maintained in the laboratory under regulated temperature and humidity for normal larval development and healthy larvae.
– The hatched larvae which feed on seedlings are provided with new set of mustard plants in the cup.
– The pupa that developed is collected by camel-hair brush and used for further multiplication.

# 5 Cotton Aphid, *Aphis gossypii*

## *5.1 Materials Required*

- Brinjal seeds
- Earthen pots
- Cylindrical iron frame
- Muslin cloth
- Polythene sheets
- Camel hair brush

## *5.2 The Aphids Can Be Reared as Follows*

– Brinjal seeds are sown in earthen pots, and one to two seedlings are maintained per pot.
– Apterous adult aphids of approximately uniform size and age are collected and introduced on the top leaves of the brinjal seedlings (25 days old).
– Each plant is covered with cylindrical iron cage encircled with muslin cloth on the sides and transparent polythene sheet on the top.
– Culture of *A. gossypii* can be maintained continuously.

# 6 Bhendi Leafhopper, *Amrasca devastans*

## *6.1 Materials Required*

- Bhendi seeds preferably of the variety "Arka Anamika"
- Earthen pots (0.2 m dia)
- Cylindrical iron frame
- Muslin cloth
- Polythene sheets
- Aspirator (glass with rubber tubing)

## *6.2 Procedure*

– Bhendi seeds are sown in earthen pots.
– Then the plants are allowed to grow and are covered with cylindrical mylar film cages.
– Leafhoppers are collected from the fields using aspirators and are released inside.
– The leafhoppers are allowed to feed continuously and multiply.

# 7 Green Leafhopper, *Nephotettix* spp., and Brown Planthopper, *Nilaparvata lugens*

## *7.1 Materials Required*

- Paddy seeds
- Cement pots/troughs
- Cylindrical iron frame
- Muslin cloth
- Polythene sheets
- Aspirator

## *7.2 Procedure*

– Paddy seedlings are sown in pots of 2 × 3 sq. ft. with closer spacing.
– Then the plants are allowed to grow and are covered with cylindrical mylar film cages.

– Green leafhoppers/brown planthoppers are collected from the fields using aspirators.
– The collected insects are released inside the cages.
– They are allowed to feed and multiply continuously.

# 8 Whiteflies, *Bemisia tabaci*

## *8.1 Materials Required*

- Brinjal or cotton seeds
- Earthen pots
- Cylindrical iron frame
- Muslin cloth
- Polythene sheets
- Camel hair brush

## *8.2 Procedure*

– Brinjal or cotton seeds can be sown in earthen pots (0.3 m dia).
– Then the plants are allowed to grow and are covered with cylindrical mylar film cages (0.3 m ht).
– Whitefly adults are collected from the fields using aspirators and are released inside, at least six pairs per pot.
– They are allowed to feed continuously and multiply. Observations on life cycle recorded.

**Illustrated Example 1:** Finger millet stem borer, *Sesamia inferens* Walker (Fig. 1)

**Illustrated Example 2: Broad-bean aphids *Aphis fabae* Scopoli and other aphids** (Fig. 2)

**Fig. 1** Techniques and materials used for mass rearing finger millet stem borer on artificial diet. (**a**) Artificial diet in plastic boxes for larval growth and development. (**b**) Plastella cups with pupae for adult emergence. (**c**) Corrugated paper strips for matured larvae to pupate. (**d**) Plastic boxes with artificial diet inoculated with neonate larvae. (**e**) Cardboard sticks wrapped with plastic films for moths to oviposit. (Source Lingappa 1978)

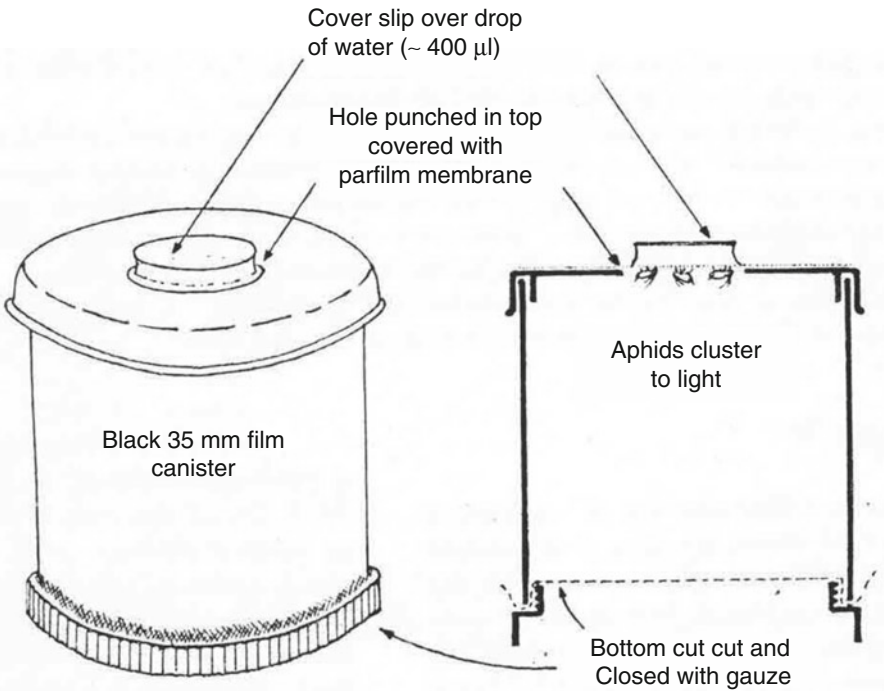


**Fig. 2** Diagram showing feeding chamber made out of black, plastic 35 mm film canister. (Source: Miles and Harrewijn 1991)

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# Part II
# Instrumentation in Host Plant Resistance Studies

# Laboratory Equipments Used for Host–Plant Resistance Studies


**K. P. Kumar and Christine Ilemut**

**Abstract** To measure response of arthropods to the host or non-host plants, sensitive and sophisticated equipments have been developed and used for understanding host–plant relationships. To unravel the intricate relationships between the plant and the insects, the use of select equipments has been provided.

**Keywords** Responses of arthropods · Characteristics of the plant · Measurement · Equipments

## 1 Introduction

To elucidate intricate and subtle interactions in host–plant relationships, a list of select important equipments and their uses is given below:

## 2 Electroantennogram

- This equipment measures the output of the antennae to the brain in response to a particular odour.
- The insect pheromones can be screened.
- Host–plant interactions/tritrophic interactions can be measured (Fig. 1).

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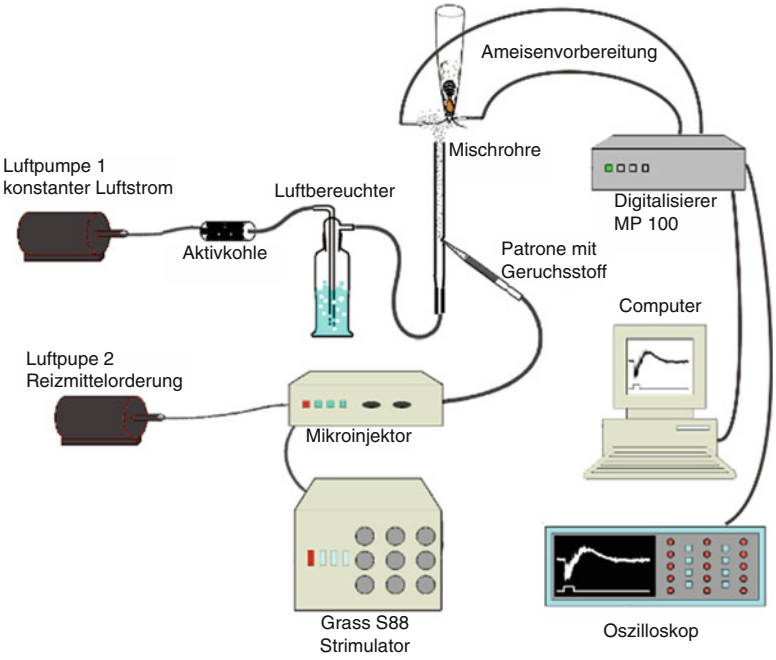


**Fig. 1** A schematic representation of electroantennogram. (Source File: G O López-Riquelme 2010)

## 3 EthoVision

- For tracking and analysis of insect movements and activity can be assessed
- Parameters: distance, velocity, turn angle, angular velocity, patterns of special movements, etc. (Fig. 2)

**Gradient Thermocycler/PCR Machine** For amplification of DNA (Fig. 3).

**DNA Electrophoresis Units** Resolution of DNA molecules and PCR-amplified DNA bands by horizontal electrophoresis (Fig. 4).

**UV Transilluminator** To observe and interpret ethidium bromide-stained agarose gels.

**Programmable Refrigerated Microcentrifuge** Centrifugation of samples in microvolumes and at controlled temperature. Temperatures ranging from 0 to 40 °C can be maintained.

**Programmable High-Speed Refrigerated Centrifuge** To centrifuge different samples at controlled speed and temperature. Speed: up to 30,000 rpm.

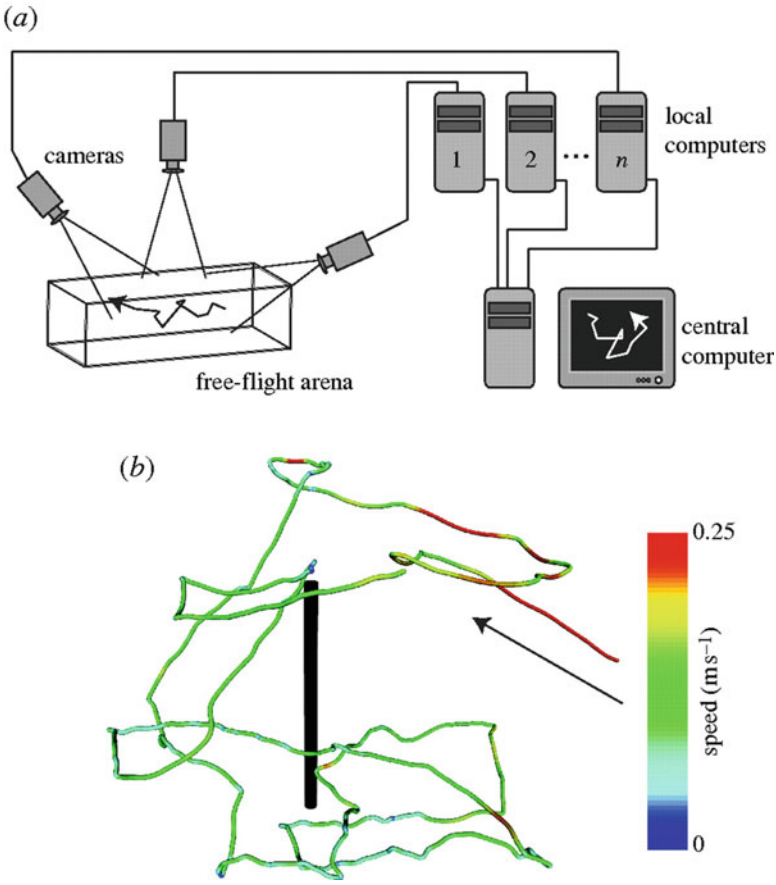


**Fig. 2** (**a**) Schematic of the multi-camera tracking system. (**b**) A trajectory of a fly (*Drosophila melanogaster*) near a dark, vertical post. Arrow indicates direction of flight at onset of tracking. (Source: Straw et al. 2010)

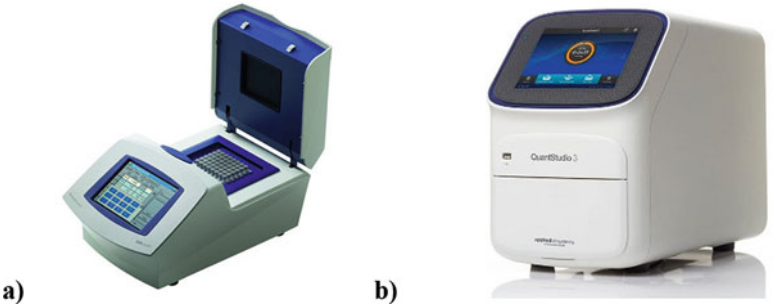


**Fig. 3** (**a**) Thermocycler PCR and (**b**) real-time PCR. (Source: Labgear International and Biocompare.com)

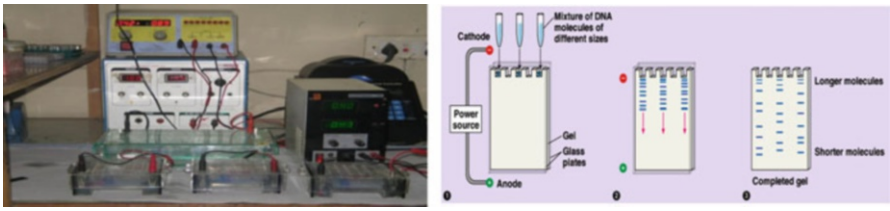


**Fig. 4** Instruments used for running gel electrophoresis. (Source: Pearson Education publishing as Benjamin Cummings)

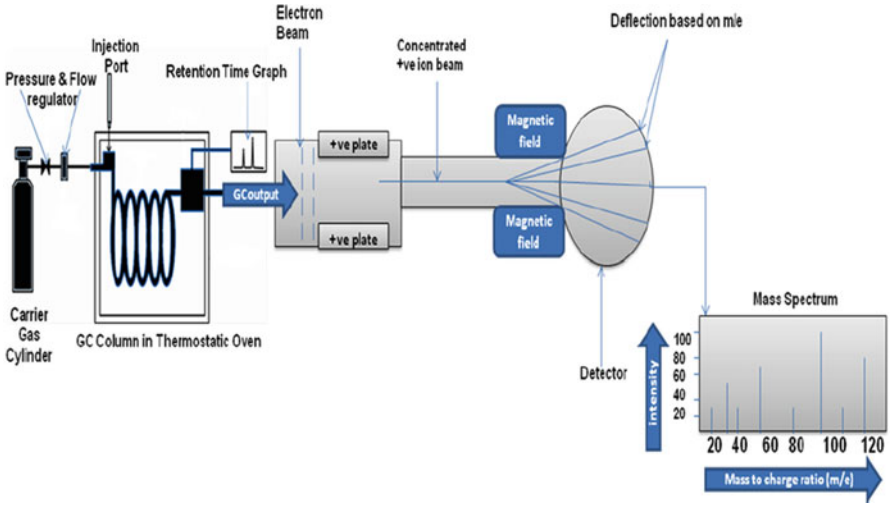


**Fig. 5** Schematic diagram of GC–MS setup. (Source: Kumar et al. 2015)

**UV-Vis Spectrophotometer** Quantitative estimation of biomolecules under visible and UV light (190–900 nm).

**High-Performance Liquid Chromatograph (HPLC)** This equipment has the unique ability to separate and quantify the residues of polar, nonvolatile and thermolabile chemical compounds. This instrumental setup can also be used for alkaloid metabolite extraction and analysis from plant and animal sources.

**Gas Chromatograph–Mass Spectrometer (GC–MS)** Mass spectrometry is a powerful analytical technique used to identify unknown compounds, quantify known compounds and elucidate structural and chemical properties of molecules based on molecular weight. This equipment has now almost become indispensable for studies related to host–plant relationships (Fig. 5).

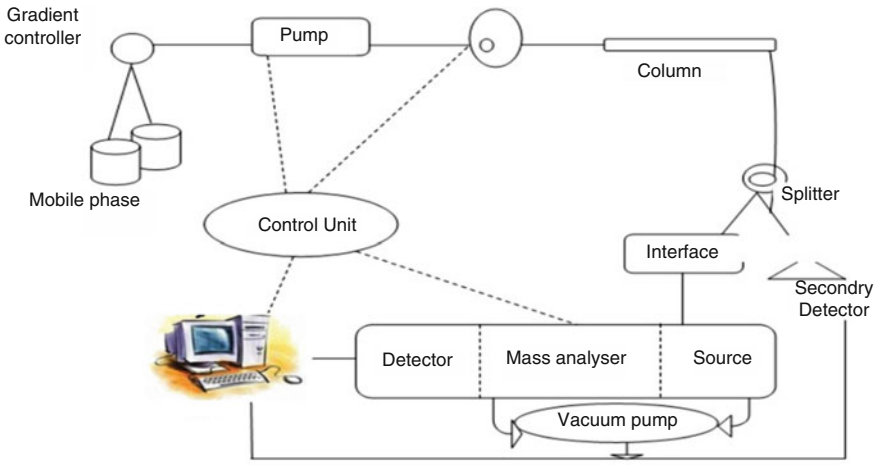


**Fig. 6** Schematic diagram of LC–MS setup. (Source: Parasuraman et al. 2014)

**Liquid Chromatography–Mass Spectrometry (LC–MS)** Liquid chromatography–mass spectrometry is an analytical technique combining the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS). Currently coupled chromatography–MS systems are being commonly used in chemical analysis because the individual strength of each technique is enhanced synergistically. While liquid chromatography separates mixtures with multiple components, mass spectrometry facilitates structural identity of the individual components with high molecular specificity. Therefore, LC–MS has tremendous potential in understanding molecules and their chemistry that mediate between the plants and phytophagous insects (Fig. 6).

**Plant Growth Chambers** This too helps in raising the insect culture under controlled temperature and RH, and the culture of insect can also be maintained with this tool.

**Leaf Area Metre** To estimate the leaf area consumed by an insect or arthropod.

**Stereo Zoom Microscope** For recording major morphological characters of different stages of plants and insect pests and also for population counts, this microscope can be used.

**Phase Contrast Microscope** For studying minute detailed characters of plants and insects, this microscope is very helpful.

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# Electroantennogram for Recording Olfactory Responses of an Insect to Plant Volatiles


**T. N. Madhu and B. Doddabasappa**

**Abstract** The electroantennogram (EAG) technique for recording olfactory responses of an insect to plant volatiles has been described in this chapter. The procedure is useful in detecting the plant chemicals to which the insects respond positively or negatively.

**Keywords** Electroantennogram · Neurology · Plant volatiles · Chemical profiles

## 1 Introduction

The electroantennogram (EAG) is used as a bioassay technique to establish the potential of plant volatiles in olfactory stimulation of an insect. The EAG records the change in the potential for olfaction between the tip of an antenna and its base in response to stimulation by a plant odor.

1. The test insect is anesthetized with carbon dioxide and its head is incised.
2. The antenna of the insect is inserted into the tip of a glass pipette.
3. The EAG readings are recorded with glass capillary electrodes filled with electrolyte (NaCl 3.75 g/KCl 0.175 g) (see Fig. 1).
4. The indifferent electrode (IE) is placed in the pedicellus, and the recording electrode (RE) is pierced through the ventral tip of the funiculus.
5. The IE is inserted into the occipital opening and through the scapus into the pedicellus. With this setup, movement artifacts are eliminated, and desiccation is prevented, and the dissected antenna has an effective lifespan of 1–2 h.
   (Note: The electrodes are connected via Ag-AgCl (chlorinated silver wire) to the recording instruments).

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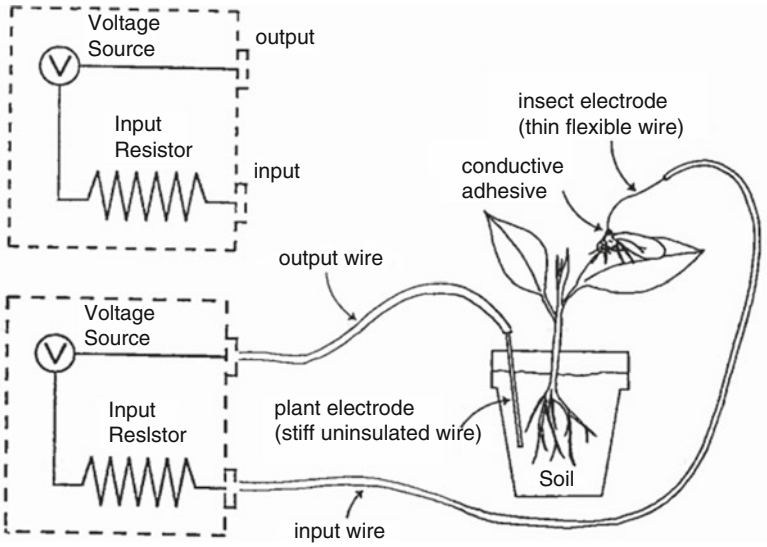


**Fig. 1** Electric feeding monitor system for sap-sucking insects. (Source: Walker and Janssen 2000)

6. The continuous flow of charcoal-filtered air is maintained at a flow rate of 80 cm $S^{-1}$ from a glass tube positioned within 5 mm of the antennal preparation in the odor delivery system and response evaluation system.
7. The test chemicals are dissolved in paraffin oil. Each solution (25 ml) is pipetted onto a piece of filter paper placed in a Pasteur pipette attached to a syringe.
8. The tip of the pipette is inserted through a hole in the glass tube, and the syringe plunger is depressed to pass 1 ml of air through the pipette into the air stream. The duration of the pulse is 1 s.
9. The antenna is stimulated at 2 min intervals with each test chemical followed and preceded with a standard and control.
10. The amplitude of the response to the test compound is expressed as a percentage of the mean of the two adjacent standard response amplitudes. The differences in volatility between the test compounds are relative.

**Additional Information** The antennal olfactory receptor system in certain phytophagous insects is sensitive for the detection of odor components of green plants and shows different sensory sensitivity for individual odor components. In rice, volatiles extracted as steam distillates from rice plants significantly affect the behavior and biology of the brown planthopper. The volatiles of resistant and susceptible rice varieties act as feeding deterrents and attractants, respectively. In a multi-choice test conducted inside plastic cages, more females settled and fed on tillers of susceptible rice than on the resistant variety.

**Illustrated Example 1:** Attraction of frit fly *Oscinella frit* to oats (Fig. 2)

**Illustrated Example 2:** Adult spittlebug feeding on an intact leaf blade and on roots of *Brachiaria* sp. (Fig. 3)

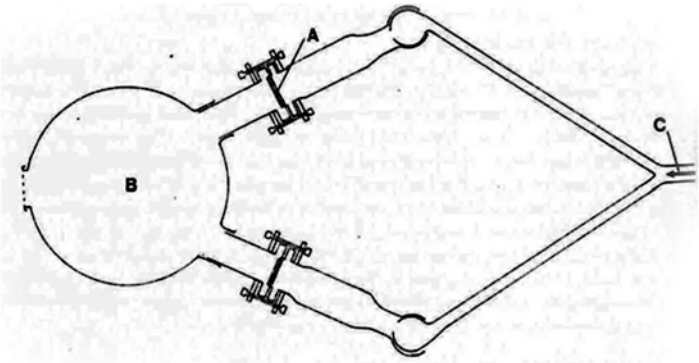


**Fig. 2** Glass Y-tube olfactometer: A, metal sieve plate of Micropore filter holder; B, glass sphere (1 liter) containing flies; C, direction of airflow. (Source: Hamilton et al. 1978)

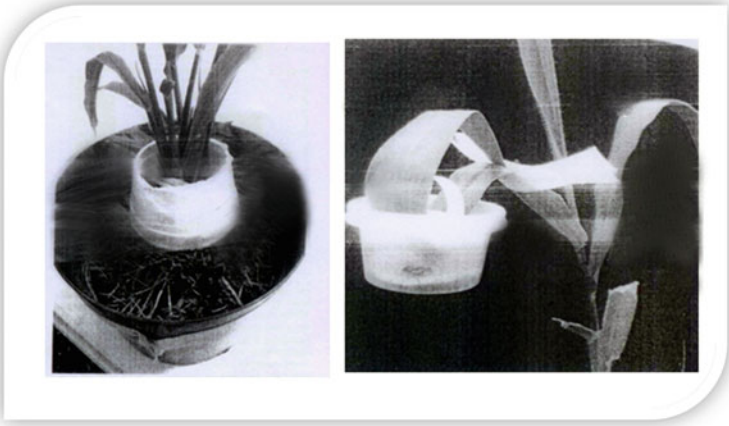

**Fig. 3** (**a**) Adult spittlebug feeding on an intact living leaf blade. (**b**) Adult spittlebug feeding on roots of *Brachiaria* sp. (Source: Lina M. Aguirre et al. 2012)

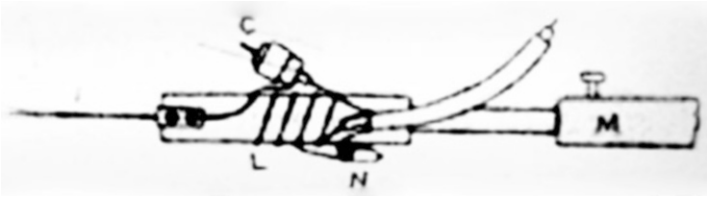


Figure to show radio frequency probe used to cut mouth parts of aphids. $C$ = 3–30 pF concentric capacitor; $L$ = copper inductor coil; $N$ = neon tuning indicator; $M$ = micromanipulator, with a tungsten needle at front

**Illustrated Example 3:** A tool to incise mouth parts of aphids (Source: Downing and Unwin 1977)

**Acknowledgment** The authors would like to thank authorities of Central Institute for Cotton Research, Maharashtra, and College of Horticulture, Kolar, for their encouragement and support.

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# Standardization of Feeding Tests in Phytophagous Insects to Host and Nonhost Plants


**G. Basanagouda and K. R. M. Bhanu**

**Abstract** To distinguish host or nonhost plant of a phytophagous insect, it is important that a standardized procedure is in place. Disc method used for chewing and biting insects is described in this chapter. The statistical methods that can be employed are also mentioned.

**Keywords** Host plant · Nonhost plant · Leaf-disc method · Feeding stimulants

## 1 Introduction

Herbivorous insects exhibit specific behavioural responses to host and nonhost plants. All plants contacted elicit behaviour from phytophagous insects: some are rejected, some accepted. But this occurs in a series of sequential events. Although host plants are in the latter category, they are not all equally preferred. Furthermore, some nonhosts are acceptable as well. This raises the question as to where the boundary should be drawn between host and nonhost plants (Bernays Elizabeth and Chapman Reginald 2007). Probably the best answer is the ecological one: host plants are those on which the insect completes normal growth and development in nature. The insects' physiological host range, however, includes some nonhost plants, because many of these elicit feeding and are nutritionally adequate. It follows that screening by the sensory and central nervous systems must be sufficiently, broadly tuned to accept a wider group of plants naturally found. Perhaps a continuum of preferences exists, with rejected nonhosts located beyond some hypothetical threshold of acceptability. This threshold is poorly defined and fluctuates with environmental conditions and prior feeding experience.

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Assessing the feeding capacity or feeding rate on plant in quarantine terms is necessary for finding feeding preferences by insects on plants. Quantitative measurement of feeding is done by disc test, which allows quantitative comparisons of preference for different plant species (using leaf discs) or chemicals (using filter paper discs) Visser (1986). This test is demonstrated by Jermy et al. in 1968. By one plant or a control chemical common, the preferences of many plant species or chemicals can be compared quantitatively for herbivorous insects.

Evidence that all host plants are not equally preferred has been obtained using this method. For example, Jermy et al. (1968) experimented on the food preference of tobacco hornworm in *Manduca sexta*, a solanaceous feeder in nature but it also can feed on other plants when deprived of taste organs. The corn earworm (*Heliothis zea*) is a polyphagous feeding on several plants. Both species of larvae were raised on laboratory on artificial diet. Seven plants were used in the experiment. The food selection behaviour of larvae was examined by leaf disc test (Jermy 1961, 1987). Leaf discs, 17 mm in diameter, were punched from fresh leaves of each plant species selected for study. The leaf discs were fixed on a pin between two pieces of celluloid film (2 × 2 mm, 0.2 mm in thickness) at such a distance from the tip of the pin that when stuck into the paraffin substrate, the leaf discs were held horizontally 5 mm above it. The larvae moving on the substrate easily reached the disc and initiate feeding on the leaf discs. Paper food container cups (9.5 cm in diameter and 8 cm high) with a 1–1.5 cm thick paraffin layer on the bottom were used for the tests. To maintain humidity, a moist sheet of filter paper was placed on the wax layer, and the cup was covered. Wire screening placed on the filter paper protected it from being eaten by the larvae. Four discs punched out from the leaves of each of the three plant species which were to be tested (i.e. 12 for each cup) were arranged on supporting pins in ABCABC fashion around the edge of the cup (Fig. 1). The arrangement of disc was so arranged that it insured a larva leaving one disc would encounter both other plants before again coming to the first plant. Furthermore, if a larva situated on a disc from plant A went into its characteristic searching motion, it would encounter a disc from B or C with equal probability. Quantification was done by recording the time when the consumption of any of the three plant species first reached 50% of the total disc area of the plant in a given individual test. This particular time was designated as $T_{50}$. This method of recording ensures that a caterpillar has completely consumed at least two of the four discs (50%) and has encountered both other plants. The workers also incorporated a correction factor for leaves unequal thickness.

The results are summarized in Fig. 2. There were no significant differences in preferences among the three plants by the diet-fed and the *Nicotiana*-fed larvae. This indicates that *Lycopersicon*, *Nicotiana* and *Solanum* plants are equally acceptable to these animals; however, preferences were modified significantly in the other two groups of larvae in the direction of preferring the plants on which they were raised.

In Fig. 3, young second instar larvae were placed individually in small plastic cups on pieces of *Pelargonium* and *Taraxacum* leaves and were reared until the last instar. The sixth instar larvae were tested individually using *Pelargonium*, *Taraxacum* and *Canna* leaf discs. The acceptability of *Canna* by these larvae is normally quite low, and it was included in the preference test to determine whether

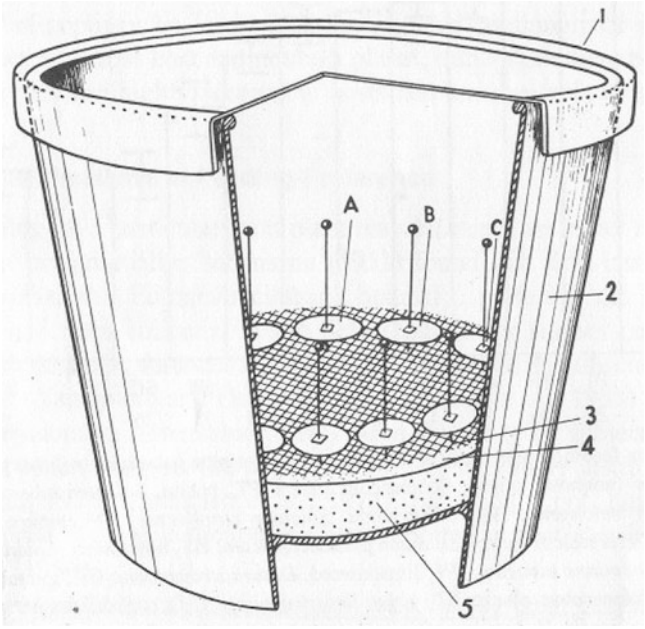


**Fig. 1** The disc test. Discs cut with a cork borer from leaves of species A, B and C are mounted on pins and held by small acetate squares 1 cm above the wax substrate. 1, cover; 2, plastic or paper cup; 3, wire screen; 4, moist filter paper; 5, paraffin wax layer. The caterpillar is placed in the centre; feeding is scored as area of disc eaten. (Source: Jermy et al. 1968)

or not exposure to the other two plants also produced changes in the acceptability of *Canna*.

The data, summarized in Fig. 4, clearly demonstrated that even though larvae were exposed to these nonhost plants for as long as four instars, acceptability did not increase.

Stadler and Hanson (1978) modified the method of Jermy et al. (1968) for conducting studies on induction of preference for artificial diets in the tobacco hornworm, *M. sexta*. A 0.1 ml aliquot of the extracts was applied to the filter paper discs. This corresponds to the extract of 80 mg of fresh leaves or about twice the weight of a leaf disc of the same size. Pure chemicals were dissolved in the appropriate solvent, and 0.1 ml was applied per disc. Controls were of solvent alone. After evaporation of the organic solvents, 0.1 ml of distilled water was used for wetting the discs. Four discs of each of two treatments and one control were mounted on pins arranged in an alternating fashion near the circumference of the test arena, which was a plastic container (diameter 11 cm, height 9 cm) with a layer of paraffin wax (1 cm thick) on the bottom. Some tests were performed with discs of the diet 1 mm thick, using 2% agar discs as controls. Other conditions for the experimental set-up were maintained by Jermy et al. (1968).

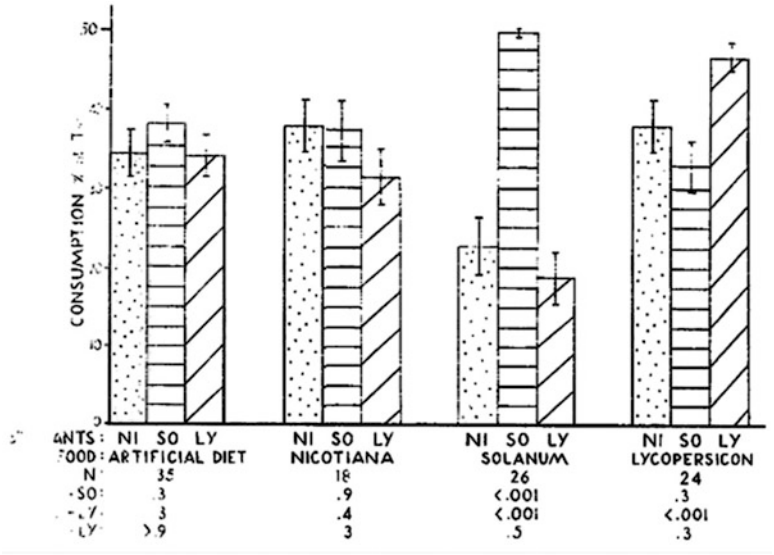


**Fig. 2** Food preference of *Manduca* larvae reared on artificial diet or on host plants. The bars at the top of the first column signify the standard error. Abbreviation in Figs. 3, 4 and 5. P, probability level; N, number of replicates; NI, *Nicotiana*; SO, *Solanum*; LY, *Lycopersicon*; PE, *Pelargonium*; TA, *Taraxacum*; CA, *Canna*; BR, *Brassica*. (Source: Jermy et al. 1968)

**Illustrated Example 1:** Feeding preference in flea beetle, *Altica lythri* Aube (Phillips 1977)

Stadler and Hanson (1978) conducted studies on food discrimination in *Manduca sexta*. In these experiments, field-collected animals showed the same preferences as did animals cultured in the laboratory for many generations. Rearing larvae on leaf species, an artificial diet or homogenized leaves added to artificial diet induced a preference for that food in subsequent choice tests. Extracts of these foods using organic solvents elicited feeding choices resembling those evoked by the food themselves. Water extracts were effective as stimulants or deterrents, but responses to them differed considerably from responses to the foods.

Thus induction of preference was shown to be influenced by a specific nutrient compound: 15 artificial diets were tested; 3 were successful, including a completely defined medium. Various components of the diets were tested for feeding preferences, both as omissions from the main diet and as pure compounds. Some were stimulatory; most were neutral or slightly deterrent; a few were strongly deterrent. The data suggest that food discrimination depends on the perception of a complex chemical message comprised of both polar and non-polar compounds, with the latter being of somewhat greater importance.

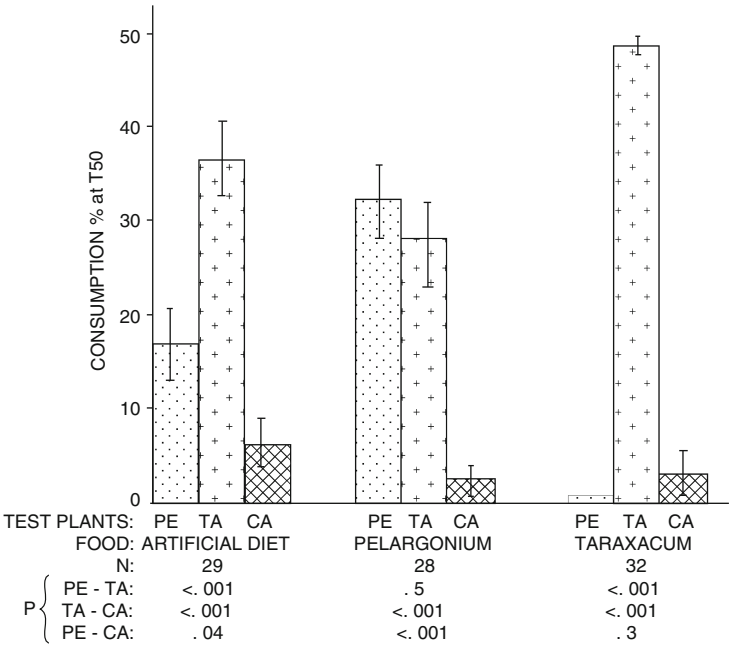


**Fig. 3** Food preference of *Heliothis* larvae reared on artificial diet is on host plants. For details refer to Fig. 2. (Source: Jermy et al. 1968)

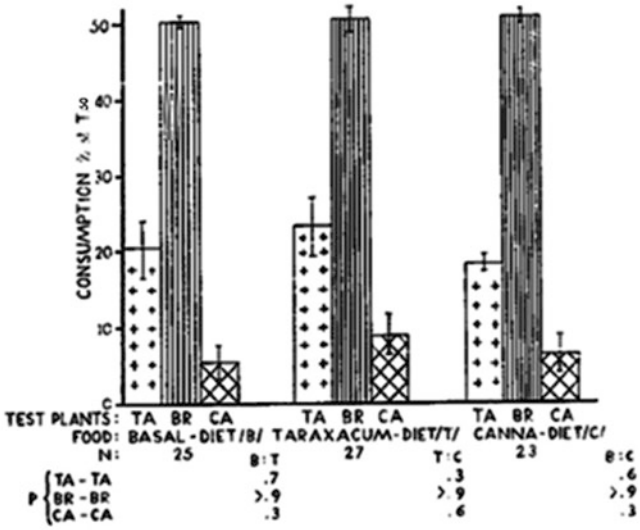


**Fig. 4** Food preference of *Manduca* larvae reared on artificial diet or on diet mixed with *Taraxacum* or *Canna* leaves. Refer to Fig. 2 for details. (Source: Jermy et al. 1968)

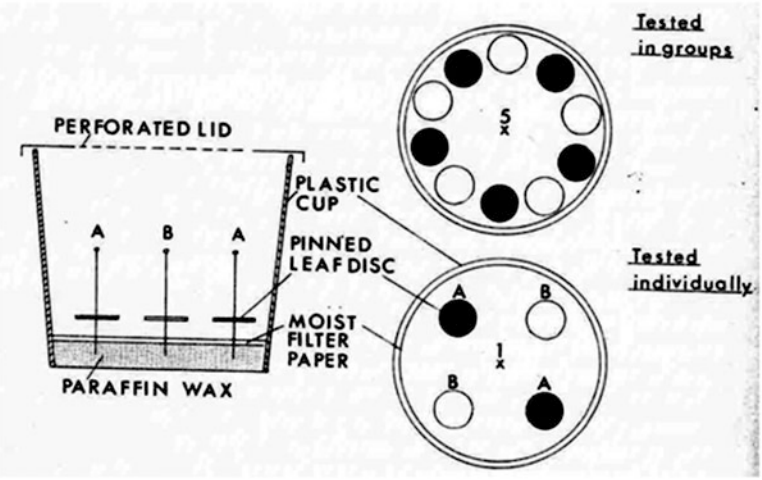


**Fig. 5** Arrangement of 1 cm leaf discs in 5–6 h preference tests. Adults and larvae were starved for 24 h prior to testing and placed in the centre of the plastic cup at the start of the test. (Source: Phillips 1977)

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# Wind Tunnel: A Tool to Test the Flight Response of Insects to Semiochemicals


**S. Subhash and P. R. Shashank**

**Abstract** Currently, chemical communication among insects is being increasingly exploited for pest management. In chemical communication, plant volatiles play a key role. A detailed description of olfactometer and wind tunnel is furnished in this chapter, suitable for pyralid moths. The data can be subjected to ANOVA. For confirmatory results, further tests need to be conducted. Wind tunnel results are indicative and detect the presence of chemicals involved in the communication between two individual insects, usually of opposite sex.

**Keywords** Chemical communication · Pheromones · Wind tunnel · Laboratory test

## 1 Introduction

Semiochemicals are chemical substances or mixtures that carry a message within or between species. It is usually used in the field of chemical ecology embracing pheromones, allomones, kairomones, attractants, and repellents. Insect pheromones are the main research target in the semiochemicals, because of potential practical application in agriculture. A wind tunnel is an olfactometer used for bioassay of olfactory stimuli. Wind tunnel tests have been widely used in insect pheromone research to study plant volatiles such as kairomones and synomones (Baker and Linn 1984; Kainoh et al. 1980; Ichiki et al. 2011).

Two types of wind tunnels are used in entomological research: cylindrical and rectangular types. A majority of the studies on pheromone and plant volatiles use cylindrical tunnel because the sex pheromone or volatile compound sample is hung

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from the ceiling of the tunnel and do not vary the concentration even at the corners of the tunnel. Rectangular tunnel is used to study the responses of parasitoids to plant volatiles.

Sabelis and van de Baan (1983) used a Y-tube olfactometer and determined that predacious mites responded to the odors of plants infested with spider mites. This was the first demonstration of a tri-trophic interaction in which predators or parasitoids are attracted to plants infested with herbivore prey. Studies on the effects of plant volatiles on the behaviors of natural enemies were conducted with olfactometers and wind tunnels as indicated by Van Driesche and Bellows (1996).

## *1.1 Structure of Wind Tunnel (Kainoh 2011)*

- A laboratory with a ventilation system is ideal for setting up a wind tunnel.
- Temperature may be maintained at 25 °C (temperature can be controlled by adjusting the air-conditioning system and during the winter to increase the room temperature to 25 °C by electric heater).
- Electrode steam humidifier fixed on the wall of the tunnel is used to maintain humidity greater than 50–60% R.H. This humidifier is used even in midwinter when the outdoor temperature is below 0 °C. Insects do not respond well below 50% R.H.

**Cylindrical or Rectangular** An ideal air current is produced with a cylindrical wind tunnel rather than a rectangular one because air currents are retarded at the corners of a rectangular tunnel. If insects fly into the corner of a tunnel, they may perceive lower concentrations of the odor coming from the upwind end.

**Pulling-Air and Pushing-Air Type** One type of tunnel is the pulling-air type, and another is the pushing-air type. Pushing-air type tunnels do not disturb the plume. Opening the window for insect handling does not disturb the airstream in the pushing-air type tunnel. Therefore, insects on the releasing platform directly perceive the odor immediately after being released without any disturbance in the airstream. In such experiments, a laminar airstream of incense smoke is observed even with the windows open. In the pulling-air type wind tunnel, insects on the releasing platform perceive disturbed air movement when released, but the air current gradually becomes normal after the window is closed. In addition, air should not leak from the tunnel wall, and all windows must be tightly closed.

One disadvantage of a pushing-air type wind tunnel is lack of even laminar flow inside the tunnel. Care must be taken to maintain a balance of wind pressure in both the pushing-fan side and exhaust-fan side. A stable laminar flow cannot be achieved unless there is a good balance in both the inlet and outlet of the tunnel.

**Air Movement (Wind Speed)** Wind speed is an important factor in wind tunnel experiments. Most studies in the literature use a wind speed of 25 to 30 cm/sec. To measure the wind speed in the tunnel, an anemometer is fixed at the upper wall of the tunnel.

**Charcoal Filter** Charcoal filter was attached to the wind tunnel between the centrifugal blower and the wind tunnel. The filter consists of six charcoal filter panels installed in a zigzag pattern; each panel (30 × 50 cm, 3 cm thick) is filled with charcoal particles.

**Honeycomb Structure (or Mesh)** Turbulence in the airstream must be controlled for insect flight in a wind tunnel. A honeycomb structure fixed at the upwind end can facilitate laminar flow of the air.

**Light Source** In wind tunnel tests with sex pheromones of nocturnal moths, light intensity is a significant parameter. In total darkness, there was no attraction of the moths, but during light condition with intensity, attraction was noticed. In a light condition at 3.5 *lx*, the attraction was 38%, and male catches were not stable. For diurnal insects, wind tunnel experiments for *Aphidius colemani* Vierek used a light intensity of 150 *lx*, because female *A. colemani* did not show good orientation toward the odor source (herbivore-damaged plant) under lighter conditions and flew upward to the ceiling of the tunnel at 2000 *lx*. However, the tachinid fly *Exorista japonica* (Townsend) readily flew to the target plant under full-light conditions (>2000 *lx*) (Ichiki et al. 2011; Hanyu et al. 2009).

Six tubes of 40 W each can maintain light conditions similar to sunlight, and the light intensity can be changed with a voltage converter from 0 to 6000 *lx*. Under the ceiling panel, a plastic light diffuser was placed to scatter the light throughout the chamber.

**Visual Ground Patterns** Flying nocturnal moths watch ground patterns when orientating to female sex pheromones as demonstrated by a moving-floor wind tunnel. Using this type of moving-floor wind tunnel, the flight speed of the moths is regulated and sustained flight experiments performed. Optomotor anemotaxis is the term used to explain the behavior of male moths orientating to female moths, in which they visually monitor progress and react to this feedback. There are several ways to show moving patterns to insects. In many laboratories, a movable floor pattern to the wind tunnel is not added because the system is too costly. Instead, green and ochre color strips (15 cm wide) are placed to represent the soil and plants.

**Data Recording** The software "The Observer (ver. 5)" (Noldus Information Technology, Wageningen, the Netherlands) is used to record the behavior of insects in a wind tunnel (Hanyu et al. 2009).

Each behavioral event (walking, flying, stationary, or grooming) and location (release site, floor, wall, ceiling, target) can also be recorded, and then the duration; average time on the release site (latency); total time flying in the wind tunnel; total time walking on the floor, wall, or ceiling; and other parameters are calculated from these recordings.

The use of video camera to record the flight of insects is gaining prominence. The camera is placed at the downwind end, so one can record all behavioral events from the releasing site to the target. To record the behavior of small insects, two cameras must be set in the tunnel, one near the releasing site and another near the target site (Fig. 1 and Table 1).

**Fig. 1** An observer recording the behavior of the insects using wind tunnel

**Table 1** Visual observation can also be recorded for some insects like moths; the following table can be used to record behavioral observations of pyralid moths

Treatment code:
Date:
Age of the male:
Observed by:
Experiment starting time:
Experiment closing time:

| Moths | Behavioral categories | | | | | |
|---|---|---|---|---|---|---|
| | Starting flight | Catching plume (male moth flying upwind along the plume) | Close to source (hovering around within 10 cm of the test compound) | Source contact | Duration of remaining close to source | Other behavioral observations |
| 1 | | | | | | |
| 2 | | | | | | |
| 3 | | | | | | |
| 4 | | | | | | |
| 5 | | | | | | |
| 6 | | | | | | |
| % Response | | | | | | |

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# Part III
# Techniques in Host Plant Resistance Studies

# Techniques for Determining the Brown Plant Hopper *Nilaparvata lugens* (Stål) Resistance in Rice in Vietnam


**Huynh Thanh Loc and Nguyen Van Hoa**

**Abstract** In Vietnam select rice germplasm was screened against brown plant hopper adopting resistant level index (IRRI 1996) and standard seedling box test (IRRI 1996). MTL649 and OM 10043 rice varieties not only gave higher yields but also possessed both bph4 and bph18 genes. Of 34 rice varieties evaluated using molecular markers RG457 and RM190, 5 exhibited BPH resistance that are heterozygous genotypes with the band size varying from 200 to 600 bp.

