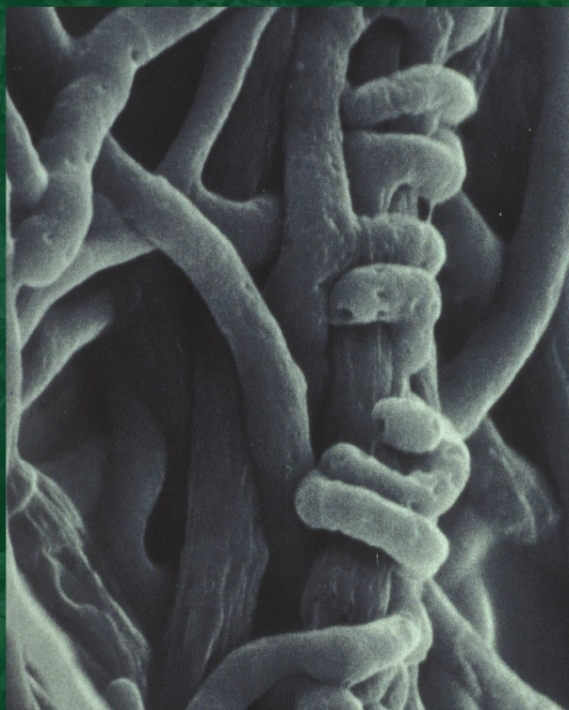
Ulrich Gisi
Ilan Chet
M. Lodovica Gullino
Editors

PLANT PATHOLOGY IN THE 21ST CENTURY 1
Contributions to the 9th International Congress

Recent Developments in Management of Plant Diseases



Springer

Recent Developments in Management of Plant Diseases

Plant Pathology in the 21st Century: Contributions to the 9th International Congress

For other titles published in this series, go to
http://www.springer.com/series/8169

Ulrich Gisi · Ilan Chet · M. Lodovica Gullino
Editors

Recent Developments in Management of Plant Diseases

Springer

Editors
Ulrich Gisi
Syngenta Crop Protection AG
Stein
Switzerland

Ilan Chet
Hebrew University
Rehovot
Israel

M. Lodovica Gullino
Università degli Studi di
Torino, Italy

ISBN 978-1-4020-8803-2 e-ISBN 978-1-4020-8804-9
DOI 10.1007/978-1-4020-8804-9
Springer Dordrecht Heidelberg London New York

Library of Congress Control Number: 2009927088

Contents

Introduction

In addition to being scientifically interesting, plant pathology covers very practical aspects such as the study of symptoms, causes and mechanisms of disease development as well as the development of methods to manage plant diseases. Reliable disease management is the foundation for both the quantity and quality of plant products available to the world population and contributes to maintain the health and beauty of our landscape. Considering that an average of 14% of the produced crops are lost to plant diseases and another 6–12% loss after harvest, particularly in developing countries, it is easy to understand the need of measures to manage plant diseases.

Plant disease management remains an important component of plant pathology and is more complex today than before including new innovation in diagnostic kits for early and more precise diagnosis, the discovery of new modes of action of chemicals with low environmental impact and biological control agents with reliable and persistent activity as well as the development of new plant varieties with durable disease resistance.

This book is a collection of the invited lectures given at the 9th International Congress of Plant Pathology (ICPP 2008), held in Torino, August 24–29, 2008 and is part of a series of volumes on Plant Pathology in the twenty-first Century. It focuses on new developments of disease management and provides a very much updated overview of the state of the art given by world