**Keywords** Brown plant hopper · Resistance · Genes · Molecular markers · Vietnam

## 1 Introduction

Rice production in Vietnam in the Mekong and Red River deltas is important for the food supply in the country and national economy. Vietnam is one of world's richest agricultural regions and is the second largest (after Thailand) exporter worldwide and the world's seventh largest consumer of rice. Vietnam's land area of 33 million ha has three ecosystems that dictate rice culture. These are the southern delta (with its Mekong Delta dominating rice coverage), the northern delta (the tropical monsoon area with cold winters) and the highlands of the north (with upland rice varieties).

The brown plant hopper (BPH), *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae), is a plant hopper species that feeds on rice plants (*Oryza sativa* L.). BPH is among the most important pests of rice, and rice is the major staple crop for about half the world's population. In the context of rapid industrialization and urbanization, rice production in Vietnam has achieved a considerable progress. However, the change of ecological system with an emphasis of using widely Chinese varieties (especially hybrid rice varieties), using numerous crops per year, high seed rate pesticides and nitrogen application in the South of Vietnam are the reasons leading to the change of the situation of rice plant hoppers and virus diseases.

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**Fig. 1** Adult brown plant hopper (BPH), *Nilaparvata lugens*, on rice

**Fig. 2** Population of brown plant hopper (BPH), *Nilaparvata lugens*, on rice

Prior to the 1970s, BPH outbreaks had been largely confined to northeast Asia (Korea, Japan, China). However, with intensification of rice production during the Green Revolution of the 1970s and 1980s, plant hoppers became a major threat to rice productivity in tropical Asia and the South Pacific (Sogawa 1982). In response to plant hopper damage at that time, the International Rice Research Institute (IRRI) evolved a member of varieties with BPH resistance (Khush 1979; Brar et al. 2009).

Since the early 1990s, plant hopper populations had been relatively low and outbreaks generally rare. However, since about 2002, outbreaks have reached an unprecedented scale and frequency throughout tropical and subtropical Asia. Hopper densities have surpassed those experienced at the height of the Green Revolution, and major losses have occurred in China, Thailand and Vietnam (Catindig et al. 2009; Cheng 2009). Several National Agricultural Research and Extension Systems (NARES) are again looking to host plant resistance as a possible solution to these outbreaks (Brar et al. 2009). However, although a number of resistant varieties are currently deployed in the field, a systematic understanding of the genetics of resistance among these varieties is often lacking (Figs. 1 and 2).

## 2 Case Studies

### 1. Study of Growth Characteristics, Yield and Brown Plant Hopper (*Nilaparvata lugens* Stål) Resistant Capacity of the Rice Varieties in Thừa Thiên-Huế

Pham Thi Thanh Mai et al. (2012) used the brown plant hopper (BPH)-resistant rice varieties supplied by the Plant Resources Center, Science Institute (PRCSI) of Agronomy, Hanoi. The rice varieties were cultivated and studied in summer-autumn crop of Thừa Thiên-Huế to determine their growth characteristics and yield. Simultaneously, they also conducted BPH artificial infection to initially assess their adaptation to conditions in local planting and their resistant capacity to BPH populations. Results showed that IRRI 352, BG 367-2, SaiDuongKienAn and LocNuoc grew well, developed and resisted the BPH populations of Thừa Thiên-Huế. IRRI 352, BG 367-2, Sai An Kien An and LocNuoc were the four short duration and high-yielding rice varieties, with morphological features comparable to natural conditions in ThừaThiên-Huế. These four rice varieties are the important materials for growing and regenerating the BPH-resistant rice with higher yields (Tables 1, 2 and 3).

**Table 1** Resistant level of rice varieties to PBH in Thừa Thiên-Huế Province under in vitro conditions

| Sl. No | Varieties | 5 days after treatment | | 7 days after treatment | |
|---|---|---|---|---|---|
| | | Resistant level | Description | Resistant level | Description |
| 1 | IRRI352 | $2.5^{cd}$ | K | $4.2^{de}$ | KV |
| 2 | BG367-2 | $2.2^{de}$ | K | $4.3^{de}$ | KV |
| 3 | KhauHangNieu | $2.7^{bcd}$ | K | $4.8^{cd}$ | NV |
| 4 | KhauSet | $2.7^{bcd}$ | K | $4.2^{ed}$ | KV |
| 5 | KhauNieuKenTap | $2.2^{de}$ | K | $6.2^{b}$ | N |
| 6 | KhauVan | $3.2^{b}$ | KV | $4.5^{cde}$ | KV |
| 7 | KhauPang | $2.2^{de}$ | K | $3.8^{e}$ | KV |
| 8 | SaiDuongKienAn | $2.0^{de}$ | K | $3.7^{e}$ | KV |
| 9 | LocNuoc | $1.8^{e}$ | K | $3.8^{e}$ | KV |
| 10 | HaiHoanhLun | $2.8^{bc}$ | K | $5.3^{c}$ | NV |
| 11 | TN1(infected variety control) | $5.5^{a}$ | NV | $8.3^{a}$ | NN |

*K* resistant, *KV* slightly resistant, *NN* heavily infected, *N* infected, *NV* slightly infected; (**): Difference is significant at 1%; Means followed by same alphabetical superscript are statistically on parat ($P < 0{,}05$)

***Resistant level index*** (IRRI 1996)

*<1: Highly resistant*
*1.0–3.0: Resistant*
*3.1–4.5: Slightly resistant*
*4.6–5.5: Slightly infected*
*5.6–7.0: Infected*
*7.1–9.0: Heavily infected*

**Table 2** Morphological indicators of rice varieties studied

| Sl. No | Varieties | Duration of life (days) | Germination ratio (%) | Height of tree (cm) | The ability to branch out | Length of flower (cm) |
|---|---|---|---|---|---|---|
| 1 | Khang Dan | 96 | $97.67^{a}$ | $103.67^{cd}$ | $8.00^{a}$ | $21.77^{e}$ |
| 2 | IRRI352 | 96 | $96.33^{a}$ | $93.00^{c}$ | $7.00^{abc}$ | $24.27^{bc}$ |
| 3 | BG367-2 | 94 | $95.67^{a}$ | $95.33^{c}$ | $8.00^{a}$ | $21.93^{de}$ |
| 4 | KhauHangNieu | 102 | $82.00^{d}$ | $77.67^{d}$ | $6.00^{cde}$ | $21.94^{de}$ |
| 5 | KhauSet | 102 | $93.00^{b}$ | $117.67^{a}$ | $5.33^{ef}$ | $25.00^{b}$ |
| 6 | KhauNieuKenTap | 102 | $92.33^{b}$ | $78.33^{d}$ | $6.67^{abcd}$ | $22.47^{de}$ |
| 7 | KhauVan | 96 | $91.67^{b}$ | $110.67^{abc}$ | $4.00^{f}$ | $24.77^{bc}$ |
| 8 | KhauPang | 102 | $88.00^{c}$ | $106.67^{b}$ | $5.00^{ef}$ | $24.81^{bc}$ |
| 9 | SaiDuongKienAn | 96 | $97.00^{a}$ | $96.33^{c}$ | $6.33^{bcde}$ | $23.87^{c}$ |
| 10 | LocNuoc | 96 | $96.67^{a}$ | $107.00^{b}$ | $7.67^{ab}$ | $24.90^{b}$ |
| 11 | HaiHoanhLun | 102 | $96.67^{a}$ | $106.67^{b}$ | $5.00^{ef}$ | $26.53^{a}$ |

(**) Difference is significant at 1%; Means followed by same alphabetical superscript are statistically on parat ($P < 0{,}05$)

2. **Brown Plant Hopper Genes of Popular Rice Varieties in the Mekong Delta (2008–2011)**

The rice varieties from 2008–2010 collection were tested by Le Xuan Thai et al. (2012) for resistance to brown planthopper (bph), evaluated the yield and the adaptation of promising rice varieties, and tested for the presence of bph genes by molecular techniques. The results showed that there were 77 varieties owning only bph4 gene, 44 varieties owning only bph18 gene and 31 varieties possessing both bph4 and bph18 genes. Regarding the evaluation of the yield and adaptation of the varieties in Long An, Can Tho and An Giang, MTL512, MTL649, MTL657 and OM10043 had the high yields and were highly adapted to all-trial site conditions. MTL649 and OM10043 varieties did not only had the higher yields but also possessed both bph4 and bph18 genes. These varieties would be the important genetic resources for producing as well as breeding in the Mekong Delta, Vietnam (Table 4; Figs. 3 and 4).

3. **Brown Plant Hopper Resistance in Rice Varieties by (*Oryza sativa* L.) Using Molecular Markers RG457 and RM190**

Nguyen Thi Diem Thuy (2012) screened 34 rice varieties in which two standard resistant varieties (PTB33 and OM4495) and one infected variety (TN1), obtained from Biotechnology Research and Development Institute, Can Tho University and Mekong Delta Rice Institute, were included. The varieties were evaluated for resistance to brown plant hopper using molecular markers RG457 and RM190 and standard seedling box test (IRRI 1996). Using molecular marker RM190, there were 25 resistant varieties and 9 infected varieties to BPH with the band size of about 130 bp and 120 bp, respectively. Using RG457 marker, there were 5 varieties showing resistant heterozygous genotype with the band size of about 200, 250, 350 and 600 bp; 9 varieties carrying homozygous resistance with band size of about

**Table 3** Indicators, the productivity and yield of the rice varieties

| Sl. No | Varieties | No. of flower/m2 | No. of seed/ flower | No. of fertility seed/flower | Fertility seed rate (%) | Weight of 1000 seeds (g) | Theory yield (×100 kg/ha) | Actual yield (×100 kg/ha) |
|---|---|---|---|---|---|---|---|---|
| 1 | Khang Dan | $346.87^{a}$ | $127.20^{a}$ | $110.70^{a}$ | $86.99^{a}$ | $20.57^{f}$ | $78.98^{a}$ | $63.77^{a}$ |
| 2 | IRRI352 | $297.37^{cde}$ | $102.97^{bc}$ | $88.13^{b}$ | $85.75^{a}$ | $25.50^{bc}$ | $66.81^{b}$ | $58.33^{b}$ |
| 3 | BG367-2 | $321.57^{abcd}$ | $106.33^{b}$ | $90.26^{c}$ | $84.96^{a}$ | $22.12^{e}$ | $64.17^{b}$ | $57.03^{b}$ |
| 4 | KhauHangNieu | $314.60^{bcd}$ | $100.30^{bcd}$ | $65.35^{cd}$ | $65.17^{bc}$ | $26.83^{a}$ | $55.15^{c}$ | $40.47^{c}$ |
| 5 | KhauSet | $333.67^{ab}$ | $86.10^{e}$ | $47.37^{e}$ | $55.13^{d}$ | $26.73^{ab}$ | $42.17^{ef}$ | $37.10^{c}$ |
| 6 | KhauNieuKenTap | $283.80^{e}$ | $94.63^{d}$ | $62.32^{d}$ | $65.93^{bc}$ | $27.99^{a}$ | $49.50^{cd}$ | $36.33^{cd}$ |
| 7 | KhauVan | $323.77^{abc}$ | $103.83^{b}$ | $63.80^{cd}$ | $61.60^{cd}$ | $23.84^{d}$ | $49.26^{cd}$ | $32.73^{d}$ |
| 8 | KhauPang | $283.80^{e}$ | $95.70^{cd}$ | $68.60^{c}$ | $71.71^{b}$ | $24.49^{cd}$ | $47.65^{de}$ | $38.33^{c}$ |
| 9 | SaiDuongKienAn | $292.97^{de}$ | $125.73^{a}$ | $106.14^{ab}$ | $84.43^{a}$ | $22.12^{e}$ | $69.21^{b}$ | $56.93^{b}$ |
| 10 | LocNuoc | $346.13^{a}$ | $102.06^{bcb}$ | $87.17^{b}$ | $85.48^{a}$ | $21.52^{ef}$ | $64.86^{b}$ | $57.13^{b}$ |
| 11 | HaiHoanhLun | $315.33^{bcd}$ | $84.19^{e}$ | $47.77^{e}$ | $56.89^{d}$ | $26.96^{a}$ | $40.56^{f}$ | $37.27^{c}$ |

(**) Difference is significant at 1%; Means followed by same alphabetical superscript are statistically on parat ($P < 0.05$)

**Table 4** Yield of resistant rice varieties to brown plant hoppers in Long An, Can Tho and An Giang provinces at summer crop in 2011 (tons/ha)

| Sl. No | Varieties | Long An | Can Tho | An Giang | Average |
|---|---|---|---|---|---|
| 1 | MTL145 | 5.25 | 5.60 | 4.56 | 5.14 |
| 2 | MTL512 | 6.08 | 5.08 | 4.78 | 5.31 |
| 3 | MTL544 | 35.42 | 4.92 | 4.68 | 5.01 |
| 4 | MTL602 | 3.58 | 5.95 | 4.01 | 4.51 |
| 5 | MTL613 | 3.83 | 5.86 | 4.76 | 4.82 |
| 6 | MTL614 | 5.67 | 6.04 | 5.69 | 5.80 |
| 7 | MTL620 | 4.50 | 5.09 | 4.32 | 4.64 |
| 8 | MTL645 | 3.75 | 4.63 | 4.58 | 4.32 |
| 9 | MTL649 | 4.92 | 5.54 | 4.91 | 5.12 |
| 10 | MTL650 | 4.17 | 4.89 | 4.63 | 4.56 |
| 11 | MTL651 | 5.33 | 6.15 | 4.66 | 5.38 |
| 12 | MTL657 | 5.17 | 6.71 | 4.14 | 5.34 |
| 13 | HD1 | 3.17 | 5.91 | 5.39 | 4.82 |
| 14 | OM10043 | 5.00 | 5.42 | 4.23 | 4.88 |
| 15 | OMCS2000 | 3.25 | 5.46 | 3.42 | 4.04 |
| 16 | VND95-20 | 4.33 | 5.60 | 3.78 | 4.57 |
| Average | | 4.59 | 5.63 | 4.30 | 4.84 |
| LSD 5% | | 1.25 | 1.05 | 0.62 | 1.06 |
| F | | ** | ** | ** | ns |

**Significant difference at $P = 0.01$; *ns* not significant difference

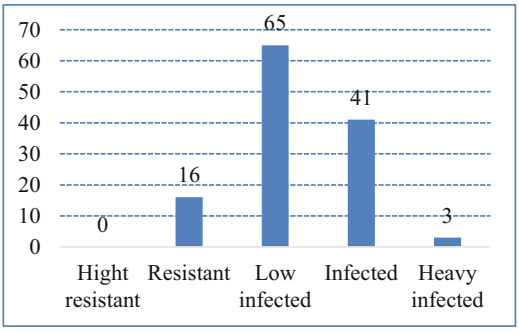


**Fig. 3** Number of resistant varieties to brown plant hopper

200, 250 and 350 bp; and 20 varieties carrying infected homozygous genotype with the band size of about 200 and 600 bp. Of the 34 rice varieties, 13 including OM4495 were found carrying two plant hopper resistance genes of bph4 (Bph3) and Bph10 linked with two molecular markers RG457 and RM190; PTB33 and OM2395 carried only Bph10 resistance gene linked with RG457; 12 varieties carried bph4 (Bph3) gene linked with molecular marker RM190; and seven varieties including standard plant hopper-infected variety TN1 were without any plant hopper resistance genes. Rice varieties OM6377, OM4103 and AS996 carrying resistance genes bph4 (Bph3) and Bph10 were slightly infected and resistant to brown plant hopper from levels 3 to 5 (Tables 5 and 6; Figs. 5 and 6).

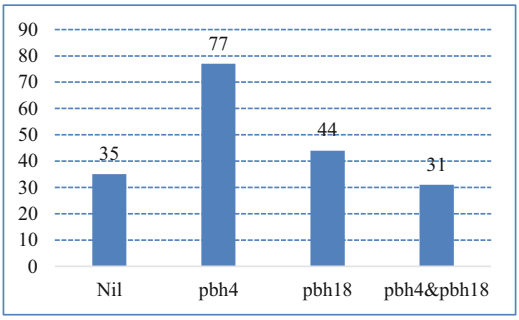


**Fig. 4** Number of varieties containing the BPH-resistant genes

**Table 5** Information of primers RG457 and RM190

| Marker | Sequence | Gene | Temperature |
|---|---|---|---|
| RG457FL | 5′GCAGTGGCAGATGGGATCGT 3′ | *Bph10* | 62 °C |
| RG457RL | 5′GCTCCGAAATCCCAAGCGAT 3′ | Nguyen Thi Lang et al. (1999) | |
| RM190FL | 5′CTTTGTCTATCTCAAGACAC 3′ | *bph4 (bph3)* | 50 °C |
| RM190RL | 5′TTGCAGATGTTCTTCCTGATG 3′ | Jairin et al. (2007) | |

**Table 6** Rice varieties with resistant genes bph10 and bph4

| Sl. no | Varieties | RG457 | RM190 | Resistant level | Description |
|---|---|---|---|---|---|
| 1 | OM4495 | ++ | ++ | 7–9 | Heavily infected |
| 2 | IR50404 | +− | ++ | 7–9 | Heavily infected |
| 3 | OM1364 | ++ | ++ | 7–9 | Heavily infected |
| 4 | OM6004 | ++ | ++ | 5–7 | Infected |
| 5 | OM5451 | ++ | ++ | 5–7 | Infected |
| 6 | OM5472 | ++ | ++ | 5–7 | Infected |
| 7 | OM6976 | +− | ++ | 5–7 | Infected |
| 8 | OMCS2000 | ++ | ++ | 5–7 | Infected |
| 9 | OM576 | ++ | ++ | 5–7 | Infected |
| 10 | OM6377 | +− | ++ | 5 | Slightly infected |
| 11 | OM4103 | +− | ++ | 5 | Slightly infected |
| 12 | AS996 | +− | ++ | 3–5 | Slightly resistant |

++ homozygous genotype, +− heterozygous genotype

***Resistant level index*** (IRRI 1996)

*<1: Highly resistant*
*1.0–3.0: Resistant*
*3.1–4.5 Slightly resistant*
*4.6–5.5: Slightly infected*
*5.6–7.0: Infected*
*7.1–9.0: Heavily infected*

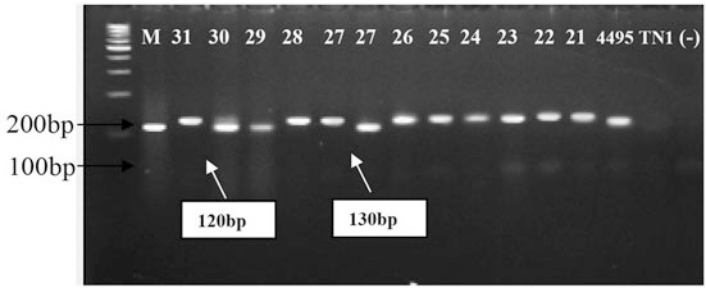


**Fig. 5** Result of PCR-RM190

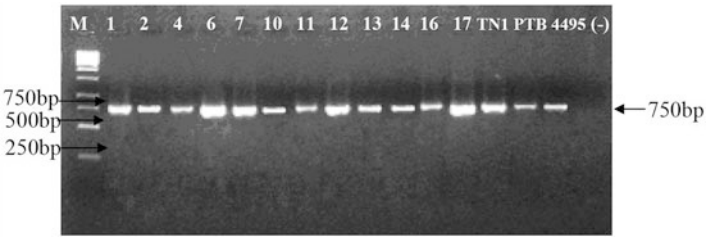


**Fig. 6** Result of PCR-RG457

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# Resistance Screening Techniques for Major Insect and Mite Pests in Vegetable Crops


**R. Srinivasan and Mohamed Rakha**

**Abstract** Host plant resistance is an important component of the integrated pest management (IPM) strategies. However, it was not fully exploited for managing the most challenging insect and mite pests of vegetable crops in the tropics and subtropics. In our attempts to develop pest-resistant vegetable varieties, the World Vegetable Center (WorldVeg) has screened a vast germplasm for major pest species in vegetable crops in the past few decades. To achieve that, there is a need for more accurate and more efficient techniques to assess the resistance or susceptibility of vegetable germplasm. Resistance to borer, defoliators, and storage pests can be generally measured based on the percentage damage that they cause. However, resistance based on population size and pest life stages can be also assessed to understand antixenosis and antibiosis factors for some insects such as whiteflies, sweet-potato weevil, and bean flies. Since aphids occur in large numbers, scoring the population using a rating scale simplifies the screening methodology and enabled us to evaluate a large number of genotypes. Scoring based on damage scales has been found to be the most reliable method of screening for resistance against thrips, leafhoppers, and mites. These scores can be directly used for statistical analysis or converted to the area under the infestation pressure curve (AUIPC). Various statistical analyses including but not limited to analysis of variance (ANOVA) or an analysis based on mean (*m*) and standard deviation (sd) can be used to group the screened genotypes into various resistance and susceptible categories. In addition, the use of biophysical and biochemical traits for pest screening as well as its role for elucidating mechanisms of resistance will be discussed in this chapter.

**Keywords** Vegetable crops · Screening techniques · Major pests · Biophysical and biochemical traits

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## 1 Introduction

Host plant resistance is an important component of the integrated pest management (IPM) strategies. However, it was not fully exploited for managing the most challenging insect and mite pests of vegetable crops in the tropics and subtropics. With more than 61,235 accessions of 440 species from 151 countries, the World Vegetable Center (WorldVeg) genebank includes globally important vegetables and legumes such as tomato, onion, peppers, cabbage, soybean, and mung bean as well as more than 10,000 accessions of traditional vegetables. In our attempts to develop pest-resistant vegetable varieties, the WorldVeg has screened a vast germplasm for major pest species in vegetable crops in the past few decades. More than 10,000 soybean and mung bean accessions were screened for resistance to the bean fly, *Ophiomyia phaseoli* (Tryon), and two other agromyzids, *O. centrosematis* (de Meijere) and *Melanagromyza sojae* (Zehntner) (Chiang and Talekar 1980). They were also screened for their resistance to bruchids [*Callosobruchus chinensis* (L.) and *Callosobruchus maculatus* (F.)] (reviewed by War et al. 2017). Over 1000 sweet potato accessions have been screened for sweet-potato weevil, *Cylas formicarius formicarius* F. (Talekar 1987). Germplasm screening initiated in the WorldVeg in the 1980s revealed high levels of *Helicoverpa armigera* (Hübner) resistance only in the wild *Lycopersicon* species, particularly *Solanum habrochaites* and *Solanum pennellii* (Talekar et al. 2006). However, recently we found high resistance in *Solanum galapagense*, *Solanum cheesmaniae*, and *Solanum pimpinellifolium* accessions against whitefly (*Bemisia tabaci* Genn.), spider mite (*Tetranychus urticae* Koch), and *Tuta absoluta* (Meyrick) (Rakha et al. 2017 a, b, c). We have also identified resistant sources for thrips in onions (Njau et al. 2017) and aphids in okra (Abang et al. 2014 & 2016). To achieve that, there is a need for more accurate and more efficient techniques to assess the resistance or susceptibility of vegetable germplasm. In this chapter, we have summarized the simple and effective screening assays in the field and laboratory to identify the sources of resistance to major insect and mite pests in vegetable crops.

## 2 Resistance Screening Techniques

### *2.1 Borers*

For example, tomato fruit borer (*H. armigera*); eggplant fruit and shoot borer (*Leucinodes orbonalis* Guenée); okra shoot and fruit borer (*Earias vittella* Fab.); legume pod borer (*Maruca vitrata* Fab.); lima-bean pod borer (*Etiella zinckenella* Treitschke)

There are two stages in the screening program, namely, preliminary screening and advanced screening.

**Table 1** Damage rating scale for borer insects

| Damage level (%) | Rating |
|---|---|
| No damage | Highly resistant |
| 0–10.0 | Resistant |
| 10.1–20.0 | Moderately resistant |
| 20.1–30.0 | Moderately susceptible |
| 30.1–40.0 | Susceptible |
| 40.1 and above | Highly susceptible |

In preliminary screening, at least ten plants per accession will be planted in a single row/bed. All the test accessions will be compared with a standard (known) susceptible accession, which acts as the check. The damage scoring usually relies on the natural infestation, and hence the susceptible accession should be planted at regular space intervals (for instance at every ten rows) as well as all over the experimental plot. If the natural insect population pressure is lower, it has to be supplemented from laboratory rearing. The total number and damaged number of shoots/fruits/pods will be recorded on at least five plants in each accession, at regular time intervals (for instance, every week or every 10 days). After recording the data, the damaged shoots/fruits/pods should be harvested from the plants. The data must be recorded from the same plant at every observation, and hence it is suggested to tag the plants before commencing the observation. From this data, the percentage damage for the whole plant will be calculated, and the accessions will be grouped for resistance using the following scale (Table 1), developed by Kashyap and Verma (1986).

In case of advanced screening, only those accessions which were rated as moderately resistant to highly resistant in the preliminary screening will be selected. The selected accessions will be planted in replications, following the randomized block design. The observation and scoring procedures are same as the preliminary screening.

## *2.2 Defoliators*

For example, beet armyworm (*Spodoptera exigua* Hübner); common armyworm (*Spodoptera litura* Fab.)

The accessions to be screened should be planted in alternate rows with the susceptible accession in 4X1 m plots, replicated for four times, and the crop should be exposed to infestation by the ambient armyworm population. The insect damage will be recorded by counting the number of damaged leaves and the total number of leaves from a randomly selected 1 $m^2$ area of each plot at regular intervals. The plants will be ranked based on the mean percent damage differentiated by statistical analysis – analysis of variance (ANOVA) (AVRDC 1977).

## 2.3 Sucking Insects: Aphids

For example, green peach aphid (*Myzus persicae* Sulzer) on peppers; cowpea aphid (*Aphis craccivora* C.L.Koch); cotton aphid/melon aphid (*Aphis gossypii* Glover)

Resistance screening for sucking insects including aphids also involves both preliminary screening and advanced screening.

The aphid population on 20 fully expanded leaves/leaflets selected at random across all plants in each plot will be counted. The plants will be monitored at regular intervals (every week). The starting date depends on the crop. In pepper, for instance, it could be started with 12-week-old plants and continued up to 25 weeks. Aphid population counts will be obtained for each plot. Mean plot counts for each week will be calculated across 20 leaflets or leaves. The score and count data from plots will be expressed as the area under the infestation pressure curve (AUIPC) and will be calculated using the following formula (Shaner and Finney 1977):

Area under pest infestation pressure curve

$$\sum_{i=1}^{n} [(Y_i + Y_{i+1})/2](t_{i+1} - t_i)$$

$n$: number of assessment times
$Y$: number of insects at time $t$

AUIPC will be subjected to a statistical analysis based on mean ($m$) and standard deviation (sd) (Table 2 and Fig. 1).

## 2.4 Sucking Insects: Leafhopper

For example, cotton leafhopper (*Amrasca devastans* Dist.) on eggplant

The characteristic symptom of leafhopper attack is phytotoxemia (hopperburn) caused by desapping of leaves by nymphs and adults. The hopperburn is caused by the removal of fluids from vascular tissues of the plant with simultaneous injection of toxic substances from the leafhopper into the plant. Hence, screening for leafhopper resistance is mainly done based on the hopperburn symptoms. At least ten plants in each accession will be graded, based on the following four grades:

**Table 2** Resistance category based on mean ($m$) and standard deviation (sd)

| AUIPC ($n$) | Rating |
|---|---|
| $n < (m - 2\text{sd})$ | Highly resistant |
| $(m - 2\text{sd}) < n < (m - \text{sd})$ | Resistant |
| $(m - \text{sd}) < n < (m)$ | Moderately resistant |
| $(m) < n < (m + \text{sd})$ | Moderately susceptible |
| $(m + \text{sd}) < n < (m + 2\text{sd})$ | Susceptible |
| $n > (m + 2\text{sd})$ | Highly susceptible |

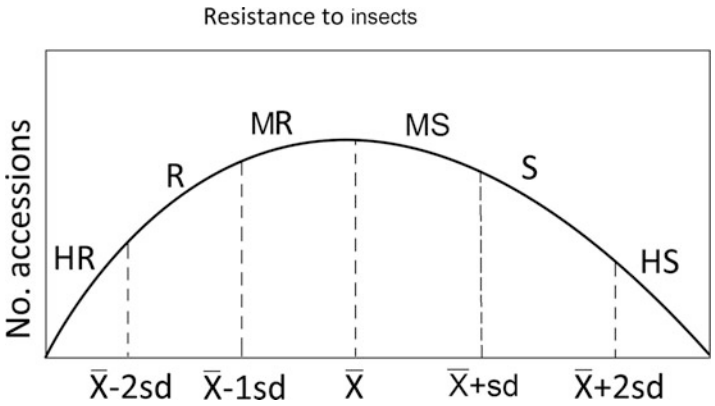


**Fig. 1** Graphic illustration of various categories of resistance based on mean ($\bar{x}$) and standard deviation (sd). *HR* highly resistant, *R* resistant, *MR* moderately resistant, *MS* moderately susceptible, *S* susceptible, *HS* highly susceptible

**Table 3** Resistance category based on mean hopperburn index

| Hopperburn index | Rating |
|---|---|
| 0.1–1.0 | Resistant |
| 1.1–2.0 | Moderately resistant |
| 2.1–3.0 | Susceptible |
| 3.1–4.0 | Highly susceptible |

Grade 1: Undamaged leaves
Grade 2: Few leaves on lower position of the plant curling, crinkling, and slight yellowing
Grade 3: Crinkling and curling all over, yellowing, bronzing, and browning leaves in the middle and lower positions
Grade 4: Extreme curling, yellowing, bronzing and browning, and drying of leaves and defoliation and stunted growth

Then, the hopperburn index or leafhopper resistance index will be calculated using the following formula (Nageswararao 1973):

$$[(P_1 \times \mathrm{G1}) + (P_2 \times \mathrm{G2}) + (P_3 \times \mathrm{G3}) + (P_4 \times \mathrm{G4})]/(P_1 + P_2 + P_3 + P_4)$$

where G is the leafhopper injury grade and $P_i$ is the total number of plants in $G_i$.

Based on the hopperburn index, the accessions can be rated as resistant or susceptible, as given below in Table 3.

## 2.5 Sucking Insects: Whitefly

For example, sweet-potato whitefly (*Bemisia tabaci* Genn.) on solanaceous crops

Two types of assessments, viz., choice and no-choice tests are generally used to evaluate whitefly resistance in solanaceous crops (Romanow et al. 1991). In a choice bioassay, whiteflies are given the choice between two or more different host plants from which it chooses a more preferred host. In the test, ten pairs of adult non-viruliferous whiteflies will be collected with a handheld aspirator for each tested plant, and adults will be placed in the cage (Fig. 2). Adult whiteflies will be removed from the plants by a handmade vacuum aspirator after counting adult of whiteflies on each plant 3 days after introduction by gently turning the plants. The numbers of egg, nymph, and puparium can be also counted under the stereomicroscope (10×) 3, at 10 and 15 days, respectively, after infestation, but it is very time and labor intensive. Log transformation will be used to normalize data before analysis. Choice bioassays are effective in measuring the most preferred host. There are several factors involved in how whitefly chooses a host plant such as color, trichomes, pH, semiochemicals, nitrogen availability, and amino acid composition (Berlinger et al. 1983; van Lenteren and Noldus 1990; Bentz et al. 1995; Blackmer and Byrne 1999; Bleeker et al. 2009; Rakha et al. 2017a, b). However, choice tests alone do not indicate how the whiteflies might survive on non-preferred plants if that is their only option.

In a no-choice bioassay, whiteflies can be kept in small clip-on cages attached to the leaves (Fig. 3), allowing for determination of antixenosis and/or antibiosis and thus allowing the selection of plants with the highest levels of resistance. However, the no-choice procedure is very time and labor intensive and not easily applicable to large plant populations required in breeding for polygenic traits. In this test, two or three clip-on cages (2.5 cm in diameter and 1.0 cm high) will be placed on each plant. Five pairs of non-viruliferous adult whiteflies will be collected with a hand-held aspirator and inserted into each clip-on cage. Dead and alive adult whiteflies and eggs will be counted on 1 or 4 days after whitefly introduction (Firdaus et al.

**Fig. 2** Whitefly choice assay in eggplant

**Fig. 3** Whitefly no-choice assays in tomato

2012; Rakha et al. 2017a). The leaflets can be cut from the plant to facilitate egg counting under the stereomicroscope (10×). An arcsin (Sqrt) transformation will be applied to normalize adult mortality data, whereas a Sqrt ($x$ + 1) transformation will be applied to egg number before data analysis.

## *2.6 Sucking Insects: Thrips*

For example, chili thrips (*Scirtothrips dorsalis* Hood) on hot and sweet peppers; onion thrips (*Thrips tabaci* Lindeman)

**Based on Thrips Population** The number of thrips can be estimated by sampling three apical leaves from at least five plants in each accession at regular intervals. The buds will be collected in glass vials containing a drop of acid fuchsin in 50% ethyl alcohol. The samples will be filtered with Whatman #2 filter paper in the laboratory and the thrips will be counted using microscope. Acid fuchsin will be added to facilitate the identification of thrips on filter paper background (Kumar et al. 1996). The data on thrips population will be pooled and AUIPC will be calculated.

**Based on Thrips Damage (Pepper)** Rating for thrips damage is more reliable and efficient than estimating thrips populations in screening pepper accessions for resistance to thrips (Maharijaya et al. 2011). The thrips infestation based on damage symptoms will visually be rated in five plants at random from each accession. The rating should be done at weekly intervals, for 8–10 weeks using the following scale in Table 4 (Kumar et al. 1996). Thrips damage in pepper can be also tested through leaf disc and detached leaf tests. In leaf disc test, fully opened leaves will be used to take leaf discs (4 cm in diameter) by a leaf punch and placed in Petri dishes on water agar (15 g/l agar) with the lower (abaxial) side upward. Ten starved female adult thrips will be placed on each leaf disc using a wet brush. To prevent thrips from escaping, dishes will be closed using air-permeable plastic and placed in growth rooms with controlled conditions (24 °C, 16 h light, 70% RH). The extent of "silver

**Table 4** Damage rating scale for thrips in pepper

| Score | Symptoms |
|---|---|
| 0 | No symptoms |
| 1 | Terminal 3–4 leaves showing tiny eruptions in interveinal area of leaf |
| 2 | Terminal 3–4 leaves showing upward curling along leaf margin |
| 3 | Severe scarring of terminal and a few basal leaves |
| 4 | Stunted plants, leaves severely curled, and leaf area greatly reduced |
| 5 | Plants with no leaves and only stem remaining |

**Table 5** Damage rating scale for thrips in onion

| Score | Symptoms |
|---|---|
| 0 | No damage |
| 1 | 20% leaf area damaged |
| 2 | 40% leaf area damaged |
| 3 | 60% leaf area damaged |
| 4 | 80% leaf area damaged |
| 5 | 100% leaf area damaged |

damage" and destruction by thrips feeding, oviposition, and secretion will be rated together using a relative scale from 0 (no damage) to 3 (severe damage) 2 days after inoculation (Maharijaya et al. 2011). In the detached leaf tests, it will be similar to leaf disc test, except that intact leaves from each accession will be placed with their petioles in wet Oasis (2 × 5 × 4 cm) and put in a jar. Jars will be closed by air-permeable plastic and placed in growth rooms with controlled conditions, and damage of thrips will be rated as indicated above.

**Based on Thrips Damage (Onion)** Seeds of selected onion accessions will be sown in flats (50 cm long, 20 cm wide, 6 cm high). Each flat could accommodate four accessions of eight plants each. Plants will be maintained in the greenhouse for 3 months at 27 ± 3 °C. At the end of 3 months, when the thrips damage is high, each accession will be evaluated twice at an interval of 10 days for the degree of thrips damage using the following scale in Table 5 (AVRDC, 1996).

The thrips population or the damage scores can be subjected to the statistical analysis based on mean (*m*) and standard deviation (sd) (Table 2).

## *2.7 Sweet-Potato Weevil*

The test accessions should be planted between two weevil source rows, which contain a susceptible cultivar. This cultivar should be planted 10 weeks earlier and then infested with laboratory-reared weevils. At harvest stage, roots will be cut open, and the number of weevils (larvae + pupae + adults) inside will be counted. The percentage of damaged roots should also be assessed by separately weighing the damaged and healthy portions of each root. For each accession, the mean number of

insects per kilogram of roots and the mean percentage of damaged roots will be subjected to a statistical analysis based on mean (*m*) and standard deviation (sd) (AVRDC 1975; Talekar 1987), as described above for whitefly.

## 2.8 Bean flies

For example, *Ophiomyia phaseoli*, *O. centrosematis*, and *Melanagromyza sojae*

Seeds of each accession (mung bean/soybean) should be planted in a single row on a 5 × 0.75 m raised bed. Standard cultural practices can be followed, without any insecticidal applications. Four to 6 weeks after planting, 10 plants (or 50% of the plants when there are less than 20 plants per plot) will be sampled. The number of larvae and pupae found in each plant should be recorded through dissecting the plants. At each sampling date, the mean number of insects (larvae + pupae) found in each ten plant sample will be subjected to a statistical analysis based on mean (*m*) and standard deviation (sd) (AVRDC 1984). This screening methodology is the "plant-destructive" methodology, which cannot be used for screening the segregating populations, because the plant has to be maintained in the field for further advancing of subsequent generations. Hence, the plants should be stimulated to produce lateral branches, which will be used for scoring the insect population.

## 2.9 Bruchid

For example, *Callosobruchus chinensis*

Seeds of each accession (mung bean) should be confined in glass vials (2.5 cm diameter and 12 cm height), and five pairs of 1- to 3-day-old adults will be released over the seeds. The vials have to be covered with muslin cloth and maintained at 30 °C and 70% RH. Ten days after the release, the adult insects will be removed from the seeds, and the number of eggs laid on the seeds will be recorded. The seed samples should be transferred back to the incubator; 3 weeks later, the number of emerging first-generation adults will be recorded. Simultaneously, the number of undamaged as well as bruchid-damaged seeds will also be recorded. The accessions free of first-generation adults will further be evaluated (AVRDC 1990).

## 2.10 Mites

For example, red spider mite, *Tetranychus evansi* Baker and Pritchard, *T. cinnabarinus* Boisd; two-spotted spider mite (*Tetranychus urticae* Koch) on tomato and pepper; broad mite, *Polyphagotarsonemus latus* Banks on pepper

**Table 6** Damage rating scale for red spider mite in tomato

| Score | Damage symptom(s) |
|---|---|
| 0 | No damage |
| 1 | First attack of mites with a few small feeding patches |
| 2 | Large feeding patches (<25% leaf area) |
| 3 | Feeding patches in >25% leaf area |
| 4 | Entire leaf with feeding marks but still green |
| 5 | Necrotic and chlorotic area, the leaf begins to shrivel |

The methodology is similar to aphids, and the counting of mites and their eggs should be done under a dissecting microscope.

The screening can also be done based on the rating of damage symptoms by mites.

### 2.10.1 Red Spider Mite in Tomato

The seedlings of selected accessions (at least ten plants are required for each accession) will be prepared. The seedlings of each accession will be planted in a single row without any replication. It is advised not to spray any pesticides; if other insect pests become very serious, a specific pesticide which would not interfere the mite population can be applied. Starting from 15 days after transplanting, the plants should be scored for mite damage at regular intervals (set it as either 10 or 15 days). Five plants should be scored randomly in each accession using the following scale (Nihoul et al. 1991). Two leaves (preferably one in the top and other in the middle canopy) should be chosen for scoring based on the scale in Table 6.

Mean leaf damage index (MLDI) could be calculated for each accession by adding the value of individual leaves and dividing by the number of leaves sampled. The MLDI for each observation can be calculated over the season, and those accessions which consistently recorded lower MLDI should be selected. The selected accessions should be subject to advanced screening trial following randomized block design.

### 2.10.2 Two-Spotted Spider Mite in Tomato

Leaf damage in the choice assays can be conducted using seedling trays. Seed will be sown in seedling trays in the plastic house. Ten seedlings per accession will be tested in single row. Each test accession will be surrounded by the susceptible check. The trays will be moved from the plastic house to growth rooms under controlled conditions (26 °C, 16 h light, 70% RH). One week after moving to the growth rooms, the plants will be covered by a net (60 mesh/cm$^2$) and mass infested with a very high density of spider mites from bean leaves. Each plant will be inoculated with about 100 to 150 spider mite adults, eggs, and nymphs. When the bean leaves

**Table 7** Damage rating scale for broad mite in pepper

| Scale | Symptoms |
|---|---|
| 0 | No symptom on any leaf |
| 1 | Bronzing followed by crinkling of freshly emerged leaves |
| 2 | Crinkling of new leaves, followed by crinkling and elongation of older leaves |
| 3 | Necrosis on the growing point and dropping of old leaves |
| 4 | Heavy necrosis of growing point |
| 5 | Heavy necrosis of growing point and defoliation of infested plants |

wilted, the net will be removed from the plants, because the spider mites will be moved onto the tomato plants. Leaf damage will be scored 7 to 10 days after spider mite infestation using the above scale (Nihoul et al. 1991).

### 2.10.3 Broad Mite in Pepper

At least ten plants per accession should be planted in a single row. The plants should be observed weekly, and the damage can be assessed on a scale of 0–5 (Table 7). The ratings will be subjected to a statistical analysis based on mean (*m*) and standard deviation (sd) (AVRDC 1998).

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# Screening Pigeon Pea, *Cajanus cajan* (L.) Millsp., Against Major Insect Pests


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**Abstract** Screening pigeon pea germplasm against insect pests and the development of resistant varieties/hybrids require entomological and plant breeding techniques. Research on the development of tolerant or resistant varieties have been initiated by scientists toward a sustainable pest control strategy. This chapter focuses on the screening techniques in pigeon pea for host plant resistance against major pests.

**Keywords** Pigeon pea · Screening · Germplasm · Insect pests

## 1 Introduction

Pigeon pea is an important pulse crop. It is commonly called red gram or *arhar*. It is grouped into two categories: late maturing (*Cajanus indicus* var. *bicolor*) and early maturing (*Cajanus indicus* var. *flavus*) (Singh et al. 2009). More than 300 insect species are recorded to feed on pulses globally. The major pests are gram pod borer (*Helicoverpa armigera* Hubner), pod bug (*Clavigralla gibbosa* Spinola), plume moth (*Exelastis atomosa* Walsingham), spotted pod borer (*Maruca vitrata* Geyer), and red gram pod fly (*Melanagromyza obtusa* Malloch). On an average, 2.4 million tonnes of pulses worth Rs 6000 crores are lost annually due to insect pests (Reddy 2009). In India alone, the gram pod borer, *Helicoverpa armigera* (Hubner), and the pod fly, *Melanagromyza obtusa* (Malloch), are the most economically important. In certain years *H. armigera* caused 90–100% yield loss in India (Yelshetty and Sidde Gowda 1998) (Figs. 1, 2, 3, 4, 5 and 6).

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**Fig. 1** Gram pod borer (*Helicoverpa armigera* Hubner) larval damage and adult moth

**Fig. 2** Plume moth (*Exelastis atomosa* Walsingham), plume moth larva

**Fig. 3** Red gram pod fly (*Melanagromyza obtusa* Malloch) maggot damage

**Fig. 4** Red gram pod fly (*Melanagromyza obtusa* Malloch) pupa and adult

**Fig. 5** Spotted pod borer (*Maruca vitrata* Geyer), larval damage and adult moth

**Fig. 6** Pod bug (*Clavigralla gibbosa* Spinola), eggs and adult techniques for genotype screening against insect pests of pigeonpea

## 2 Based on Larval Count or Larval Infestation

This method can be used for evaluation against pod borer, spotted pod borer, plume moth, and other borers. This is the most convenient method to study the relative susceptibility of a given number of genotypes. Each genotype is grown with defined area and replications. Observations are taken at weekly intervals from the time of appearance to disappearance of the pest on each genotype following RCBD. The data are documented on larval numbers on five randomly selected plants from each replication for each genotype at 50% pod filling. The number of larvae per plant is worked out for each genotype, and resulting data is statistically analyzed by ANOVA and LSD (Khorasiya et al. 2014).

## 3 Based on Number of Maggots and Pupae

As pod fly maggots reside inside the pods without showing any external symptoms, counting the maggots or pupae per pod will be an efficient method. For this, 50 pods are taken from each of the 10 randomly selected plants from each replication. Each pod is split opened and the number of maggots and pupae counted. The average number per pod is calculated and statistically analyzed by ANOVA and LSD (Singh et al. 2017b) (Fig. 7).


**Fig. 7** Pigeon pea pods damaged by major insect pests

## 4 Based on Egg Counts per Plant

The variations in the number of eggs in the test genotypes may be attributed to the ovipositional preference of the pod borer on pigeon pea genotypes. Egg counts are taken on ten randomly selected plants from each replication for each genotype at 50% pod filling (Cheboi et al. 2016).

## 5 Based on Pod Damage

This is the most commonly used methods for screening against all pod borers and pod wasp, *Tanaostigmodes cajaninae*. Under this technique, 50 pods are collected for each of the 10 randomly selected plants per replication. From these collected pods, the number of infested/damaged pods is counted. The percentage pod damage is calculated using the formula

$$\text{Pod damage } (\%) \frac{\text{Number of damaged pods}}{\text{Total number of pods}} \times 100$$

(Source: Cheboi et al. 2016)

Data is analyzed after arcsine transformation. The correlation and regression analyses are carried out for each genotype (Sharma et al. 2003; Kooner and Cheema 2006; Cheboi et al. 2016).

## 6 Pest Susceptibility Rating

This technique is used for screening against pod borers, modified based on percentage pod damage. In this method, test genotypes are grown with the infester rows (highly susceptible genotype, having greater than 50 percentage pod damage). Fifty pods are collected from each of the ten randomly selected plants per replication. From these collected pods, the number of infested or damaged pods is counted. The percentage pod damage is calculated. The pest susceptibility rating (PSR) for each genotype is calculated following Abott (1925) which is given below:

$$\text{Pest susceptibility } (\%) \frac{\text{P.D. of infester} - \text{P.D. of test entry}}{\text{P.D. of infester}} \times 100$$

where P.D. = mean of percentage pods or grains damaged (Source: Abott 1925; Kooner and Cheema 2006).

The pest susceptibility percentage is then converted to 1 to 9 scale as follows:

| Pest susceptibility rating (PSR) | Pest susceptibility (%) | Category |
|---|---|---|
| 1 | 100 | Highly resistant |
| 2 | 75 to 99.9 | Resistant |
| 3 | 50 to 74.9 | Moderately resistant |
| 4 | 25 to 49.9 | Moderately resistant |
| 5 | 10 to 24.9 | Moderately susceptible |
| 6 | −10 to 9.9 | Moderately susceptible |
| 7 | −25 to −9.9 | Susceptible |
| 8 | −50 to −24.9 | Highly susceptible |
| 9 | −50 to less | Highly susceptible |

Source: Kooner and Cheema (2006) and Sreekanth et al. (2017)

## 7 Based on Seed Damage

This method is used for screening against pod-sucking bug, pod fly, and pod borer. Identifying the damage-causing insect pests is the basic prerequisite for this technique. Each genotype is grown with defined area and replications. At pod maturity, 50 pods from 10 randomly selected plants are harvested from each replication. After threshing and cleaning, the damaged and undamaged seeds are separated and examined for the type of insect pests causing the damage. Percentage seed damage is calculated by

$$\text{Seed damage } (\%) = \frac{\text{Number of seeds damaged}}{\text{Total number of seeds examined}} \times 100$$

(Source: Minja et al. 1999)

Data is subjected to analysis of variance after arcsine transformation. The correlation and regression analysis for the different pigeon pea genotypes is computed (Minja et al. 1999).

## 8 Detached Leaf Assay

This technique is used for screening against *H. armigera* neonate larvae. *H. armigera* larvae feed on leaves in the absence of reproductive structures. Further, because of temporal variations in flowering in the genotypes and nonuniformity of insect pest density, it is difficult to screen genotypes under field conditions. So, Sharma et al. (2005) designed the "detached leaf assay" screening against *H. armigera*, wherein the individual trifoliate leaves are cut with scissors and immediately planted in a slanting manner in 3% agar-agar medium in a 250 ml plastic vial to avoid drying of the leaves. Each genotype is replicated following

completely randomized design. A definite number of neonate larvae are used in each replication for each genotype. The experiments are stopped when the larvae consume around 80% of the leaf area in the susceptible control or when there are large differences in leaf consumed between the susceptible and resistant genotypes. The genotypes are scored for leaf feeding by visual observations on a 1 to 9 scale (1 = <10% leaf area damaged, 2 = 11–20, 3 = 21–30, 4 = 31–40, 5 = 41–50, 6 = 51–60, 7 = 61–70, 8 = 71–80, 9
= > 81% leaf area damaged) (Sharma et al. 1992). The number of surviving larvae after the termination of feeding period is recorded and individually placed in 25 ml plastic vials to avoid cannibalism. The weights of surviving larvae are taken few hours after isolating them from the food. Data on the percent larval survival and mean weight of larvae are used for evaluating the genotypes.

## 9 Based on the Number Damaged or Webbed Inflorescence

This method is followed for screening against spotted pod borer, *Maruca vitrata*, as larvae feed in webbed twigs/pods. The inflorescence damage due to spotted pod borer is recorded on five twigs per plant and from ten randomly selected plants per replication at peak flowering stage of the crop blooms (Anantharaju and Muthiah 2008).

## 10 Screening Against Tur Pod Bug

### *10.1 Screening Based on Oviposition Behavior*

The experiment is conducted under screen house conditions. For recording the data on oviposition, six pairs of adults are released on each genotype (one pair/plant) at peak flowering stage per replication. The pods receiving eggs are tagged in each genotype. The data is recorded on total number of eggs laid on different plant parts (leaves, flower buds, flowers, and pods), number of eggs per cluster, hatching percentage, egg laying pattern, and oviposition period throughout the season. The data are analyzed by following factorial CRD (Chitralekha et al. 2017).

### *10.2 Based on Nymphal and Adult Count*

Ten plants of each genotype are randomly selected from each replication, and a number of nymphs and adults are counted at weekly intervals. The average numbers are compared on each genotype for analysis (Singh et al. 2017a).

### *10.3 Based on Seed Damage*

Each genotype is grown with defined area and replications. At pod maturity, 50 pods from randomly selected 10 plants are harvested per replication. After threshing and cleaning, the damaged and undamaged seeds are separated and examined for the type of damage by insect pests. Percentage seed damage is calculated as

$$\text{Seed damage } (\%) = \frac{\text{Number of seeds damaged}}{\text{Total number of seeds examined}} \times 100$$

(Source: Minja et al. 1999)

Analysis of variance is computed after adopting arcsine transformation. The correlation and regression analysis on different pigeon pea genotypes is calculated (Minja et al. 1999).

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# Screening Soybean, *Glycine max* L. Merri. Against Major Insect Pests


**K. S. Jagadish, Dymtro Kravtsov, Ranvir Singh, and C. G. Arun Kumara**

**Abstract** In this chapter, screening procedures for soybean germplasms against 12 economically important pests are described. The screening procedures are based on more than one criterion-like level of infestation, number of insects per unit plant, weight loss of the plant part damaged, duration of life stages of the target species, and others. Experimental procedures for determining mechanism of resistance against aphids and bruchid beetle are also summarized. For each screening and mechanism of resistance procedure, statistical data analysis is indicated.

**Keywords** Screening · Soybean · Germplasms · Insect pests

## 1 Introduction

Soybean (*Glycine max*) is an important pulse crop rich in protein. Soybean, being an excellent source of protein and oil, is a two-dimensional crop, as it contains about 40–42% high-quality protein and 20–22% oil. Fat-free (defatted) soybean meal is a significant and cheap source of protein. Gangrade (1976) reported over 99 insect species attacking soybean crop at Jabalpur, MP, India. Vieira et al. (2011) observed that when *Bemisia tabaci* occurs in large populations, the plants are weakened by the extraction of large amounts of sap. Efficient genotype screening technique is a prerequisite for exploiting host plant resistance against insect pests (Figs. 1, 2, 3, 4 and 5).

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Pheno-phases of soybean for screening against pests attack

| Stage no. | Pheno-phase | Description |
|---|---|---|
| *Vegetative stages* | | |
| VE | Emergence | Cotyledons above the soil surface |
| VC | Cotyledon | Unifoliolate leaves unrolled sufficiently so the leaf edges are not touching |
| V1 | First node | Fully developed leaves at unifoliolate nodes |
| V2 | Second node | Fully developed trifoliolate leaf at node above the unifoliolate nodes |
| V3 | Third node | Three nodes on the main steam with fully developed leaves beginning with the unifoliolate nodes |
| V(n) | nth node | (n)number of nodes on the main stem with fully developed leaves beginning with the unifoliolate nodes. n can be any number beginning with 1 for V1, first-node stage |
| *Reproductive stages* | | |
| R1 | Beginning bloom | One open flower at any node on the main stem |
| R2 | Full bloom | Open flower at one of the two uppermost nodes on the main stem with fully developed leaf |
| R3 | Beginning pod | Pod 5 mm (3/16 inch) long at one of the four uppermost nodes on the main stem with a fully developed leaf |
| R4 | Full pod | Pod 2 cm (3/4 inch) long of the four uppermost nodes on the main stem with a fully developed leaf |
| R5 | Beginning seed | Seed 3 mm (1/8 inch) long in pod at one of the four uppermost nodes on the main stem with a fully developed leaf |
| R6 | Full seed | Pod containing a green seed that fills the pod cavity at one of the four uppermost nodes on the main stem with a fully developed leaf |
| R7 | Beginning maturity | One normal pod on the main stem that has reached its mature pod color |
| R8 | Full maturity | Ninety-five percent of the pods that have reached their mature pod color. Five to ten days of drying weather are required after R8 before the soybeans have less than 15% moisture |

Source: Fehr and Caviness (1977)

**Fig. 1** Stem tunneling by stem fly (*Melanagromyza sojae* Zehntner) and pupa

**Fig. 2** Southern green stink bug (*Nezara viridula* L.) adult and nymphs

**Fig. 3** *Helicoverpa* feeding on soybean leaves

**Fig. 4** Soybean pod damaged by borer

**Fig. 5** Soybean leaf folder, *Nacoleia vulgalis* [*Omiodes indicata*] Fabricius

## 2 Screening Against Stem Fly, *Melanagromyza sojae* Zehntner

**For screening, each genotype is grown in three replications. Sampling is done at weekly intervals starting from 15 days of sowing till crop senescence. For recording pest incidence, 30 plants are randomly uprooted from each replication. Plants are split open vertically and examined for the presence of maggots. Incidence on respective genotype is calculated using the formula**

$$\text{Infestation}(\%) = \frac{\text{Number of plants with infestation}}{\text{Total number of plants}} \times 100$$

(Source: Jadhav et al. 2013; Patel 2013)

For each infested plant, data is also recorded on plant height and length of the maggot tunnel caused by the maggot and percent tunnel damage:

$$\text{Percent tunnel damage} = \frac{\text{Length of the tunnel}}{\text{Plant height}} \times 100$$

(Source: Jadhav et al. 2013; Patel 2013)

### 2.1 *Screening Against Girdle Beetle,* Obereopsis brevis *Gahan*

Each genotype is grown in three replications. Sampling is done at 10 days intervals starting from first appearance of the girdle beetle till crop senescence. For recording incidence, 1 meter row is randomly selected from each replication. Plants are split

open vertically and examined for the presence of grubs. Percent incidence on respective genotype is calculated by

$$\text{Infestation}(\%) = \frac{\text{Number of plants with infestation}}{\text{Total number of plants}} \times 100$$

(Source: Netam and Kanwar 2013)

## 2.2 *Screening Against Whitefly,* Bemisia tabaci *Gennadius*

The data are recorded at the time of peak infestation. Ten plants are randomly selected from each replication per genotype. The counts are made for the number of eggs, larvae, and pupae from three leaves selected from lower, medium, and upper parts of the plant. The level of resistance is confirmed based on the level of infestation as described below.

| Number of whitefly | Scale | Category of resistance |
|---|---|---|
| Less than 10 eggs + larvae + pupae on 2.85 $cm^2$ | 1 | Very resistant |
| 11–20 eggs + larvae + pupae on 2.85 $cm^2$ | 2 | Resistant |
| 21–35 eggs + larvae + pupae on 2.85 $cm^2$ | 3 | Moderately resistant |
| 36–50 eggs + larvae + pupae on 2.85 $cm^2$ | 4 | Susceptible |
| More than 51 eggs + larvae + pupae on 2.85 $cm^2$ | 5 | Very susceptible |

Source: Gulluoglu et al. (2010)

## 2.3 *Screening Against Pod Bug,* Riptortus linearis *Fabricius*

**Choice Test**

For this test, ten genotypes are placed randomly in a meshed cage (length, 120 cm; width, 120 cm; and height, 100 cm). For each genotype, three replications can be maintained. The material used for making the mesh cage is nontranslucent to the adults of *R. linearis*. Two newly emerged adults of *R. linearis* are released onto each plant (30 per cage) at R5 growth stage of soybean. Seeds are observed under a binocular microscope. The data are recorded on number of punctures on seed, number of damaged seeds/plant, number of seeds/plant, and seed weight/plant. Percent damage is calculated by

$$\text{Pod damage}(\%) = \frac{\text{Number of pods damaged}}{\text{Number of total pods}} \times 100$$

(Source: Krisnawati et al. 2016; Krisnawati et al. 2017)

$$\text{Seeds damage}(\%) = \frac{\text{Number of seeds damaged}}{\text{Number of total seeds}} \times 100$$

(Source: Krisnawati et al. 2016; Krisnawati et al. 2017)

Observations are also made on plant height, number of branches/plant, number of nodes/plant, number of pods/plant, number of seeds/plant, seed weight per plant, number of damaged pods/plant, and number of damaged seeds/plant. Correlation and regression are also computed (Krisnawati et al. 2016; Krisnawati et al. 2017).

**No-Choice Test**

One plant per genotype is placed in a meshed cage (50 cm height and dia 26 cm), with three replications per genotype. Artificial infestation is done by releasing one pair of newly emerged *R. linearis* per cage, at R5 growth stage of the soybean plant. At maturity, pods are harvested and seeds collected. Seeds are examined under a binocular microscope. The data is recorded on number of punctures on the seed, number of damaged seeds/plant, number of seeds/plant, and seed weight/plant. Percent damage is calculated by the formula

$$\text{Pod damage}(\%) = \frac{\text{Number of pods damaged}}{\text{Number of total pods}} \times 100$$

(Source: Krisnawati et al. 2016; Krisnawati et al. 2017)

$$\text{Seeds damage}(\%) = \frac{\text{Number of seeds damaged}}{\text{Number of total seeds}} \times 100$$

(Source: Krisnawati et al. 2016; Krisnawati et al. 2017)

Observations are also made on plant height, number of branches/plant, number of nodes/plant, number of pods/plant, number of seeds/plant, seed weight per plant, number of damaged pods/plant, and number of damaged seeds/plant. Correlation and regression analysis are run for the yield attributes.

The criterion of resistance is based on the method suggested by Chiang and Talekar (1980).

| | |
|---|---|
| X>X̄+2SD | Highly susceptible |
| X̄>X>X̄+2SD | Susceptible |
| X̄>X>X̄−1SD | Moderately resistant |
| X̄-1SD>X>X̄−2SD | Resistant |
| X<X̄−2SD | Highly resistant |

Source: Krisnawati et al. (2016) and Krisnawati et al. (2017))

## 2.4 *Screening Against Pod Bug,* Riptortus pedestris *Fabricius*

For screening against pod bug, ten pods are collected from each resistant genotype. Seeds are removed and only empty pod shells are collected. Empty shell from resistant genotypes is filled with seeds collected from susceptible genotypes. Five replications are maintained for each genotype (ten pods/replicate) in plastic boxes (0.3 m × 0.2 m). In each box, five nymphs of 3rd–4th instar are released. Seeds are separated after 4 days of infestation and stained in 1% acid fuchsin for 5 min. The seeds are then examined under a stereomicroscope (40×) for number of stylet punctures on pod shell and seeds. The fuchsin will stain the stylet punctures as red, which can be easily examined under a stereomicroscope (Suharsono and Sulistyowali 2012).

## 2.5 *Screening Against Redbanded Stink Bug,* Piezodorus guildinii *Westwood, and Green Belly Stink Bug,* Dichelops melacanthus *Dallas*

The soybean genotypes are screened based on the biological parameters of the pod bug. For obtaining the uniform insect culture, egg masses are collected and placed in Petri dishes containing moistened muslin paper and one pod of one genotype. After hatching, 5 s instar nymphs are collected and transferred into another Petri dish (representing one replication) lined with filter paper, containing two pods of one of the soybean genotypes collected from R5 stage. Fifteen replications per genotype are maintained. The pods are changed once every 2 days. Data are recorded on the duration of the nymphal stages (N2–N5), the developmental period (egg to adult), the adult weight (at 24 h of age), the mortality in each nymphal instar, and the mortality in nymphal stage and adult longevity (Silva et al. 2013; Canassa et al. 2017).

## 2.6 *Screening Against Southern Green Stink Bug,* Nezara viridula *(L) (Hemiptera: Pentatomidae)*

Data are recorded on the number of bugs from ten randomly selected plants from each replication. Observations are also made on leaf and pod trichome density, plant height, and days to maturity. Correlation and regression analysis are calculated among morphological characteristics and pod bug population (Namayanja et al. 2000).

## 2.7 *Screening Against Southern Stink Bug,* Nezara viridula *Linnaeus, for Field Resistance*

The genotypes are screened based on the following parameters (Jackai et al. 1988).

**Based on Population Density**

For screening against pod bug, each genotype is grown with pre-defined number of replications. Observations on population density are recorded in each replication. Sampling is done using drop-cloth (DC) technique, from flowering till crop maturity. For easy separation of the species, third or older instars are counted (Jackai et al. 1988).

**Based on the Crop Yields**

Ten plants are randomly selected from each replication per genotype. A definite number of pods are selected from each plant from top to bottom. The pods are scored based on the level of damage. Scores are averaged for each genotype. In addition, six plants are randomly selected from each replication at crop maturity. Each plant is cut into two parts (20 cm from the ground level and an upper portion), and each part is collected in a separate bag. The samples are brought to the laboratory. The pods and seeds are examined for damage by the pod bug. The genotypes are screened based on the extent of pod damage.

**Damage Score**

| Damage symptoms | | | | Score |
|---|---|---|---|---|
| Only slight/no pod damage* | 0–10% | Little or no seed abortion** | | 1 |
| Low pod damage | 11–30% | Seed damage/abortion | <30% | 3 |
| Moderate damage to pods | 31–50% | Seed damage/abortion | <50% | 5 |
| Pod damage severe on upper half of plant | 51–70% | Slight damage to other half of pod | | 7 |
| Pods heavily damaged throughout plant | >70% | Mostly without seeds | | 9 |

*Pod damage means pod shriveling, wrinkling, or premature drying, following stink bug feeding
**Seed damage/abortion means seeds completely or partly damaged in such a way that this can be detected by pressing on pods or visually as in the case of severe pod damage (Source: Jackai et al. 1988)

**Percent Pod Damage**

$$\text{Pod damage}(\%) = \frac{\text{Number of pods damaged}}{\text{Number of total pods}} \times 100$$

(Source: Jackai et al. 1988)

**Percent Seed Damage**

$$\text{Seeds damage}(\%) = \frac{\text{Number of seeds damaged}}{\text{Number of total seeds}} \times 100$$

(Source: Jackai et al. 1988)

**Seed Damage Index**

$$\text{Seed damage index} = \frac{\text{Number of damaged seeds}}{\text{Total number of pods sampled}} \times 100$$

(Source: Jackai 1982)

**Grain Yield/ha**
Yield data is worked out at the end of the season.

## 2.8 *Screening Against Defoliators, Tobacco Leaf Caterpillar,* Spodoptera litura *Fabricius, and Soybean Semilooper,* Chrysodeixis acuta *Walker*

Each genotype is grown in three replications. At weekly intervals, larval numbers are counted on ten plants randomly selected from each replication per genotype. The observations are also made on biophysical characters at 25 and 40 days after sowing. The following biophysical characters are measured during the experiment (Sasane et al. 2018):

*Leaf succulence*: It is expressed as relative water content (RWC).

Leaf samples are taken from each genotype. Leaf fresh weight is recorded. Same sample is allowed to float on the surface of the water for 4 h; the sample leaf is then weighted to record turgid weight. The same leaf sample is then oven-dried and dry weight is recorded. Relative water content is calculated using the following formula:

$$\text{RWC}(\%) = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}} \times 100$$

(Source: Barrs and Weatherley 1962; Sasane et al. 2018)

*Leaf thickness*: Leaf thickness is expressed as specific leaf weight (SLW):

$$\text{Specific leaf weight}\,(\text{SLW}) = \frac{\text{Dry leaf weight}(\text{gm})}{\text{Leaf area}(\text{cm}^2)} \times 100$$

(Source: Amanullah 2015; Sasane et al. 2018)

*Leaf area*: Leaf area is measured by using a planimeter. Percent leaf area damaged is worked out by using the following formula:

$$\text{Leaf area damage}(\%) = \frac{\text{Leaf area eaten}}{\text{Total leaf area}} \times 100$$

(Source: Sasane et al. 2018)

**Trichome Density**

Leaves are collected at 25 and 40 days after sowing. Samples are kept in acetic acid/alcohol (2:1) solution for one night, for the purpose of removing the chlorophyll. After that leaves are transferred in 90% lactic acid in small vials. For recording of the trichome density, leaves are mounted on a slide in a drop of lactic acid and examined under the microscope (10×). The trichomes are counted on abaxial leaf surface from randomly selected microscopic fields and recorded as trichome density (no./cm$^2$) (Khan et al. 1986; Sasane et al. 2018).

The correlation coefficient is calculated between population densities of different defoliators and different biophysical traits. At the end of the season, yield per hectare is worked out.

## 2.9 *Screening Against Defoliator,* Spodoptera litura *Fabricius*

Individual plants of each genotype are grown in pots. After 26 days of sowing, the pots are transferred to nylon cages, with one plant per cage, and the same is replicated for each genotype. After 28 days of sowing, the plants are infested with second instar larvae of *S. litura* (two larvae per plant). The larvae are allowed to be fed for 1 week, leaves are randomly selected from each plant, and damaged leaves are scored.

| Score | Leaf damage |
|---|---|
| 0 | No damage |
| 1 | 1–25% leaf damage |
| 2 | 26–50% leaf damage |
| 3 | 51–75% leaf damage |
| 4 | >76% leaf damage |

The leaf damage intensity was calculated as follows:

$$P = \sum \frac{ni \times vi}{ZN} \times 100$$

where

P = Leaf damage intensity
ni = Number of damaged leaf with score i
vi = Score (i, 0–4)
Z = The highest score (4)
N = Total number of leaves

(Source: Bayu et al. 2018)

Based on the score, the resistance is categorized as below.

| | |
|---|---|
| X>X̄+2SD | Highly susceptible |
| X̄>X>X̄+2SD | Susceptible |
| X̄>X>X̄−1SD | Moderately resistant |
| X̄-1SD>X>X̄−2SD | Resistant |
| X<X̄−2SD | Highly resistant |

Source: Chiang and Talekar (1980) and Bayu et al. (2018)

## 2.10 *Screening Against Aphid,* Aphis glycines*, Based on "Damage Index" (DI)*

The experiment is conducted under greenhouse condition using the choice test. Three plants per genotype are grown in pot (5 liters, vol.). The pots of all genotypes are placed randomly in the greenhouse. Two of the three plants are inoculated with two wingless aphids at V1 stage of soybean genotype having partially expanded trifoliate. The aphid populations are counted 10 days after inoculation when the plants are in V3 stage. In addition, 4 weeks after inoculation, plants from each genotype are visually examined and rated based on the following scale.

| Visual rating scale to establish the damage index (DI) | |
|---|---|
| Score | Aphid population and damage symptoms |
| 0 | No aphids, plant appears normal and healthy |
| 1 | Less than 100 aphids per plant, plant appears normal and healthy |
| 2 | 101–300 aphids per plant, mostly on the young leaves and the tenderstem at top of plant, plant appears normal and healthy |
| 3 | 301–800 aphids per plant, leaves slightly curly and shiny, young leaves and stemscovered with aphids |
| 4 | More than 800 aphids per plant, plants stunted, leaves severely curled, yellow, and covered with sooty mold and cast skins |

Source: Zhuang 1999; Mensah et al. 2005

Based on the score, damage index is calculated using the following formula:

$$\text{Damage Index(DI)}(\%) = \sum \frac{(\text{Scale value} \times \text{Number of plants in the category})}{(4 \times \text{total number of plants evaluated})} \times 100$$

| Damage index (DI) | Class of resistance |
|---|---|
| 30% or less | Resistant |
| More than 30% | Susceptible |

Source: Zhuang (1999) and Mensah et al. (2005)

The 30% break point is chosen on the basis of observations that a soybean genotype with a DI value less than 30% never showed symptoms of damage under high aphid pressure until the end of the season (Zhuang 1999; Mensah et al. 2005). Correlation is computed between aphid population and damage index.

## 2.11 *Screening Against Aphid,* Aphis glycines*, Based on "Aphid Index"*

The experiment is laid out in a choice test. Each genotype is grown in pots (5 liters, vol.) with replications. Each plant is artificially infested with four wingless aphids. Data is recorded on the aphid population and aphid damage rating at 5–9 days after infestation.

| Aphid population rating | |
|---|---|
| Score | Aphid population |
| 0 | No aphids |
| 3 | High-aphid density (>100 aphids per plant) |

| Plant damage rating | |
|---|---|
| Score | Plant damage |
| 0 | No perceptible damage |
| 3 | Severe leaf distortion and stunting, or plant death |

Aphid index is worked out by using the following formula:

$$\text{Aphid Index} = \text{Aphid population(score)} \times \text{Aphid damage(score)}$$

The average of aphid index is calculated for each genotype and having values from 0 to 9.

(Source: Hill et al. 2004)

## 2.12 *Screening Against Aphid,* Aphis glycines*, Based on Damage Rating*

The experiment is conducted under greenhouse conditions using the choice test. One plant is grown in one pot (5 liters, vol.) for each genotype. There are six replications for each genotype. The pots of all genotypes are placed randomly in greenhouse. Each plant is inoculated with ten wingless aphids at R2 stage of soybean genotype having partially expanded trifoliate. Damage rating is assigned for each genotype, after every day of inoculation, by using a 1–5 scale as shown in the table below. The scale is a modification of the damage scales reported by earlier workers (Heng-Moss et al. 2002; Hill et al. 2004; Pierson et al. 2010)

| Damage rating | Damage symptoms |
|---|---|
| 1 | 10% yellowing discoloration, leaf distortion, plant stunting, and desiccation |
| 2 | 11–30% yellowing discoloration, leaf distortion, plant stunting, and desiccation |
| 3 | 31–50% yellowing discoloration, leaf distortion, plant stunting, and desiccation |
| 4 | 51–75% yellowing discoloration, leaf distortion, plant stunting, and desiccation |
| 5 | >76% of the leaf area with yellowing discoloration, leaf distortion, plant stunting, desiccation, or dead tissue |

Source: Heng-Moss et al. (2002), Hill et al. (2004) and Pierson et al. (2010)

The genotypes are categorized based on the damage rating as described below.

| Damage rating | Class of resistance |
|---|---|
| >4 | Highly susceptible |
| >3 but <4 | Moderately susceptible |
| >1 but <3 | Moderately resistant |
| =1 | Highly resistant |

Source: Heng-Moss et al. (2002)

## 2.13 *Mechanism of Resistance Against Aphid*

**Antibiosis**

Each genotype is grown in pots (5 liters, vol.) with one plant per pot. Twenty wingless aphids per plant are released on youngest fully expanded trifoliate at R1 stage of the plants. Aphids are confined to the plants using tubular plastic cages. The parent aphids are removed 48 h after inoculation. The number of progeny is counted 8 days after inoculation, thereafter at weekly intervals till 35 days.

**Tolerance**

Each genotype is grown in pots (5 liters, vol.) with one plant per pot. Twenty wingless aphids per plant are released on youngest fully expanded trifoliate at R1 stage of the plants. Aphids are confined to the plants using tubular plastic cages. The parent aphids are removed 48 h after inoculation. An un-infested control is also kept for each genotype. Weekly observations are taken for aphid population, and damage ratings are assigned for each genotype and the control.

The scale is a modification of the damage scales reported by earlier workers (Heng-Moss et al. 2002; Hill et al. 2004; Pierson et al. 2010).

| Damage rating | Damage symptoms |
|---|---|
| 1 | 10% yellowing discoloration, leaf distortion, plant stunting, and desiccation |
| 2 | 11–30% yellowing discoloration, leaf distortion, plant stunting, and desiccation |
| 3 | 31–50% yellowing discoloration, leaf distortion, plant stunting, and desiccation |
| 4 | 51–75% yellowing discoloration, leaf distortion, plant stunting, and desiccation |
| 5 | >76% of leaf area with yellowing discoloration, leaf distortion, plant stunting, desiccation, or dead tissue |

Source: Heng-Moss et al. (2002), Hill et al. (2004) and Pierson et al. (2010)

The dried plants are harvested, and data is recorded on yield parameters like number of pods per plant, number of seeds per pod, average seed weight, average dry weight of pods, dry weight of stem, and total plant biomass. Infested genotypes having plant damage ratings and yield parameters similar to their respective control plants are selected as tolerant (Pierson et al. 2010).

**Antixenosis**

For examining the antixenosis, choice test is performed. All genotypes are grown together in a single pot (10 liters, vol.) in a circular fashion. When the plants reach to V1 stage, adult aphids are placed in the center on a filter paper. The aphid populations are counted on each genotype after 24 h of release (Diaz-Montano et al. 2006).

## *2.14 Screening Against* Callosobruchus maculatus *Fabricius (Coleoptera: Bruchidae)*

For screening, 50 seeds are taken from each genotype. Their initial weight is recorded. After weighing each genotype, the seed is kept in a Petri dish (0.3 m dia). Two pairs of 1–2 day-old adult of *C. maculatus* are released per Petri dish. They are allowed to mate and oviposit. Adults are removed after 72 h. A number of eggs laid per genotype seed are recorded. Eggs are observed for the next 60 days for the number of adults emerging. The seed weight is recorded after infestation. The data are recorded on the following parameters:

**Adult Emergence**

$$\text{Adult emergence}(\%) = \frac{\text{Number of adults emerged}}{\text{Total number of eggs laid}} \times 100$$

*Development period*: Time taken from oviposition to adult emergence

**Growth Index**

$$\text{Growth Index} = \frac{\text{Adult emergence}(\%)}{\text{Development period}}$$

**Seed Weight Loss**

$$\text{Seed Weight loss}(\%) = \frac{\text{Initital weight} - \text{weight of infected seeds}}{\text{Initial weight}} \times 100$$

(Source: Sharma and Thakur et al. 2014)

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# Techniques for Determining Mechanisms of Resistance: Antixenosis for Feeding


**L. Vijay Kumar, P. N. Guru, and B. S. Rajendra Prasad**

**Abstract** Entomologists have designed several experimental setup to measure feeding responses of insects to host–plants. One method each for chewing and sucking pests has been described in this chapter. The experimental techniques should be easy to execute, repeatable, and precise. It should be applicable to a wide range of insects with the same or different types of mouthparts. The test should generate quantitative data amenable for statistical analyses.

**Keywords** Screening technique · Feeding preferences · Chewing insect · Sucking insect

## 1 Introduction

Antixenosis is defined as the resistance mechanism employed by the plant to deter or reduce colonization by insects. Generally insects orient themselves toward or away from plants for food and oviposition and/or for shelter. To study the preference or non-preference of test plants by chewing and sucking insects, leaf disc method and sap feeding test, respectively, can be employed.

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## 2 Leaf Disc Method for Chewing Insects

This method is suited for mandibulate insects. In this method, leaf disc of known size is excised from the foliage of test plant, and they are placed in a petri dish at equidistance on a moist filter paper. The second or third instar larvae or any other chewing insects are allowed for feeding. After a given period of time, the area fed by the insect will be measured and calculated.

Munakata (1977) investigated chemicals which act as feeding deterrents against tobacco cutworm, *Spodoptera litura*. Leaf discs were punched with a cork borer from leaves of susceptible food plants. The discs were immersed in acetone solutions of test samples with pure acetone as a control. After air-drying the discs were placed in polythene dishes with test larvae of tobacco cutworm. After half the areas of the controlled discs were usually eaten, within 2 h at which time the consumed areas of all discs were measured. The consumed area of treated discs was expressed as a percentage of the consumed area of controlled discs and was used as an index of the antifeedent activity of the samples.

## 3 Sap Feeding Test for Sucking Insects

This method is suited for homopterans or sap feeders. In this method, the extract of foliage or fruit is taken in a feeding vial, and they are kept for feeding. Based on the quantity fed by the insect, the preference is assessed.

Both the above tests can be conducted under two different conditions, namely, (i) free-choice condition and (ii) no-choice condition/confined feeding.

### *3.1 Free-Choice Condition*

Preference of the larvae or any chewing insect among the selected accessions is ascertained by leaf disc method. Leaf disc of 250 $mm^2$ size is excised from a 30-day-old plant from the second leaf beneath the terminal bud of each accession and is placed at equidistance on moist filter paper in a 150 mm dia petri dish. Single third instar larvae pre-starved for 6–12 h are released at the center of the petri dish. The leaf area consumed is calculated by using graph sheet.

Chakravarthy and Lingappa (1985) evaluated different concentrations of methanol extracts of *Lablab niger* (field bean) under choice conditions following the procedures of Munakata and Okamoto (1964) to the pod borer *Adisura atkinsoni* Moore. The first instar *Adisura* larvae were more sensitive in discriminating nonhost from the host. The first instar larvae preferred 10% concentration of the plant extracts over higher concentrations.

### *3.2 No-Choice/Confined Feeding*

Single 3rd instar larva
from the stock culture is pre-starved for 6 h and then allowed to feed individually on the leaf disc kept separately in 100 mm petri dishes. The area of leaf fed by the larva after 12, 24, 48, and 72 h is measured. This experiment can be conducted in the glass house also by allowing individual insect on the test plant enclosed by a screening cage.

## 4 Feeding Bioassay

Different techniques for insect feeding have been deployed to study the role of naturally occurring plant chemicals in the insect's choice of host and determine the resistance mechanisms in crop plants. The basic design in studying antixenosis of plant substrates is to present a choice of different substrates to the insects. The substrates may be whole plants, excised plants, leaf discs, or artificial substrates with an incorporated resistance factor. Since the behavioral effect of a chemical may or may not be independent of its nutritional value, these two properties must be experimentally separated before evaluating the chemical as a feeding stimulant or deterrent. This can be carried out by running short-term assays to avoid post-ingestional effects of food. In the long-term tests, consumption effect on insect growth and digestion of the compound can be measured separately to assess the antibiosis nature of the phytochemicals. Test plant materials are typically compared with control or other test substances in dual-choice, multiple-choice, or no-choice tests. The number and arrangement of substrates vary greatly with species, cage design, and objectives of the experiment.

## 5 Chewing Insects

**Dual-choice arena test:** This is used to assess the relative antixenosis mechanism of the resistance.

**Antixenosis in cowpea to the legume pod borer**, *Maruca testulalis* (*vitrata*) (Geyer) (Pyralidae: Lepidoptera)

- A round plastic container or "arena" (18.5 cm diameter × 7.5 cm depth) with a 25-cm-thick piece of Styrofoam fitted at the bottom is used.
- A filter paper of similar size, moistened to keep the arena slightly humid, is placed on top of the Styrofoam.

- Before the filter paper is installed, it is marked with a pencil into four equal parts, and pods of two varieties are placed in each part to provide a choice for the legume pod borer.
- The pod segments are arranged concentrically in an alternating sequence of susceptible and resistant varieties or cultivars.
- Four third- or fourth-instar larvae of *M. testulalis* reared on artificial medium are introduced at the center of each arena and allowed to choose between the test varieties for 72 h.

Feeding measurements, viz., the feeding ratio (FR, fraction of feeding out of four) and the feeding severity (FS, extent of feeding), are recorded. Thereafter, a feeding index (FI) to estimate the insect's preference for and its consumption of the test varieties is computed as FI = (FR × FS). FI varies from 1 to 4. To make comparisons among varieties against the control plant, a preference ratio (PR) is computed as follows.

$$\text{PR} = 2\,(\text{FI of test plant}) - (\text{FI of control} + \text{FI of test plant}).$$

The Preference Ratio (PR) has a minimum value of 0 and a maximum value of 1, where PR > 1 indicates a preference for the test plant, PR < 1 indicates a preference for the control plants, and PR = 1 indicates no preference.

**Sap Feeders**

Determining feeding behavior of plant hoppers and leafhoppers using improved electronic monitoring system developed by Kawabe et al. (1981) has now become quite popular. This device detects the changing electrical impedance in the insect and the substrate in which the insect is probing. To accomplish this, a small alternating current or direct current voltage is applied across the insect and the substrate which results in a small flow of charge. When the insect probes into a plant or diet, the electric circuit is completed. A complex signal originating from the insect and the substrate is amplified, and the waveform patterns within the signal are then subsequently correlated with the various phases of feeding: probing, salivation, and ingestion.

## 6 Assessment of Feeding Behavior of Brown Plant Hopper on Rice

The electronic monitoring system is used for assessing the feeding behavior of the brown plant hopper to determine antixenosis resistance in rice varieties (Velusamy and Heinrichs 1986).

- The insects are starved but water satiated for 24 h.
- A gold wire (20 m dia) is then attached with electroconductive paint to the dorsum of 1-day-old female brachypterous brown plant hopper.

- The opposite end of the gold wire is attached to a larger wire leading to the input of a current detection amplifier.
- The insect is placed on the leaf sheath of a potted rice variety or cultivar.
- The final amplifier is adjusted to 500 mV full-scale output. The speed of the strip-chart recorder is maintained at 2mV $min^{-1}$.
- The sequence of brown plant hopper feeding on susceptible and resistant rice cultivars will be electronically recorded as waveforms.
- The insect may make more frequent and shorter probes and they are recorded.

## 7 Evaluation of Feeding Activity of Hoppers in Rice (Honeydew Experiment)

Feeding activity of hoppers can be studied through two different methods.

### *7.1 Ninhydrin method*

Ninhydrin reacts with amino acids present in the honeydew exuding from the hoppers and produce purple color. Filter papers are used to collect the honeydew.

### *7.2 Bromocresol method*

Whatman No. 1 filter paper is dipped in bromocresol solution (2 mg bromocresol green in 1 ml of ethanol) and allowed to dry for 1 h and dipped again. Once the filter paper turns slight yellow, it is placed at the base of the plant. A cage is fixed around the plant, and five BPH are released and allowed to feed for 24 h. If honeydew drops on filter paper, blue spots develop. If the concentration of honeydew is high, blue spots turn white in the center with blue edges. The area of spot that develops is measured and quantified with honeydew proportion (Fig. 1).

**Illustrated Example:** Response of aphids to plant extracts:

Aphids were collected from laboratory reared on potato stem cuttings in a controlled-environment chamber at 25 °C and L16:D8 regime. The impact of extracted sesquiterpene on settling behavior of *M. persicae* was determined by test arena constructed from a hollow polythene stopper with two circular holes (1 $cm^2$ each) removed from the bottom. A Parafilm M (American Can Co.) sachet, containing 20% sucrose in distilled water, was placed over the holes converting them into feeding areas for the aphids. A small (1 × 5 mm) curved strip of Parafilm was placed at the center of each circular feeding area. In each test arena, the strip on

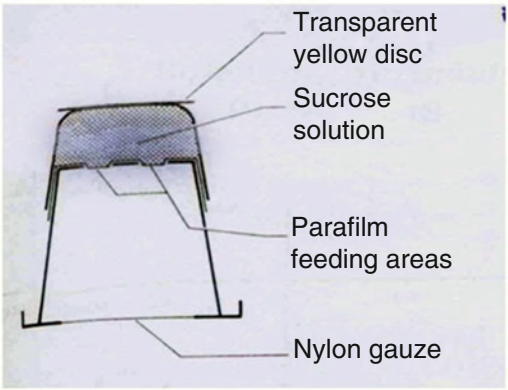


**Fig. 1** Bioassay assembly for aphid settling behavior. Test sample applied to a folded strip of Parafilm attached to the center of feeding areas. (Dirk et al. 1987)

feeding areas "S" received the sesquiterpene mixture and the other feeding area denoted "C" served as a control. The sesquiterpene mixture was dissolved in carbon tetrachloride and mixed in a 1:1 ratio with a 2.5% solution of Carboset (B.F. Goodrich Co.) in acetone. After solvent evaporation, the Carboset provided for a slow release of volatiles. The control mixture consisted of carbon tetrachloride, Carboset, and acetone in concentrations equal to the sesquiterpene treatment. Four adult apterae aphids were introduced in each test arena which was subsequently inverted and placed on a plastic lid. The lid had an open area for aeration covered with a fine mesh screen. The tests were conducted in a controlled environment cabinet (25 °C) with continuous illumination. The number of aphids present on each of the feeding areas was counted after 3, 6, and 10 h. The data were analyzed using chi-square on the null hypothesis that the aphids present on the feeding areas were distributed equally in a test arena (Dirk et al. 1987).

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# Techniques for Determining Mechanisms of Resistance: Antixenosis for Oviposition


**K. R. Manikandan and A. Arasu**

**Abstract** In the host selection process, oviposition by adult female is a crucial step. It establishes the fact that the insect can utilize the plant for reproduction. The ovipositional preference of an adult can be tested under choice/no-choice conditions. Nonparametric and parametric statistical tools can be deployed to deduce inferences from the experiments.

**Keywords** Ovipositional preference · Choice conditions · No-choice conditions · Reproduction

## 1 Introduction

The ovipositional preference of adult insects can be measured by both free-choice and no-choice/confinement conditions. Laboratory choice experiments may use filter papers soaked in plant extracts, whole plants or plant parts. Studies on oviposition are conducted with a simultaneous choice, and preferences are estimated on the basis of relative number of eggs laid on the test plants.

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## 1.1 Free-Choice Conditions

Fresh shoots or twigs or the seedlings are used for the ovipositional preference study. The fresh shoot of each accession is excised and such shoots of all the test accessions are kept immersed together in nutrient medium (agar and water 1:100) in a 100 ml conical flask inside a plastic container (45 cm ht and 45 cm dia) in five replications and a pair of pre-mated adult insects are released. Number of eggs laid and percent egg hatching in each accession are calculated after 12, 24, 48 or 72 h.

## 1.2 No-Choice/Confinement Condition

Fresh shoot of each of the accession is excised, and the twigs are kept immersed individually in nutrient solution. The conical flask (250 ml vol) having the shoot of each accession is enclosed in a big glass trough (30 cm × 25 cm × 12.5 cm), and a pair of pre-mated adults are released; five such shoots are maintained for each accession individually. 10% sucrose solution enriched with vitamin mixture is provided as diet for the adults. After 12, 24, 48, and 72 h, the number of eggs laid on each accession is counted, and percent egg hatching is recorded.

### 1.2.1 Illustrated Example 1: Oviposition by *H. virescens* (Table 1; Fig. 1)

**Table 1** Effect of antennal amputation (ant. amp) and acid treatment of tarsi (t.tr.ac.) on oviposition by *H. virescens* ($N = 25$)

| Treatment | $\bar{X} \pm$ SD eggs laid |
|---|---|
| Control | 179 ± 177 a, b |
| Ant. amp. | 66 ± 91 b |
| Fore t. tr. ac. | 132 ± 135 a, b |
| Mid t. tr. ac. | 285 ± 153 a |
| Hind t. tr. ac. | 193 ± 167 a, b |
| All t. tr. ac. | 190 ± 181 a, b |

Means not followed by the same letters significantly different, $P < 0.05$, SNK multiple range tests
Source: Ramaswamy et al. 1987

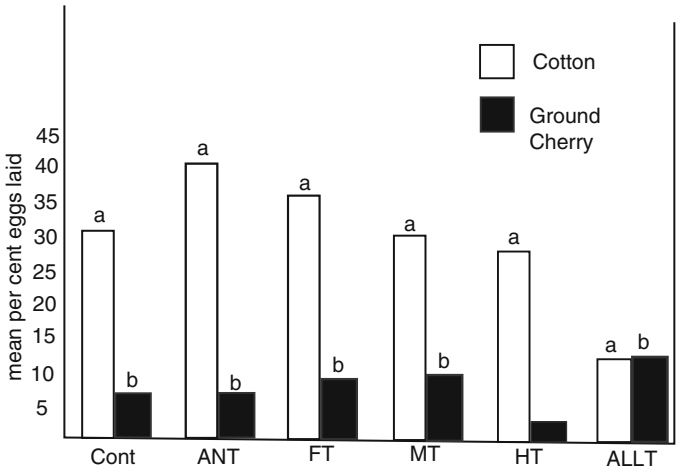


**Fig. 1** Oviposition preferences of *H. virescens* to cotton and ground cherry in a choice situation. Treatments *CONT* control, *ANT* antennectomized, *FT* acid-treated fore tarsi, *MT* acid-treated mid tarsi, *HT* acid-treated hind tarsi, *ALLT* all tarsi treated with acid. Bars without similar letters at to significantly different ($P < 0.05$, t-test). Statistical comparison within treatments only $N = 25$. (Source: Ramaswamy et al. 1987)

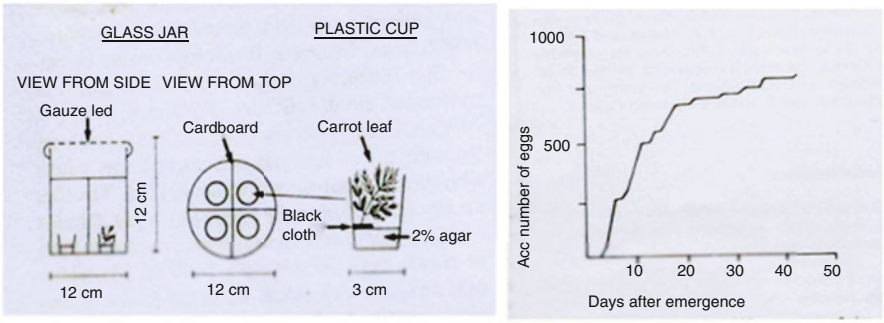


**Fig. 2** (**a**) Diagram of choice chamber used for egg-laying experiments with the carrot fly (*Psila rosae*). (**b**) Accumulated number of eggs laid by one group of nine female carrot flies. (Source: Eilenberg 1987)

### 1.2.2 Illustrated Example 2: Egg-Laying Behavior of Female Carrot Flies (*Psila rosae*) (Fig. 2)

### 1.2.3 Bioassay to know the oviposition preference for chemical stimulants of *Brassica* sp. by the diamondback moth, *Plutella xylostella* (Plutellidae: Lepidoptera) (Reed et al. 1989).

- In this experiment, the glucosinolates from the three *Brassica* spp. (*Brassica napus* L., *B. juncea* (L.) Czerniak, and *Sinapis alba* L.) are extracted to determine the ovipositional preference of the diamondback moth.
- Compounds from aerial portions of 4–6-week-old plants have to be extracted and fractionated using ion-exchange liquid chromatography.
- The activity of glucosinolates is neutralized by myrosinase or sulfatase enzymes, which degrade glucosinolates.
- Bioassays are conducted in clear plastic chambers (30 × 30 × 20 cm) with screened tops.
- A drawer in the side of the chamber should be added for the insertion of an 18.5-cm-diameter Whatman No.1 filter paper disk which contains the extracts that are bioassayed.
- The disks should be allowed to dry before exposing them to moths.
- Twenty-five newly emerged (0–24-hour-old) male and female moths are placed in a bioassay chamber with 10% sucrose solution.
- The moths are allowed for oviposition for 1–6 days.
- All bioassays are choice tests with equal-size areas treated with different solutions for comparison.
- At the end of the experiment, the filter papers should be removed, and the eggs are counted.
- The data can be log-transferred and analyzed by two-way ANOVA.

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# Techniques for Determining Mechanisms of Resistance: Antibiosis


**N. R. Prasanna Kumar and Thimmanna**

**Abstract** The adverse effects of feeding continuously on a plant for the insect are measured by antibiosis. The test is conducted under confined conditions. The antibiotic factor manifests on the growth and development of the insect. In this chapter, procedures for the tests have been outlined for the sesame shoot webber and the rice planthopper. The indices associated with tests of antibiosis are provided.

**Keywords** Antibiotics · Growth and development · Growth indices · Parafilm sachets

## 1 Introduction

Often antibiosis mechanism of resistance is assessed under no-choice tests, with the insects confined on plants or plant materials inside a cage. Such tests are performed mostly in the greenhouse or in the laboratory, sometimes under field conditions. Meridic or artificial diets can also be used in antibiosis tests.

Antibiosis is defined as the influence of crop cultivar on growth and development of insect. If the cultivar is susceptible, it favours the growth and development of insects. If it is resistant, it disturbs the growth and development of insects (Sharma and others. 2005).

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## 2 Lepidopteran Insects

Well-grown test plants are selected for antibiosis study. Neonate larvae are released individually in the screening cages enclosing the foliage of the test plant. The larvae are observed once in 2 days, given with fresh foliage whenever needed, and percent larval mortality; pupation percentage and pupal weight; larval, pupal and adult longevity; and adult emergence including malformation if any are recorded. The influence of the test plant on the growth and development of insects is assessed by calculating various indices as mentioned below:

$$\text{Growth index} = \frac{\text{Percentage of pupation}}{\text{Average duration of larval period}}$$

$$\text{Larval pupal index} = \frac{\text{Average larval period on standard host} \times \text{Average pupal period on standard host}}{\text{Average larval period on test host} \times \text{Average pupal period on test host}}$$

$$\text{Pupal index} = \frac{\text{Average pupal wt.(mg) on test host}}{\text{Average pupal wt (mg) on standard host}}$$

$$\text{Adult index} = \frac{\text{Average adult longetivity on test host}}{\text{Average adult longetivity on standard host}}$$

$$\text{Survival index} = \frac{\text{Average adult emergence per cent on test host}}{\text{Average adult emergence per cent on standard host}}$$

$$\text{Ovipositional index} = \frac{\text{Average number of eggs laid on test host}}{\text{Average number of eggs laid on standard host}}$$

## 3 Antibiosis of Selected Sesame Accessions Against Shoot Webber, *Antigastra catalaunalis*

Selected sesame accessions (30, 45 and 60 days old) are tested for their antibiotic effects, if any, against *A. catalaunalis*. The selected accessions are raised in earthen pots, and five neonate larvae are released individually on the plants at the respective age. The released larvae are caged and observed once in 2 days and allowed to pupate. Larval, pupal and adult longevity, pupation percentage, pupal weight and adult emergence percentage including malformation, if any, are recorded (Balaji and Selvanarayanan 2009).

Based on the above observations, developmental indices as mentioned above were computed by the following methods described by Dubey et al. (1981).

## 4 Sap-Feeding Insects

The known number of insects (nymphal stage) is released in a test plant. Then the nymphal, adult period, mortality at various insect stages is being observed. Based on the results, the resistance will be calculated.

## 5 Hoppers

Several methods have been developed for assessing the antibiosis resistance of rice varieties to the brown planthopper. These methods include hopper feeding, survival of nymphs and population growth. The feeding activity of hoppers is determined by measuring the amount of honeydew excreted. This technique has proved useful for determining the level of resistance of rice varieties to the brown planthopper, whitebacked planthopper and green leafhopper.

## 6 Quantifying Honeydew Excretion

Honeydew excreted by the brown planthopper is used as a criterion for determining the amount of sap ingested by the insect on resistant and susceptible rice cultivars. The quantity of honeydew excreted is low on resistant cultivars and high on susceptible cultivars.

**Materials Required** Potted rice cultivars (susceptible and resistance), filter paper, Petri dish, plastic cup, cellophane tape, cotton plug, hoppers and 0.001% ninhydrin solution.

**Procedure**

- The experiment is set up as shown in the diagram using 40–50-day-old potted, susceptible and resistant variety.
- Known number of planthoppers (2–3 pairs) are released into plastic cup.
- The planthoppers excrete honeydew on filter paper.
- The filter paper is sprayed with 0.001% ninhydrin in acetone solution.
- The areas of honeydew spots that are bluish or purple are measured, and that indicated the intensity of feeding by the planthoppers.
- Each treatment including the control is replicated several times.
- The amount of honeydew excreted is highly correlated with weight gain over the same time period for a given rice variety.

**Note** Using the parafilm sachets, the quantity of honeydew excreted by one insect during a 24-h period can be worked out. This is used as a parameter in comparing the insect's feeding activity on susceptible and resistant crop varieties (Fig. 1).

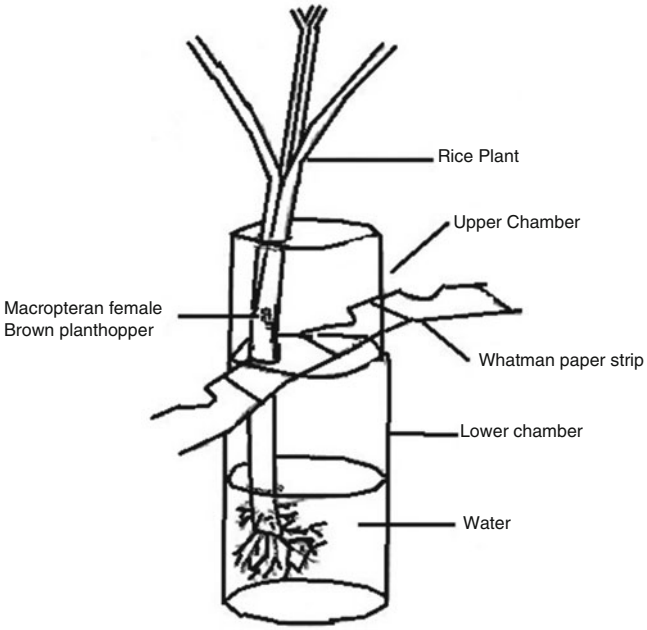


**Fig. 1** Laboratory set-up for estimating hopper-excreted honeydew on filter paper. (Source: Paguia et al. (1980))

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# Techniques for Determining the Mechanisms of Resistance: Tolerance


**G. P. Muthuraju and Yanal Ahmad Al Kuddsi**

**Abstract** Scientists have designed different techniques to measure tolerance; measurement of tolerance under field conditions is most desired. Few examples of plants exhibiting tolerance against specific species of pest insects have been given. Measurement of tolerance against rice brown planthopper is described.

**Keywords** Plant vigor · Tolerance · Recovery · Durability

## 1 Introduction

Tolerance refers to the ability of a plant to yield in spite of abiotic and biotic constraints. The basic difference between resistance and tolerance is that the former stems from the insect response to certain specific host characteristics, whereas the latter are expressed from a plant's response to the insect attack. Under field conditions, tolerant plants are desirable because often they are more durable and stable than resistant plant (see Vasantharaj David and Ramamurthy 2011).

## 2 Recovery Resistance

On crops like castor (*Ricinus communis*), the semilooper, *Achaea janata* Linn. (Noctuidae: Lepidoptera), often completely defoliates the plant, particularly in the breed varieties/selections/hybrids, under field conditions. However, the plant during the course of its growth puts forth new leaves and recovers. This ability to recover

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**Fig. 1** Pest of sugarcane: early shoot borer, *Chilo infuscatellus* Snellen. (Source: TNAU Agritech portal)

from insect damage varies with the genotype. This type of resistance is useful and of value to farmers and is of practical significance.

Tolerance usually results from one or more of the following factors:

1. General vigor of the plants
2. Regrowth of damaged tissues
3. The strength of the stems and the resistance to lodging
4. Production of additional branches
5. Efficient utilization by the insect of non-vital plant parts
6. Lateral compensation by neighboring plants

Different techniques have been developed to evaluate the plant characteristics most commonly associated with insect tolerance. These characteristics include increases in the size and growth rate of plant leaves, stems, petioles, roots and seed, or fruit. If determinations are made in the seedling stage, plant survival is a common measurement of tolerance (Figs. 1 and 2).

## 3 Example: Brown Planthopper, *Nilaparvata lugens*

1. The seeds are sown in rows in a standard seed box (60 × 40 × 40 cm).
2. Twenty-five seeds of each test entry are sown in a 12 cm row.
3. Seven days after sowing (DAS), when the seedlings are in the two-leaf stage, the seed boxes are placed in a water pan inside a screened room.
4. The weeds are removed and the seedlings are thinned to about 20 per row.
5. Five cm water is to be maintained into the wooden/plastic/concrete plan.
6. Ten brown planthopper nymphs cultured on susceptible variety are uniformly distributed on to the each test seedling by holding the base of the plant and lightly tapping the plants and blowing on them.
7. The plant damage is assessed on 28 days after seeding.


**Fig. 2** Sweet sorghum stem borer (*Chilo partellus*). (Source: ICAR, NAIP)

8. Weight loss (dry) in plants due to brown planthopper feeding will be estimated by the functional plant loss index (FPLI)

$$\text{FPLI} = 1 - \left[\frac{\text{Dry wt of infestedplants}}{\text{Dry wt of uninfested plants}}\right] * \left[1 - \frac{\text{Damage rating}}{9}\right] * 100$$

9. The regression of FPLI *(y)* on brown planthopper dry weight *(x)* will be computed for each test variety, and a pooled regression over all varieties is calculated.
10. The tolerance index based on the brown planthopper dry weight on the test varieties is also used for the assessment of tolerance, where:

$$\text{Tolerance} = \frac{\text{Brown planthopper dry weight on test variety}}{\text{Brown planthopper dry weight on susceptible varieties}}$$

**Illustrated Example 1: Applying High-Throughput Technology to Identify and Decipher Resistance Mechanism (Fig. 3)**

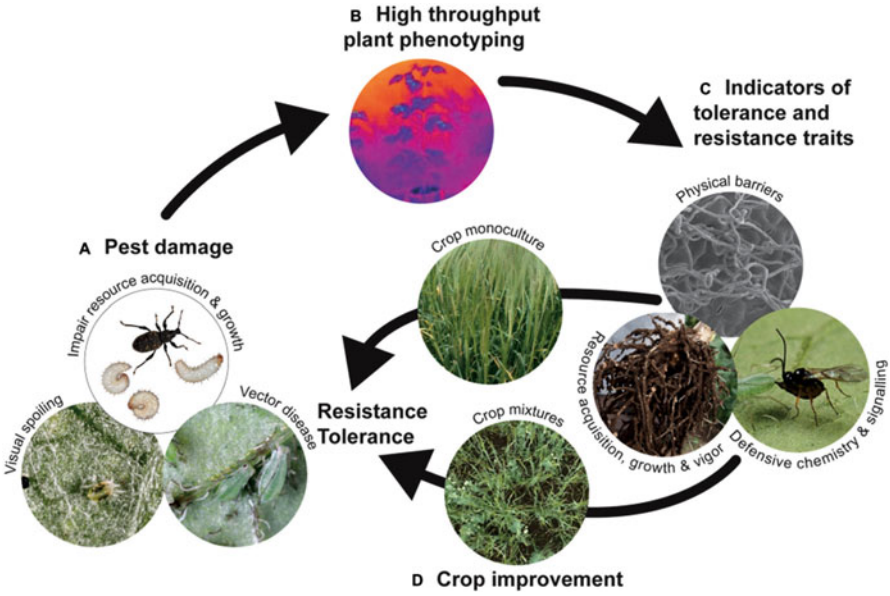


**Fig. 3** A strategy for effective crop protection against target insect pests. (A) Identify the appropriate defense strategy (resistance or tolerance) based on the damage and threat posed by the pest; (B) develop high-throughput phenotyping (HTP) technologies, particularly new imaging methods, for screening large plant populations to (C) identify appropriate indicators of resistance and tolerance traits; indicators could include reflectance properties that provide information about leaf surface characteristics and physical barriers and thermal and absorption data that provides information about stomatal conductance and water status and therefore indicate photosynthetic activity and plant vigor and absorption/reflectance data that characterizes leaf pigment composition and metabolic changes underpinning defense signalling (e.g., attracting natural enemies); (D) traditionally, desirable traits are characterized in germplasm monocultures, but phenotyping traits in crop mixtures is a potential route for durable pest control, particularly under environmental change. (Source: Mitchell et al. 2016)

**Acknowledgment** The authors would like to thank authorities of College of Horticulture, Mysore and General Commission for Scientific Agricultural Research, Damascus, Syria for their encouragement and support.

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# Techniques for Evaluation of Biophysical Factors of Resistance in Crop Plants


**N. Muthukumaran and R. Promoth Kumar**

**Abstract** Biophysical plant characters confer resistance or susceptibility to the phytophagous insect attack. The quantification of the role of biophysical features is key to designing and developing cultivars' resistance/tolerance to pests under field conditions. While enumerating the data on the role of plant physical characters, a data sheet is provided to record field observations. Data to indicate the extent to which biophysical features are associated with resistance or susceptibility are also given.

**Keywords** Biophysical plant features · Resistance · Tolerance · Correlation and regression analysis

## 1 Introduction

The relationship between plants and insects is an ancient and fascinating one. Over the years, plants have developed numerous strategies to make them resistant to predation by insect herbivores. Insects, at the same time, have figured out ways of circumventing or detoxifying the defense mechanisms in plants. This interrelationship is dynamic and ongoing.

The study of the interrelationships between the various insect and plant species was labeled "coevolution" by Ehrlich and Raven in 1964 (Ehrlich and Raven 1964). One model to look at coevolutionary dynamics is of an interspecies arms race, where each insect and plant species tries to develop a weapon system that the others cannot match. The weapons in this case are ecological, biochemical, and morphological adaptations (Dan Stein 1991).

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## 2 Examples of Morphological Traits That Confer Resistance

All plants must assume forms that allow certain metabolic functions to take place, including photosynthesis and orderly growth. But in addition, some plants have acquired morphological adaptations that make them at least partially resistant to insect predation. These adaptations can be classified into the following categories. But there are no resistance adaptations that hold true for all plants, and specific examples are hard to point out with certainty.

### *2.1 Color*

Certain colors are less attractive to a given insect species. For example, imported cabbage worm is less attracted to red-colored *Brassica* species (cabbages, broccoli, and related species). Cucumber beetles do less damage on reddish-colored varieties of leaf lettuce and are attracted to certain hues of yellow.

### *2.2 Shape*

While it is impossible to generalize what shapes resist insect feeding better, shape does play a role in avoiding predation. For example, one study noted that thick rooted turnips were less damaged by turnip maggots. Another study showed that onions with leaves having a narrow angle of contact are more attractive to thrips than onion varieties with loose leaves.

### *2.3 Thickening of Cell Walls and/or Rapid Growth*

In response to predation, some plants create tougher leaves or callous tissue. Both corn and soybeans have been noted to increase growth in response to feeding by certain aphids (Dale Norris and Marcos Kogan 1980).

### 2.4 Hairiness

Many plants utilize trichomes (plant hairs) to protect themselves against predation. A few examples of insects that are at least partially suppressed by plant hairiness include bean aphid and potato leafhoppers on beans, two-spotted spider mite on strawberry, and whitefly on tomatoes, peppers, and potatoes.

### 2.5 Surface Waxes

Waxy leaf surfaces provide protection against some insects, cabbage flea beetle on *Brassicas*, for example, but may encourage other insects (i.e., cabbage aphids) (Dale Norris and MarcosKogan 1980).

### 2.6 Anatomical Adaptations

For example, corn with very tight husks is somewhat resistant to corn earworm, *Helicoverpa zea*. Corn varieties with tough, resilient stalks can tolerate burrowing by corn borers without breaking and causing little or no yield loss. A variety of wheat with a solid stem does not allow sawfly larvae to bore through the stems and reach their feeding sites (Roger 1984). In addition to the above, many other characters such as frego bract, petiole length, leaf toughness, leaf angle to the stem, stem color, and stem thickness are also found to influence insect resistance.

## 3 Estimation of Biophysical Traits in Crop Varieties

### 3.1 Trichome Density

Trichomes are present on upper and lower surfaces of leaf, stem, petiole and bracts, or calyx. Length and breadth of trichomes are measured using micrometer. Further, a number of trichomes per unit area are also estimated. One-millimeter-long transverse section is cut from the respective plant part of the selected accessions. The sectioned samples are observed under a compound microscope (10× magnification), and the

number of trichomes is counted and expressed as trichome density per mm length. The types of trichomes present in the selected accessions may be characterized by micrometric measurements of their length and breadth.

## *3.2 Plant Height*

Plant height is measured from ground level to the growing tip of the main stem at different phenophases of the plant.

## *3.3 Internodal Distance*

Length of the top five internodes in five plants from each accession is measured using a graduated scale, and the average internodal length can be computed.

## *3.4 Leaf Length and Breadth*

The length and breadth of the top, middle, and bottom leaves in five randomly selected plants per accession are measured on 45 days after sowing.

## *3.5 Thickness of Leaf*

Using micrometer, the thickness of the leaf is estimated.

## *3.6 Capsule Characters*

Capsule length, capsule breadth, locule length, locule breadth, locule wall thickness, number of seeds per capsule, number of capsules per plant, and weight of the matured capsule may be recorded from ten capsules each, in plants selected at random per accession.

## 4 Estimation of Trichome-Mediated Resistance: Entrapment Test

Glandular trichomes on the surface of certain crop varieties trap the neonate insects when they are moving. Such trapping eventually leads to death of the insects. This entrapment may be estimated by the following procedure:

- Young, fully expanded leaflets from 35-day-old test plants are excised and placed individually, adaxial side up on a moist filter paper spread at the bottom of 80 mm plastic petri dish.
- On each leaflet, ten neonates are placed, using a fine camel hairbrush, on the adaxial leaf surface, and the lid is placed on top to avoid desiccation.
- The larvae are gently prodded with a camel hairbrush at 6 and 12 h after placement. If no reaction is evident, the neonate is designated trapped and dead.
- In control, leaflets excised from each test plant are gently swabbed on both sides using moistened cotton with 95% ethanol to break the trichome heads and to remove the trichome exudates.
- These leaflets are rinsed in distilled water to remove ethanol.

## 5 Evaluation of Biophysical Bases of Resistance (Impedance Test)

- Young, fully expanded leaflets from 35-day-old test plants are excised and placed individually adaxial side up on a foam sheet.
- Two foam strips are kept on the foam sheet parallel to each other leaving a gap of 1 cm. The inner sides of the foam strips are smeared with wax to avoid larval climbing.
- One, the third instar larva is allowed to crawl on the leaf between the foam strips from one end to another, and the time taken by the larva to travel is recorded.
- In control, leaflets excised from each test plant are gently swabbed on both sides using cotton moistened with 95% ethanol to break the trichome heads and to remove the trichome exudates.
- These leaflets are then rinsed in distilled water to remove ethanol.
- The relative time taken by larva to crawl on the leaf surface of test accessions with and without trichomes is recorded to study the impedance by trichomes.

**Example**

- Eight different cultivars of field bean, *Lablab niger* Medick, encompassing resistance, susceptible, and highly susceptible groups are sown. The following 11 plant morphological characters are recorded to correlate plant characters and borer resistance.

Plant morphological characters recorded with classification of each morphological character

| Plant character | Classes under each character with quantified value in parenthesis |
|---|---|
| Foliage color | Light green (I), green (2), dark green (3) |
| Days to flower | 60(1),61–80(2),81–100(3),101–140(4),141–160(5) |
| Flower color | Light pink (I), pink (2), white (3) |
| Plant type | Erect (I), spreading (2), semi-spreading (3), and highly spreading (4) |
| Inflorescence type | Raceme arising from leaves at lower nodes and flowers borne on the floral axis (I), axillary, flowering branch arises in the axil of each trifoliate leaf (2) |
| Inflorescence length | Less than 5 cm (I), 5–20 cm (2), 21–35 cm (3) |
| Pod color | Green (I), dark green (2), white (3), red (4), dark red (5) |
| Pod shape | Sickle (I) and straight (2) |
| Pod texture | Soft (I), slightly hard (2), and hard (3) |
| Fragrance | Present (I) and absent (2) |
| Pod form | Flat (1) and inflated (2) |

Source: Chakravarthy (1977)

- Each plant character is suitably divided into classes to accommodate all variations under each character.
- Further the plant characters are arbitrarily quantified (Table 1) for the purpose of statistical analysis. The data are subjected to stepwise regression analysis.
- Fifty flowers are labeled at the peak activity of pod borers, especially the dominant borer species. Observations on color development, pod age, surface area, and pod texture are recorded on 50 flowers (pre-labeled) daily till the pods mature fully.
- The buds, flowers, and pods surrounding the labeled flowers are gently removed mechanically to facilitate oviposition on labeled pods. For the same reason, the dried-up petals covering the tender pods are also removed.
- Based on color development, the pods are grouped into the following stages: (a) 100% green, (b) 75% green, (c) 50% green, (d) 25% green, and (e) 100% red.
- As the pods develop, they gradually turn red from green. The surface area is recorded from five pods at each stage of color development for each select genotype (Chakravarthy 1977) (Fig. 1).

**Table 1** Correlation of morphological characters of select *Lablab niger* cultivars with ovipositional response and larval boring

| Cultivars | % pods bored ($X_1$) | Av. No. of eggs laid ($X_2$) | Foliage color[a] | Days to flower | Flower color | Plant type | Inflorescence type | Inflorescence length | Pod color[b] | Pod shape | Pod texture[b] | Fragrance[b] | Pod |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. | | | | | | | | | | | | | |
| 2. | | | | | | | | | | | | | |
| 3. | | | | | | | | | | | | | |
| 4. | | | | | | | | | | | | | |
| 5. | | | | | | | | | | | | | |
| 6. | | | | | | | | | | | | | |
| 7. | | | | | | | | | | | | | |
| 8. | | | | | | | | | | | | | |

Source: Chakravarthy (1977)

[a]Morphological characters entered regression with ($X_2$)

[b]Morphological characters entered regression with ($X_1$)

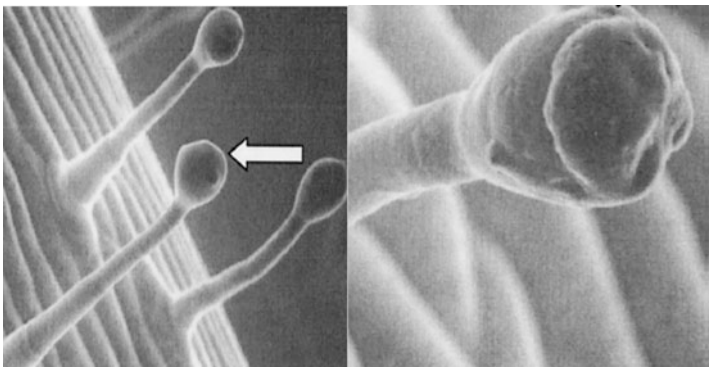

**Fig. 1** Environmental scanning electron micrographs of stem and leaf glandular trichomes of glandular-haired genotypes of alfalfa (Source: Shockley and Backus 2002)

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# Techniques for the Evaluation of Biochemical Factors of Resistance in Crop Plants


**K. Balaji and A. Thanga Hemavathy**

**Abstract** The analysis of plants for determining biochemical profile is crucial for elucidating the role of different chemicals in host–plant relationships. The procedures for estimating sugars, lipids, tannins, alkaloids, proteins, amino acids, enzymes and hormones are enumerated in this chapter. The role of antimetabolites/anti-nutritional elements is also highlighted.

**Keywords** Biochemicals · Preparation of plant samples · Anti-nutritional factors · Hormones

## 1 Introduction

Different chemical constituents of plants that are the byproducts of primary and secondary metabolism make the plant resist against insects. It will adversely affect the growth, development and metabolic processes of insect species. Primary metabolic products like carbohydrates, sugars, proteins, enzymes, lipids and certain organic acids play an important role in this process. Apart from different plant secondary metabolic products, other compounds like alkaloids, terpenoids, flavonoids, glycosides, phenolic compounds, essential oils, isothiocyanates, coumarins, tannins and aromatic fatty acids are also having an important role in plant defence. Plant analysis is the determination of the concentrations of vital elements in a sample from a particular part of a plant sampled at a particular stage. The plant analysis is mainly for total chemistry or quantitative analysis and semi-quantitative or rapid tissue analysis.

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## 2 Sampling

The sample should be a true representation. The selection of plant parts in definite stage of plant growth is crucial. The physiologically matured plant parts should not be selected as it will not undergo rapid changes in nutrient composition. The plant part selected, time of sampling and the number of plants sampled vary from species to species. The sampling should be done just prior to or at the time the plant begins reproductive stage of growth. The sampling is done when the plants are damaged by insects and may be compared with non-infested plant. Plants infested with diseases or plants with physiological disorders should not be sampled.

**Steps Followed During the Preparation of Plant Samples**

- The plant samples should be transferred to muslin cloth and then to the laboratory as quickly as possible. For long distance transportation, samples should be transported under refrigerated conditions, preferably within 2 days.
- The samples are washed by sponging with a piece of cotton wool using 0.1% detergent solution to remove the waxy coating (if needed) and soil dust particles on leaves. Then, the samples are washed with HCl (0.1 N) to remove metallic contaminations added through pesticide sprays. Washing should be done as quickly as possible. Delayed contact of leaf with detergents and acids may leach out nutrients such as K and Ca from the samples. The samples are washed in running water and rinsed with deionised/distilled water. The excess moisture is wiped out and the samples are placed in a paper bag.
- The samples are dried in an oven at 65–70 °C for 48 h to remove moisture and to inactivate the enzymes.
- The samples need to be grinded at this temperature using a Wiley mill or a homogeniser with stainless steel blades to prevent contamination with metal ions such as iron, zinc and copper. After grinding, the samples are mixed thoroughly and dried again at 70 °C to remove the moisture.
- The samples are stored in screw-type plastic or glass vial to prevent contamination.

## 3 Estimation of Biochemical Compounds

Chemical compounds of plants profoundly influence behaviour and reproduction of insects utilising plants as hosts. A direct correlation may exist between the plant age, the concentration of the chemical constituent and the growth and development of an insect. Understanding the relationships between specific chemical compounds and insects at different life stages will be most fruitful in developing management plans for the pest.

## 3.1 Estimation of Sugars

**Preparation of Oven-Dried Sample**

- The samples are dried at 32 °C in a hot air oven for 24–48 h.
- The sample is powdered using pestle and mortar.
- The sample is sieved through a 100 mesh screen and stored in sealed containers at 4 °C, until analysis.

Reducing and total sugars are estimated following Nelson (1944), with slight modifications.

**Reagents**

- **Copper reagent – A (Nelson's reagent – A):** 25 gm sodium carbonate (anhydrous), 25 gm Rochelle's salt (sodium potassium tartrate), 20 gm sodium bicarbonate and 200 gm sodium sulphate are dissolved in 800 ml distilled water, and solution is made to 1 litre using distilled water. This reagent is filtered and stored at room temperature.
- **Copper reagent – B (Nelson's reagent – B):** 15 gm copper sulphate is dissolved in a small quantity of distilled water and made up to 100 ml, and few drops of concentrated $H_2SO_4$ are added.
- **Copper reagent – A + B (Nelson's reagent – A + B):** 96 ml copper reagent A and 4 ml copper reagent B are mixed to make up 100 ml of reagent A + B.
- **Arsenomolybdate colour reagent:** 25 gm ammonium molybdate is dissolved in 450 ml distilled water. Later, 21 ml concentrated $H_2SO_4$ is added to prepare the final reagent.
- 3 gm sodium arsenate is dissolved separately in 25 ml water and added to the above solution and placed in an oven at 37 °C for 24–48 h.
- The reagents are stored in a glass stoppered brown bottle at room temperature.
- 1 N $H_2SO_4$: 4.9 ml conc. $H_2SO_4$ is diluted in 100 ml distilled water.
- 1 N NaOH: 4 gm NaOH is dissolved in a little quantity of distilled water, and the final volume is made up to 100 ml.
- Standard glucose solution: A stock glucose solution containing 1 mg glucose per ml in water is prepared. The sample is diluted to 1:10 to prepare 100 μg glucose per ml as a standard solution.

**Estimation**

- For the estimation of total sugars, non-reducing sugars are hydrolysed by 1 ml of 1.0 N $H_2SO_4$ to 0.5 ml of aliquot and heated in boiling water bath for 30 min.
- After cooling under running water, one to two drops of phenolphthalein indicator are added.
- Later, 1.0 N NaOH is added dropwise to neutralise the acid in the hydrolysate till it develops pink colour.
- Later, 1.0 N $H_2SO_4$ is added to make it colourless; finally, the volume is made up to 10 ml with distilled water, and the absorbance is read at 510 nm.

- Reducing sugars are estimated in 0.4 ml of aliquot by adding 1.0 ml of A + B reagent.
- The mixture is heated for 20 min and cooled under running water.
- 1 ml of arsenomolybdate solution is added, and the final volume is made up to 10 ml using distilled water.
- The absorbance is read at 510 nm. A standard graph is constructed using a glucose solution as a standard in the range of 20–100 µg.

## *3.2 Estimation of Total Soluble Sugars by Anthrone Method*

**Principle**

The anthrone reaction is the basis of a rapid and convenient method for the determination of hexoses, aldopentoses and hexuronic acids either free or present in polysaccharides. Carbohydrates are dehydrated by conc. $H_2SO_4$ to form furfural. Furfural condenses with anthrone (10-keto-9, 10-dihydro-anthracene) to form a blue-green-coloured complex which is measured colorimetrically at 630 nm.

**Reagents**

- 2.5 N HCl.
- **Anthrone reagent:** Dissolve 200 mg anthrone in 100 ml of the cold 95% $H_2SO_4$. Prepare fresh solution before use.
- **Standard glucose**: Stock – dissolve 100 mg in 100 ml water working standard – 10 ml of stock diluted to 100 ml with distilled water. Store refrigerated after adding a few drops of toluene water.

**Procedure**

- Weigh 100 mg of the sample into a boiling tube.
- Hydrolyse by keeping it in a boiling water bath for 3 h with 5 ml of 2.5 N HCl, and cool to room temperature.
- Neutralise it with solid sodium carbonate until the effervescence ceases.
- Make up the volume to 100 ml and centrifuge.
- Collect the supernatant and take 0.5 and 1 ml aliquots for analysis.
- Prepare the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard. “0” serves as blank.
- Make up the volume to 1 ml in all the tubes, including the sample tubes by adding distilled water.
- Then add 4 ml anthrone reagent.
- Heat for 8 min in a boiling water bath.
- Cool rapidly and read the green to dark colour at 630 nm.
- Draw a standard graph by plotting concentrations of the standard on the X-axis versus absorbance on the Y-axis.
- From the graph, calculate the amount of carbohydrates present in the sample tube.

**Calculation**

Amount of carbohydrates present in the sample (% mg): sugar value from the graph (mg) × total volume of extract (ml)

Aliquots sample used (0.5 or 1 ml): weight of sample (mg) × 100

**Note**

Cool the contents of all the tubes on ice before adding ice-cold anthrone reagent.

## *3.3 Estimation of Cellulose (Updegraff 1969)*

Cellulose, a major structural polysaccharide in plants, is the most abundant organic compound in nature and is composed of glucose units joined together in the form of the repeating units of the disaccharide cellobiose with numerous cross-linkages.

**Principle**

Cellulose undergoes acetolysis with acetic/nitric reagent forming acetylated cellodextrin which gets dissolved and hydrolysed to form glucose molecules on treatment with 67% $H_2SO_4$. This glucose molecule is dehydrated to form hydroxymethyl furfural which forms green-coloured product with anthrone, and the colour intensity is measured at 630 nm.

**Materials**

**Acetic/nitric reagent:** Mix 150 ml of 80% acetic acid and 15 mL of concentrated nitric acid.

**Anthrone reagent:** Dissolve 200 mg anthrone in 100 mL concentrated sulphuric acid. Prepare fresh and chill for 2 h before use.

**Procedure**

- Add 3 mL acetic/nitric reagent to a known amount (0.5 g or 1 g) of the sample in a test tube and mix in a vortex mixer.
- Place the tube in a water bath at 100 C for 30 min.
- Cool and the centrifuge the contents for 15–20 min.
- Discard the supernatant.
- Wash the residue with distilled water.
- Add 100 mL of 67% sulphuric acid and allow it to stand for 1 h.
- Dilute 1 mL of the above solution to 100 mL to 1 mL of this diluted solution; add 10 mL of anthrone reagent and mix well.
- Heat the tubes in a boiling water bath for 10 min.
- Cool and measure the colour at 630 nm.
- Set a blank with anthrone reagent and distilled water.
- Take 100 mg cellulose in a test tube and proceed from step 6 for standard. Instead of just taking 1 ml of the diluted solution (step 7), take a series of columns (say 0.4–2 ml, corresponding to 40–200 μg of cellulose) and develop the colour.

**Calculation**
Draw the standard graph and calculate the amount of cellulose in the sample.

## *3.4 Estimation of Hemicellulose (Goering and Van Soest 1970)*

**Reagents**

- **Neutral detergent solution:** Weigh 18.61 g disodium ethylene diamine tetra acetate and 6.81 g sodium borate decahydrate. Transfer to a beaker. Dissolve in about 200 mL distilled water by heating, and add a solution (about 100–200 mL) containing 30 g of sodium lauryl sulphate and 10 mL 2-ethoxy ethanol. To this, add a solution (about 100 mL) containing 4.5 g disodium hydrogen phosphate. Make up the volume to 1 litre and adjust the pH to 7.0.
- Decahydronaphthalene.
- Sodium sulphite.
- Acetone.

**Estimation**

- To 1 g of the powdered sample in a refluxing flask, add 10 mL cold neutral detergent solution.
- Add 2 mL decahydronaphthalene and 0.5 g sodium sulphite.
- Heat to boiling and reflux for 60 min.
- Filter the contents through a sintered glass crucible (G2) by suction and wash with hot water. Finally, give two washings with acetone. Transfer the residue to a crucible, and dry at 100 °C for 8 h.
- Cool the crucible in a desiccator and weigh.

**Calculation**

Hemicellulose = neutral detergent fibre (NDF) − acid detergent fibre (ADF)

## *3.5 Estimation of Total Soluble Protein Content*

- The desired plant parts are homogenised with prechilled acetone in a pestle and mortar.
- The slurry is filtered using Whatman No.1 filter paper and washed with chilled acetone.
- The acetone powder is air-dried overnight and then stored in sealed containers in a deep freezer.

**Reagents**

- **Solution A:** 20 gm of anhydrous carbonate ($Na_2Co_32H_2O$) and 4 g sodium hydroxide are dissolved in 1000 ml distilled water.
- **Solution B:** 1 ml of 1.35 sodium potassium tartrate and 0.1 ml of 5.5% $CuSo_4$ and 5 $H_2O$ solutions are mixed together.
- **Solution C:** 50 ml solution A is mixed with 1 ml solution B just before use.
- **Folin-Ciocalteu reagent (FCR):** The commercial FCR is diluted 1:1 before use.
- **Standard bovine serum albumin (BSA) solution:** A stock BSA solution is prepared containing 2 mg BSA/ml of working standard solution.

**Sample Extraction**

- 100 mg of oven-dried, powdered sample is extracted in 10 ml of 0.1 M sodium phosphate buffer (pH 7.0) for 1 h on a magnetic stirrer at room temperature.
- The extract is then centrifuged at 10,000 rpm for 20 min, and the supernatant is used for the estimation of total soluble protein content (Lowry et al. 1951).

**Estimation**

- A known volume of aliquot sample is taken and made up to 1 ml with distilled water. To this, 5 ml of solution C is added and mixed well.
- After 10 min, 0.5 ml of FCR is added and mixed immediately.
- The blue colour developed is read at 660 nm after 30 min against a reagent blank in a colorimeter.
- A standard graph will be constructed using BSA solution as a standard in the range of 20–120 μg.
- The total protein content is expressed as mg per gram of oven-dried sample.

## *3.6 Estimation of Lipid (Fobes et al. 1985)*

- For estimation of lipid, nearly 5 gm of leaflets are required.
- Fresh weight of a leaf is recorded. Total leaf surface area will be measured using the leaf area metre.
- The leaves are gently swirled in 200 ml chloroform for 60 sec, and the chloroform + lipid solution is decanted and then filtered through Whatman No. 2 paper.
- Filtrate will be rotary evaporated under vacuum, and the purified extract is removed from the collection vessel with −10 ml of chloroform.
- The remaining leaf material is dried at 70 °C in a forced-air oven, and dry weight is measured subsequently.
- When the leaves are washed with three successive aliquots of chloroform, the first wash removes 95% of the recovered lipid, and the second removes the remaining 5%.

- Lipid extraction from halved leaves showed the reproducibility of lipid recovery to be ±7.5%.
- 100 µl aliquots are removed from each lipid sample and placed in a tarred aluminium weight boat. The sample is dried at 100 °C and later cooled, and the final weight is recorded.
- The lipid content of plant sample is expressed as mg lipid/gm dry weight and µg lipid/$cm^2$ leaf area.
- The calculation of sample dry weight includes the dry weight of leaves after lipid extraction (gm) plus the mg of extracted epicuticular lipid.
- Data are analysed by a linear regression model, the regression lines are fitted, and the correlation coefficients are calculated.

**Lipid analysis by TLC (Silva Fernandes et al. 1964)**

- Chloroform is removed by rotary evaporation, and the residue is redissolved in hexane.
- The sample is then applied to a column (13 × 1.1 cm) containing 10 g alumina (grade II) and eluted with:

  (a) 75 ml hexane – to give a fraction of hydrocarbons
  (b) 50 ml hexane/diethyl ether (50:50) – to give a fraction enriched in sterols
  (c) 100 ml methanol – to give a fraction enriched in polar lipids

- Flavonoids particularly obvious in the samples are adsorbed irreversibly at the top of the alumina column and do not recover.
- The column fractions are dried using rotary evaporator and redissolved in 4.0 ml $CHC1_3$.
- Duplicate 100 µl aliquots are dried in tarred aluminium weight boats and weight of lipid determined by difference.
- Lipid composition is determined by TLC on silica gel G (Whatman) using the solvent mixture of hexane/diethyl ether/acetic acid at 40:10:1, respectively.
- Compounds are detected by:

  (a) Exposure to iodine vapours
  (b) Spraying with orcinol spray to detect sugars
  (c) Spraying with ferric chloride spray (0.05% $FeCl_3$-$6H_20$ in water/sulphuric acid/acetic acid, 90:0.5:5), to detect sterols (red colour on heating the plate)

- The major components of epicuticular lipids are hydrocarbons and sterols.

## *3.7 Estimation of Total Soluble Amino Acids*

Total soluble amino acids in the extract are estimated following the ninhydrin method procedure of Moore and Stein (1948).

- **0.2 M citrate buffer, pH 5.0:** 21 gm citric acid is dissolved in 200 ml of 1.0 N NaOH in 500 ml volumetric flask, and make up the final volume to 500 ml with distilled water.
- **Preparation of ninhydrin reagent:** 20 gm of ninhydrin is dissolved in 500 ml methyl cellosolve (ethylene glycol monomethyl ether). 800 mg of hydrated stannous chloride is dissolved in 500 ml of 0.2 N citrate buffer, pH 5.0. These two solutions are mixed to get ninhydrin reagent.
- **Diluents solution:** Equal volumes of distilled water and n-propanol are mixed to get the diluent solutions.
- 1 ml of ninhydrin reagent is added to 1.0 ml of extract and boiled in a specimen tube over water bath for 20 min.
- The specimen tubes are cooled under running water, and the volume is made up to 10 ml with the diluent solution. It develops purple colour and is read at 570 nm.
- A standard curve is prepared with glycine to calculate the quantity of total soluble amino acids.

## *3.8 Estimation of Total Phenol Content*

**Reagents**

- **Folin-Ciocalteu reagent** – commercial-grade reagent is diluted to 1:1 with water.
- 20% sodium carbonate solution – 20 gm of $Na_2CO_3$ is dissolved in water and made up to 100 ml.
- **Standard catechol solution** – 1 mg catechol per ml is added in water, and the solution is diluted in 1:10 ratio to obtain 100 μg catechol per ml as working standard solution.
- 100 mg sample is extracted in 10 ml warm 80% ethanol for 1 h at room temperature and then centrifuged at 6000 rpm for 15 min.
- The supernatant is dried to evaporate on a water bath, and the residue is dissolved in 5 ml water.
- The alcohol-free extract is used for the estimation of total phenols.
- Aliquot samples of 0.1 ml are diluted to 3 ml with water, and add 0.5 ml of FCR added and mix well.
- Exactly after 3 min, two ml 20% sodium carbonate solution is added and kept in a boiling water bath for 1 min. After cooling under running tap water, the absorbance is read at 650 nm, against the reagent as blank in a colorimeter.
- A standard graph is constructed with catechol as a standard in the range of 20–100 μg.
- The total phenol content will be expressed as mg/gram of oven-dried sample.

## 3.9 Estimation of Total Tannin Content

**Reagents**

- **Vanillin-HCl reagent** – 8% hydrochloric acid in methanol and 4% vanillin in methanol are mixed in equal volumes just before use.
- **Standard catechin solution** – 1 mg catechin is added per ml in methanol and diluted at 1:10 ratio to obtain 100 µg catechin per ml working standard solution.
- 100 mg of oven-dried powdered sample is extracted with 5 ml methanol for 24 h at room temperature with occasional stirring.
- The above extract is centrifuged at 5000 rpm for 10 min. The supernatant is used for the estimation of total tannins.
- 1 ml aliquot is added with 5 ml of vanillin-HCl reagent and mixed well.
- After incubation for 20 min, the absorbance is read at 500 nm as against a blank (reagent) in a colorimeter.
- A standard graph is constructed using catechin as a standard in the range of 0.2–2.0 mg.
- The total tannin content is expressed as the mg/gram of oven-dried sample.

## 3.10 Estimation of Enzymes

- Leaf samples (200 mg) are homogenised in a chilled pestle and mortar with 1 ml cold 0.1 M phosphate buffer (pH 6.5).
- The extract is centrifuged at 6000 rpm for 15 min at 4 °C, and the supernatant is used as an enzyme extract.
- The peroxidase and polyphenol oxidase activities are assayed by following the procedure given by Srivastava (1987), and activity is expressed as changes in absorbance per min per g of fresh weight.

**Peroxidase Estimation**

- Peroxidase is analysed by the alkaline PAGE procedure as described by Dadlani and Varier (1993) and staining procedure of Reddy and Gasber (1971).

**A. Preparation of Extraction Buffer (Tris-Hydrochloric Acid, pH 7.5)**

- The specified quantity of Tris-base is dissolved in 50 mL distilled water, adjusted to the desired pH using concentrated HCl with the final volume of 100 ml.
- The quantity of Tris-base to be dissolved for the specified pH is given below.

| Tris-HCl buffers | pH | Quantity (gm) |
|---|---|---|
| Tris-extraction buffer | 7.5 | 1.21 |
| Separating gel buffer (1.875 M) | 8.8 | 22.69 |

**B. Stock Acrylamide Solution**

- Acrylamide 30 g and bisacrylamide 0.8 g are dissolved in distilled water, and final volume is made up to 100 ml.

**C. Ammonium Persulphate (APS) 10%**

- Ammonium persulphate (1.0 gm) is dissolved in 10 ml distilled water and then prepared fresh every time.

**D. Stacking Gel Buffer (Tris-HCl, pH 6.7) 0.6 M**

- Tris (6.0 gm) is dissolved in 50 ml distilled water, and the pH is adjusted to 6.7 with concentrated HCl, and the volume is increased to 100 ml with distilled water.

**E. Electrode Buffer**

- Tris base – 9.0 gm.
- Glycine – 42.3 gm.
- Finally, 3000 ml is prepared by dissolving both the components in distilled water.

**Preparation of Gel**

- Running gel/separating gel (8%)
- Stock gel solution – 8.0 ml
- Tris-HCl buffer (pH 8.8) – 7.5 ml
- 10% APS – 0.4 ml
- Distilled water – 14.5 ml
- Tetramethylethylenediamine – 10 ml

**Casting of Gel**

- Glass plates, comb, spacers and the electrophoresis apparatus are cleaned thoroughly.
- The spacers are placed on the edges in between the plates and clamped tightly.
- The stacking gel mixture is poured along the sides of the plate and allowed to solidify, and the edges are sealed.
- The separating gel mixture is gently mixed without forming bubbles and carefully poured in between the glass plates.
- A layer of distilled water is added above the gel and allowed to polymerise.
- The tracking gel mixture is mixed gently and poured in between the glass plates above the separating gel.
- The comb is placed between the plates, and polymerisation takes place in about 30 min.
- After polymerisation, the comb and clips are removed carefully, and the glass plates with the polymerised gel are placed in the electrophoresis apparatus.
- The electrode buffer is added to the tank, and the air bubbles are removed.

**Sample Preparation**

- The freshly collected samples (0.5 gm) are washed with freshwater and kept on blotting paper to remove excess water.
- The samples are grind using a prechilled pestle and mortar.
- The extraction buffer (0.2 ml) is added and grinded very finely.
- Then the grind materials are transferred into a clean Eppendorf tube and placed in the refrigerator for 2 h at 10 °C.
- Later, the samples are centrifuged in a refrigerated centrifuge at 12,000 rpm for 20 min.
- 0.2 ml of supernatant from each sample is taken into a separate Eppendorf tube, and 0.2 ml of buffer solution is added and mixed well.
- For better mixing of the two solutions, the samples are boiled in boiling water bath for 10 min and then cooled for loading.

**Electrophoresis**

- Electrophoresis is done using a Bio-Rad electrophoresis unit.
- A uniform sample of 0.1 ml is loaded to each well.
- The gel assembly is immersed in the electrode buffer; care should be taken while doing so that the upper tank is filled with electrode buffer.
- The unit is connected to the power pack fitting the electrodes into the sockets of identical colour.
- Electrophoresis is carried out at a constant current of 2.0 mA per well till the tracking dye crossed the stacking gel.
- Later, the current is increased to 2.5 mA per well.
- The electrophoresis is stopped after the tracking dye reached the bottom of the gel.

**Preparation of the Staining Solution and Staining**

(i) Solution A – 100 ml ammonium chloride (6%)
(ii) Solution B – benzidine 100 mg dissolved in 1 ml acetone

- Staining solution – solution B is added drop by drop to solution A while stirring by hand.
- After mixing the solution, it is added to the gel (electrophoresis) and incubated for 30 min at 35 °C.
- During incubation, the gel is shaken for 30 min using a rocker shaker, and 1–2 ml hydrogen peroxide is added drop by drop to gel.
- The gel is transferred to a 100 ml solution of 7% acetic acid for 4 h, which stopped the staining process and enhanced banding resolution.

**Evaluation and Documentation**

The gel is placed over a transilluminator and photographed. The gels with enzyme bands are analysed for intensity of banding and relative mobility (Rm) of each band as calculated by the following formula.

Rm = distance travelled by the band/distance travelled by tracking dye

**Polyphenol Oxidase**
After native electrophoresis, the gel is equilibrated for 30 min in a 0.1 M phosphate buffer (pH 7.0) containing 0.1% p-phenylenediamine, followed by 10 mM catechol in the same buffer (Jayaraman et al. 1987). The addition of catechol is followed by a gentle shake, which results in the appearance of discrete bands of a dark brown colour.

## 4 Rapid Tests for Phytochemical Analysis (Siddiqui et al. 2009)

### *4.1 Tannins*

- To 200 mg plant material, 10 ml of distilled water is added, and the contents are filtered.
- To 2 ml of filtrate, $FeCl_3$ blue is added.
- Black precipitate indicates the presence of tannin and phenols.

### *4.2 Alkaloids*

- To 200 mg plant material, 10 ml of methanol is added, and the contents are filtered.
- To 2 ml filtrate, 1% HCl is added and steamed.
- 6 ml of Mayer's reagent/Wagner's reagent/Dragendorff's reagent is added.
- Creamish/brown/red/orange precipitation indicates alkaloids.

### *4.3 Saponins*

- To 0.5 ml of filtrate, 5 ml of distilled water is added.
- Frothing persistence indicates the presence of saponins.

### *4.4 Terpenoids*

- 2 ml filtrate is added with 2 ml acetic anhydride and concentrated $H_2SO_4$.
- Blue-green ring indicates the presence of terpenoids.

### *4.5 Cardiac Glycosides (Keller-Kiliani Test)*

- 2 ml filtrate is added with 1 ml of glacial acetic acid, $FeCl_3$ and concentrated $H_2SO_4$.
- Blue-green precipitate indicates the presence of cardiac glycoside.

### *4.6 Steroids (Liebermann-Burchard Reaction)*

- To 200 mg plant material, 10 ml chloroform is added, and the contents are filtered.
- 2 ml filtrate is added with 2 ml acetic anhydride and concentrated $H_2SO_4$.
- Blue-green ring indicates the presence of steroids.

### *4.7 Flavonoids*

- To 200 mg plant material, 10 ml chloroform is added, and the contents are filtered.
- 2 ml filtrate is added with concentrated HCl and magnesium ribbon.
- Pink-red colour indicates the presence of flavonoids and glycoside.

## 5 Estimation of Plant Hormones

### *5.1 Estimation of Indole Acetic Acid*

Indole acetic acid is reacted with trifluoroacetic acid and acetic anhydride to convert it into indole-a-pyrone which is measured fluorimetrically.

**Extraction of Sample**

- Freeze a known quantity 5 g of plant material in liquid nitrogen and grind to a fine powder using a pestle and mortar. Continue grinding with 10 ml methanol redistilled to a fine suspension.
- Filter the homogenate through a G4 glass filter under suction into a 100 ml flask.
- Extract the material on the filter twice by adding 100 ml methanol and then once with 5 ml. Evaporate the filtrate in a rotary evaporate at 30 °C to an aqueous residue.
- To the aqueous residue, add 10 ml of cold 0.5 M $K_2HPO_4$ solution so that PH reaches to about 8.5.
- Transfer to a suitable separating funnel and shake with 10 ml diethyl ether each time it discards the lipid fraction. Adjust the aqueous layer to PH 3 by adding about 3 ml of 2.8 M phosphoric acid. Extract IAA with 10 ml diethyl ether.

- The quantity of 10 ml diethyl ether is then extracted with 10 ml cold 50 mM $K_2HPO_4$ solution and is adjusted to 3 with phosphoric acid (0.28 M), and the IAA is passed into a final 100 ml diethyl ether.
- The ether is then evaporated in a few minutes under reduced pressure. Dissolve the residue in known volume (5 ml) cold distilled water.

**Estimation**

- Pipette out 1 ml of the above methanolic extract each in four different test tubes.
- To each tube, add 1 ml of methanol containing 0, 10, 20 or 30 ng of IAA, respectively.
- Dry the contents in each tube completely under reduced pressure and cool to 0 °C.
- To each flask, add 0.2 ml of ice-cold trifluoroacetic acid-acetic anhydride reagent and mix.
- Place the tubes on ice for exactly 15 min to ensure the complete conversion of IAA into indole alpha pyrone. Stop the reaction by adding 3 ml water.
- A blank may be prepared occasionally by adding first 3 ml water to one of four aliquots and 05 ml reagent after 15 min.
- Take the readings in a spectrophotofluorimeter using an excitation at 440 nm and emission at 490 nm for low concentration samples.
- Calculate the amount in unknown.

## *5.2 Estimation of Ethylene (Teitel et al. 1989)*

The ethylene evolved is measured in a gas chromatography based on the adsorption principle on activated silica gel.

**Procedure**

- Place the fruits in conical flask or cylinders.
- Seal the mouth with rubber septum or gasket.
- Incubate for 1 h at 20 °C.
- Withdraw gas samples with hypodermic syringes and inject into GLC.
- For standard, inject pure ethylene into empty conical flasks or cylinders of same volume and satisfy identical assay conditions. Remove the same volume of internal atmosphere as that of sample from the flask inject into GLC and measure ethylene peak height.

**Calculation**

The quantity of ethylene produced is expressed as microlitre ethylene per kg material.

# 6 Estimation of Anti-Nutritional Factors

## *6.1 Trypsin Inhibitor (Kakade et al. 1974)*

The trypsin inhibitor activity is measured indirectly by inhibiting the activity of trypsin. A synthetic substrate (BPNA) is subjected to hydrolysis by trypsin to produce yellow coloured p-nitroanilide.

The degree of inhibition by the extract of the yellow colour production is measured at 410 nm.

**Reagents**

- 30% glacial acetic acid (v/v).
- **Trypsin:** Dissolve 6.25 mg lyophilised trypsin and make up to 25 mL with 0.001 M HCl. Dilute 2 mL of this solution to 2 mL for assay.
- **Substrate:** Benzoyl-DL-arginine-p-nitroanilide (BAPNA).
- Completely dissolve 40 mg BAPNA in 0 mL of dimethyl sulphoxide, and then make up to 100 mL with Tris-HCl buffer pH 8.2.
- Tris-HCl buffer pH.2: Weigh 6.05 g Tris(hydroxymethyl)aminomethane and 2.94 g $CaCl_2.H_2O$, dissolve in 900 mL water, adjust to pH 8.2 with diluted HCl and make up to 1000 mL with distilled water.

**Sample Extraction**

- Extract 0.5 g sample in 25 mL water by grinding in prechilled mortar and pestle.
- Extract the ground sample in a refrigerator for 2–3 h with occasional shaking for complete extraction of TI.
- Centrifuge the homogenous at 12,000 rpm for 20 min at 4 C.
- Dilute 1 ml of the supernatant in to 10 ml with distilled water and use as TI source.

**Procedure**

- Pipette out 0–1 ml of extract in duplicate of test tubes, one to serve as endogenous (E) and the other test (Test).
- Make up the volume to 2 ml with buffer in the endogenous set.
- Add 1 ml of trypsin solution (20 microgram) to each tube in the test set. Pipette out into a separate test tube 1 ml of buffer and 1 ml of trypsin solution for standards.
- Incubate all the tubes in water bath at 37 °C.
- After a few minutes, add 2.5 ml of substrate (1 mg BPNA) to each tube.
- Allow to reaction to proceed for 10–60 min at 37 °C.
- Stop the reaction by adding 0.5 ml of 0% glacial acetic acid.
- Read the absorbance at 410 nm in a spectrometer.
- Determine the protein content in the extract by the method of Lowry et al.

**Calculation**

- Find out the T-S absorbance.
- Plot the absorbance against the volume of extract.
- Determine the aliquot size of the extract required to inhibit 50% trypsin activity (S/2). That aliquot size is considered to be one unit of trypsin inhibitor.
- One unit of activity corresponds to that amount of trypsin inhibitor in microgram protein which gives 50% inhibition of enzyme activity under experimental conditions.
- The trypsin inhibitor activity is expressed as trypsin inhibitor units per gram sample or per mg protein.
- The dilutions of trypsin inhibitor source were made in such a way that 0.5 ml produces 50% inhibition.

## *6.2 Estimation of Gossypol (Bell 1967)*

Gossypol reacts with phloroglucinol under highly acidic condition to produce reddish brown product which is measured at 550 nm.

**Reagents**

- Ethanol.
- Phloroglucinol reagent.
- Dissolve 5 g phloroglucinol in 100 ml of 80% ethanol.

**Sample Extraction**

- Weigh 5 g of fresh tissue, cut into small pieces and plunge into small boiling 95% ethyl alcohol (15–20 ml) for 5 minutes.
- Collect the extract by filtering. Repeat the extraction with residue and combine the extracts. Dilute the extract with 40% ethanol and adjust the extract with 1 N HCl to pH 3.0. Mix the contents with 1.5 volume of diethyl ether at 40 °C using a separating funnel.
- Save the ether phase and wash with two changes of distilled water.
- Evaporate the ether extract in vacuo to dryness and redissolve the residue in a known volume of 95% ethanol.

**Procedure**

- Pipette out different aliquots (1.2 ml) of the gossypol extract in ethanol in test tubes.
- Add 0.5 ml of phloroglucinol reagent followed by 1 ml conc. HCl to each tube.
- Incubate for 30 min with occasional shaking at room temperature.
- Make the volume of solution to 10 ml with 80% ethanol.
- Read the absorbance of colour at 550 nm against a reagent bottle.
- Prepare a standard graph with gossypol acetate.

## 6.3 Estimation of Phytic Acid (Wheeler and Ferrel 1971)

- The phytic acid is extracted with trichloroacetic acid and precipitated as ferric salt.
- The iron content of this precipitate is determined calorimetrically and the phytate phosphorus content calculated from this value assuming a constant 4 Fe:6P molecular ratio in the precipitate.

**Reagents**

- 3% trichloroacetic acid
- 3% sodium sulphate in 3% TCA
- 1.5 N NaOH
- 3.2 NaOH
- $FeCl_3$ (dissolve 583 mg $FeCl_3$) in 100 ml of 3% (TCA)
- 1.5 M potassium thiocyanate (KSCN)
- Standard Fe $(NO_3)_3$ solution

**Estimation**

- Weigh finally ground (40 mesh) sample estimated to contain 5–30 mg phytate into a 125 ml Erlenmeyer flask.
- Extract in 50 ml 3% TCA for 30 min with mechanical shaking or with occasional swirling by hand for 45 min.
- Centrifuge the suspension and transfer a 100 ml aliquot of the supernatant to 40 ml conical centrifuge tube.
- Add 4 ml of $FeCl_3$ solution to the aliquot by blowing rapidly from the pipette.
- Heat the contents in a boiling water bath for 45 min.
- If the supernatant is not clear after 30 min, add one or two drops of 3% sodium sulphate in 3%TCA and continue heating.
- Centrifuge (10–15 min) and carefully decant the clear supernatant.
- Wash the precipitate twice by dispersing well in 20–25 ml 3%TCA, heat in boiling water for 5–10 min and centrifuge.
- Repeat washing with water.
- Disperse the precipitate in a few ml of water and heat in boiling water for 30 min.
- Bring volume to approximately 30 ml with water and heat in boiling water for 30 min.
- Filter hot through a moderately retentive paper Whatman No 2.
- Wash the precipitate with 60–70 ml hot water and discard the filtrate.
- Dissolve the precipitate from the paper with 40 ml hot 3.2 $NHNO_3$ into 100 ml volumetric flask.
- Wash the paper with several portions of water, collecting the washings in the same flask.
- Cool flask and contents to room temperature and dilute to volume with water.
- Transfer a 5 ml aliquot to another 100 ml volumetric flask and dilute to approximately 70 ml.

- Add 20 ml of 1.5 M KSCN dilute to volume, and read colour immediately at 480 nm.
- Run a reagent blank with each set of samples.

**Standard**

- Weigh accurately 433 mg Fe $(NO_3)_3$ and dissolve in 100 ml water in a volumetric flask.
- Dilute 2.5 ml of this stock standard and make up to 250 ml in volumetric flask.
- Pipette out 2.5, 5, 10,15 and 20 ml of this working standard into a series of 100 ml volumetric flasks and proceed to step 16.

**Calculation**

Find out the μg in the test from the standard curve, and calculate the phytate (P) as per the equation.

$$\frac{\mu g\ Fe\ x15}{\text{Weight of sample (g)}}$$

## 6.4 Estimation of Silica (Kilmer V. J. Silicon 1965)

**Reagents**

- 20% acetic acid
- Ammonium molybdate
- 20% tartaric acid
- $Na_2SO_3$
- $NaHSO_3$
- 50% NaOH
- $SiO_2$ (99.99%)
- $Na_2CO_3$

**Setting Up Standard Curve**

- Transfer 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 ml Si standard solution to a 50 ml volumetric flask, respectively.
- Add 30 ml acetic acid (20%) and 10 ml ammonium molybdate solution (54 g/L, pH 7.0). Shake up to mix thoroughly. Keep for 5 min and then add 5 ml 20% tartaric acid and 1 ml reducing solution. Adjust to 50 ml with 20% acetic acid.
- The reducing solution was made by mixing solution A (2 g of Na2SO3 and 0.4 g of 1-amino-2-naphthol-4-sulfonic acid in 25 ml of dd$H_2O$) and solution B (25 g of $NaHSO_3$ in 200 ml of double distilled (dd) $H_2O$) and adjusted to 250 ml with dd$H_2O$.
- Store in a tightly stoppered plastic bottle in the dark. Thirty minutes later, measure the absorbance at 650 nm.

- **Sample preparation:** Flag leaves, stems and hulls collected from each line in either replication were dried in oven at 70 °C for 7 days. Each sample was ground and sifted through a 60 mesh sieve. They were then dried at 60 °C for 48 h.
- Two 100 mg samples were weighed from each of 498 hull, flag leaf and stem powders.
- **Sample pretreatment:** Put each 100 mg sample into a 100 ml polyethylene tube. Add 3 ml 50% NaOH and cover it with a loose-fitting plastic cap.
- Gently vortex and then autoclave at 121 °C for 20 min. Transfer to volumetric flask and adjust to 50 ml with dd$H_2O$.

**Sample Determination**

- Transfer 1 ml sample solution to a 50 ml volumetric flask. Add 30 ml 20% acetic acid and 10 ml ammonium molybdate solution (54 g/L, pH 7.0).
- Shake up to mix thoroughly. Keep for 5 min and then immediately add 5 ml 20% tartaric acid and 1 ml reducing solution.
- Adjust to 50 ml with 20% acetic acid. Thirty minutes later, measure the absorbance at 650 nm.

**Preparation of Standard Solution**

- Put 1 g ultrapure SiO2 (99.99%) in a muffle furnace. Heat slowly to 1000 °C and keep for 1 h at 1000 °C. Cool to room temperature (about 3 h).
- Weigh 0.1 g pretreated SiO2 and put into a nickel crucible. Add 2 g $Na_2CO_3$. Heat slowly to 1000 °C such that a lucent melt is formed. Cool to room temperature.
- Take the nickel crucible out from the muffle furnace. Add 5 ml boiling dd$H_2O$ into the nickel crucible, and transfer the melt from the nickel crucible completely into a 300 ml plastic cup.
- Add 150 mL dd$H_2O$. Stir until the chemical is completely dissolved. Transfer to a 1000 ml volumetric flask. Adjust to 1000 ml. Transfer the solution to a 1000 ml plastic bottle, tightly stoppered and stored at room temperature. The solution contains 0.1 mg/ml SiO2.

## *6.5 Estimation of Lignin (Goering and Van Soest (1970)*

Modified procedure is the determination of acid detergent lignin (ADL) (procedure to obtain acid detergent fibre (ADF) for ADL analysis):

- Dry 16 ml glass tubes at 100 °C for at least an hour.
- Weigh tubes to four or five decimal places in groups of ten at a time.
- Dry finely ground samples at 60 °C and weigh about 250 mg into weighed tubes. (Grinding was carried out in a Wiley mill with a 1 mm screen (Mertens 1992).
- Add 10 ml acid detergent fibre solution (2% cetyltrimethylammonium bromide in 1 litre 0.5 M $H_2SO_4$) to the sample. Put cap on tube. Vortex.
- Reflux over a steady heat (water bath) for 1 hour at 95–100 °C, and vortex every 10 min. Centrifuge for 10 min at 3000 rpm and remove supernatant by suction.

- Add 15 ml hot distilled water to residue; vortex. Centrifuge for 10 min at 3000 rpm and discard supernatant. Repeat this step three times.
- Add 15 ml acetone to the residue. Vortex. Centrifuge for 10 min at 3000 rpm and discard supernatant. Repeat this step one more time.
- Evaporate residual acetone in water bath at 60 °C.
- Dry tubes at 90 °C overnight.
- Weigh tubes and residue using hot weighing technique to four or five decimal places and calculate ADF (Mertens 1992) (ADL procedure). A protective mask *must* be worn when handling 12 M $H_2SO_4$ in this assay.
- Label glass microfibre filters (GMF) with marker pen (both sides), dry at 100 °C overnight and weigh ten tubes at a time one to five decimal places.
- Add 1.5 ml 12 M $H_2SO_4$ to tubes containing residues (in fume cupboard) and digest at 30 °C for 60 min mixing carefully every 10 min.
- Following digestion, the acid-insoluble residue was collected by filtration using 45 mm Buchner funnels with preweighed 55 mm Whatman GF/C glass microfibre filters.
- An extensive washing with water and a final acetone rinse (twice) were used prior to drying the samples overnight at 100 °C.
- Weigh the filters and residue to five decimal places.
- Ash at 450 °C for 6 h. Weigh GMF with ash to five decimal places.
- ADL was determined as the difference in weight of the residue before and after ashing, with correction for ashing losses from GMF2.

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# Techniques for Determining the Repellent and Antifeedant Activity to Phytophagous Insects


**N. E. Thyagaraja and A. T. Rani**

**Abstract** Repellents are potent compounds that prevent phytophagous insects from feeding on the plant. A procedure has been detailed out in this chapter to test the repellent against the test insect. The diamondback moth (DBM) has been picked up as an example here. Using the bioassay, the antifeedant properties of the test plant can also be studied.

**Keywords** Secondary metabolites · Insect-plant interactions · Repellent · Attractant

## 1 Introduction

One of the novel means of protecting plants from herbivores would be to prevent them from feeding on plants. In the course of their growth and development, plants emanate certain chemicals that may repel or resist from feeding on plants. Plants have evolved over 400 million years ago and have acquired effective defensive mechanisms that ensure survival under adverse environmental condition such as stress or against natural enemies. Besides a number of morphological protective mechanisms, plants have developed highly sensitive chemical defense mechanism against herbivorous feeders. These defense mechanisms do not cause immediate death but affect common biochemical and physiological functions. Until a few decades ago, plant secondary metabolites were considered substances with no specific function, which only reflected an aspect of biodiversity. Recent chemical,

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ecological studies have shown that many of these secondary metabolites play an important role in plant-insect interactions (Wada and Manakata 1968). Some compounds, either separately or synergically, make up a chemical defense barrier in the plant against certain pests and diseases.

## 2 Tests for Repellent Response

Repellency tests are conducted following the method proposed by Talukder and Howse (1993, 1994)

- Cut the filter paper approximately 12 cm in diameter and place in a circular plate.
- The fruiting bodies or shoot or leaf of selected resistance and susceptible cultivars are cut into circular pieces (4 × 4 cm dia) and placed contiguously on the large circular plate (0.3 m dia).
- Thirty test insects (feeding stage) are released on each plate.
- Insects present on each shoot/leaf/fruit are counted at hourly intervals for 5 h after treatment.
- The data were converted to express percentage repulsion (PR) using the following formula:

$$\text{PR}\ (\%) = (N_C - 50) \times 2$$

where:

$N_C$ – percentage of insects present in the susceptible plant (control).
Positive values (+) indicated repellency
Negative values (−) indicated attractancy
Five replications are made of each treatment

Class repellency rate (%)

| Repellency (%) | Repellency class |
|---|---|
| > 0.01–0.10 | 0 |
| 0.10–20.00 | I |
| 20.10–40.00 | II |
| 40.10–60.00 | III |
| 60.10–80.00 | IV |
| 80.10–100.00 | V |

## 3 Test for Antifeedant Response – For Example, *Plutella xylostella* (Plutellidae: Lepidoptera)

The antifeedant activity of different hosts or nonhost plants against fourth instar larvae of DBM can be studied based on the leaf area consumed and reduction in the larval weight gain after feeding for 48 or 72 h.

Leaf disc bioassay method was employed for studying the antifeedant property against fourth instar DBM larvae. The modification involved the utilization of leaf discs in a uniform area (16 sq cm) measured prior to the commencement of the treatment. Two fourth instar larvae of DBM starved for 6 h were weighed and introduced into each petri dish (9 × 9 cm). A control was maintained with each treatment. Each treatment was replicated 15 times.

The observations on the leaf area consumed by each set of larvae and the reduction in weight gain were recorded after feeding for 48 h. The leaf area consumed by larvae was measured by employing the graphical method. The percent antifeedant activity was calculated using

$$\text{Percent antifeedant activity} = \frac{\text{Percent leaf area left unfed in treatment} - \text{Percent leaf area left unfed in control}}{100 - \text{Percent leaf area left unfed in control}} \times 100$$

The antifeedant activity was also measured in terms of reduction in the weight gain in larvae that fed on treating leaves as compared to the weight gain in control larvae using the formula:

$$\text{Percent reduction in weight gain} = \frac{\text{Weight gain in control larvae} - \text{Weight gain in treated larvae}}{\text{Weight gain in control larvae}} \times 100$$

Some of the methods are also outlined in another chapter titled "Techniques for Determining Mechanisms of Resistance: Antixenosis for Feeding" in this volume.

**Acknowledgment** The authors would like to thank authorities of College of Agriculture, Hassan and University of Agricultural Sciences, GKVK, Bengaluru, for their encouragement and support.

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# Evaluation of Insect Resistance Using Tissue-Cultured Plants


**Amalahyacinth and Chand Asaf**

**Abstract** Evaluation of insect resistance in plants using callus from plant tissue culture helps in understanding the nature and causes of resistance. Under controlled environmental conditions, tissue culture is an ideal way of evaluating resistance. Growth and feeding in insects are inhibited by callus of several insect-resistant plants. The methods to evaluate larval feeding preference and larval growth on callus are discussed in this chapter.

**Keywords** Plant tissue culture · Callus · Insect resistance · Bioassay · Pest management

## 1 Introduction

Tissue culture is in vitro aseptic culture of cells, tissues, organs, or whole plant under controlled nutritional and environmental conditions often to produce the clones of plants. The resultant clones are true to type of the selected genotype. The controlled conditions provide the culture an environment conducive for their growth and multiplication. These conditions include the proper supply of nutrients, pH medium, adequate temperature, and proper gaseous and liquid environment.

Plant tissue culture technology is being widely used for large-scale plant multiplication. Apart from their use as a tool of research, plant tissue culture techniques have in recent years become a major industrial importance in the area of plant propagation, disease elimination, plant improvement, and production of secondary metabolites (Altaf Hussain et al. 2012). Small pieces of tissue (named explants) can be used to produce hundreds and thousands of plants in a continuous process. A single explant can be multiplied into several thousand plants in relatively short time period and space under controlled conditions, irrespective of the season and weather

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on a year-round basis. Endangered, threatened, and rare species have successfully been grown and conserved by micropropagation due to the high coefficient of multiplication and small demands on the number of initial plants and space.

## 2 Techniques of Plant Tissue Culture

### *2.1 Micropropagation*

Micropropagation starts with the selection of plant tissues (explant) from a healthy, vigorous mother plant. Any part of the plant (leaf, apical meristem, bud, and root) can be used as an explant. The whole process can be summarized into the following stages.

#### 2.1.1 Stage 0: Preparation of Donor Plant

Any plant tissue can be introduced in vitro. To enhance the probability of success, the mother plant should be ex vitro cultivated under optimal conditions to minimize contamination in the in vitro culture (Cassells and Doyle 2005).

#### 2.1.2 Stage I: Initiation Stage

In this stage, an explant is surface sterilized and transferred into nutrient medium. Generally, the combined application of bactericide and fungicide products is used. The selection of products depends on the type of explant to be introduced. The surface sterilization of explant in chemical solutions is an important step to remove contaminants with minimal damage to plant cells (Husain and Anis 2009). The most commonly used disinfectants are sodium hypochlorite, calcium hypochlorite, ethanol, and mercuric chloride ($HgCl_2$). The cultures are incubated in growth chamber either under light or dark conditions depending on the method of propagation.

#### 2.1.3 Stage II: Multiplication Stage

The aim of this phase is to increase the number of propagules (Saini and Jaiwal 2002). The number of propagules is multiplied by repeated subcultures until the desired (or planned) number of plants is attained.

#### 2.1.4 Stage III: Rooting Stage

The rooting stage may occur simultaneously in the same culture media used for multiplication of the explants. However, in some cases, it is necessary to change

media, including nutritional modification and growth regulator composition, to induce rooting and the development of strong root growth.

#### 2.1.5 Stage IV: Acclimatization Stage

At this stage, the in vitro plants are weaned and hardened. Hardening is done gradually from high to low humidity and from low light intensity to high light intensity. The plants are then transferred to an appropriate substrate (sand, peat, compost, etc.) and gradually hardened under greenhouse.

### *2.2 Production of Callus*

Dip mature seed kernels in a beaker containing 70% ethanol for 90 s. Then transfer to a sterile beaker with 2 grams of laboratory detergent in 100 ml 5.25% sodium hypochlorite solution. Swirl for 20 min and then rinse 5–7 times in sterile distilled water. Keep seeds over a moist filter paper on a petri dish and incubate at 25 °C for 24–48 h. Excise the embryos using sterile dissecting needles, swirl for 5 min in 5.25% sodium hypochlorite, and rinse 5–7 times in sterile distilled water. Place the embryos on a cell culture medium in a petri dish.

MS-3 medium (Murashige and Skoog 1962) is widely used for callus induction. The media is prepared with a pH of 5.8 and steam sterilized in an autoclave at 120 °C for 20 min. Cool the medium and transfer the developing callus (3 weeks) in fresh media using a sterile spatula.

### *2.3 Disinfection of Insect Eggs*

Allow gravid female moths to lay eggs on sterile filter papers. Disinfect the eggs in 70% ethanol for 15 s and subsequently in 0.1% mercuric chloride solution for 15 min. Wash these eggs in sterile distilled water and keep in petri dish lined with moist sterile filter paper (Michael Smith et al. 1994). Incubate at 27 °C until they hatch. Transfer these newly hatched larvae to petri dishes with plant callus.

### *2.4 Evaluation of Resistance Using Tissue Culture*

The evaluation of insect resistance using plant callus is generally based on larval feeding preference and larval growth on callus initiated from susceptible and resistant plant varieties. Callus tissues of several insect-resistant plants exhibit resistance to insect feeding and growth. Maize callus tissue exhibits resistance to the fall army worm, *Spodoptera frugiperda*; the southwestern corn borer, *Diatraea grandiosella*;

**Fig. 1** Banana tissue culture plants. (Source: Indiamart, 2010)

the European corn borer, *Ostrinia nubilalis*; and the corn ear worm, *Helicoverpa zea*, similar to the resistance shown by whole plant foliage (Williams and Davis 1985). Callus tissues from fall army worm resistant Bermuda grass cultivars also exhibit resistance equivalent to whole plant foliage (Croughan and Quisenberry 1989). Caballero et al. (1988) used callus tissue from rice plants to evaluate their resistance to the yellow stem borer, *Scirpophaga incertulas*; the striped stem borer, *Chilo suppressalis*; and the rice leaf folder, *Cnaphalocrocis medinalis* (Fig. 1).

## 3 Bioassays

### *3.1 Choice Test*

To determine whether neonate larvae of stem borers orient and settle preferentially on callus initiated from susceptible or resistant plants, larval orientation and settling responses can be measured following the methodology of Williams et al. 1987.

Place five pieces of callus (0.5 g) of each test cultivar in a circular manner equidistantly from the center of a petri dish. Transfer 50–100 blackhead stage eggs or neonates carefully to the center of each petri dish. Keep the petri dish in complete darkness and record the number of larvae present on each callus at 1, 6, 12, 24, and 48 h after infestation.

### *3.2 Consumption and Utilization of Food*

Prestarve third instar larva reared on callus of susceptible plant for 4 h. Individually weigh the larvae and allow to feed only on callus of test variety. After 24–48 h, stop feeding the larvae but provide water for 4 h to excrete all food matter from gut. Record the weight of individual larvae feeding callus of each test variety and calculate the percent increase in body weight.

### 3.3 Growth and Development

To determine the growth of larvae feeding on callus initiated from susceptible and resistant plants, infest the petri dishes containing approximately 500 mg to 1 g of callus with 3–5 neonate larvae. Maintain the larvae in a growth chamber at 27 °C and 12:12 (L:D) until they pupate. Record the percent larval survival and larval weight 7–15 days after infestation. Also record the length of larval development period, percent larvae becoming pupae, and pupal weight. The higher the growth index, the more suitable the callus is for insect growth.

The callus tissue from insect susceptible and resistant plants has been used to determine growth of stem borer larvae feeding on them (Khan 1994).

### 3.4 Control of Contamination in Callus Cultures

Frequently, callus cultures have become contaminated with bacteria and fungi following infestation with insect larvae. The contamination affects larval survival and growth. With high levels of contamination, entire experiments have sometimes been jeopardized or even lost. Williams et al. 1994 modified the procedures to better control contamination without adversely affecting the ability to distinguish between leaf feeding resistant and susceptible corn genotypes (Fig. 2).

### 3.5 Materials and Methods

Initiate callus from two corn hybrids that are resistant to leaf feeding by southwestern corn borer and fall armyworm, Mp704 X Mp707 and Mp707 X Mp708, and two susceptible corn hybrids, Ab24E X Tx601 and SC229 X. Follow the procedures as described by Williams et al. (1983) to initiate callus from mature corn kernels. Swirl the kernels in a sterile beaker with 2 g laboratory detergent and 100 ml 5.25% sodium hypochlorite for 20 min. Rinse twice in sterile distilled water and then swirl for 5 min in a solution of 700 ml ETOH. Rinse the seeds five times in sterile distilled water, and soak for 3 min in a solution of 100 mg gentamicin and 1.2 g sorbic (2,4-hexadienoic acid) per liter of sterile distilled water. Maintain the seeds on germination paper moistened with the gentamicin and sorbic acid solution for 48 h at 25 ° C. Excise the embryos using forceps and a scalpel. Place the embryos in petri plates on Murashige and Skoog medium supplemented with 20 g/liter sucrose, 8 g/liter agar, 15 mg/liter 2,4 dichlorophenoxyacetic acid (2,4-D), and 0.15 mg/liter of zeatin (6-[4-hydroxy 3-(methylbut-2-enylaminopurine). Maintain the callus at 27 ° C with a photoperiod of 12:12 (L:D) and transfer to a maintenance medium after 4 weeks. Transfer the callus to fresh medium at 4-week intervals thereafter. The maintenance medium differs from the initiation medium in that 2,4-D was reduced to

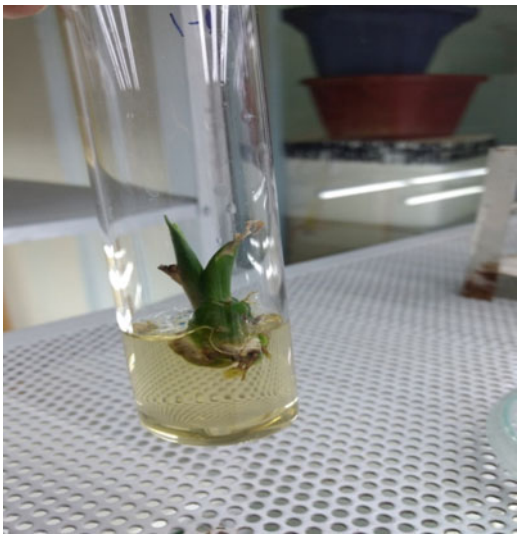

**Fig. 2** Tissue-cultured plantlets of tuberose

5 mg/liter and zeatin solution was reduced to 0.1 mg/liter. Place approximately 500 mg of callus on maintenance medium in petri plates, 15 plates per hybrid, and infest each plate with 1 neonate fall armyworm larva.

Prepare a mixture of 750 ml sterile distilled water, 7500 mg agar, 450 mg sorbic acid, and 45 mg gentamicin and heat to 82 ° C. Pour the mixture in 10 ml aliquots into 30 ml plastic cups and allow to cool. Place 500 mg callus and 1 neonate fall armyworm larva in each of 15 cups per hybrid. Arrange the petri plates and diet cups in a randomized complete block design with 15 replications and place in a growth chamber at 29 ° C with a photoperiod of 12:12 (L:D). After 7 days, weigh the larvae from the uncontaminated plates and cups. Conduct an additional experiment a month later, using callus of three hybrids, Mp707 X Mp70B, Ab24E X Tx601, and SC229 X Tx601. Place 500 mg of callus in 14 cups per hybrid and infest each one with a neonate southwestern corn borer larva following the same procedure as in the first experiment except that the larvae allowed to feed 14 days before weighing. Analyze the larval weights from the two experiments using least squares to fit linear models because of the large number of missing values. In the first experiment, analyze the larval weights for petri plates and cups separately. Determine the significance of differences among means by Fisher's Protected LSD ($P = 0.05$).

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# Germplasm Screening and Evaluation Techniques Against Insect Pests


**M. Saravanaraman and G. D. Prahalada**

**Abstract** Standardized screening and evaluation procedures are required to precisely locate resistant/tolerant genotypes in a germplasm. Such procedures ensure comparison of data from different geographical location screening, and evaluation procedures are done in the field, greenhouse, and laboratory depending on the requirements. Depending on the goals set or desired, the precision and comprehensiveness of screening procedures vary. Germplasm screening procedures for certain key pests on few major crops are described in this chapter. These are quantitative in nature, suitable for statistical analyses.

**Keywords** Germplasm screening · Evaluation parameters · Standardization · Statistical analysis

## 1 Introduction

Crop plants continuously encounter biotic stresses which hamper the ultimate yield and survival of the crop plants. Insects directly or indirectly cause injury by feeding on almost all parts of the plant from root to panicle (Atwal and Dhaliwal 2015). On the other hand, plants also have evolved several effective defense mechanisms to fight against insect attacks and show adaptive responses to prevent or reduce the damages caused by insects (Dangle and Jones 2001). Although synthetic insecticides initially showed the potentiality to control harmful pests to minimize their damage to crops, later extensive pesticide application resulted in increased cost of crop production, reduced populations of natural enemies of insect pests, lead to the

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development of pesticide-resistant races of insects, and polluted the environment (Kavitha and Reddy 2012). Hence, the development of insect resistant cultivars is considered as one of the most economical and effective measures for insect management (Prahalada et al. 2017). Germplasms need to be properly screened for insect resistance and evaluated using suitable evaluation techniques. This will help the breeder to develop insect-resistant cultivars to mitigate the problems caused by insects on crop plants and to secure the yield loss incurred because of insect attacks.

Germplasm screening techniques should be reliable, easy to execute, rapid, and comprehensive, i.e., it should embrace interactions between the insect and the plant. The screening technique should be standardized and equally applicable in varying ecosystem conditions.

The screening can be broadly carried out under the following categories (Smith et al. 1994):

I. Laboratory screening
II. Greenhouse screening
III. Field screening

## 2 Laboratory Screening

Laboratory screening is highly reliable than field or greenhouse screening since the environmental influence is greatly avoided. However, it is practically difficult to maintain whole plants for resistance evaluation under laboratory conditions. Hence, excised leaflets or fruiting structures are generally used, which is more apt for evaluating the preference or non-preference of few accessions rather than screening a bulk germplasm. Based on the area fed or damaged, dry weight of tissues of check and test accessions upon feeding are some of the techniques. Measuring area fed or damaged, estimating dry weight of check and test accessions upon feeding are some of the techniques.

## 3 Greenhouse Screening

In the greenhouse, breeding materials can be screened rapidly by infesting plants at the seedling stage. This technique is economic in space, time, and labor. It can be used for screening cultivars of grain and forage crops like rice, wheat, sorghum, and alfalfa. Greenhouse screening can also be used for confirming field response of cultivars to the pest and/or pathogen.

## 4 Field Screening

Field screening needs to be conducted in an endemic area. The germplasm to be evaluated should be planted at the proper period so that the evaluation can be done at the peak period of infestation/activity.

### *4.1 Natural Occurrence of the Insect*

In general, the natural occurrence of the insect in endemic areas will envisage the evaluation process. Such evaluations are conducted during the different seasons to ensure the resistance levels at different population levels of the infesting insect pest.

### *4.2 Artificial Infestations*

In certain conditions, when the natural population of the insects is less to cause sufficient damage in all the accessions, resistance rating may be ambiguous. Under such circumstances, artificial infestation techniques are adopted. Depending upon the crop and the concerned insect pest, eggs or larvae or adults are released in the test plots. For such purpose, mass rearing of the test insects on natural host plants or on artificial diets under laboratory conditions is the best method to obtain a sufficient, homozygous population. Eggs of the test insects in agar-based suspensions may be injected into the test accessions as in the case of corn earworm, *Helicoverpa zea* (Boddie). Alternatively, for inoculating larval stages onto plants, plastic larval plant inoculators called bazooka inoculators may be used (Smith et al. 1994).

## 5 Screening Rice Germplasm for Resistance to Insect Pests

Screening rice germplasm for insect resistance has been widely attempted, and successful results have been obtained at the International Rice Research Institute (IRRI) and other institutes. Based on these, standard screening technologies have been formulated.

### *5.1 Screening Rice Germplasm for Resistance to Brown Plant Hopper (Heinrichs et al. 1985)*

Screening rice accessions for resistance against brown plant hopper, *Nilaparvata lugens* (Stal.) (Delphacidae:Lepidoptera), can be done under greenhouse or field conditions.

### 5.1.1 Greenhouse Screening

Seedbox Technique

There are two types of seedbox techniques, namely, (a) conventional seedbox screening techniques (SST) and (b) modified seedbox screening technique (MSST), which are widely followed.

A. Conventional Seedbox Screening Techniques (Panda and Khush 1995)

Conventional seedbox screening test is a rapid method for screening a large number of rice germplasm (test entry) for qualitative resistance to the brown plant hopper. The test cultivars are raised in wooden or metal boxes as shown in Fig. 1.

Standard Seedbox

- The seeds are sown in rows in a standard seedbox (60 × 40 × 40 cm).
- About 25 seeds of each test entry are sown in a 12 cm row.
- In the standard seedbox, 39 entries can be evaluated.
- On the 7th day after sowing (DAS), when the seedlings are at the two-leaf stage, the seedboxes are placed in a water pan (with 5 cm water level) inside a room screened preferably with wire mesh.
- The weeds are removed and the seedlings are thinned out to about 20 per row.

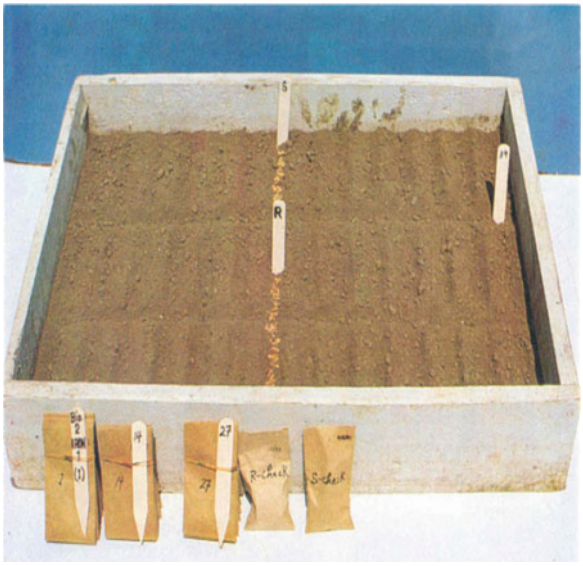


**Fig. 1** Conventional seedbox used for BPH bioassay. (Heinrichs et al. 1985)

- After 10 days, ten brown plant hopper nymphs cultured on susceptible variety are released onto each test seedling by holding the base of the plant and lightly tapping the plants and blowing on them.
- The entries are scored when 90% of the susceptible check seedlings in that box are wilted/dead.
- Standard Evaluation System (SES) of the International Rice Research Institute (IRRI) scale (IRRI 1996) can be used to scale seedling damage.

| Score | Visual symptom |
|---|---|
| 0 | No damage |
| 1 | Very slight damage |
| 3 | First and second leaf of most plants with orange tips – slight stunting |
| 5 | Pronounced yellowing and half of the plants wilting, pronounced wilting |
| 7 | More than half of the plants wilting or dead and the remaining plants stunted |
| 9 | All the plants dead |

Compartment Seedbox

- Boxes having several compartments with dimension of 114 × 69 × 7 cm are used.
- Seeds are sown in each compartment to avoid/reduce the mixing of seeds.
- About 180 accessions can be evaluated.
- The entries are scored when 90% of the susceptible check seedlings in that box are wilted/dead. IRRI SES described above can also be used to score the accessions.

B. Modified Seedbox Screening Technique (MSST)

- In this technique, older seedlings can be infested.
- Insects are released at 10 DAS at three to five Brown planthopper (BPH) per seedling.
- Entries are graded as per IRRI SES score when susceptible check is rated at Grade 7 (which generally occur about 28 DAS).
- The observations are recorded from 28 DAS onward at 2-day interval.

### 5.1.2 Field Screening

The varietal resistance has been challenged and well appreciated by evaluating their resistance in the field. For BPH, field screening has been also carried out as shown in Fig. 2, to study their field level of resistance.

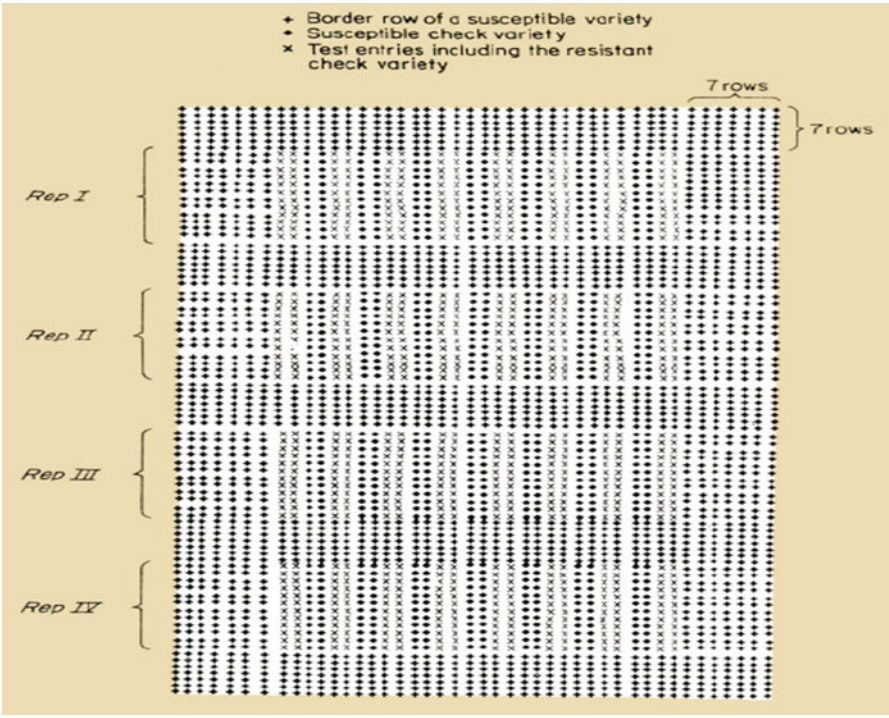


**Fig. 2** Schematic diagram showing the field screening layout for BPH bioassay. (Heinrichs et al. 1985)

A. Resurgence Technique

- Resurgence-inducing chemicals (decamethrin 30 g a.i./ha or methyl parathion or triazophos 500 g a.i./ha) may be sprayed on the susceptible plants planted along the border on 20 days after transplanting (DAT) and repeated at 10-day interval up to 70 DAT.
- The accessions are scored as per the below-mentioned SES score, when plants in the susceptible checks start wilting.

**SES Scale**

| Score | Visual symptom |
|---|---|
| 0 | No damage |
| 1 | Slight yellowing of few plants |
| 3 | Leaves partially yellow but with no "hopperburn" |
| 5 | Leaves with pronounced yellowing and stunting or wilting, 10–25% of plants with hopperburn remaining stunted |
| 7 | More than half of the plants wilting or with hopperburn, remaining plants stunted |
| 9 | All the plants dead |

B. Polythene Barrier Technique

- Test entries are enclosed using 75-cm-high polythene sheets (top opened), erected on 30 DAT, and infested with BPH.
- Resurgence-inducing chemicals are sprayed over the entire plot at 10 DAT.
- The accessions are graded based on the damage caused by adopting the SES rating scale.
- The detailed layout of the polyethylene barrier technique for field screening is shown in Fig. 3.

C. Microplot Technique

- In the experimental field, small plots of 1.5 × 1.5 × 1 m dimension are prepared as shown in Fig. 4.
- Seedlings of the test accessions and a susceptible check are transplanted at 21 DAS with the spacing of 20 × 10 cm in the plots.
- Fiberglass mesh cages (1.5 × 1.5 × 1 m) are placed over these small plots.
- At 15 DAT, to kill the natural enemies and to induce resurgence of BPH, chemicals such as decamethrin need to be sprayed.
- Subsequently, two pairs of BPH/hill are released at 20 DAT, and the microplot is enclosed with the cage. Totally 70 pairs/cage are released.
- When 50% of plants of the susceptible check show wilting or "hopperburn," the damage on the test accessions is recorded, and the accessions are rated as per SES scale (IRRI 1996).

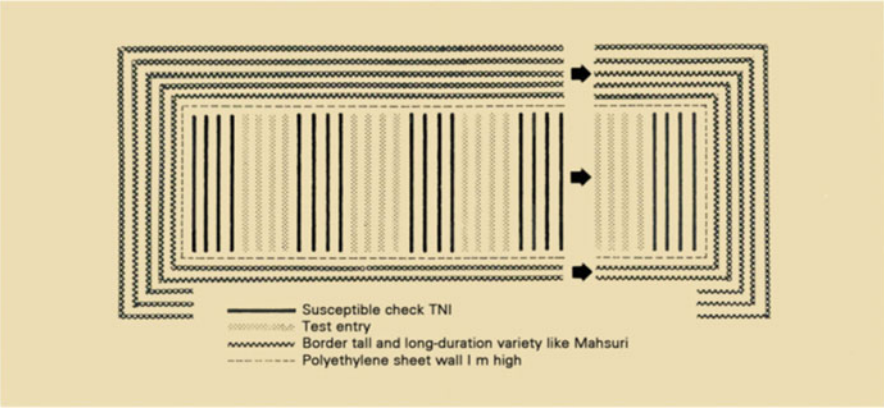


**Fig. 3** Schematic diagram showing the layout of Polythene barrier technique for BPH bioassay. (Heinrichs et al. 1985)

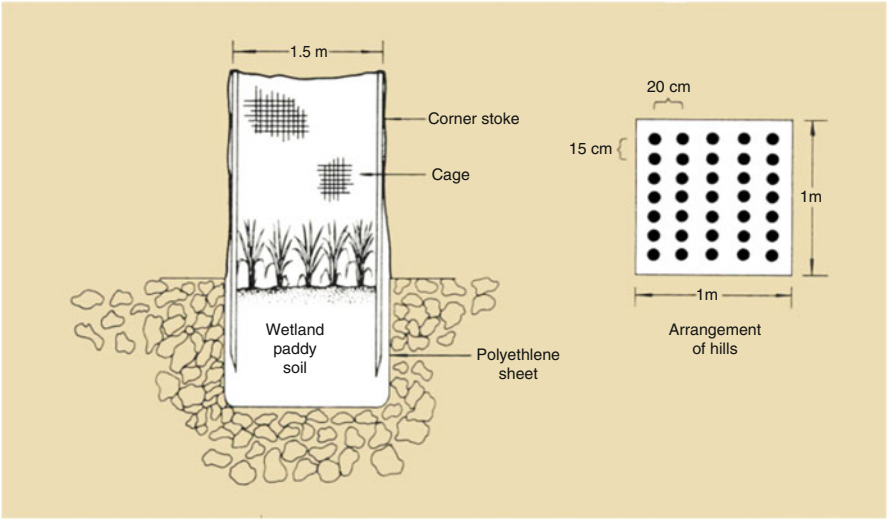


**Fig. 4** Schematic diagram showing the layout of Micro-plot technique for BPH bioassay. (Heinrichs et al. 1985)

## 5.2 *Screening Rice Germplasm for Resistance to Yellow Stem Borer*

The larva of yellow stem borer feeds on the stem and causes drying of the central shoot known as "deadheart" in the young seedlings and drying of the panicle in grown-up plant called "white ear" (Fig. 5). Damage ranges from 30% to 80%. Screening rice accessions for resistance against yellow stem borer, *Scirpophaga incertulas* (Pyralidae:Lepidoptera) is done in the greenhouse or screenhouse. Resistance against stem borer is evaluated based on the percentage of "deadheart" or "white ear."

### 5.2.1 Greenhouse Screening

- Egg masses of stem borer collected from the field can be used to obtain neonates.
- Instead, adult moths collected from light traps are allowed inside oviposition cages containing susceptible rice plants for egg laying.
- Cutoff portions of the leaves with freshly laid eggs are placed in jars with a screen cover and observed for larval emergence.
- Wooden seedbox with dimension of 60 × 40 × 10 cm with 5-cm-deep soil needs to be used for raising the seedlings.
- Totally 2 g of seed/entry is sown in a 20 cm row with 10 cm gap between the rows.

**Fig. 5** Photograph showing yellow stem borer, *Scirpophaga incertulus* and "white ear" symptom

- One susceptible check and one resistant check should be maintained.
- TN1 is the common susceptible check, and W1263 can be used as resistance check for stem borer.
- After 14 DAS, the seedlings are transplanted in plant beds with a spacing of 20 × 20 cm.
- One row should be maintained for each entry.
- One seedling/hill has to be transplanted and properly labeled.
- After 14 DAT (28 DAS), the plants are infested with newly hatched larvae of stem borer at 10 larvae/row.
- The number of deadhearts with larvae inside is recorded on 2nd and 4th weeks after infestation. Observations should be recorded only after recording 25% damage in case of the susceptible check.

$$D = \frac{\text{Dead heart in test entry } (\%)}{\text{Dead heart in S-check } (\%)} \times 100$$

**SES Scale**

| Scale | Deadheart (%) |
|---|---|
| 0 | No |
| 1 | 1–20 |
| 3 | 21–40 |
| 5 | 41–60 |
| 7 | 61–80 |
| 9 | 81–100 |

As above, the percentage of white ear is recorded by releasing the larvae at booting stage and recording the observations around 90 DAT. In field condition, 3000 entries are tested in 1 ha area.

### 5.2.2 Screenhouse Screening

It is the best procedure for evaluating rice germplasm against the yellow stem borer.

- A screenhouse of 28 × 22 × 2.5 m can accommodate six beds (25 × 2.5 m), which are separated by pathways. Six hundred entries can be evaluated in a non-replicated trial in this screenhouse.
- Fourteen-day-old seedlings of ten rice entries are transplanted in the plant beds with 20 × 20 cm spacing.
- For every 20 rows of test entries, 1 row each of the susceptible and the resistant checks are planted.
- For an initial screening test, one row of each entry is planted.
- For retesting, entries will be replicated three times in a randomized complete block design (RCBD).
- On 30 DAT, newly hatched larvae are transferred using a fine camel-hair brush onto the youngest leaf or auricles at the rate of one larva per tiller in the sown entries.
- Deadhearts are counted for 4 weeks after larval infestation at weekly intervals.
- The test is considered valid when at least 50% of the tillers get infested by the borer larvae.

The percentage of "deadheart" for each entry is computed as:

$$\text{Deadheart}\ (\%) = \frac{\text{Number of Deadhearts counted}}{\text{Total number of tillers observed}\ (\text{Healthy} + \text{infected} + \text{damaged})} \times 100$$

## 5.3 *Screening Rice Germplasm for Resistance to Leaf Folder*

Rice leaf folder or roller is considered one of the major harmful insect pests of rice. They do harm by rolling leaf and reducing photosynthesis area (Fig. 6). Screening for rice folder, *Cnaphalocrocis medinalis* Guenee, can be carried out in cages in the greenhouse.

- Earthen pots may be used to sow ten seeds/entry per pot.
- The pots are to be placed in metal trays with water (flooded condition).
- After 10 DAS, thinning has to be done to maintain only five seedlings/pot.
- After 14 DAS, around 120 such pots are enclosed in a large net cage with a dimension of 1.74 × 1.4 × 0.75 m.
- Ten pairs of adult moths (3 days old) are released inside the cage.
- The adults are fed with 5–10% honey solution kept in the corner of the cage.
- After 21 DAS, the cage has to be removed, and the plants are allowed to grow well with more sunlight.
- At 35 DAS, the damage evaluation has to be done when the S-check shows 60% of symptoms on the leaf.
- TN1 or CR1009 is used as susceptible check.

**Fig. 6** Photograph showing leaf folder, *Cnaphalocrocis medinalis* Guenee and its causal symptom

**SES Scale**

| Score | Visual symptom |
|---|---|
| 0 | No damage |
| 1 | Up to 1/3 of the leaf area scrapped |
| 2 | Above 1/3 and up to half of leaf area scrapped |
| 3 | Above 1/2 of the leaf area scrapped |

The number of leaves in each grade is counted and "*R*" (damage rating) is computed using the following formula:

$$R = \frac{\frac{(G1)\times 100\big)\ \text{x}\ 1}{\text{Total no.of leaves observed}} + \frac{(G2\ \text{x}\ 100)\ \text{x}2}{\text{Total no.of leaves observed}} + \frac{(G3\ \text{x}\ 100)\ \text{x}3}{\text{Total no.of leaves observed}}}{6}$$

where in *G*1 is the total number of leaves with grade 1, *G*2 total number of leaves with grade 2, and *G*3 total number of leaves with grade 3.

Damage rating (*R*) for each test entry and the susceptible check (TN1) are calculated. Then the adjusted damage rating (*D*) for each test entry is determined based on the extent of damage in the susceptible check by the following formula:

$$D = \frac{R \text{ of test entry}}{R \text{ of S-check}} \times 100$$

The damage rating has to be fit into the 0–9 SES scale as follows:

**SES Scale**

| Scale | Damage (%) |
|---|---|
| 0 | No |
| 1 | 1 to 10 |
| 3 | 11 to 30 |
| 5 | 31 to 50 |
| 7 | 51 to 75 |
| 9 | Above 75 |

## 5.4 *Screening Rice Germplasm for Resistance to Gall Midge*

Rice gall midge, *Orseolia oryzae* (Wood-Mason), infests the crop and causes "silver shoot" (Fig. 7). Screening rice germplasm for resistance to gall midge is based on the extent of damage. The screening can be done both in laboratory and field conditions.

### 5.4.1 Field Screening

- The select rice accessions are planted in fields in a RCBD. Each entry is replicated thrice in 3 rows of 15 clumps with a spacing of 25 × 20 cm between rows and plants, respectively.
- After every ten rows of test entries, a row of susceptible check (usually TN1) is planted.
- The accessions are raised by following the recommended package of practices.
- To maintain optimum pest infestation and population, relative humidity needs to be maintained by providing a water level of 4 inch above the ground.
- For evaluating the rice entries against gall midge attack, the method suggested by Prakasa Rao (1975) can be followed.
- The silver shoots are recorded twice at 30 days after planting (DAP) and 50 DAP.
- The data on total number of hills and number of infested and healthy hills will be recorded.

**Fig. 7** Photograph showing typical 'silver shoot' symptom caused by rice gall midge

- The percentage of hills and tillers damaged is computed, and the results are converted to 0–9 scale (IRRI 1996).

$$\text{Damaged plants } (\%) = \frac{\text{Number of infested plants}}{\text{Total number of plants}} \times 100$$

$$\text{Silver shoots } (\%) = \frac{\text{Number of infested tillers}}{\text{Total number of tillers}} \times 100$$

The percentage of infested plants will be converted into 0–9 scales using standard evaluation score (SES) for rice (IRRI 1996).

| Scale | Infested plants (%)/silver shoot (%) |
|---|---|
| 0 | None |
| 1 | Less than 1% |
| 3 | 1–5% |
| 5 | 6–15% |
| 7 | 16–50% |
| 9 | 51–100% |

### 5.4.2 Laboratory Screening

The method suggested by Vreden and Arifin (1977) can be followed.

- The study involves raising seedlings of the available accessions and popular rice cultivars in a plastic tray/box of 60 × 45 × 10 cm dimension.
- In each tray/box, at both the sides, a row of susceptible check, TN1, is raised (15 plants/row).
- Once seedlings are 10 days old, the boxes are placed in a shallow iron tray measuring 123 × 85 × 21 cm containing water to provide better aeration, and the entire tray is covered by nylon mesh.
- In each box, 20–30 entries can be accommodated.
- When the plants attain 15–20 days age, 30–50 of gall midges/box should be released using an aspirator.
- After 2 days of infestation, water can be sprayed on the plants using hand atomizer at 2–3 h intervals for 2 days. This is to provide favorable condition for hatching and to increase relative humidity.
- After 4 weeks of release, percentage of damaged plants will be recorded using the following formula.

$$\text{Damaged plants } (\%) = \frac{\text{No.of damaged plants}}{\text{Total no.of plants}}$$

Then, the percentage of infested plants will be converted to 0–9 scale using the standard evaluation score (SES) for rice (IRRI).

### 5.4.3 Screening Rice Germplasm for Resistance to Biotypes of Gall Midge

Biotypes in insect pests are comparatively infrequent because of the insects' own complex physiology and the pest resistance in host plants being often related to the host-finding behavior. The biotypes are distinguished on the basis of their interaction with differential host varieties or clones of the host plants. The biotypes are not necessarily similar to the geographical races or insect populations which may be distinguishable on the biological grounds. The development of biotypes is related to severe selection pressure by resistance crop variety. Differential varieties distinguish biotypes or pathotypes (races) by their qualitative differences in reactions to different insect or pathogen strains. In the simplest case showing unequivocal specificity of virulence, variety A is resistant to biotype 1 but susceptible to biotype 2, whereas variety B is susceptible to biotype 1 but resistant to biotype 2. In this example, A and B are differential varieties.

Methodology

- A set of 14 standard differentials under 4 groups were identified to characterize the prevailing gall midge biotypes in India by the Indian Institute of Rice Research (IIRR), Hyderabad.
- In the selected test locations, 20–25 old seedlings of each differential are planted in 3 rows consisting of 20 hills in each row with a spacing of 20 × 15 cm between rows and plants, respectively.
- The level of infestation is increased by maintaining constant water level of 5 inches in the field and applying 25% excess nitrogenous fertilizer (urea).
- One row of each differential will be harvested at 30 days after planting to provide fresh sprouts for infestation.
- The observations on damaged plants on hill basis and number of healthy and infested tillers in all 20 hills are recorded at 30 and 50 days after transplanting.
- The percentage of damaged plants and silver shoots was worked out as:

$$\text{Damaged plants } (\%) = \frac{\text{No.of damaged plants}}{\text{Total no.of plants}}$$

$$\text{Silver shoots } (\%) = \frac{\text{No.of infested tillers}}{\text{Total no.of tillers}}$$

- Each entry under four groups rated as either resistance (*R*) with less than 10% plant damage or susceptible with higher damage.
- Based on their pattern of resistance or susceptibility, the biotypes in test locations are differentiated as biotype 1 (R-R-R-S), biotype 2 (S-R-R-S), biotype 3 (R-S-R-S), biotype 4 (S-S-R-S), biotype 5 (R-R-S-S), and biotype 6 (R-S-S-S).
- Further, the percent silver shoot at 50 days transplanting in each differential is converted to 0–9 scale using IRRI SES for rice developed at IRRI (IRRI 1996).

## 6 Screening Sorghum Germplasm for Resistance to Stem Borer

The following is the rapid screening method for evaluating resistance to sorghum stem borer, *Chilo partellus* (Crambidae:Lepidoptera).

- Sorghum seedlings are sown in microplots (3 × 1 m) with spacing of 15 cm between rows and 10 cm between plants. An RCBD with three replications is adopted for this evaluation.
- Test plants are infested with stem borers reared on a kabuli bean diet.
- The larvae are dispensed onto the plants using bazooka larval inoculator at the rate of three to four per stroke.
- When the plants are 9–10 days old, plants are artificially infested by applying one stroke of the bazooka containing larvae and carrier into the leaf whorl of each plant.
- Leaf feeding is scored 7 days after infestation (DAI) on a visual rating scale of one to nine (1, highly resistant; 9, highly susceptible), and "deadhearts" are recorded at 14 DAI (Fig. 8).
- In this method, 12,000 entries per year can be screened.
- This method is valuable for the initial screening of germplasm for resistance (Table 1).

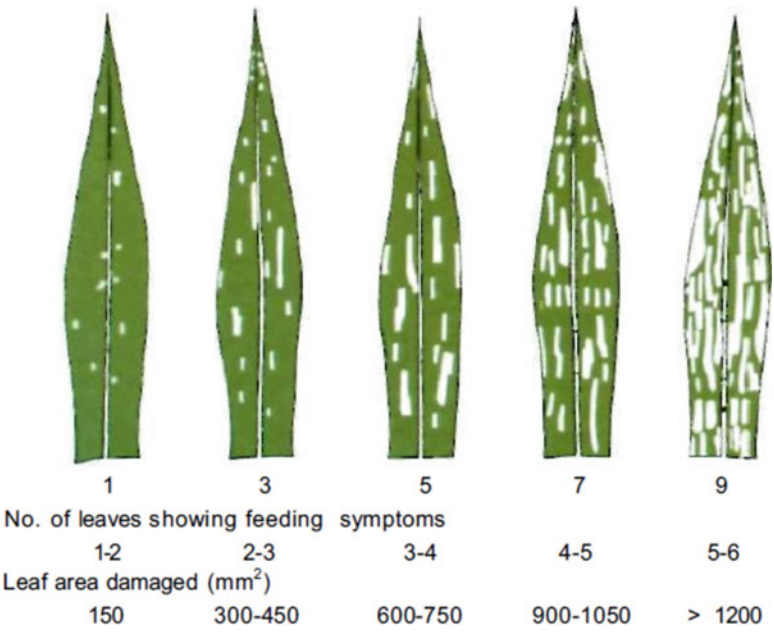


**Fig. 8** Leaf damage rating scale for *Chilo partellus*

**Table 1** Visual damage rating scale for leaf feeding, deadheart and chaffy and broken panicles, due to stem borer (Sharma et al. 1992)

| Score | No. of leaves with feeding symptoms | Leaf area eaten ($mm^2$) | Deadhearts/chaffy/broken panicles (%) |
|---|---|---|---|
| 1 | 1–2 | <150 | <10 |
| 2 | 1–2 | 150–300 | 10–20 |
| 3 | 2–3 | 300–450 | 21–30 |
| 4 | 2–3 | 450–600 | 31–40 |
| 5 | 3–4 | 600–750 | 41–50 |
| 6 | 3–4 | 750–900 | 51–60 |
| 7 | 4–5 | 900–1050 | 61–70 |
| 8 | 4–5 | 1050–1200 | 71–80 |
| 9 | 5–6 | >1200 | >80 |

## 7 Screening Chickpea Germplasm for Resistance to Pod Borer

- Pod borers (*Helicoverpa armigera*) make holes in the fruit of chickpea and eat the seeds (Fig. 9).
- Generally, chickpea germplasm is screened under natural field conditions, preferably in areas where pod borer incidence is predominant.
- Each chickpea entry has to be raised in a row of 5 m length.
- An RCBD with three replications may be adopted.
- Distance of 30 and 15 cm between rows and plants should be maintained, respectively.
- Whenever, the natural population of pod borers is negligible, test plants are infested with pod borers reared on chickpea-based artificial diet.
- At the harvest of the crop, pod damage needs to be recorded in each replicate after counting the total number of pods and pods damaged by the pest from five randomly selected plants.
- Damage caused by pod borer is calculated and converted into percent damage by using the following equation:

$$\text{Pod damage } (\%) = \frac{\text{Number of damaged pods}}{\text{Total number of pods}} \times 100$$

The percent damage of any test entry is compared with that of the check variety, by using the following formula:

$$\text{Pest susceptibility percentage} = \frac{\text{P.D. of check} - \text{P.D. of test entry}}{\text{P.D. of check}} \times 100$$

where P.D. is the mean percentage of pods damaged

**Fig. 9** Photograph showing the Pod borer (*Helicoverpa armigera*) on Chick pea and its damage

The pest susceptibility percentage is then converted to a 1-to-9 rating scale described by Lateef and Sachan (1990) with slight modification as follows:

| Susceptibility | Pest susceptibility (%) |
|---|---|
| 1 | 100 |
| 2 | 76 to 99 |
| 3 | 50 to 75 |
| 4 | 25 to 49 |
| 5 | 10 to 24 |
| 6 | −10 to 9 |
| 7 | −25 to –9 |
| 8 | −50 to –24 |
| 9 | Less than −50 |

## 8 Screening Castor Germplasm for Resistance to Leafhopper

Leafhopper is one of the serious pest of oilseeds especially castor (Fig. 10). Screening castor (*Ricinus communis* L.) genotypes for resistance against green leafhopper, *Empoasca flavescens* F., is carried out by adopting the following procedures:

- A suitable land with good irrigation facility is selected for conducting the experiments.
- The selected genotypes are screened by adopting RCBD with necessary replications.
- The leafhopper population is recorded at 10-day interval from five to ten plants per genotype.
- The number of nymphs and adults of leafhopper are counted in three leaves per plant, each one from lower, middle, and upper portions.
- Entries will be classified into different groups as follows:

**Fig. 10** Photograph showing Castor leafhopper

**Table 2** Field reaction of select castor genotypes to leafhoppers

| | Mean number of leafhoppers/three leaves/plant[a] | | | |
|---|---|---|---|---|
| | Observation on date | | | |
| Genotypes | 1st | 2nd | 3rd | 4th |
| 1…10 | | | | |
| SEm± | | | | |
| C.D at 5% | | | | |
| Mean | | | | |

[a]Note: Observations should coincide with the peak numbers/activities of the pest

| No. of leafhoppers/three leaves | Injury grade | Category |
|---|---|---|
| 0 | No injury | Resistant |
| 1 | Hopperburn up to 10% | Moderately resistant |
| 2 | Hopperburn up to 11–25% | Moderately susceptible |
| 3 | Hopperburn up to 26–50% | Susceptible |
| 4 | Hopperburn above 50% | Highly susceptible |

- Data is subjected to transformation before analysis by ANOVA.
- Data recording can be done as per the model given in Table 2

## 9 Screening Sesame Germplasm for Resistance to Shoot Webber and Capsule Borer

Screening sesame accessions for resistance to shoot webber and capsule borer, *Antigastra catalaunalis* Dup. (Fig. 11), can be carried out in both greenhouse and field conditions.

**Fig. 11** Photograph showing Sesame shoot webber and capsule borer

## *9.1 Greenhouse Screening (Visual Grading System)*

- Seeds of each entry are sown in nursery bags individually.
- These nursery bags are kept inside a screening cage made up of a wooden frame of 2 m × 1 m × 1 m dimension covered with nylon mesh all around.
- The seedlings are thinned at the rate of two/bag on the fourth day after germination, and subsequently one/bag is maintained from eighth day onward.
- On 15th day after germination, the plants are subjected to infestation by releasing 10 pairs of adults per 50 accessions.
- Sugar solution (10%) is provided as food for the moths.
- The female moths, after mating with the males, are allowed to oviposit on the plants.
- The experiment is replicated thrice, and 15 days after release, the accessions are scored based on the intensity of damage and grouped into different resistant categories as described by Sridhar and Gopalan (2002) (Table 3).

## *9.2 Field Screening (Balaji and Selvanarayanan 2009)*

- Seeds of the test entries are sown on the ridges of 2 m length with a spacing of 30 cm between rows and 30 cm between plants.
- Ten plants are maintained per replication.
- A known susceptible check, namely, SVPR-1 (Vijai Anandh 2003), is maintained at one row for every five rows of the test accessions as "infestor" rows.
- Totally two rows of the susceptible check are also maintained around the experimental field as "infestor" crop.
- Recommended agronomic practices are followed except plant protection measures.

**Table 3** Grade chart for seedling screening under greenhouse conditions

| Extent of damage | Grade | Resistance rating |
|---|---|---|
| Partial loss of one or two leaves or no damage at all | 1 | Highly resistant |
| Partial folding and loss of chlorophyll of one or more leaves of most plants | 3 | Resistant |
| Folding of four or more leaves and feeding or half of the plants damaged | 5 | Susceptible |
| Most of the leaves folded and damaged or all plants dead | 7 | Highly susceptible |

**Table 4** Scoring sesame accessions for resistance to *A. catalaunalis*

| Damage (%) | | | | |
|---|---|---|---|---|
| leaf | Flower | Capsule | Score | Resistance rating |
| 0–2 | 0–1 | 0–1 | 1 | Highly resistant |
| 2–4 | 1–2 | 1–2 | 3 | Resistant |
| 4–6 | 2–3 | 2–3 | 5 | Moderately resistant |
| 6–8 | 3–4 | 3–4 | 7 | Susceptible |
| >8 | >4 | >4 | 9 | Highly susceptible |

**Table 5** Cumulative scoring of sesame accessions for resistance against *A. catalaunalis*

| Cumulative score | Score | Resistance rating |
|---|---|---|
| 0–1 | 1 | Highly resistant |
| 1–3 | 3 | Resistant |
| 3–5 | 5 | Moderately resistant |
| 5–7 | 7 | Susceptible |
| >7 | 9 | Highly susceptible |

- Incidence of *A. catalaunalis* is recorded by observing five plants selected randomly per replication at weekly interval.
- Mean percent damage to the leaf, flower, and capsule is computed by counting the total number of leaves, flowers, and capsules and the number of infested leaves, flowers, and capsules, respectively.
- Leaf damage, flower damage, and capsule damage are recorded from 15 DAS, 36 DAS, and 50 DAS onward, respectively, and the respective mean percentage is worked out.
- Based on the damage assessed at various stages, the accessions are categorized by adopting the score chart formulated by Sridhar and Gopalan (2002), but with few modifications.
- As the damage on reproductive parts such as flowers and capsules influences yield more than the leaf damage, lesser flower and capsule damage and more leaf damage are equated to a particular score as presented below (Table 4).

After arriving at the cumulative score based on percent damage on leaf, flower, and capsule for a particular accession, score (1–9) is allotted by referring to the score chart, and resistance rating is made (Table 5).

### 9.2.1 Procedure to Evaluate Resistance Against *A. catalaunalis*

As a hypothetical model, resistance rating of an accession having 2%, 12%, and 1% leaf, flower, and capsule damage, respectively, is described below:

Step 1. By referring to score chart for leaf (2%), flower (2.5%), and capsule (1%) damage, the scores 3, 5, and 1 are given.

Step 2. Cumulative score for the damage is calculated, i.e., 3 + 5 + 1 = 9, and mean 9/3 = 3 is the cumulative score.

Step 3. Mean cumulative score 3 is referred to grade chart which fall in the group between >1 and 3 referring grade 3.

Step 4. Grade 3 is read against the resistance level, which represents the category resistant (R).

## 10 Screening Groundnut Germplasm for Resistance to Leaf Miner and Leafhopper (Anonymous 1986)

### *10.1 Evaluating Leaf Miner Resistance in Groundnut Germplasm Based on Area of Leaflets Dried Due to Mining (ICRISAT System)*

- Leaf miner is one of the most import pests of groundnut. Symptoms include small blister-like mines seen on the upper leaf surface near the midrib. As the feeding advances, the mines increase in size, and the entire leaflet becomes brown, rolls, shrivels, and dries up as shown in Fig. 12. In severe cases, the affected crop presents a burnt-up appearance. At later stages, larvae web the leaflets together and feed on them, by remaining within the folds.
- The entries are grown in rows in replicates.
- Ten plants are selected at random for each entry.
- From each such selected plant, ten leaflets that are heavily mined by the leaf miner are selected at random, and percent leaflet area destroyed is worked out.
- The following rating scale is used for allotting injury values:

| Rating | Area dried due to mines (%) |
|---|---|
| 1 | 0 |
| 2 | 1–10 |
| 3 | 11–20 |
| 4 | 21–30 |
| 5 | 31–40 |
| 6 | 41–50 |
| 7 | 51–60 |
| 8 | 61–80 |
| 9 | 81–100 |

**Fig. 12** Photograph showing the symptom of groundnut leaf miner

## *10.2 Evaluating Leaf Miner Resistance in Groundnut Germplasm Based on Percent Leaflet Damage (AICRP System)*

- Test entries are grown in a row of 4 m length with a row-to-row distance of 60 cm and plant-to-plant distance of 15 cm.

| Damaged leaflets (%) | | Leaflet area damaged (%) | |
|---|---|---|---|
| Actual | Rating (A) | Actual | Rating (B) |
| 0–10 | 1 | 0–10 | 1 |
| 11–20 | 2 | 11–20 | 2 |
| 21–30 | 3 | 21–30 | 3 |
| 31–40 | 4 | 31–40 | 4 |
| 41–50 | 5 | 41–50 | 5 |
| 51–60 | 6 | 51–60 | 6 |
| 61–70 | 7 | 61–70 | 7 |
| 71–80 | 8 | 71–80 | 8 |
| 81–90 | 9 | 81–90 | 9 |
| 91–100 | 10 | 91–100 | 10 |

- Each entry is replicated thrice.
- Soybean is grown as "infestor" crop after every five rows of test entries.
- The following observations are recorded:

  (a) The total number of leaflets and number of damaged leaflets from five randomly selected plants from each row are recorded. The percentage of damaged leaflets is calculated, and the damage rating is done as described below.

  (b) Twenty leaflets are collected at random from each row, and the area of damage is recorded. The percentage of leaflet area damaged is assessed, and the damage rating is done as described below.

Further, the severity index can be calculated by the following formula:

$$\text{Severity Index} = \frac{A \times B}{100}$$

where $A$ is the mean rating for percent damaged leaflets and $B$ mean rating for percent leaflet area damaged.

## *10.3 Evaluating Leafhopper Resistance in Groundnut Germplasm*

Feeding by leafhopper causes folding of leaflets which lead to the development of yellow coloration in a triangular fashion at the tip of the leaflets (Fig. 13). This injury is used as a criterion for assessing the relative susceptibility of groundnut entries.

- Test entries are grown in a row of 4 m length with a row-to-row distance of 60 cm and plant-to-plant distance of 15 cm.
- Each entry is replicated thrice.
- The leaflets showing yellow tip symptoms are counted from ten randomly selected leaflets in each plant.
- Ten plants per row are observed for recording the damage.

Injury rating is done based on percent yellow tip leaflets using a one-to-ten scale as given below:

| Yellow tip leaflets (%) | Rating |
|---|---|
| 0–10 | 1 |
| 11–20 | 2 |
| 21–30 | 3 |
| 31–40 | 4 |
| 41–50 | 5 |
| 51–60 | 6 |
| 61–70 | 7 |
| 71–80 | 8 |
| 81–90 | 9 |
| 91–100 | 10 |

**Fig. 13** Photograph showing the hopperburn symptom caused by groundnut leafhopper

**Fig. 14** Photograph showing the hopperburn symptom caused by Cotton leafhopper

## 11 Screening Cotton Germplasm for Resistance to Sap Feeders and Bollworms (Rao 1973)

### *11.1 Evaluation of Resistance in Cotton Germplasm Against Leafhoppers,* Empoasca devastans

- Test entries are grown in rows of 6 m length with a spacing of 75 cm between rows and 30 cm between plants.
- Bhendi is used as "infestor" crop and sown at the rate of one row for four rows of cotton entry.
- Ten plants per entry are observed to record the population of nymphs and adults in the three leaves per plant, each one from the top, middle, and bottom regions, respectively.
- The leafhopper population is recorded on 30, 45, and 60 days after sowing.
- The damage caused by leafhopper is shown in Fig. 14, and it is assessed in each entry on 45 and 60 days after sowing (DAS) based on the following grading:

1. Leaves free from crinkling or curling with no yellowing, bronzing, and drying of leaves
2. Few leaves on lower portions of the plant curling, crinkling, and slight yellowing
3. Crinkling and curling all over; yellowing, browning, and bronzing in the middle and lower portion; plant growth hampered
4. Extreme curling, yellowing, browning, and bronzing, drying of leaves and defoliation, stunted growth

The injury index is calculated as per the following formula:

$$\text{Injury index} = \frac{G1 \times P1 + G2 \times P2 + G3 \times P3 + G4 \times P4}{P1 + P2 + P3 + P4}$$

where "*G*" represents the number of the grade of injury and "*P*" the population under that grade for each entry.

Based on the index, the entries may be grouped into different categories of resistance as follows:

| Injury index | Resistance rating |
|---|---|
| 0.1–1.0 | Resistant |
| 1.1–2.0 | Moderately resistant |
| 2.1–3.0 | Susceptible |
| 3.1–4.0 | Highly susceptible |

## *11.2 Evaluation of Resistance in Cotton Germplasm Against Whitefly,* Bemisia tabaci *Genn. (Fig. 15)*

- Test entries are grown in rows at 20 plants per row per entry.
- Ten plants per entry are observed to record the population of nymphs on the third, fifth, and seventh leaf from the terminal end of the main shoot in each plant.
- The whitefly population is recorded from 55 DAS to 110 DAS at weekly intervals.

**Fig. 15** Photograph showing white fly, *Bemisia tabaci* population on cotton leaves

- The mean data on population and damage symptoms at maturity are tabulated, and the resistance reaction is graded as given below:

| Nymphs/ leaf | Symptoms | Damage scale | Resistance rating |
|---|---|---|---|
| 0 | Leaves healthy | 0 | Highly resistant |
| 1–25 | Leaves showing yellow spots on the upper surface below which the nymphs are feeding, mild appearance of honey dew on the leaves below the attacked leaves, visible during early morning/late evening | 1 | Resistant |
| 26–50 | Large yellow spots on the leaves and general yellowing. A layer of honey dew uniformly present on the leaves below the attacked leaves, 10–15 circular spots of reddish brown mold or black sooty mold on such leaves | 2 | Moderately resistant |
| 51–100 | Uniform growth of black sooty mold on the leaves below the attacked leaves. Attacked leaves pale yellow, unhealthy, and droop from the margins | 3 | Susceptible |
| Above 100 | Leaves fully blackened due to sooty mold. Leaves droop and may drop | 4 | Highly susceptible |

## *11.3 Evaluation of Resistance in Cotton Germplasm Against Bollworms (Fig. 16)*

- Test entries are grown in rows at 20 plants per row per entry.
- Ten plants per entry are observed to record the total number of squares, bolls, as well as those infested by bollworms in each plant.
- The infestation pattern can be visualized in Fig. 16 and is recorded from 35 to 40 DAS to 110 DAS at weekly intervals.

**Fig. 16** Photograph showing cotton bolls infested by cotton bollworms

- The mean percent infestation for the entire season is calculated, and the resistance rating is done as given below:

| Infestation (%) | Resistance rating |
|---|---|
| 0.1–10 | Resistant |
| 10.1–20 | Moderately resistant |
| 20.1–30 | Susceptible |
| >30 | Highly susceptible |

## 12 Screening Sugarcane Germplasm for Resistance to Borers (Rajendran et al. 1998) and Sap Feeders

### *12.1 Evaluation of Resistance in Sugarcane Germplasm Against Shoot Borer,* Chilo infuscatellus *Snell*

- The total number of tillers and the infested/affected tillers is counted in the test genotypes or clones in the unit area of not less than 40 $m^2$ or in not less than two rows of 8 m length, and the percentage of incidence is worked out.
- Normally for one acre, not less than five such random samples should be taken, and the mean is computed.
- Incidence should be assessed at least three times, viz., first, second, and third months, after planting.
- The typical symptom of a shoot borer is called "deadheart" (Fig. 17). These "deadhearts" were removed after each counting. The cumulative incidence by shoot borer is calculated by using the following formula:

$$\text{Cumulative incidence} = \frac{A1 + A2 + A3}{T3 + A1 + A2} \times 100$$

**Fig. 17** Photograph showing the 'deadheart' symptom caused by *Chilo infuscatellus* Snell (sugarcane shoot borer)

where:

*A*1 is the number of affected tillers at first month after planting.
*A*2 is the number of affected tillers at second month after planting.
*A*3 is the number of affected tillers at third month after planting.
*T*3 is the total number of tillers at third month after planting.

Based on the cumulative incidence, the genotypes are rated as follows:

| Cumulative incidence (%) | Resistance rating |
|---|---|
| 0–15 | Less susceptible |
| 15.1–30 | Moderately susceptible |
| 30.1 and above | Highly susceptible |

## 12.2 *Evaluation of Resistance in Sugarcane Germplasm Against Internode Borer,* Chilo sacchariphagus indicus *Kapur*

- The total number of tillers or canes and the infested/affected tillers or canes as shown in Fig. 18 is counted in the test genotypes or clones in a unit area, and percentage of incidence is computed.
- Simultaneously, in the same sampled area, the total number of affected nodes in the canes is counted, and percentage of intensity of infestation is computed.
- The infestation index is then arrived by the following formula:

$$\text{Infestation index} = \frac{\text{Per cent incidence (cane basis)} \times \text{Per cent intensity (node basis)}}{100}$$

**Fig. 18** Photograph showing the typical symptom caused by *Chilo sacchariphagus indicus* Kapur (internode borer)

Based on the infestation index, the genotypes are rated as follows:

| Infestation index | Resistance rating |
|---|---|
| 0–2.0 | Resistant |
| 2.1 to 4.0 | Less susceptible |
| 4.1 to 6.0 | Moderately susceptible |
| Above 6.1 | Highly susceptible |

## 12.3 *Evaluation of Resistance in Sugarcane Germplasm Against Top Borer,* Scirpophaga excerptalis *Walker (Fig. 19)*

- The total number of tillers or canes and the infested/affected tillers or canes is counted in the test genotypes or clones in a unit area, and percentage of incidence is computed.
- Artificial infestation may be done, if natural incidence is negligible.

Based on the incidence percentage, the genotypes are rated as follows:

| Infestation index | Resistance rating |
|---|---|
| 0–15.0 | Resistant |
| 15.1–30.0 | Less susceptible |
| 30.1–60.0 | Moderately susceptible |
| Above 60.1 | Highly susceptible |

**Fig. 19** Photograph showing *Scirpophaga excerptalis* Walker (sugarcane top borer)

**Fig. 20** Photograph showing tip dying and yellow color leaves caused by *Melanaspis glomerata* Green (sugarcanescales)

## *12.4 Evaluation of Resistance in Sugarcane Germplasm Against Scale Insects,* Melanaspis glomerata *Green*

- The leaves of infested canes show signs of tip drying and unhealthy pale green color, and with continued infestation, these turn yellow (Fig. 20).
- An artificial infestation technique is followed, if natural incidence is negligible.
- Severely infested cane pieces are tied in the standing canes, when the crop is about 6 months old.
- Scale insects are allowed to multiply, and the genotypes or clones are evaluated based on cane drying and visual grading of pest infestation as per the following rating scale (David et al. 1986).

| Infestation | Resistance rating |
|---|---|
| When only a few insects are seen on any of the internodes without a well-established colony (very light) or when the encrustation of the pest covers only about 1/4 of an internode (light) | Less susceptible |
| When the encrustation of the pest covers nearly 1/2 of an internode (moderate) | Moderately susceptible |
| When the encrustation of the pest covers 3/4 of an internode (severe) or more than 3/4 of an internode (very severe) or when the canes show drying due to pest attack | Highly susceptible |

# 13 Screening Tomato Germplasm for Resistance Against Fruit Borer, *Helicoverpa armigera* Hubner (Selvanarayanan 2000)

Screening tomato germplasm for resistance against fruit borer, *Helicoverpa armigera* (Hubner), is carried out in areas where the natural incidence is quite predominant.

- The larvae of this pest scrape the tomato foliage until early or late second instar stage. The larva bores into the fruit making it unfit for marketing. In severe cases of infestation, more than 80% of fruits get damaged (Fig. 21).

**Fig. 21** Photograph showing the typical symptom caused by *Helicoverpa armigera* Hubner (Tomato fruit borer)

- Seedlings of each accession may be raised in individual earthen pots or in separate seedbeds.
- 25-day-old seedlings are planted with a spacing of 40 cm × 100 cm.
- Each accession is grown in a row of 4 m length.
- One row of 40-day-old seedlings of marigold (*Tagetes erecta* L.) plants is grown as "infestors" around the trial plot. The flowering of both the test crop and the trap crop is thus synchronized to augment the larval load on the test accessions.
- Need-based irrigation and organic fertilization should be done.
- Chemical plant protection needs to be avoided.
- The variety CO 3, serving as the susceptible check, needs to be planted at 1 row for every 16 rows of the test accessions.
- Incidence of the larval population per plant is recorded at weekly intervals on five plants selected at random in each accession from 1 week after transplanting till final harvest.
- The following scale can be used for resistance rating of the accessions, based on larval population pressure per plant.

| Larval population per plant | Grade | Resistance rating |
|---|---|---|
| 0.0–0.30 | 1 | Resistant (R) |
| 0.31–0.60 | 2 | Moderately resistant (MR) |
| 0.61–0.90 | 3 | Susceptible (S) |
| >0.91 | 4 | Highly susceptible (HS) |

- Fruit infestation per plant is recorded at weekly intervals on five plants selected at random in each accession from 1 week after appearance of first fruit till final harvest.
- The following scale can be used for resistance rating of the accessions, based on the percent fruit damage,

| Larval population per plant | Grade | Resistance rating |
|---|---|---|
| < 15.0 | 1 | Resistant (R) |
| 15.1–30.0 | 2 | Moderately resistant (MR) |
| 30.1–45.0 | 3 | Susceptible (S) |
| >45.1 | 4 | Highly susceptible (HS) |

# 14 Screening Cucurbits for Resistance to Pumpkin Beetles and Fruit Fly

## *14.1 Evaluation of Resistance in Cucurbitaceous Genotypes Against Pumpkin Beetles,* Aulacophora hilaris *(Boisd.) (Fig. 22)*

- Test genotypes are raised in pots at ten seedlings per pot with necessary replicates.
- Screening is done under insect-proof caging of dimension 8 m × 3 m × 2 m.
- Seedlings raised in pots are kept inside the cage, and ten beetles are released inside the cage at one beetle per plant when two cotyledonary leaves of each seedling are fully expanded.
- Ten percent excess beetles are released to compensate escape or mortality.
- A susceptible check is also enclosed in each cage.
- Genotypes are scored for resistance reaction when the susceptible check shows complete damage and scored as per the following scale.

| Score | Extent of damage | Resistance rating |
|---|---|---|
| 1 | No damage | Immune |
| 2 | Slight damage – 25% of the cotyledonary leaf area consumed | Resistant |
| 3 | Slight to medium damage – 50% of the cotyledonary leaf area consumed | Tolerant |
| 4 | Medium damage – 75% of the cotyledonary leaf area consumed | Susceptible |
| 5 | Severe damage – more than 75% of the cotyledonary leaf area damaged | Highly susceptible |

**Fig. 22** Photograph showing *Aulacophora hilaris* (Pumpkin beetle)

**Fig. 23** Photograph showing the typical symptom caused by cucurbits fruit fly

## *14.2 Evaluation of Resistance in Cucurbitaceous Genotypes Against Fruit Fly,* **Bactrocera cucurbitae** *Coq.*

- This is the most destructive insect pest of cucurbits.
- Maggots feed on near-ripe fruits, riddle those fruits, and pollute the pulp as shown in Fig. 23.
- Test genotypes are sown in rows at 50 cm spacing within row and at 2 m spacing between rows.
- Resistant and susceptible genotypes should be sown on alternate hills.
- The number of fruit fly-infested fruits in each plant should be recorded for 4 weeks from the initiation of fruit set.
- If the natural incidence is less, laboratory-reared adults may be released in the field.
- To attract fruit flies, a slurry of jaggery can be sprayed on the crop.
- Resistance rating is made at harvest stage based on infestation of fruit fly as given below.

| Fruit damage (%) | Resistance rating |
|---|---|
| 1–10 | Highly resistant |
| 11–20 | Resistant |
| 21–50 | Moderately resistant |
| 51–75 | Susceptible |
| 76–100 | Highly susceptible |

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# Part IV
# Ecological and Climatographic Factors in Host Plant Resistance Studies

# Effects of Plant Phenological Factors on Insect Pest Infestation


**Santosh Kulkarni, A. K. Chakravarthy, and Naveen Kumar**

**Abstract** For understanding the relations between the crop plant and pest population, phenology of crop is basic and important. Crop phenology can be divided into definite pheno-phases, and numbers of pest species against each pheno-phase can be recorded. This will be help in making screening germ plasms against pests precise. The temporal relationships between the plant and the insect help in making pest management more efficient and cost-effective.

**Keywords** Phenology · Pest population · Screening · Timing of control intervention

## 1 Introduction

Insect migration, the blooming of flowers and trees and the seasonal occurrence of insects are examples of phenological events. A sequence of phenological events such as blooming time can be used as a biological calendar to anticipate the order and time when various insect pests reach vulnerable stages.

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## 2 Plant Phenology and Insect Pests

- Three to four different varieties of cotton with a susceptible check need to be raised following recommended package of practices in randomised block design. Three to four replications or treatments can be improvised.
- Five plants per replicate from central rows can be labelled at germination for monitoring phenological events in insects.
- The entire life cycle of the plants can be divided into germination, vegetative, squaring, flowering, boll formation and boll maturation stages.
- The number of days required to complete each phase can be converted to the amount of °C required by calculating day degrees following Falcon and Smith (1973).

$$\text{Day degrees for 1 day} = \frac{\text{Max}^{\circ}\text{C} - \text{Min}^{\circ}\text{C}}{2} - 10^{\circ}\text{C}$$

- Each square on emergence on five test plants should be tagged to provide date records for each phenological phase.
- Infestation of pests on the four to five varieties of cotton should be documented.
- The following methods can be deployed to sample different species of insects:

  (i) Surface grasshopper, *Chrotogonus trachypterus* (Blanchard): Infestation is considered positive by its partial or full damage on cotyledons or shoots on whole plot basis.
  (ii) Jassid, *Amrasca biguttula biguttula* (Ishida): Population of jassid nymphs is counting on ten fully-formed leaves from the upper half canopy of plants, as about 90% jassids are found concentrated on expanded leaves at top portions of plants.
  (iii) Whitefly, *Bemisia tabaci* (Genn.): Whitefly population per plant on the four varieties is recorded using a 0.5 $m^3$, wooden, foldable cage, covered with black muslin cloth on three sides and a glass pane on the fourth. Number of whiteflies alighting on the glass pane is encountered.
  (iv) Aphid, *Aphis gossypii* (Glov.): Population of apterous individuals is recorded on three random leaves per plant for a total of 5–10 plants, as the case may be.
  (v) Leaf-feeders, *Sylepta derogata* (Fab.), *Myllocerus undecimpustulatus* (Desbr.) and *Anomis flava* (Fab.): Population of the aforementioned leaf-feeders are determined on the whole plant basis.

(vi) Spotted bollworms, *Earias insulana* Boisd. and *Earias vittella* Fab.: Infestation of pests on shoots can be in terms of percentage plants damaged. The shed fruiting structures will be collected; sorted into a square, flower and boll; and dissected for determining causes of shedding. Shedding of reproductive parts in cotton can be ascribed to pests like *Earias* spp. or to pink bollworm or to physiological factors or natural factors. Damage by *Earias* spp. is identified by the presence of larvae or irregular, large holes in the fruiting structures plugged with excreta. Shedding due to pink bollworm is identified by the presence of larva or small pinholes at the base of the fruiting structure. If the shed fruiting structures are undamaged with no larva or damage symptoms, the shedding can then be ascribed to physiological factors. Fruiting bodies shed are collected from five or three plants per sowing date per variety and then examined in the laboratory. The data for each fruiting structure can be analysed by ANOVA followed by t test.

(vii) Pink bollworm, *Pectinophora gossypiella* (Saund.): The pink bollworm incidence on fruiting structures is determined as for *Earias* spp. The bolls are debracted and loculi separated and dissected to express infestation on loculi basis also. Unharvestable bolls are examined for the carry-over population of the pink bollworms.

Whether factors and physical, biochemical and ancillary plant growth characters change with pheno-phases, it is desirable to study their effects on pest infestation. For determining physical plant characteristics, samples of leaf and shoot from the top 15 cm portion (active feeding sites for most insects) of the plants and 3-week-old bolls are drawn from each plot. The following methods are used to record physical and chemical characters.

## 3 Physical Characters

- Leaf area is determined by graphical method, and thickness is measured by micrometry.
- Mirror-type camera lucida can be used for measuring hair angles.
- Number of gossypol glands/$cm^2$ leaf area can be counted under stereo-binocular microscope ($\times$ 10).
- The data sets can be analysed utilising appropriate statistical tests (Fig. 1).

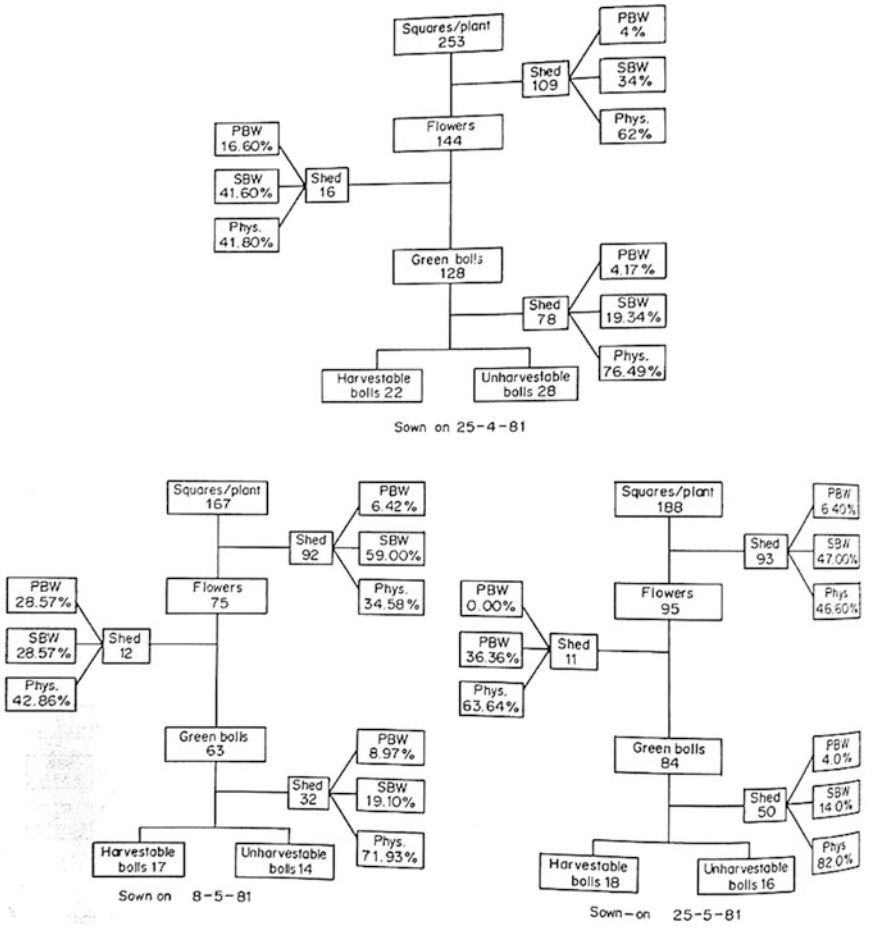


**Fig. 1** Insect pest damage in relation to phenology of cotton plants on three dates of sowing. *PBW* pink bollworm, *SBW* spotted bollworm, *PHYS* physiological drop; percentage values in blocks indicate the proportion of shedding of reproductive parts. The central vertical bars indicate the total number of reproductive parts at the beginning of each pheno-phase. The F414 Hirsutum cultivar was sown on three dates. More details are in the paper. (Source: Chakravarthy et al. 1985)

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# Determining Stability of Insect-Resistant Plants in Varying Climatic Regimes


**Subhash B. Kandakoor and V. Sridhar**

**Abstract** Climate change may result in the breakdown of resistance. Climate change that facilitates the introduction of susceptible cultivars in newer regions may favor insect pests. On the contrary, chemical composition of plants may change in view of global warming, and certain insect pests may benefit from reduced host defenses; as a result climate change can also modify resistance performances of crop varieties to insect pests; it may induce increased or decreased feeding, depending on the situation and the pest. Introduction of suitable new varieties of crops or crops that take advantage of the new environmental conditions should be done with at most care and field trials. This is one of the adaptive methods suggested as a response to climate change. Cultivating suitable pest-resistant plants is critically important for feeding human generations in the future.

**Keywords** Climate change · Host-plant interaction · Breakdown of resistance · Adaptive methods

## 1 Introduction

Climate change is a variation either in the mean state of the climate or variability in its components, persisting for an extended period. The region or area experiences unusual climatic events. It encompasses temperature increase, sea-level rise, changes in precipitation patterns, and increases in the frequency of extreme weather events. These changes have drastic impacts on the economy of countries like India that is dependent on agriculture, in a major way (see Sharma et al. 2010).

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Host plant resistance to insects is one of the most environment-friendly components of pest management. However, climate change may alter the interactions between the insect pests and their host plants. Global warming may also change the flowering or blooming times in plants, leading to ecological consequences such as the introduction of new insect pests and attainment of a pest status by non-pest insects. However, many plant species in tropical countries withstand the changes due to climate. Global warming may result in the breakdown of resistance to certain insect pests. Sorghum varieties exhibiting resistance to sorghum midge, *Stenodiplosis sorghicola* (Coq.), in India become susceptible to this pest under high humidity and moderate temperatures near the equator in Kenya. There will be increased impact of insect pests which benefit from reduced host defenses as a result of the stress caused by the lack of adaptation to sub-optimal climatic conditions. The chemical composition of some plant species changes in direct response to biotic and abiotic stresses. As a result, such plant tissues are less suitable for growth and survival of insect pests. However, problems with new insect pests occur if climatic change favors the introduction of insect susceptible cultivars or crops. The introduction of new crops and cultivars to take advantage of the new environmental conditions is one of the adaptive methods suggested as a possible response to climate change. A recently concluded study reported that phytophagous feeders specific R-gene resistance can be enhanced by increased atmospheric $CO_2$ concentrations (Johnson and Tobias 2018).

Example: High temperature modifies resistance performances of rice varieties to brown planthopper, *Nilaparvata lugens* (Stal) (Delphacidae: Homoptera).

To investigate the effect of temperature on the resistance characteristics of rice varieties with different resistance genes to brown planthopper (BPH), *Nilaparvata lugens* (Stal), the resistances of IR26 (Bph 1) and (Bph2) to the BPH population of Mandya, Karnataka, India, are evaluated by using the standard seedling screening techniques (SSST) developed by the International Rice Research Institute (IRRI), Philippines.

## 2 Procedure

**Insects** Adults of BPH can be collected in rice fields in Mandya, Karnataka, India and can be maintained on susceptible rice variety TN 1 at 26 ± 1 °C and photoperiods of 12 L: 12D.

## 3 Resistance of IR 26 and IR 36 to BPH Under Different Temperatures

- Germinating seeds are sown in a plastic tray (45 cm × 30 cm × 10 cm) filled with soil for 4 cm in depth.
- The tray will be divided equally into 9 rows, 15 germinating seeds of the testing rice variety are shown in 1 row, and 3 rows are arranged in an array of TN 1, IR 26, and IR 36, respectively.
- Three replicates are established for each rice variety in a tray.
- There will be a total of ten trays or reapplications for each rice variety in the experiment.
- Damage grades of rice seedling under different temperatures are evaluated by SSST.
- The tray (45 cm × 30 cm × 8. 5 cm) will be maintained at five different temperatures (22 ± 1 °C, 25 ± 1 °C, 28 ± 1 °C, 31 ± 1 °C, and 34 ± 1 °C), respectively, and two trays will be set up at each temperature.
- After TN 1 reaching grade 9, the survival seedling of IR 26 and IR 36 is recorded daily until more than 90% IR 26 and IR 36 seedlings died, and the durations for durable resistance of IR 26 and IR 36 will be computed.

**Illustrated Example** Bt sweet corn hybrids expressing Cry1ab genes in differential climatic divisions (Fig. 1).

The effects of climate change on *Bt* crop-pest interactions and insect resistance to Bt crops remain unexplored to date. The relationship of temperature anomaly and Bt adoption with field evolved resistance to Cry1Ab Bt sweet corn in *Helicoverpa zea* was analyzed. Increased Bt adoption during 1996–2016 decreased *H. Zea* populations, but increased temperature anomaly buffers population suppression. Temperature anomaly and its interaction with elevated selection pressure from high Bt acreage might have geared up the Bt resistance development. *H. Zea* damage to corn ears, kernel area consumed, mean instars, and proportion of late instars in Bt varieties increased with Bt adoption and temperature anomaly, through additive or interactive effects. Risk of Bt-resistant *H. zea* extending was high given Bt adoption and the expected increase in pest overwintering and migration. The study found that challenges posed by climate change for Bt biotechnology-based agricultural pest management and incorporating evolutionary processes influenced by climate change into Bt resistance management programs are urgently required (Venugopal and Dively 2017).

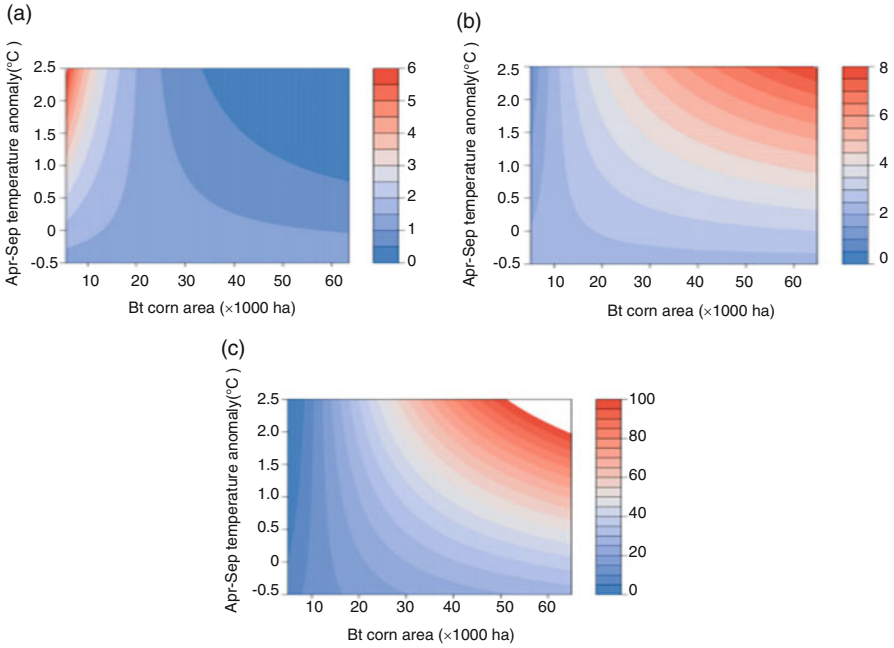


**Fig. 1** Interactive impacts of temperature anomaly and Bt corn acreage in agricultural districts of Maryland, USA, 1996–2016 on (**a**) *Helicoverpa zea* population abundance and indicators of field evolved resistance to Bt corn. Bt corn acreage was estimated as the product of total area planted corn for each year in each agricultural district and the national average percentage of Bt corn for that year. Temperature anomaly for each year during the study period was used as a predictor. Contour plots depict the predictions from linear mixed effects models (LMMS) with each dependent variable scaled on the right axis with lower values in blue and higher in red. (Source: Venugopal and Dively 2017)

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# Role of Plant Volatiles, Pest-Resistant Varieties and Transgenics in Tri-trophic Interactions


**Chacko Jobichen and V. Selvanarayanan**

**Abstract** The interaction between plants, parasites and parasitoids (tri-trophic interaction) is of great significance in developing newer pest-resistant crop varieties. One of the important defence mechanisms of a plant infested by an insect pest is to release volatiles that can attract parasitoids. These volatiles are broadly classified as herbivore-induced plant volatiles (HIPVs). HIPVs are also involved in communication between neighbouring plants and different parts of the same plant. The volatiles send clues to the other community members at different trophic levels that influence their interactions. Many parasitoids rely on these volatiles to detect the presence of their hosts. When pests attack plants, plants try to attract predators and parasitoids of the attacking herbivores with the help of the volatile chemicals that can provide various information like location, activity and developmental stage of the attacking herbivore. The release of pest-resistant varieties of various crops also influences the tri-trophic interactions which may result in changing the behaviour of pests/predators/parasitoids. This chapter elaborates the various plant volatiles and their role in the tri-trophic interaction. The introduction of various pest-resistant (transgenic) varieties and how they influence these tri-trophic interactions is also discussed.

**Keywords** Plant volatiles · Resistant varieties · Tri-trophic interaction · Transgenics

## 1 Introduction

A crop plant, when infested by an insect pest, will use all its chemical arsenal to ward off or wane the pest. Such herbivore-induced plant volatiles (HIPVs) can attract the parasitoids of the infesting pest. There are various types of volatiles released by

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plants from various parts including flowers, leaves, fruits and roots (Loreto and Schnitzler 2010). These released volatiles make the condition of the plant known to the other community members at different trophic levels (Dicke and Baldwin 2010) and also mediate the interactions between organisms at various trophic levels. Many parasitoids rely on these volatiles to detect the presence of their hosts. As part of the survival strategy, herbivore insects will remain inconspicuous, but they involuntarily give away their position by attacking the plants (Zhu et al. 2015).

When pests attack plants, plants try to attract predators and parasitoids of the attacking herbivores with the help of the volatile chemicals that can provide various clues such as location, activity and developmental stage of the attacking herbivore. Previous studies reported that tobacco, cotton and maize plants release distinct blends of HIPVs in response to damage by herbivores (De Moraes et al. 1998).

The release of various pest-resistant varieties also affects the type and quantity of volatiles released by plants. Resistant plant varieties could change the quality of the herbivores and also limit their availability to the parasitoids. These changes eventually impact the tri-trophic interactions which may result in changing the behaviour of pests/predators/parasitoids. When green leaf volatile mute plants are planted in their native habitats, certain flea beetles that used to colonize these plants are unable to recognize the presence of the plant and hence not colonized. In another instance, certain lepidopteran herbivores use green leaf volatiles as feeding stimulants. The larvae placed on the volatile mute plants were unable to recognize the presence of food, and they didn't start feeding the plant (Baldwin 2010).

Plant volatiles are mainly classified into four categories: terpenes, fatty acid derivatives, amino acid derivatives and phenylpropanoid/benzenoid compounds. Several species- or gene-specific volatiles are not included in this classification (Fig. 1).

Volatile terpenes are the most abundant ones, and they are generally present in many plant essential oils. These volatiles are responsible for odours associated with citrus, mints and conifers. Terpenes are classified based on the number of C5 units present in the molecule. Lower terpenes like monoterpenes (C10), sesquiterpenes (C15) and diterpenes (C20) are mainly present in nature as volatiles. Various functional groups attached to these terpenes help to increase their volatility (Singh and Ram 2014).

A large variety of volatile aliphatic and aromatic compounds are found in plants, containing alcohol, aldehyde, ketone, acid or ester functional groups. These compounds are usually present in lower concentrations in plants. But they are associated with the distinctive odours of fruits and flowers and hence have great aesthetic and commercial importance. The primary function of these volatiles is to attract pollinating organisms and seed dispersers. Most organisms are capable of associating the odours from these volatiles to a particular reward. Some plants like orchids are capable of sending deceptive volatiles that mimic other herbivores so that they can attract the pollinators. Some other volatiles are repulsive to humans because of their unpleasant odour, but they are highly attractive to certain animals and insects. Certain plant groups like the carrot families mimic the stench and appearance of rotting carcasses or dung to attract the insects. Some plants even mimic the smell of

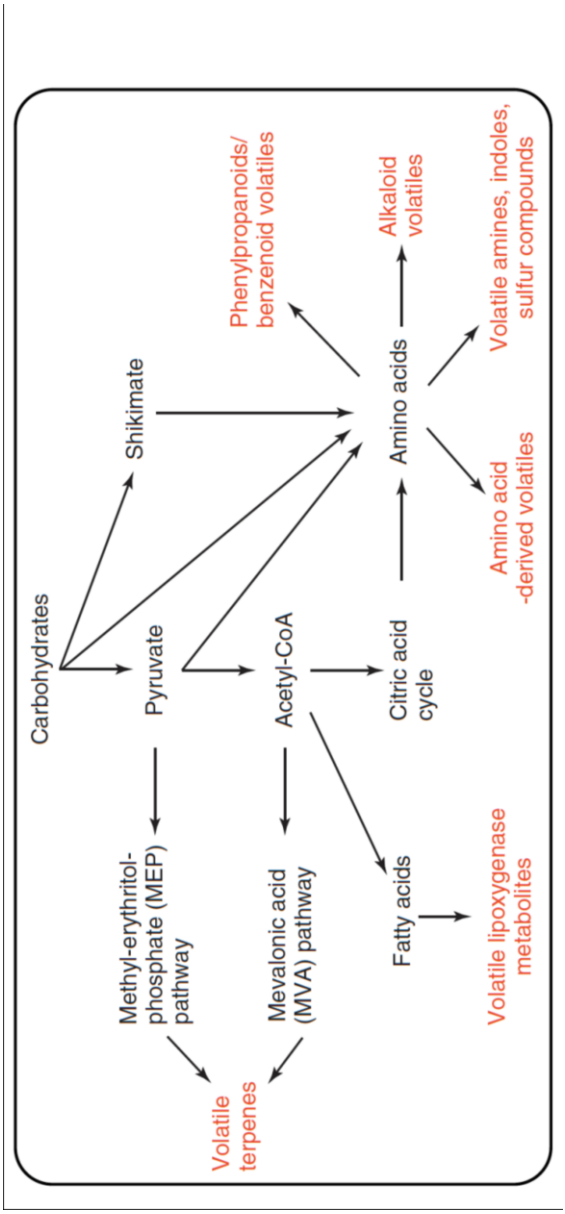


**Fig. 1** Generalized pathway for the synthesis of plant volatiles (volatile compound names are in red). (Adapted from Rosenkranz et al. 2016)

wet soil to attract insects seeking water. The ability to make these olfactory associations is sufficiently valuable that organisms tolerate instances when it is exploited. Compounds such as indole are offensive at high concentrations, but they provide a pleasing odour in the diluted form (Rosenkranz et al. 2016).

Apart from the above functions, plant volatiles serve several physiological and ecological functions in plants. Many of the volatile terpenes are nonspecific toxins active against a wide range of organisms including bacteria, fungi, plants and animals. Monoterpenes are known to inhibit the growth of competing plants, and they are also toxic to plant pathogens and insects. Similarly, many other volatile terpenes are localized to young tissues of the plants, and they act as feeding deterrents to herbivores.

The volatile compounds are also involved in physiological functions in plants. Isoprene is a volatile known for its thermal protective function in photosynthesis (Loreto and Schnitzler 2010). Isoprene is also implicated in quenching the reactive oxidative species in plant tissue (Loreto and Velikova 2001).

## 2 Techniques for Collecting and Evaluating Plant Volatiles

The headspace gas samples are mainly collected using two techniques, viz. dynamic and static methods. The difference between these techniques is the use of headspace gas circulation in the case of dynamic, while it is absent in the static technique. The volatiles are concentrated on a sorbent matrix in both techniques. A popularly used method for dynamic headspace sampling is the closed-loop stripping method. In this method, a plant or plant part is enclosed in a sealed system, and the air trapped within is continuously circulated through the sorbent matrix resulting in a highly concentrated volatile sample. This concentrate is used for low-volume solvent desorption and analysis by gas chromatography. An example of static headspace sampling is the solid-phase microextraction (SPME) that utilizes adsorbent-coated fibres to trap volatiles, which can then be thermally desorbed inside the injector port of a gas chromatograph. Another method is the use of ‘electronic noses’, which are chemical sensors combined with neural network computing (Bartlett et al. 1997).

Proton-transfer-reaction mass spectrometry (PTR-MS) is an online measurement technique used to detect the plant volatiles. It provides a sensitive, noninvasive and rapid detection of volatiles in the PPTV/PPBV (parts per trillion by volume/parts per billion by volume) range (Hansel et al. 1995; Tholl et al. 2006). This system can be connected to a dynamic, controlled cuvette system, to screen volatile compounds in real time. A high-throughput system having several dynamic cuvettes connected to trace gas analysers can be used to define plant phenotypes according to their disease resistance and stress tolerance in the future (Niederbacher et al. 2015).

## 3 Role of Plant Volatiles in Tri-trophic Interactions

Volatile compounds play a major role in plant communication and indirect defence. The infested plants attract the enemies of the herbivores via this tri-trophic interaction. This was first reported in the spider mite-infested Lima beans calling carnivorous mites for help (Dicke and Sabelis 1988). Similar phenomenon was also reported in several other plants, predator and parasitoids. Volatiles are also involved in plant–plant communication by which a plant under herbivore attack can alert its neighbouring plants, so that they can prepare for the pest attack (Heil 2014). However, the mechanism by which the plants receive the volatile signals is still poorly understood.

A recent study has shown the importance of silicon (Si) in plant defences that operate in a direct manner against herbivores. Liu et al. (2017) reported that in rice (*Oryza sativa*), this is mediated by the jasmonate signalling pathway. In this study, they tested the effect of Si supplementation versus Si deprivation to rice plants on HIPV production following feeding by the important pest, rice leaf folder (*Cnaphalocrocis medinalis*). The results indicated that the effects of Si on HIPVs are modulated by the jasmonate pathway. Further, this work also demonstrated that silicon alters the HIPV blend of herbivore-infested rice plants. They suggest that Si treatment to crops can enhance induced, indirect defences and associated biological control of pests because of the strong attraction of parasitoids by the HIPVs produced by +Si plants (Liu et al. 2017).

## 4 The Specificity of HIPVs in Various Plant Species

Host-induced plant volatiles' main function is to attract the carnivorous natural enemies of the herbivores that are currently infesting the plant (Turlings et al. 1990, 1995; Arimura et al. 2009; De Rijk et al. 2013). A study on olfactory responses of the parasitoid *Cardiochiles nigriceps* to three species of host plants (tobacco, maize and cotton) attacked by two closely related herbivore species, the tobacco budworm (*Heliothis virescens*: host) and the corn earworm (*Helicoverpa zea*: nonhost) was reported by De Moraes et al. (1998). They found that tobacco, cotton and maize plants each released distinct blends of HIPVs in response to damage by these herbivores. The parasitoid showed the ability to distinguish differences in HIPV blends so that it can identify the infestation by its host, *H. virescens*, from that by the nonhost *H. zea*. Similarly, a braconid parasitoid (*Aphidius ervi*) shows specificity to its host pea aphid (*Acyrthosiphon pisum*) when compared to a nonhost (the black bean aphid, *Aphis fabae*) that attacks the broad bean plants (Du et al. 1998; Guerrieri et al. 1999). Another study has shown that *Cotesia vestalis* that attacks diamondback moth (DBM) (*Plutella xylostella*) larvae showed high preference for DBM larvae-infested cabbage plant volatiles over either infested, artificially damaged or nonhost (cabbage white butterfly (CWB) larvae (*Pieris rapae*))-infested

cabbage plant volatiles. In another case, there are reports of predatory arthropods and parasitic wasps responding to volatiles released by plants attacked with non-prey or non-host herbivores (Takabayashi et al. 2005).

## 5 Introduction of Pest-Resistant Varieties (Transgenic Crops) and Their Influence in the Tri-trophic Interactions

Genetic manipulation of host plants is done by incorporation of toxin genes (Brar and Khush 1993). One notable example is the toxin genes, from *B. thuringiensis*, introduced in the crop plants to combat lepidopterous pests (Tabashnik 1994). By introducing the toxin genes, there is a risk of insects developing resistance to toxins in such transgenic crops (Tabashnik et al. 2013). The strategy mainly proposed for reducing the resistance risks is to provide refuges with conventional crops (Jin et al. 2015) which might slow down the resistance development in insects. So far, there is no evidence of direct adverse effects on natural enemies. While there are incidence of secondary pests outbreaks in these transgenic plants which needed to be controlled by traditional insecticide sprays. There was a concern that these gene transfer techniques might be damaging to natural enemies (Verkerk et al. 1998). But recent studies have reported that natural enemies were not affected when fed by pests reared on Bt crops, and these Bt plants are safe to be incorporated into integrated pest management system (Tian et al. 2015).

Genetic engineering is also used to provide the plants with the capacity to emit the target volatiles. For example, genetically modified tobacco plants produce higher amounts of terpenoids (diterpene cembratriene-ol) that modulate the insect behaviour (War et al. 2011; Lücker et al. 2004). Similarly, *Arabidopsis* engineered with a gene for overexpression of strawberry nerolidol synthase produces (3S)-(E)-nerolidol that attracts a predatory mite (Kappers et al. 2005). Transgenic maize plants with an engineered gene TPS10 produce various sesquiterpene hydrocarbons that attract the parasitic wasp (Schnee et al. 2006). Efforts to genetically engineer plants that can produce terpenoids will be more prominent in the future as a technique for reducing the pest infestation. Similar studies were reported in the case of soybean varieties that can resist the soybean aphid (Hagenbucher et al. 2014). Host plant resistance to soybean aphid also influences its interaction with its natural enemies (Jeremy et al. 2012). This study reported that higher densities of the aphid parasitoid *Binodoxys communis* (Hymenoptera: Braconidae) were reported in susceptible soybean plots in comparison with resistant plots.

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# Economic and Ecological Values of Resistant Plants


**A. K. Chakravarthy, E. V. Jose Luis, S. Onkara Naik, and B. Rajkumar**

**Abstract** As a sole method, resistant cultivars help keep the population of key pests under check. Resistant cultivars under field conditions can obviate the need for application of insecticides. Resistant cultivars can well combine with natural enemies, cultural practices, mechanical and physical devices/barriers, microbial, insecticides, and molecular and biotechnological tools. Proven examples are provided in this chapter that successfully combines resistant varieties with other methods in pest management, conferring ecological and economic values.

**Keywords** Resistant variety · Integration · Economic benefits · Ecological value

## 1 Introduction

Herbivores feed on plants. Continuous feeding forms a selective pressure on plants affecting their growth and development. Plants through evolutionary period have developed ways to defend against herbivores and pathogens. The first significant economic contribution of host plant resistance to agriculture was made in 1890 when European vines were successfully grafted on resistant rootstock to save the French wine industry from grape phylloxera, *Viteus vitifoliae* (Fitch). With this development in the host plant resistance, there was a resurgence in interest in scientists to work on the development of resistance plant varieties in about 15 crops against 50 most important key insect pests. In the past two decades, more than 500 insect-resistant

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varieties/cultivars of rice, maize, cotton, sorghum, alfalfa, and wheat have been developed and released worldwide (Dhaliwal and Arora 2001). The insect-resistant varieties are cultivated in millions of hectares annually helping farmers to save billions of dollars in insecticide cost (Dhaliwal and Arora 2001).

## 2 Resistant Varieties: As a Lone Method

Under natural conditions, least susceptible plants only survive. In evolutionary period, these plants accumulate products that serve to protect them from herbivores and pathogens. For instance, starch reserves in roots of primitive cassava genotypes give protection against phytophages. This is because of cyanogenic glucosides. In Africa, bitter cassavas are cultivated in regions where wild pigs, baboons, and porcupines were abundant. These genotypes are also resistant to grasshoppers (Baker 1972). Improved cassavas are now cultivated in Africa, but the genotypes are susceptible to insect pests. The scientists from International Rice Research Institute (IRRI), Manila, Philippines, have done a spectacular work in developing the improved rice varieties that provide resistance to as many as four insect pests and five diseases. For instance, IR 36 is resistant to brown plant hopper, green leafhopper, stem borers, gall midge, blast, bacterial blight, and tungro virus disease. The yield of IR 8 fluctuates widely due to pests and diseases as the yield of IR 36 is alone planted in about 11 million hectares in the world and yields an additional income of one million dollars annually to rice growers and processors (Khush and Brar 1991). Rice varieties resistant to brown plant hoppers and green leafhoppers are grown over 20 million hectares in Asia. These resistant varieties can be grown with the minimum use of insecticides and are an important component of integrated pest management programmes (Panda and Khush 1995).

The red scale, *Aonidiella aurantii* (Maskell), is the most important pest of citrus and is widely distributed across the world. Plants with more oil glands have shown fairly high degree of resistance to the red scale (Habib et al. 1972). Sandhu et al. (1979) reported that red flesh, pink flesh (Gulabi), and strawberry showed resistance/tolerance to the fruit fly, *Bactrocera dorsalis* (Hendel). They attributed this to the rough, gritty, and wrinkled skin of the guava fruit. Probably, such a guava skin is deterrent to oviposition by the fruit fly. The smooth skin of Allahabad Safeda and Lucknow-49 was susceptible.

*Aonidiella aurantii*

*Bactrocera dorsalis*

*Dryocosmus kuriphilus*

Various strategies can be adopted to prolong the useful life of the resistant varieties or to develop varieties with different genes so that farmers may have access to new varieties when the resistance of the current varieties breaks down (Panda and Khush 1995). Several sorghum hybrids bred in South Africa exhibited the greatest tolerance to stem borer's damage and suffered low yield loss (Kfir et al. 2002). However, there is a danger of insect developing new biotypes if highly insect-resistant varieties are solely used, as it happened in wasp, *Dryocosmus kuriphilus* Yasumatsu in chestnut in Japan (Shimura 1972). Highly insect-resistant varieties may prove susceptible to other pests. An example is PLS-24 and PLS-16-1 *Lablab niger* (field beans) genotypes, highly resistant to lepidopterous and coleopterous borers but highly susceptible to the bean aphid, *Aphis craccivora* Koch (Chakravarthy and Lingappa 1988).

## 3 Resistant Varieties and Biological Control

By reducing pest numbers, resistant varieties help to shift the pest: predator ratio in favor of biological control (Heinrich 1994). Varietal resistance is usually compatible with biological control but may have an adverse effect on the natural enemies too. In field studies at International Rice Research Institute, Philippines, the brown plant hopper, *Nilaparvata lugens* (Stal), and spider, *Lycosa pseudoannulata* (Boesenberg and Strand), ratio increased with the level of susceptibility from ASD 7 and IR 36, both highly resistant rice cultivars to IR 42 and Triveni and moderately resistant cultivars to IR 8 and TN 1 susceptible cultivars.

*Nilaparvaata lugens*

*Lycosa pseudoannulata*

*Cyrtorhinus lividipennis*

The host plant resistance may also enhance the predatory activity. Predation rate of the mirid bugs, *Cyrtorhinus lividipennis* Reutger, when feeding on the first instar brown plant hopper nymphs increased on the resistant cultivars, IR 36, as compared to the susceptible IR 8. Combinations of host plant resistance and predation by the mirid bugs *Cyrtorhinus lividipennis* have a cumulative effect on the population increase of the green leafhopper, *Nephotettix virescens* (Distant). In case studies, the number of green leafhoppers reached only 6 on IR 29 (resistant) with the predator and 31 without the predator, while there were 91 and 200 hoppers, respectively, on susceptible IR 22.

The compatible nature of plant resistance and biological control has been demonstrated in between resistant varieties of barley and sorghum and parasitization of

the greenbug, *Schizaphis graminum* (Rondani). The movement of greenbug on the resistant sorghum cultivars exposes them to greater parasitization. The parasitoid, *Lysiphlebus testaceipes* (Cresson), was able to keep the biotype C greenbug population nearly static on both susceptible and resistant barley, when the initial population of the aphid was three per plant. But when 12 aphids and 1 female parasite were introduced per plant, the parasitoid could suppress the aphid population only on the resistant barley. Thus damage to barley was reduced by the combined effect of varietal resistance and parasitoids. There is also synergistic interaction between maize cultivars resistant to the leaf-feeding fall armyworm, *Spodoptera frugiperda* (Smith), and nuclear polyhedrosis virus (NPV) disease of fall armyworm.

Some of the soybean (*Glycine max* Linn.) varieties have shown high level of insect resistance and adequate agronomic characteristics under heavy stink bug (mainly *Nezara viridula*, *Piezodorus guildinii*, and *Euschistus heros*) attack in Brazil. Release of these varieties with release of bioagents is expected to result in an enormous improvement in the integrated pest management (IPM) programme, since stink bugs are still, almost exclusively, controlled by insecticides (Moscardi and Gomez Sosa 1996).

*Nezera virudula*

*Peizodorus quildinii*

*Euschistus heros*

Dogramaci et al. (2005) studied tritrophic interactions among the parasitoid *Lysiphlebus testaceipes*; sorghum greenbug, *Schizaphis graminum*; and the greenbug-resistant sorghum hybrids in the Northern USA. *Lysiphlebus testaceipes* was effective in controlling greenbug on sorghum via antibiosis mechanism (Cargill 7607E) to greenbugs. The parasitoid was also effective in controlling greenbugs on a greenbug-tolerant hybrid (Cargill 797) but not on susceptible sorghums.

## 4 Resistant Varieties and Physical Methods

Resistant varieties play a crucial role in crops of low economic value where in the yield fluctuates due to the biotic and abiotic factors. The use of resistant varieties is of great significance for countries like India where the land holdings are small and the farmers are not well equipped to adopt costly methods of pest suppression. Physical method of pest control is one of the oldest methods that offer several ways of managing the pests. For instance, *Lablab niger* Medick creeper type is found to be

tolerant to pod borers compared to field type *Lablab niger*. Collection and destruction of larvae would be easier on resistant/tolerant cultivars than susceptible ones (Chakravarthy 1978). Bangalore Blue and Bhokri cultivars of grape were found resistant to defoliator, *Adoretus drevanceli* (Blanch), than the Bangalore Purple, Khalili and Anab-e-Shahi, and hence the resistant cultivars offer easy picking and destruction of larvae than the susceptible cultivars (Chakrabarthy et al. 1970). Under cold storage conditions, the resistant potato cultivars keep the potato tuber moth population below the damage level than the susceptible potato cultivars. The use of light traps is the most effective tool in reducing the root grub infestation on the resistant/tolerant crop plants like groundnut, coconut, and areca nut than root grubs on the susceptible varieties. There are many physical methods available used in combination with host plant resistance as a component of integrated pest management (IPM).

## 5 Resistant Varieties and Cultural Practices

The use of resistant varieties in combination with short-duration cotton varieties has given better control of cotton boll weevil (*Anthonomus grandis* Boheman) and pink bollworm (*Pectinophora gossypiella* Saunders). The damage by the cereal leaf beetle *Oulema melanopus* (Linn.) in mixed and pure stands of resistant and susceptible wheat varieties has been evaluated. The control of cereal leaf beetle *Oulema melanopus* by using natural enemies was more effective in mixed cropping of beetle-resistant and beetle-susceptible wheat varieties than the pure stand of either one of these varieties. Most of the cultural practices are compatible with other pest management practices and have long been associated with subsistence farming as an appropriate approach to low-input cropping system. Nachiappan and Bhaskaran (1983) reported that the mango cultivars Baneshan, Chinnarasam, and Khader were resistant to mango leafhoppers, *Idioscopus* spp. and *Amritodus atkinsoni* (Leth). This has attributed due to the presence of less nitrogen but high level of phosphorus, potassium, calcium, and phenols. These results were confirmed by Khaire et al. (1987). This indicates that soil nutrition regulation can be used in managing mango leafhoppers.

*Dulema melanopus*

*Idioscopus* spp

*Amritodus atkinsoni*

## 6 Resistant Varieties and Insecticides

The efficacy of insecticides can be increased by host plant resistance. Higher mortality of plant hoppers and leafhoppers feeding on resistant rice varieties was recorded than the susceptible varieties when used in combination with different insecticides (Heinrich 1994). The mortality of brown plant hopper was higher when reared on moderately resistant ASD 7 or highly resistant cultivar Sinna Sirappu than when feeding on susceptible TN 1 cultivar. Effective control of hoppers was recorded when low dose of insecticide was applied on moderately resistant varieties. The vector of rice tungro virus, *Nephotettix virescens* (Distant), was effectively managed by the use of insecticides in combination with resistant host plant. There was no tungro infection on the resistant cultivar IR28 even without the use of insecticides.


*Nephotettix virescens*

*Sitobion avenae*

For the management of insect pests, feeding on resistant varieties requires lower concentration of insecticides than feeding on the susceptible varieties. Nymphs of the wheat grain aphid *Sitobion avenae* (Fab.) reared on resistant wheat variety. The nymphs reared on resistant variety possess antibiotic compound DIMBOA were significantly more susceptible to the insecticide deltamethrin than the nymphs reared on the susceptible wheat variety dollarbird. When the nymphs reared on the cultivar with high DIMBOA content, the LD50 of the nymphs was reduced to 91%. The aphid population was 85% on the partially resistant varieties of Brussels sprouts of that on the susceptible variety. The insecticide (malathion, LD50 55%) required to manage the pest was much lower dose on partially resistant variety than on the susceptible variety (Panda and Khush 1995).

## 7 Resistant Varieties and Vectors

Insect vectors can be potentially risky as they can spread disease-causing organisms. The banana variety AAB was to be resistant to bunchy top disease. The possible reason could be the banana variety is resistant to banana aphid *Pentalonia nigronervosa* Coquerel which is a vector of bunchy top virus. Certain species of citrus like *Citrus nobilis*, *Citrus aurantifolia*, and *Citrus rashmi* had significantly low population of aphid (*Toxoptera* spp.) from among the 22 species

of three hybrids (Anonymous 1984). Birch et al. (1992) showed the use of RFLP analysis, for distinguishing biotypes of the virus vector aphid *Amphorophora idaei* that had aided in raspberry screening. Interrupting vector-borne disease transmission by replacing insect vectors populations with nonvector forms is an old idea that may become practicable with the use of molecular techniques and genetic engineering which could supply a population of selectively modified vectors. Because natural refractoriness may prove genetically unwieldy, biotechnology may provide novel ways to disrupt transmission by adding or removing specific insect functions that block or delay host/pathogen interactions. Molecular techniques are making possible to enhance classic genetic control strategies and transformation of insect symbionts and disrupt virus-host interactions (Evans 1996).

## 8 Resistant Varieties and Biotechnological Tools

Biotechnological tools play an important role in developing the resistant crop varieties that are not easily achieved by the conventional means, as perhaps with virus resistance in some crops and insect resistance with particular pest like cereal stem borers. Current work with transgenic plants, particularly the incorporation of *Bacillus thuringiensis* delta endotoxin into the crops for the management of insect pests, appears proceeding on a vertical resistance model, based on complete resistance conferred by one or few genes. The varieties like those produced through conventional vertical resistance breeding will be susceptible to the development of pests' resistance. Further, they may undervalue the benefits of integrated pest management approach.

Many will now agree that the risk of resistance development to *Bacillus thuringiensis*-engineered transgenic plants is considerable and probably greater than that of a *Bacillus thuringiensis* formulation topically applied to a crop on a need-based integrated pest management basis, simply because of its more continuous and extensive expression. Indeed, some entomologists have come together to express their concern that use of transgenic plants may waste a valuable resource by encouraging resistance development (Anonymous 1992).

Field trials in the USA have revealed that the maize plants with Cry I genes have given the control of the European corn borer, *Ostrinia nubilalis*. European corn borer *Ostrinia nubilalis* is the most important pest of maize in the USA and is difficult to manage by chemical means due to its inaccessibility to chemical insecticides as it spends most of its life inside the maize stalk. The plant expression of *Bacillus thuringiensis* protein is a method that can overcome the technical barriers to insect control with other agents and thus addresses an unmet need in pest management (Fischoff 1996).

Fischoff (1996) has given the plan for the implementation of insect-resistant transgenic cotton plants in the USA. But many of the same idea apply directly to the use of *Bacillus thuringiensis* cotton in other parts of the world because many of the same or closely related pests occur on cotton in all areas. Also, in the areas where

other Lepidopteran pests are seen, their natural history is often similar to that of some US pests. For instance, *Helicoverpa armigera* Hubner infests many economically important crops like cotton, pigeon pea, chickpea, sunflower, corn, chillies, tomato, and okra. Two-year study in South and Central India indicated that a number of host crops of *H. Armigera* support large populations at the same time that cotton is infested. Thus these crops may act as important sources of refuge for *Bt* cotton planting in South and Central India (Ravi et al. 2005). It is important to recognize that the plans for implementation of insect-resistant varieties will be crop and insect specific, and each example will have to be tailored to the individual cropping system.

The use of insect-resistant cotton will provide benefits to the cotton growers and to the society and environment. The resistant cotton varieties control the Lepidopteran pests, and the crop damage is reduced that they cause with an efficacy equal to or better than that of the current control methods' result in significant reduction in insecticide usage in turn reduction in cost of cultivation. This will benefit the cotton growers. Insect-resistant cotton varieties also give some secondary benefits in pest control as an indirect result of the reduction in the use of insecticides. The use of insect-resistant cotton varieties may not be suitable to the farming system in the tropics and subtropics.

Resistance problems involve more complex strategies of gene deployment, for instance, mixed or intercropped populations of resistant and susceptible plants or genetic methods to resist expression of genes to certain parts of plants or certain times. The present strategy of protecting plants with engineered, single-gene defenses may offer some real opportunities for improving pest management in the short term, particularly on crops where conventional breeding cannot achieve resistance (Waage 1996).

Yencho et al. (2000) reviewed how molecular markers can be used to increase our understanding of the mechanisms of plant resistance to insects and develop insect-resistant crops. Molecular markers can be applied to develop more durable insect-resistant crops, for instance, in potato/maize plant pest systems. Identification and characterization of rice gall midge R genes have played and continue to play an important role in the control of gall midge pests of agriculture (Harris et al. 2003).

**Ecological Results of Agriculture** In contrast to natural ecosystems, most agricultural crop production systems are ecologically unstable, non-sustainable, and energy dependent. Man has through plant domestication and cultivation practices interfered in many ways with species diversity and natural defense mechanisms of plants. Cultivated crops originated from genetically diverse plant types. However, crop plants are now grown in large, genetically homogeneous stands, a practice that decreases genetic and species diversity and increases the likelihood of economically significant insect pest infestations. Defense mechanisms of plants are re-created in resistant plants. Plant defense mechanisms include escape in space and time, incompatible biological associations, physically and chemically derived barriers, and accommodation by replacement or repair of damaged plant parts. By re-creating plant defenses, genetic resistance to insect pests plays, in an environmentally compatible manner, a vital role in the attempt to enhance ecological stability in agricultural crops.

**Effect of Insect Pest-Plant Host Relationship** Insect-resistant crop varieties suppress insect pest abundance or elevate the damage tolerance level of the plants. In other words, insect-resistant plants alter the relationship an insect pest has with its plant host. How the relationship between the insect and plant is affected depends on the kind of resistance, e.g., antibiosis, antixenosis (non-preference), or tolerance.

*Antibiosis* resistance affects the biology of the insect. So pest abundance and subsequent damage are reduced compared to that which would have occurred if the insect was on a susceptible crop variety. Antibiosis resistance often results in increased mortality or reduced longevity and reproduction of the insect.

*Antixenosis* resistance affects the behavior of an insect pest and usually is expressed as non-preference of the insect for a resistant plant compared with a susceptible plant.

*Tolerance* is resistance in which a plant is able to withstand or recover from damage caused by insect pest abundance equal to that damaging a plant without resistance characters (susceptible). Tolerance is a plant response to an insect pest. Thus, tolerance resistance differs from antibiosis and antixenosis resistance in how it affects the insect-plant relationship. Antibiosis and antixenosis resistance cause an insect response when the insect attempts to use the resistant plant for food, oviposition, or shelter.

The use of insect-resistant crop varieties is economically, ecologically, and environmentally advantageous. Economic benefits occur because crop yields are saved from loss to insect pests and money is saved by not applying insecticides that would have been applied to susceptible varieties. In most cases, seed of insect-resistant cultivars costs no more, or little more, than for susceptible cultivars. Ecological and environmental benefits arise from increases in species diversity in the agroecosystem, in part because of reduced use of insecticides. Increases in species diversity increase ecosystem stability which promotes a more sustainable system far less polluted and detrimental to natural resources.

The IPM concept stresses the need to use multiple tactics to maintain insect pest abundance and damage below levels of economic significance. Thus, a major advantage to the use of insect-resistant crop varieties as a component of IPM arises from the ecological compatibility and compatibility with other direct control tactics. Insect-resistant cultivars synergize the effects of natural, biological, and cultural insect pest-suppression tactics. The "built-in" protection of resistant plants from insect pests functions at a very basic level, disrupting the normal association of the insect pest with its host plant. The compatible, complementary role plant resistance to insect pests plays with other direct control tactics is, in theory and practice, in concert with the objectives of IPM. All crop cultivars should contain resistance to insect pests.

Plant resistance to insect pests has advantages over other direct control tactics. For example, plant resistance to insects is compatible with insecticide use, while biological control is not. Plant resistance to insects is not density dependent, whereas biological control is. Plant resistance is specific, only affecting the target pest. Often effects of the use of insect-resistant cultivars are cumulative over time. Usually the effectiveness of resistant cultivars is long-lasting.

The role of plant resistance to insects in IPM has been well defined, at least in theory. However, the specific role a resistant cultivar plays in a particular IPM

situation is crucial to successful deployment of the resistant cultivar. The impact of the resistant cultivar on standard cultural, biological, and insecticidal control methods should be well defined. Likewise, the impact of each of these control tactics on the resistant cultivar also must be defined.

Several definitions have been used to convey the relative level of resistance in a plant. However, the problem of quantifying resistance continues to be a problem influencing farmer acceptance of insect-resistant cultivars. A better way to define resistance levels in agronomically improved resistant cultivars is through quantified comparisons of insect pest damage or plant yield loss of susceptible cultivars. Once insect pest abundance or damage to yield-loss relationships has been determined, economic threshold levels can be determined and combined with factors such as crop value and insect pest control costs to develop dynamic thresholds for use by producers. Dynamic thresholds provide a description of resistance and can reduce crop loss risk because limitations are known and remedial action can be taken when necessary. By using this system to define relative differences in insect pest resistance between cultivars, it may be possible to simply indicate that a resistant cultivar has a higher economic threshold level than a traditional susceptible cultivar.

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# Part V
# Genetics, Plant Breeding and Molecular Tools in Host Plant Resistance Studies

# Molecular Marker-Assisted Selection of Plant Genes for Insect Resistance


**Vasudev Kammar and K. S. Nitin**

**Abstract** Locating and identifying genes responsible for resistance are crucial for breeding insect-resistant varieties. The molecular marker-assisted selection of plants based on plant genetics is desirable in crop improvement breeding programmes. Quantitative trait loci, marker analysis, phenotyping, and development of SCAR markers is dealt with in this chapter. The example of rice yellow stem borer is illustrated.

**Keywords** Marker-assisted selection · QTL mapping · SCAR markers · RAPD markers

## 1 Introduction

The tagging and mapping of plant genes for insect resistance has accelerated tremendously since the mid-1990s. This progress has been facilitated by construction of high-density genetic maps of certain plants and insects. Workers have utilized molecular markers in crop plants linked to genes expressing resistance to several major insect pests. In the pre-molecular age of plant resistance to insects, phenotypic evaluation determined the initial identity of a source of resistance or progeny from crosses made between resistant and susceptible parents. The marker-assisted selection (MAS) of plants based on genotype is now being used in several plant improvement programmes for hybridizations.

Demonstrating that a molecular marker is linked to a plant resistance gene, however, involves identifying a phenotypic source of resistance and isolating DNA from resistant and susceptible plants. Molecular markers are detected from

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known chromosome locations to identify DNA in a polymorphic pattern via gel electrophoresis. Genotypically, individual plants can be screened from segregating populations for linkage to putative molecular markers. Molecular markers that have been identified are linked to single major gene for resistance. They are also linked to groups of loci controlling expression of quantitative resistance known as quantitative trait loci (QTL) (Heinrichs 1994; Huang et al. 2001).

## 2 Mapping QTL and Candidate Gene Loci

Numerous direct applications of molecular marker technology to facilitate plant-breeding programmes have been recommended. Because of the large numbers of DNA markers available in populations of interest, one of the most important applications of DNA markers is to establish gene-marker linkage for the traits having qualitative inheritance. The gene-marker linkage helps to go for indirect selection based on marker inheritance for the traits that are difficult or expensive to evaluate. In the same manner, one can locate the genetic loci (otherwise called as quantitative trait loci or QTL) associated with traits having complex mode of inheritance if a genetic map of molecular markers is made available. To these maps, the already established genetic loci (otherwise called as candidate gene loci or CGL) can also be integrated. Mapping of QTL and CGL will help for indirect selection and to understand the dynamics behind the inheritance of complex traits. Several studies have been carried out to map the QTL of complex traits such as yield and yield components, drought tolerance and resistance to insect pests and pathogens (Yencho et al. 2000). Several morphological and biochemical factors contribute towards the plant resistance to insects and pathogens. The genetic loci controlling these factors can be easily located on a genetic map if the trait is measurable. Locating the genetic loci will help:

(1) To establish relationship with already identified major genes/candidate genes
(2) To associate the QTL with genes controlling the metabolic pathway of resistance factors
(3) To identify genetic loci associated with resistance to common group of insects/pathogens
(4) To identify the differential association of genetic loci with a particular resistance factor during a developmental stage of host/parasite

## 3 Plant Resistance to Insects and QTL Mapping

The mechanisms responsible for the plant resistance to insects vary, and the nature of phenotype to be measured varies among crops and insect pests. The nature and magnitude of each one are understood based on the possibility of measuring a phenotype associated with the mechanism. The first known case of QTL mapping for plant resistance to insects was in tomato, *Lycopersicon esculentum* Mill.

Among the wild species of tomato, *L. hirsutum f. glabratum* confers resistance to arthropod pests because of the presence of a principal toxic factor, viz. 2-tridecanone (2-TD), a methyl ketone compound localized in the tips of trichomes. Involving this wild species with cultivated tomato, a mapping population of 74 $F_2$ individuals were evaluated for the amount of 2-TD, calorimetrically. The degree of association between individual marker loci and absorbance values for 2-TD of 74 individuals was established based on the magnitude of correlation between marker loci and 2-TD values. The marker loci on three different linkage groups were found associated with expression levels of 2-TD. In an insect genome, there are more than one chromosome locations, from where genes can be extracted.

## 4 Case Study

Among the insect pests of rice, stem borer is considered serious, damaging the crop from seedling stage to maturity. Attempts were made to study the genetics of yellow stem borer (YSB) resistance. The study revealed the polygenic nature of the trait. The complex nature of the trait and the inherent difficulties in screening have consequently made breeding for YSB resistance a difficult task, and no adequate results have been obtained so far. An alternative to the labour-intensive and time-consuming screening procedure would be to screen the population of interest using genetic markers, which can more precisely and rapidly screen the crop germplasms against insect pests.

Marker-assisted selection is especially helpful when the characters studied are polygenic, a situation particularly common for resistance traits. In case of yellow stem borer (YSB) resistance, the detection of major quantitative trait loci could be of considerable value for insect resistance breeding programmes since their incorporation in susceptible genotypes permits a direct increase of the resistance level in the improved genotypes. Identification of markers associated with YSB resistance facilitates selection in applied breeding given the inherent difficulties in field-based screening for this pest. In this study, an attempt to identify molecular markers linked to major locus conferring YSB resistance in rice using RAPD is elaborated. Rice plants at seedling stage can be subjected for the test.

## 5 Plant Material and Phenotyping

Marker analysis was performed on an $F_2$ population obtained from a cross between two rice varieties W1263 (resistant) and CO43 (susceptible). This $F_2$ population consisted of 90 individuals that segregated for YSB resistance. Phenotyping was carried out using the methodology developed by Heinrichs (1980) with slight modifications. Young seedlings (15 days old) were transplanted in individual earthen pots and caged separately in a screen house. At 20 days after transplanting,

**Table 1** Reaction to YSB in $F_1$ and $F_2$

| Grade | F1 | F2 | |
|---|---|---|---|
| | | Deadhearts | White-ears |
| 0 | – | 19 | 3 |
| 1 | 1 | 8 | 4 |
| 3 | 15 | 29 | 23 |
| 5 | 21 | 19 | 15 |
| 7 | – | 21 | 19 |
| 9 | – | 93 | 28 |
| Total | 37 | 189 | 92 |

5 female moths were allowed to oviposit on each test plant. After 4 days, 5 egg masses per hill were retained. Damage by stem borer was scored at vegetative stage as deadhearts and at reproductive stage as white-ears and rated on Heinrichs scale as follows: 0 = none, 1 = resistant, 3–5 = moderately resistant, 7 = susceptible and 9 = highly susceptible.

Phenotypic studies both at deadheart and white-ear stages revealed that the $F_1$s were intermediate to YSB reaction in $F_2$ generation. There was unimodal distribution for the YSB reaction (Table 1) which did not fit to any Mendelian ratio and continuous variation indicating the polygenic inheritance involving few genes with major effects.

## 6 Marker Analysis

For marker analysis DNA can be extracted from young leaves as described by Gawel and Jarret (1991). Marker analysis can be done in conjunction with bulked segregant analysis. Ten $F_2$ individuals showing extreme reactions for stem borer resistance and susceptibility were used to constitute the bulks. The analysis was performed with DNA of the parents, resistant and susceptible bulks and individuals comprising the bulks and the $F_2$ mapping population.

## 7 RAPD Analysis

PCR amplification using random primers (OPERON Inc., USA) was performed. The products were analysed either on 6% polyacrylamide-urea gel or on 1.5% agarose gel. The gels were documented using AlphaImager™ 1200 (Alpha Infotech, USA), and the data were scored and subjected to statistical analysis. The markers were analysed with 90 individuals, and $\chi^2$ analysis of the $F_2$ population revealed that all the five markers had high $\chi^2$ values ranging from 43.00 to 51.90. Linkage analysis

with the $F_2$ phenotypic scores and RAPD data with MAPMAKER programme revealed that the RAPD markers $K6_{695}$, $C1_{320}$ and $AH5_{660}$ were at a distance of 12.8 cM, 15.2 cM and 14.9 cM, respectively, from the gene(s).

## 8 Development of SCAR Marker

For the use of RAPD markers in marker-aided selection, they need to be converted into more reliable and robust genetic markers. These can be developed by conversion of polymorphisms identified by RAPD into sequence-characterized amplified regions (SCARs). The identified RAPD markers $OPK6_{695}$ and $OPAH5_{660}$ were cloned into plasmid vectors and sequenced. Based on the end sequences of the marker fragments, longer primers were synthesized, and SCAR markers were generated. The markers $OPK6_{695}$ and $OPAH5_{660}$ (Fig. 1) were used to screen $F_2$ individuals and a set of germplasm, and it was seen that $OPK6_{695}$ was amplified in all the resistant cultivars, whereas the marker $OPAH_{5660}$ was amplified in all the susceptible cultivars confirming their linkage with the trait. Thus the practical utility of the identified SCAR markers, viz. $OPK6_{695}$ and $OPAH5_{660}$, in marker-assisted breeding has been well demonstrated.

**Example** Marker-assisted selection in sorghum to pests:

To develop insect-resistant varieties and transgenic use of marker-assisted selection depends on the accuracy of the resistance-screening techniques. Infester row, cage and leaf disc screening methods have been standardized to evaluate sorghum germplasm, breeding materials and mapping populations for resistance to insect pests under field and greenhouse conditions (Sharma et al. 1992, 2003).

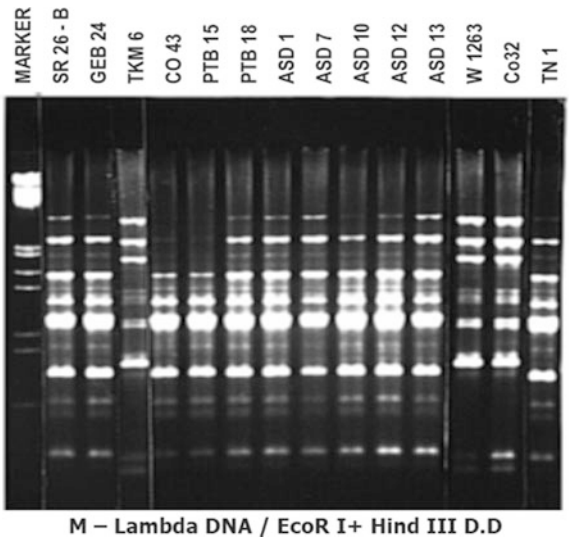


**Fig. 1** Screening of varieties for the presence of OPK 660,695

With the aid of powerful molecular genetic tools, genome-wide association studies can be conducted to understand the molecular variations in insect resistance (Chan et al. 2010; Kump et al. 2011). Genetic studies and analysis facilitate the development of molecular markers and enhance marker-assisted breeding to introgress resistance traits into economically important cultivated crops (Varshney et al. 2005; Bergelson and Roux 2010). Otherwise, insect resistance genes can be introduced into crops using transgenics.

In sorghum, marker-assisted selection requires at least six generations to transfer a trait within a species into high-yielding locally adapted cultivars through conventional breeding. The use of DNA markers offers tremendous potential for quantitative traits with low heritability. These are the most difficult characters to work in the field. The effectiveness of a marker-assisted selection (MAS) depends on phenotypic data on which the development of the marker was based. At ICRISAT, India, mapping populations have been phenotyped and genotyped for sorghum shoot fly (296B X IS 18551 and BTx 623 X IS 18551) and spotted stem borer, sorghum midge and aphid (ICSV 745 X PB 15881-3). Genetic linkage maps have been constructed to identify quantitative trait loci (QTLs) associated with resistance. Polymorphic simple sequence repeat (SSR) loci associated with resistance to shoot fly have been identified (Folkertsma et al. 2003). The QTLs are now being transferred into locally adapted hybrid parental lines via SSR-based MAS. The QTLs associated with antibiosis and antixenosis mechanisms of resistance to sorghum midge (Tao et al. 2003) and tolerance to green bug (Nagaraj et al. 2005) have also been identified. It is believed that MAS will facilitate rapid introgression of the resistance genes, and gene pyramiding, into the high-yielding varieties and hybrids.

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# *Agrobacterium*-Mediated Transformation for Insect-Resistant Plants


**B. Sunil Kumar and C. Immanuel Selvaraj**

**Abstract** *Bacillus thuringiensis* (Bt) is considered as gram-positive, aerobic, spore-forming, naturally occurring facultative soilborne bacterial pathogen and has been used for natural insect control. It produces a parasporal, persistent insecticidal protein crystals (ICPs). These ICPs are toxic in nature for a class of lepidopterans, dipterans, and coleopterans. That toxic protein differs, depending on the subspecies of Bt producing it. The most prevalent ICPs are the Cry (crystal) protein, and the other is Cty (cytolytic) protein produced by some *Bacillus thuringiensis* stains. The Cry proteins, in general were cleaved by proteolytic enzymes on intake to produce active toxins which results in osmotic imbalance, lysis of epithelial cells, and finally death due to starvation, whereas Cty proteins release vegetative insecticidal proteins (VIPs) which lead to membrane disruptions, midgut lysis, and paralysis in lepidopterans pests. The use of ICPs as a pesticide or insecticide over chemicals is more beneficial as there is less amount of environmental pollution and harmful chemical residues leaching into the soil and water bodies. It is also target specific and acts on specific class of pests and at the same time harmless to birds, fish, and mammals whose acidic gut conditions negate the bacteria's effect.

**Keywords** *Bacillus thuringiensis* (*Bt*) · Insecticidal Protein crystals (ICPs) · Major pests · Cultivated crops

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# 1 Introduction

## *1.1* Agrobacterium-*Mediated Methods*

*Agrobacterium tumefaciens*-mediated gene transfer method has been demonstrated to produce transgenic plants with new genetic properties for the plants resistant to insects. Several genes for insect resistance have been transferred through genetic engineering techniques by using delta-endotoxin coding sequences originating from *Bacillus thuringiensis* (BT)(Hilder and Boulter 1999).

*Agrobacterium tumefaciens* is a gram-negative soil bacterium responsible for crown gall disease, a neoplastic disease of many dicotyledonous plants characterized by the appearance of large tumors (galls) on the stems. Virulence is conferred by a large tumor – including plasmid (Ti plasmid) containing genes encoding plant hormones (auxins and cytokinins) and enzymes that catalyze the synthesis of amino acid derivatives termed opines. The plant hormones are responsible for de-regulated cell proliferation that accompanies crown gall growth, while the opines are secreted by the plant cells and used by bacteria as food. These genes are contained on a specific region of the Ti plasmid, the T-DNA (transfer DNA), so called because it is transferred to the plant nuclear genome under the control of virulence genes carried elsewhere on the Ti plasmid. It is this natural gene transfer mechanism that is exploited for plant transformation.

# 2 Development of Ti-Plasmid Vectors

The earliest indication that T-DNA could be used in plant transformation vector was the demonstration that DNA from *Escherichia coli* plasmid (the Tn7 transposon) could be stably transferred to the plant genome by first incorporating into the T-DNA. The transgenic plant could not be recovered from the transformed cells, either by regeneration or by grafting onto normal plants, because the hormones encoded by the T-DNA oncogenes cause unregulated and disorganized callus growth. In rare cases, shoots were derived from such callus tissue, and analysis showed that much of the T-DNA (including the oncogenes) had been deleted from the genome. An importance of T-DNA vectors was the realization that the only requirements for T-DNA transfer to the plant genome were the vir genes and the 24-bp direct repeat structures marking the left and right borders of the T-DNA. No genes within the T-DNA were necessary for transformation, and any sequence could be incorporated therein. This allowed the development of disarmed Ti plants lacking all the oncogenes, facilitating T-DNA transfer to plant cells without causing neoplastic growth.

Once suitable selectable marker has been incorporated into the T-DNA, Ti plasmids became very powerful gene delivery vectors. The wild-type Ti plasmids were unsuitable for the task due to their large size, which made them difficult to

manipulate in vitro (large plasmids tend to fragment and they lack unique restriction sites for subcloning). An early strategy to overcome this problem was the development of intermediate vectors, where the T-DNA was subcloned into a standard *E. coli* plasmid vector, allowing *in vitro* manipulation by normal procedures, and then integrated into the T-DNA sequence of a disarmed Ti plasmid resident in *A. tumefaciens* by homologous recombination (Matzke and Chilton 1981). This system was simple to use but relied on a complex series of conjugative interactions between *E. coli* and *A. tumefaciens* requiring three different bacterial strains (triparental matings). It was reported that the *vir* genes were acting trans to mobilize the T-DNA, it was soon discovered that use of large natural Ti plasmids was unnecessary. Intermediate vectors have been largely superseded by binary vectors, in which vir genes and the T-DNA are cloned on separate plasmids. These can be introduced into *A. tumefaciens* by conjugation with an *E. coli* donor or by freeze-thaw cycles or electroporation. Most contemporary *Agrobacterium*-mediated transformation systems employ binary vectors.

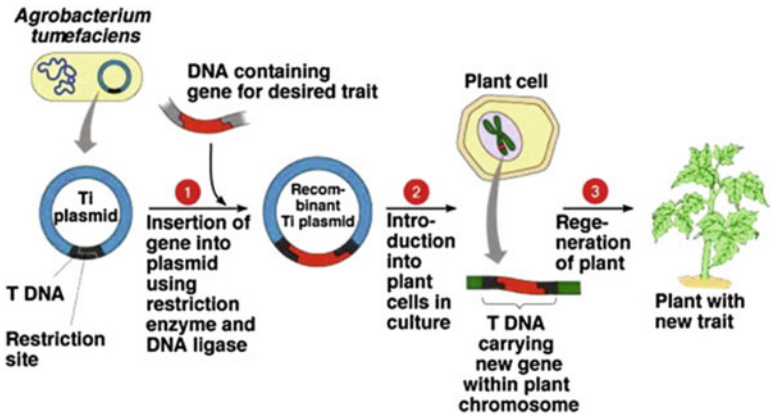


**Fig. 1** *A simple pictorial representation of Agrobacterium*-mediated transformation in sugarcane (Nadira Islam et al. 2016).

## 3 *Agrobacterium*-Mediated Transformation

1. Initiate callus from surface-sterilized explants.
2. Before transformation, divide big calli into small pieces and culture on callus induction medium for 5 days.
3. Divide big callus into 6–7 pieces and preculture a total of 100 small pieces of calli for transformation.
4. *The agrobacterium tumefaciens* strain namely, EHA105 carries the binary vector pCAMBIA1302-Ab possesses hygromycin phosphotransferase *(hpt)* and *Cry1A (b)* can be used for transformation (Lucena et al. 2014). (This *Cry1A(b)* was isolated and modified from native bacterium found in Thailand).

5. Both genes are under the control of 35SCAMV constitutive promoter.
6. Culture the bacterium in 20 mL liquid LB medium containing 50 mg/L kanamycin at 28 °C and overnight shaking at 180 rpm.
7. From this culture transfer 2 mL of suspension to 20 mL liquid LB medium containing 50 mg/L kanamycin and 100 μM acetosyringone, and keep in shaker overnight at 200 rpm.

## 4 Infection

8. After overnight culturing, centrifuge 20 mL of *Agrobacterium* suspension at 6000 rpm for 5 min, and discard the supernatant.
9. Resuspend the pellet (by gently pipetting) in 5 mL liquid callus induction medium.
10. Transfer 5 mL of suspension into a bottle containing 15 mL liquid callus induction medium (MS+ 20 g/L sucrose, 3 mg/L 2,4-D + 10% (V/V) coconut water (CW), and 100 μM acetosyringone).
11. Immerse the precultured calli in the bacterial suspension and shake at 120 rpm for 15 min at 26 °C.
12. Place the calli on tissue paper to remove the excess medium and later place on cocultivation medium.

## 5 Cocultivation and Selection

13. Transfer the infected calli onto cocultivation medium (MS + 20 g/L sucrose, 7 g/L agar, 3 mg/L 2,4-D, 10% (V/V) CW + 100 μM acetosyringone, pH, 5.7), and keep in the dark for 3 days.
14. After 3 days, wash the calli with liquid MS callus induction medium +200 mg/L cefotaxime three times to remove most of the bacteria. Then place the calli on sterile tissue paper, and culture in selective medium (MS + 20 g/L sucrose, 7 g/L agar, 3 mg/L 2,4-D, 10% (V/V) CW + 30 mg/L hygromycin, 200 mg/L cefotaxime, pH, 5.7).
15. For every 2 weeks, wash the calli one time with liquid MS + 300 mg/L cefotaxime, and subculture on the same medium. Then, after 6 weeks select the live calli and transfer to the regeneration medium.
16. Transfer the putative transformed calli to regeneration medium (MS + 20 g/L sucrose, 7 g/L agar +30 mg/L hygromycin, 200 mg/L cefotaxime, pH, 5.7) until the shoots are visible.
17. After 60 days of subculturing, transfer the putative transgenic shoots to the rooting medium (MS + 20 g/L sucrose, 7 g/L agar, 5 mg/L NAA+ 30 mg/L hygromycin, 200 mg/L cefotaxime, pH, 5.7).
18. After 60 days of growth in the culture medium, transfer the plants into pots with a soil-perlite mixture (1:1 ratio).

## 6 Confirmation of the Existence of the Transgenes by PCR Technique

19. The transgenic plants can be confirmed by using the polymerase chain reaction (PCR).
20. Use Phire Plant Direct PCR Master Mix (Thermo Scientific, Lithuania) as the PCR master mixture. This master mix is designed to perform PCR directly from different plant material without prior DNA purification.
21. Take a small piece of 3-month-old putative transgenic sugarcane leaf (approximately 2 mm in size), and place in 20 μL of dilution buffer (supplied by manufacturer); crush the leaf sample with a pipette tip, by pressing briefly.
22. Centrifuge the plant material and use 0.5 μL of the supernatant as a template. In a 20 μL PCR mixture containing 10 μL 2X Phire Plant Direct PCR Master Mix, 0.2 μl (10 μM) of each hpt (hygromycin phosphotransferase) forward primer (5′-CCTGAACTCACCGCGACG-3′) and reverse primer (5-′-AAGACCAATGCGGAGCA-TATA -3′), 0.5 μL of template, and 9.1 μL distilled water.
23. Prepare the same master mix with 10 μM Cry1A(b) for both forward (5-′-CATGGACAACAACCCAAACATCAACG-3′) and reverse primer (5-′-GTCACCTTGCTACCGAAAGTCCTCGTT-3′).
24. Set the amplification steps/PCR conditions for both primer sets, with 1 cycle of initial denaturation at 98 °C for 5 min, followed by 40 cycles of denaturation at 98 °C for 5 s, annealing at 62 °C for 5 s, and an extension at 72 °C for 20 s, followed by a final extension at 72 °C for 1 min.
25. After that, perform electrophoresis on 1% agarose gel at 50 V for 60 min with a reference ladder for the PCR-amplified products.

## 7 Southern Blot Analysis

26. Extract genomic DNA from young leaves of transgenic and untransformed control sugarcane plants by using the modified DNA extraction method described by Aljanabi et al. (1999) or by the method given by Doyle and Doyle (1990).
27. Digest the genomic DNA (50 μg per reaction) from each plant with *Nco*1 restriction enzyme for 16 h.
28. Transfer the DNA by capillary transfer (Sambrook and Russell 2001) onto a positively charged membrane (Amersham Hybond™ -N+, GE Healthcare, UK).
29. Hybridize using HyUse, a gene probe (900 bp) of Cry1A(b) labeled with DIG-dUTP (Roche Diagnostics, Germany). Follow the standard protocol for pre-hybridization and hybridization by (Sambrook and Russell 2001).
30. After 16 h of hybridization, wash the membrane with different concentrations of saline sodium citrate (SSC) buffer (2x, 1x, and 0.5x SSC).

31. Transfer the membrane into a plastic bag containing 15 ml blocking solution (10x blocking reagent: maleic acid buffer) with 1 μL (0.75 U/μL) of anti-digoxigenin-AP fab fragments (Roche Diagnostics, Germany), seal the bag, and keep in shaker for 30 min.
32. Wash the membrane twice with washing buffer (1x maleic acid and 0.3% tween 20) for 30 min.
33. After washing the membrane, expose it to X-ray film (Kodak medical X-ray film, USA) for 15 min.
34. Transfer the film into a box containing developing solution for 1–2 min. When the DNA band was visible, transfer the film immediately to a box containing fixing solution for 1–2 min, and air-dry the film for 10–15 min at room temperature.

## 8 Determination of Expression Levels of Cry1A (b) in Transgenic Sugarcane

35. Isolate total RNA from young leaves (0.1 g) of 3-month-old sugarcane by using the method described by Laksana and Chanprame (2015).
36. Quantify the concentration of total RNA using NanoDrop™ and adjust it to 1500 ng/μL.
37. Synthesize the first strand of cDNA using the total RNA as template (reaction mixture consists of 1 μg of total RNA, 2 μg oligo (dT) primer (IDT, Singapore), 0.8 mM dNTP (Thermo Scientific, Lithuania). Add RNase-free water and make the volume 12.5 μL).
38. Incubate the reaction, mix at 65 °C for 5 min, and then cool down to 4 °C for at least 2 min.
39. Then add 1x reaction buffer, 0.5 unit RiboLock RNase inhibitor (Fermentas, Lithuania), 1 mM dNTP, and 1 μL Revert Aid M-Mul VRT (Fermentas, Lithuania) to the reaction mixture tube and mix it gently, incubate the mix at 42 °C for 1 h and stop the reaction at 70 °C for 10 min, and then cool down the mix at 4 °C for 5 min.
40. Add RNase H (0.2 μL) for removal of the remaining total RNA.
41. Use the specific primers designed already for *Cry1A(b)* gene (AY742219.1) with the help of Primer3 program (http://simgene.com/Primer3). The product size was about 200 bp.
42. For real-time PCR, prepare a 20 μL reaction mixture containing 100 ng of cDNA, 10 μL 2X SensiFAST SYBR No-ROX mix buffer (Bioline Reagents Ltd., USA), and 0.8 μL of 10 μM forward and reverse primers specific to *Cry1A(b)* and actin genes.

43. Perform the amplification of both genes by using the following condition: preliminary denaturation at 95 °C for 2 min, 45 cycles of denaturing at 94 °C for 15 s, annealing at 58 °C for 15 s, and an extension at 72 °C for 20 s.
44. Compare the expressions of Cry1A(b) in transgenic sugarcane with the control sugarcane (non-transgenic). Actin was used as a reference gene.

## 9 *Agrobacterium*-Mediated Transformation in Rice

In 1994, Hiei et al. (1994) described the first efficient *Agrobacterium*-mediated transformation protocol for rice. They obtained transgenic rice plants with a transformation efficiency ranging from 10% to 30% by using a technique consisting of seven steps necessitate about 4 months. Two vital points for successful transformation were observed: the use of actively dividing embryonic callus cells derived from the scutella of mature seeds as the initial material and addition of a phenolic compound, acetosyringone, in the cocultivation steps.

Based on this method, *Agrobacterium*-mediated transformation methods have been developed with slight alterations for many rice varieties, not only *japonica* (Toki 1997; Cho et al. 1998; Yara et al. 2001) but also in *indica* (Rashid et al. 1996; Aldemita and Hodges 1996; Prasad et al. 2016; Zhang et al. 1997) and tropical *japonica* (Dong et al. 1996; Rachmawati et al. 2004). Familiarity has also demonstrated that transformation efficiencies depend on the culture response of each variety. Therefore, optimization of tissue culture conditions is essential for varieties that are more difficult to regenerate (Hiei et al. 1997). Here, varieties that can be cultured easily can be referred to as "high-regeneration" varieties, and those that are recalcitrant to cell culture are referred to as "low-regeneration" varieties.

## 10 Development of a Rapid Transformation Method for Japonica Rice

In order to improve the efficiency of transformation in rice, a quick transformation method with a modified protocol can be used (Hiei et al. 1997). In the protocol, two culture conditions were modified: (1) the media composition for emergent calli and (2) the light conditions for all culture stages apart from the co-culture period. By using a culture system that speeds up the growth of calli, the time required from callus initiation to regeneration was reduced to only 2 months and could be carried out in five or six steps. As this method was very easy, special techniques were needless. This optimized protocol has greatly facilitated routine transformation in japonica rice.

## 11 Modified Methods for Culturing Rice Varieties

Many elite japonica varieties respond poorly to conventional culture using mature seeds on MS16 or N6 medium (Murashige and Skoog 1962; Chu et al. 1975) making it difficult to obtain transgenic rice plants. Nishimura et al. (2005) have recognized a key gene controlling regeneration frequency and have developed an alternative transformation method for the elite japonica variety, "Koshihikari." Nishimura et al. (2005) succeeded in transforming a foreign gene into the elite japonica, "Koshihikari," by the conventional method with a 35–45% transformation frequency.

## 12 Indica Varieties

Most indica rice varieties show a low rate of callus growth or low-regeneration frequencies in conventional culture using mature seeds on MS16 or N6 medium. Some successes have been reported in *Agrobacterium*-mediated transformations using immature *indica* embryos as the starting materials (Aldemita and Hodges 1996). In comparison to mature seeds, preparing immature embryos is difficult and much more laborious. There are also a few reports describing *Agrobacterium*-mediated transformation using mature indica seeds (Supartana et al. 2005), but successes are limited to very specific varieties (Rashid et al. 1996; Zhang et al. 1997).

The procedures for induction of callus formation using indica embryos as starting material have been detailed out in Nishimura et al. (2006).

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# Breeding Procedures for Developing Insect-Resistant Crops


**T. Sabesan and K. Saravanan**

**Abstract** Breeding crops for pest tolerance involves basic principles of plant breeding, genetics and entomology. This chapter begins with a brief outline of breeding methods for self- and cross-pollinated crops. This is followed by techniques for emasculation and pollination to produce a hybrid. Further, various breeding procedures from simple selection, hybridization, and backcross methods of gene transfer are explained with illustrations. Plant breeding techniques involving tissue culture and transgenic and marker-assisted selection are dealt in brief. These topics give an overall glimpse to potential aspirants, to understand breeding for pest resistance/tolerance.

**Keywords** Hybrid · Crops · Gene transfer · Selection

## 1 Introduction

Collection of germplasm for identification and selection of resistant genotypes is prerequisite for transfer of resistance through conventional or modern approaches. The commonly used plant breeding methods are selection, hybridization, heterosis breeding, mutation breeding, marker-assisted breeding, and genetic engineering methods.

## 2 Hybridization

For exploiting genetic variability, gene transfer, break undesirable gene linkages, and for exploitation of heterosis, hybridization is practiced. Artificial hybridization is required to understand the inheritance of desired traits that govern the trait for

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effective transfer of the genes. Gene transfer can be affected through artificial hybridization. Hence, information on the mode of pollination and time of anthesis is essential. Selfing and crossing are the essential procedures in crop improvement process.

## 2.1 Selfing Technique

Self-pollination or selfing is done by covering the flower bud or inflorescence or the whole plant with a mesh to prevent natural cross-pollination. As the parents involved in a breeding program should be homozygous, selfing is necessary.

## 2.2 Crossing Technique

In a crop with bisexual flowers, the male reproductive part is removed, followed by transfer of pollen from desired parent. The removal of male reproductive part without affecting female reproductive organ is known as emasculation. The process of transferring of pollen from male to female parent is called cross-pollination. This process by which a hybrid is produced is called as hybridization. In monoecious crops like castor and coconut where separate male and female flowers are present in the same inflorescence, male flowers are removed. In case of dioecious crop like maize, with separate male and female inflorescence, the male inflorescence is removed.

## 2.3 Suction Method

This method is practiced in species with small flowers. Emasculation is done in the morning immediately after the opening of flowers. A thin rubber or a glass tube is attached to a suction hose to suck the anthers from the flowers. The amount of suction pressure applied should be regulated for complete removal of anthers. Vacuum emasculator is generally used. Alternatively, commercially available low-power vacuum cleaners can also be used (Sabesan *et al*. 2016 data unpublished).

## 2.4 Modified Method of Emasculation

The inflorescence can be covered with wet cloth or butter paper or a transparent poly bag, and hot air is blown by commercial hair dryer (Sabesan and Saravanan 2016 data unpublished). The heat developed inside helps in opening of spikelet and

extrusion of anthers without dehiscence. Bagging, synchronization of flowering and crossing, and tagging are the steps to be followed subsequently.

Plant breeding methods like pedigree method, bulk method, single seed descent method, and backcross methods are utilized for developing resistant varieties.

## 3 Single Seed Descent Method (SSD)

A single seed is selected randomly from each selected plant to make bulk. It is highly useful for improvement of quantitative characters (e.g., yield, earliness), rather than qualitative characters such as flesh color and pest and disease resistance.

Generally in SSD, the $F_3$ and later generations are raised from a bulk of one seed from each $F_2$ plant and subsequent generation plant in order to ensure that each $F_2$ plant is represented equally in the resultant population. This method aims at developing homozygous population in early generations without selection. Selection is practiced after attaining homozygosity.

| Steps/generations | Key points |
|---|---|
| Hybridization | Crossing of selected parents |
| $F_1$ generation | $F_1$ seeds are grown and harvested in bulk |
| $F_2$ generation | $F_2$ seeds are grown. One seed from each plant is selected randomly and mixed |
| $F_3$ generation | $F_3$ seeds are grown and harvested as above |
| $F_4$ and $F_5$ generations | The similar procedure as above is carried out |
| $F_6$ generation | $F_6$ seeds are planted. Selection for superior plants is conducted, and selected ones are harvested separately. Number of plants could range from 150 to 500 |
| $F_7$ generation | Individual plant progenies are grown, and selected progenies are harvested in bulk |
| $F_8$ generation | Preliminary yield trials and quality tests are conducted |
| $F_9$ to $F_{10}$ or $F_{13}$ generation | Coordinated yield trials and tests for resistance and quality are conducted |
| $F_{11}$ of $F_{14}$ generation | Seed multiplication for distribution |

## 4 Transfer of Dominant Gene Through Backcross Breeding

Consider variety A as a popular, well-adapted, and high-yielding variety but susceptible to a major pest. Variety B is resistant to a specific pest and has dominant gene for resistance. Generally, variety A is considered as the recurrent

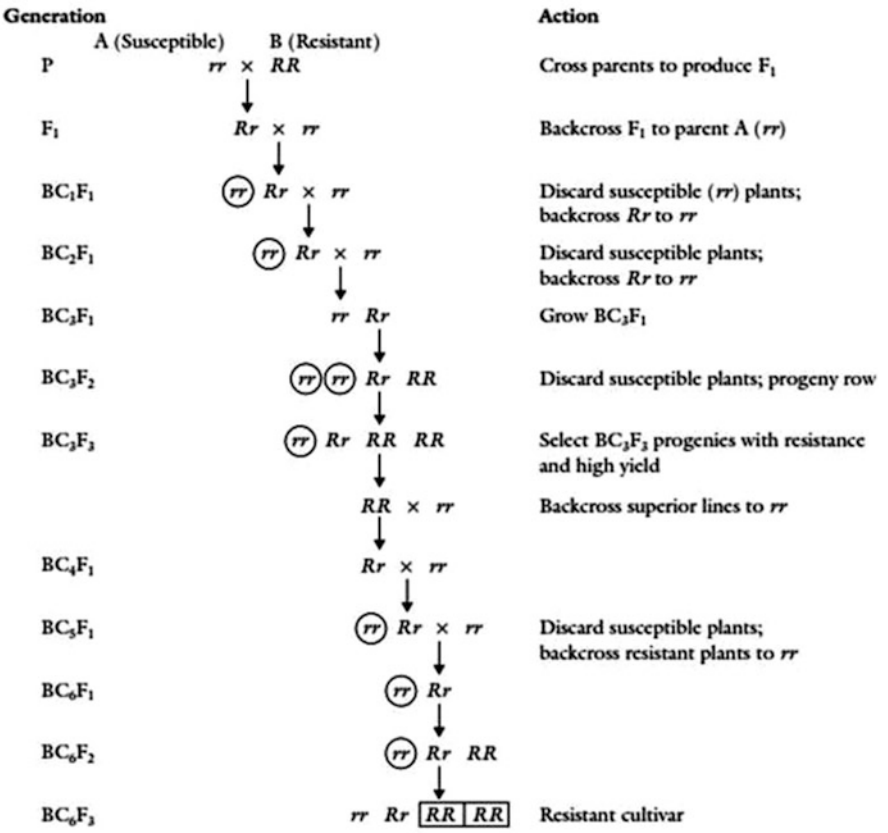


**Fig. 1** General steps in transfer of a dominant gene through backcross breeding

parent (female parent) and variety B as donor parent (male parent). The various steps in backcrossing are as follows (Fig. 1):

| | |
|---|---|
| Season 1 | Hybridization of the two varieties A and B keeping recipient variety as female parent |
| Season 2 | Raising $F_1$ generation. Selection for disease resistance is not performed. The $F_1$ generation plants are backcrossed with recipient variety A, and seeds are collected to raise $BC_1$ generation |
| Season 3 | Raising first backcross generation ($BC_1$) plants and selecting for pest resistance; selected plants are backcrossed with recurrent parent A |
| Seasons 4–7 | Raising second to fifth backcross generations ($BC_2$–$BC_5$). In each season, screening for selection of pest-resistant progenies is done as segregation for pest resistance occurs in every backcross generation. Plants that resemble recurrent parent along with pest resistance are selected. The selected progenies are backcrossed with recurrent parent A to get the next backcross generation |

(continued)

| | |
|---|---|
| Season 7 | Raising sixth backcross generation ($BC_6$), screening, and selection for pest resistance. Selected progenies that resemble recurrent parent along with pest resistance are self-pollinated and harvested separately |
| Season 8 | Raising $BC_6$ $F_2$ generation in which individual plant progenies are raised from seeds of $BC_6$ generation. Plants are selected on the basis of similarity with recurrent parent along with pest resistance and are harvested separately |
| Season 9 | Raising $BC_6$ $F_3$ generation and growing individual plant progenies from seeds of above cross. As above, plants resembling recurrent parent with pest resistance are selected but harvested in bulk |
| Yield trials | Replicated yield trials are conducted with recurrent parent as a check. The newly constituted variety should be similar to variety A for most of the important characteristics along with pest resistance |

## 5 Transfer of a Recessive Gene Through Backcross Breeding

Consider variety A as a popular, well-adapted, and high-yielding variety but susceptible to a major pest. Variety B is resistant to a specific pest which is conferred by a recessive gene. Generally, variety A is considered as the recurrent parent (female parent) and variety B as donor parent (male parent). The various steps in backcrossing are as follows:

When the desired character, i.e., pest resistance is governed by a recessive gene, continuous backcrosses cannot be performed as in dominant gene transfer method. After the first backcross and after every two subsequent backcrosses, $F_2$ generation progenies must be raised to test for pest resistance. Only $F_2$ progenies are tested for resistance as all the $F_1$ and backcross progenies are heterozygous and susceptible to the pest. The various steps for this backcross breeding are as follows (Fig. 2):

| | |
|---|---|
| Season 1 | Hybridization of the two varieties A and B keeping recipient variety as female parent |
| Season 2 | Raising $F_1$ generation. Selection for disease resistance is not performed. The $F_1$ generation plants are backcrossed with recipient variety A, and seeds are collected to raise $BC_1$ generation |
| Season 3 | Raising first backcross generation ($BC_1$) plants. As the pest resistance is controlled by recessive gene, all the plants will be susceptible, and hence, resistance is not tested for this generation. Plants raised from seeds of above crosses are selfed |
| Season 4 | Raising $BC_1$ $F_2$ generation and screening for pest resistance are carried out. The resistant plants that resemble the recurrent parent are selected and backcrossed with recurrent parent A |
| Season 5 | Raising $BC_2$ generation from the seeds of the above cross. Plants resembling recurrent parent are selected and backcrossed with recurrent parent. Resistance test is not conducted |

(continued)

| | |
|---|---|
| Season 6 | Raising $BC_3$ generation plants which are selfed to get $F_2$ seeds. Resistance test is not conducted for the sake of selection. Selection is done on the basis of resemblance to recurrent parent |
| Season 7 | Raising $BC_3$ $F_2$ generation and screening for pest resistance are conducted, and selected plants are backcrossed with variety A |
| Season 8 | Raising the $BC_4$ generation and backcross of plants selected above with recurrent parent A. Resistance test is not conducted |
| Season 9 | Raising $BC_5$ generation plants which are selfed to get $F_2$ generation seeds. Pest resistance test is not conducted in this generation |
| Season 10 | Raising $BC_5$ $F_2$ generation, screening for pest resistance, and selection of plants that resemble recurrent parents are carried out |
| Season 11 | Raising $BC_5$ $F_3$ generation in which individual plant progenies are grown. Selection is done for pest resistance and resemblance to recurrent parent A. Seeds of selected plants are bulked to constitute the new variety |
| Season 12 | Replicated yield trials are conducted with variety A as a check. The newly developed variety should be similar to variety A for most of the essential characteristics along with pest resistance |

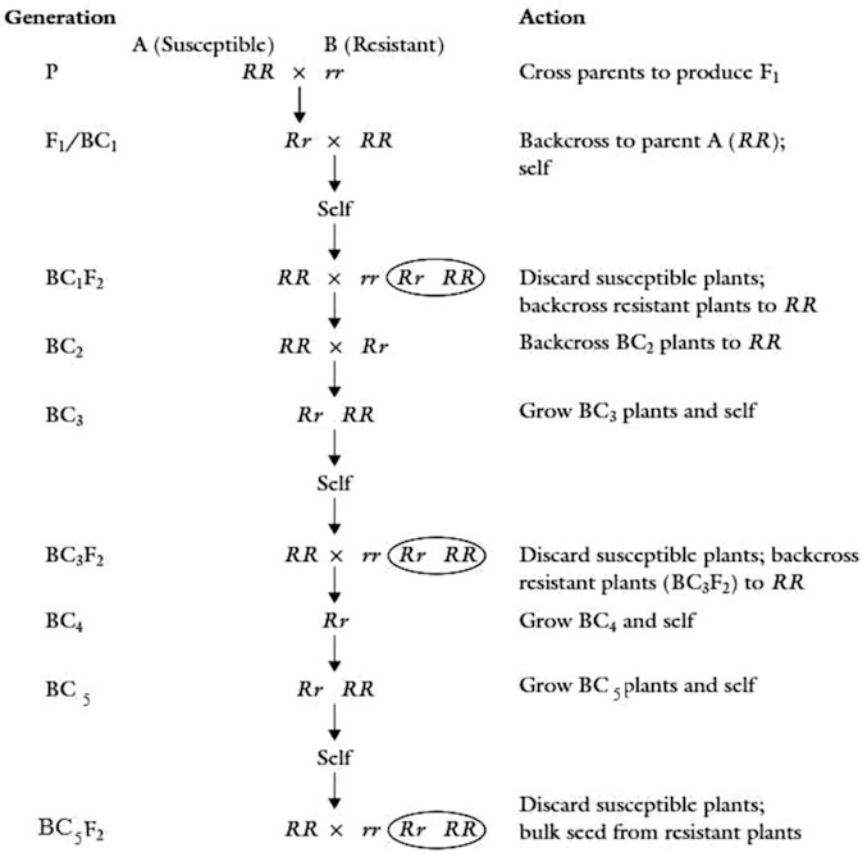


**Fig. 2** General steps in transfer of a recessive gene through backcross breeding

## 6 Wide Hybridization

Hybridization between parents belonging to different species or genera is called wide hybridization. This is followed when the genetic variability is insufficient in a crop. Crossing closely related species results in viable hybrids, while less related parents lead to poorly formed seeds which can be propagated by in vitro techniques. Several pre- and postfertilization barriers hinder the hybrid seed formation in wide hybridization.

## 7 Bridge Crossing

To overcome fertilization barriers in wide hybridization involving two incompatible parental species (e.g., $P_1$ and $P_2$), introgression of the desired trait to another genotype (e.g., $P_3$) which is cross-compatible to both the parents $P_1$ and $P_2$ is followed. Parent $P_1$ is first crossed with $P_3$, and the plants with desired traits are selected and crossed with the target parent $P_2$. This is called bridge crossing.

Polyploidy breeding involves changing the chromosome number or sets of chromosomes. Polyploidy is classified as autopolyploidy which involves two or more sets of chromosomes derived from a single species (e.g., tetraploid potato AAAA) and allopolyploidy which includes polyploids from different genomes (e.g., hexaploid wheat has three different genomes AABBDD).

For breeding crop hybrids with insect-resistant traits, the readers may consult Khush and Brar (1991), Singh (2015), and Smith (1989).

## 8 Illustrated Example: Sorghum (Eyidozehi *et al.* 2015)

Cytoplasmic male sterility systems and insect pests: Several CMS systems have been used in sorghum for hybrid production. But only the A1 CMS system has been used for producing sorghum hybrids worldwide. A2 CMS-based hybrids are only used in China (Shan *et al.* 2000). The use of a single source of male sterility narrows down the genetic base of sorghum hybrids, and there is considerable risk of insect pest and disease outbreaks in cultivars with single source of male sterility (Sharma *et al.* 2004). Resistance to *Diatraea grandiosella* and *D. saccharalis* was higher in resistant inbred line-based hybrids than the inbreds (Kumar and Mihm 1996). Similarly expression to different mechanisms and traits associated with resistance in sorghum to shoot fly, midge, shoot bug, and sugarcane aphid has been found significantly lower in CMS compared to the maintainer lines of sorghum (Dhillon *et al.* 2006a, b). Hybrids based on shoot bug, sugarcane aphid, midge, and shoot

fly-resistant CMS and restorer lines suffered less damage than the hybrid based on susceptible CMS and resistant or susceptible restorer lines, suggesting that expression of resistance to these insects is influenced by the genetic background of the CMS lines, and resistance is needed in both the parents to produce insect-resistant hybrids (Sharma *et al.* 1996; Dhillon *et al.* 2006a, b; Sharma *et al*. 2006).

**Development of CMS and Restorer Lines for Resistance to Insect Pests** The maintainer lines contain the genes that impart resistance to insect pests (Sharma *et al*. 2004). So, there is a need to diversify CMS, maintainer, and restorer lines with resistance to insect pests and diversify the CMS systems in sorghum. The A4 M cytoplasm is slightly less susceptible to shoot fly than the other CMS systems. Recovery from shoot fly damage is better in A4 M, A3, and A2 cytoplasms than the A1 cytoplasm. Shoot fly survival and development is also poor on A4 M and A4 VzM CMS systems. Initially it may be better to transfer the traits associated with resistance to shoot fly into the hybrid parents in A1 cytoplasm. Large areas under high-yielding sorghum cultivars are covered in Asia, Australia, and the Americas. Therefore, it is apparent that for host plant resistance to be an important component of pest management in sorghum, we need to transfer the insect-resistance genes into male-sterile, maintainer, and restorer lines that can be used by the public institutions and private seed industry to develop insect-resistant hybrids. Much of this material has been shared with public institutions and private seed industry over the past decade for use in sorghum improvement and for developing high-yielding hybrids with resistance to insects. To evolve and maintain stability to insect-resistant hybrids, the genes conferring resistance to insect pests must be transferred into both CMS and restorer lines (Sharma *et al.* 2004).

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# Physical and Chemical Mutagenesis Methods for Development of Insect-Resistant Crop Varieties


**K. Saravanan and T. Sabesan**

**Abstract** Selection of appropriate parents is the first step in breeding for pest resistance. Mutation breeding through physical and chemical mutagens can be resorted to, when the source of resistance is not available. This chapter gives a comprehensive look at the various options for physical and chemical mutagenesis along with their mechanism of action. Steps in mutation breeding for clonally and seed-propagated crops are explained which will enable researchers to take up resistance breeding through mutagenesis.

**Keywords** Mutation · Physical agents · Chemical agents · Resistance breeding

## 1 Introduction

Mutation refers to sudden heritable changes in the phenotype of an individual. In other words, mutations arise due to changes in DNA bases. The mutation occurs in two ways: (1) by alteration in nuclear DNA (point mutations) and (2) by change in cytoplasmic DNA (cytoplasmic mutation). Spontaneous mutation refers to a mutation that occurs in natural populations. Induced mutations refer to the artificial induction of variation using mutagenic agents called mutagens (Anonymous 1991; Agrawal 1998). Induced mutations are of two types:

1. Macromutations: Mutation with distinct morphological changes in the phenotype
2. Micromutations: Mutations with invisible phenotypic changes

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## 2 Mutation Breeding

The process of inducing desirable mutations and exploiting them for crop improvement is called as mutation breeding. It is commonly used in self-pollinated crops and in clonally propagated crops. The agent that induces mutation is called as a mutagen. It refers to the various physical or chemical agents which greatly enhance the frequency of mutations (Borojevic 1990; Chahal and Gosal 2000).

### *2.1 LD50*

The success of any mutation breeding program depends on the dosage of mutagens applied. A lower dose may not induce mutation, while a high dose can cause death of the mutated material. Hence, determination of LD50 (lethal dose), a dose that causes 50% mortality to the seeds or a safe dose where 50% of the seeds can survive, is essential. The LD50 needs to be standardized for different crops and the variety used in the study.

## 3 Characteristic Features of Physical Mutagens

1. Gamma Rays

Gamma rays have generally a shorter wavelength and hence possess more energy per photon than X-rays. Gamma radiation is usually obtained from radioisotopes in contrast to X-rays. A gamma radiation facility can be used essentially in the same manner as an X-ray machine for acute or semi-acute exposures. The most distinct advantage of gamma radiation source for prolonged treatments is that it can be placed in a greenhouse or field so that plants can be exposed as they develop over long periods of time. Cobalt-60 and cesium-137 are the main sources of gamma rays used in mutation breeding. They are stored in lead containers when not in use and operated by remote control mechanisms to irradiate plant material.

2. UV Rays

Ultraviolet light has limited penetrating ability; therefore its use is limited to treating spores, pollen grain cells, and cultured tissue. Wavelengths in the range of 2500–2800 nm are biologically most effective because this is the region of maximal light absorption by nucleic acids.

3. Beta Particles

Beta particles such as those from phosphorus-32 and sulfur-35 produce effects in tissues similar to those of X-rays or gamma rays. The penetrating ability of beta particles is lower than that of X- and gamma rays. The lower penetrating ability of

**Table 1** List of physical mutagens with mode of action

| Mutagen | Properties | Mode of action/change induced |
|---|---|---|
| X-rays | S.I., penetrating and non-particulate | Induce mutations by forming free radicals and ions. Cause all changes |
| Gamma rays | S.I., very penetrating and non-particulate | Induce mutations by ejecting atoms from the tissues. Cause all changes |
| Alpha particles | D.I., particulate, less penetrating, and positively charged | Act by ionization and excitation. Cause chromosomal breakage and gene mutations |
| Beta particles | S.I., particulate, more penetrating, and negatively charged | Act by ionization and excitation Cause chromosomal breakage and gene mutations |
| Fast and thermal neutrons | D.I., particulate, neutral particles, and highly penetrating | Cause chromosomal breakage and gene mutations |
| UV rays | Non-ionizing, low penetrating | Cause chromosomal breakage and gene mutations |

beta particles can be overcome by putting the radioisotope in a solution and administering them to the plant material. P-32 and S-35 may then be incorporated directly into cell nucleus, giving somewhat greater localization of the site of action. But, because of the variability from tissue-to-tissue and cell-to-cell, it is difficult to determine the exact dose given by an internal emitter in plant material (Broertjes and Van Harten 1988; Micke 1992).

4. Neutrons

Neutrons have been shown to be highly effective for the induction of mutation in plants, but a certain degree of confusion exists concerning the results of early experiments due to lack of adequate dosimetric techniques (Table 1).

## 4 Gamma Garden

Gamma garden is an area subjected to gamma irradiation. This area is enclosed by thick and high walls to protect the plants and animals from radiation damage. The purpose of gamma garden is to irradiate whole plants, seeds, and other propagules. The first gamma garden was built in Long Island near New York, USA. The first gamma garden in India was built in Calcutta at Bose Research Institute in 1959, at IARI in 1960, and at BARC, Trombay.

The IARI gamma garden has an area of 3 acres surrounded by a wall of 3 m height and 1 m thick. The gamma ray source consists of 6 g $^{60}$Co sealed in an aluminum capsule. The strength of $^{60}$Co is 200 curies. The sealed $^{60}$Co is kept in a lead container, since gamma- and X-rays do not penetrate lead. For irradiation, the lid of the lead container is lifted along with aluminum capsule. After irradiation the $^{60}$Co aluminum capsule is lowered into the lead container, and the lid is closed. The lead container is opened and closed with a remote control device.

### 4.1 B. Chemical Mutagen

The commonly used chemical mutagens are listed below.

| Group of mutagen | Name of chemical | Mode of action |
|---|---|---|
| Alkylating agents | Ethyl methanesulfonate (EMS) | AT GC transitions |
| | Methyl methanesulfonate (MMS) | Transitions |
| | Ethyl ethanesulfonate (EES) | GC AT transitions |
| Base analogues | 5-Bromouracil | AT GC transitions |
| | 2-Aminopurine | GC AT transitions |
| Acridine dyes | Acriflavine, proflavin | Deletion and addition |
| Others | Nitrous acid | AT GC transitions |
| | Hydroxylamine<br>Sodium azide | GC AT transitions<br>Transitions |

## 5 Mutation Breeding Procedure for Clonally Propagated Crops

Step: 1. Choice of the parent material

- Generally the best adapted variety of a crop should be chosen.
- Suppose a variety is high yielding, but susceptible to a particular pest, the objectives of mutation breeding would be to induce resistance to that particular pest in the variety.

Step: 2. Choice of mutagen

- It depends upon the plant parts to be treated. For treating vegetative parts, radiations are preferred, while for seed treatment chemical mutagens are preferred. The penetration of chemical mutagens can be enhanced by dissolving the mutagen in solvents like DMSO.

Step: 3. Mutagenic treatment

- The plant parts that are treated include seeds, pollens, buds, cuttings, or suckers.
- LD50 refers to a dose of mutagen that kills 50% of the treated individuals. Generally, the mutation treatment dosage will be based on the LD50 value.
- Duration of treatment depends on the intensity of radiations or concentrations of the chemical mutagen used. The seeds are water-soaked before treatment. After treatment the seeds or cuttings are immediately planted, and pollens are used for pollination. Plants obtained from treated seeds or cuttings are called M1 plants.

Step: 4. Handling of treated material

# 6 Mutation Breeding Procedure for Seed-Propagated Crops

## *6.1 M1 Generation*

Several hundred (500 or more) mutagen-treated seeds are space planted with wider spacing for easy identification. Generally the mutants are recessive. All the plants will be chimeras for the mutation present in a heterozygous state. About 20 seeds from each M1 plant are harvested separately (Peloquin 1982).

## *6.2 M2 Generations*

About 2000 progeny rows are grown using wider spacings. Oligogenic mutants with distinct features are identified and selected. Only 1–3% of M2 rows may be expected to have beneficial mutations.

## *6.3 M3 Generation*

Progeny rows from individual selected plants in the previous generation are grown. Inferior mutant rows are eliminated. Mutant M3 rows are harvested in bulk.

## *6.4 M4 Generation*

A preliminary yield trial is conducted with a promising mutant line selected for replicated multilocation trials.

## *6.5 M5–M8 Generation*

Selected lines are tested in coordinated multilocation trials. The best-performing line is released as a variety.

## 6.6 Advantages of Mutation Breeding

It is a cheap and rapid method of developing new varieties compared to backcross, pedigree, and bulk breeding methods. It is more effective for the improvement of oligogenic characters such as pest and disease resistance than polygenic traits. This is a simple, quick, and efficient means to introduce a new character in vegetatively propagated crops, by utilising CMS.

## 6.7 Disadvantages of Mutation Breeding

Since useful mutations are produced at a very low frequency (0.1%), a large plant population has to be screened to identify and isolate desirable mutant progenies. It has limited scope for the genetic improvement of quantitative or polygenic characters. Most of the mutations are deleterious and undesirable.

## 6.8 Achievements

A brief list of varieties developed in crops is given below.

| | |
|---|---|
| Wheat | Sharbati Sonora (gamma ray mutant of Sonara-64) |
| Rice | Jagannath (γ ray mutant of T 141) |
| Urd | Co 4 (MMS mutant of Co 1) |
| Groundnut | TG 17 |
| Cotton | MCU 7 |
| | MCU 10 (MUC4 treated with γ ray) |
| Ginger | Suravi |
| Turmeric | BSR 1 |
| Tobacco | Jayasri |

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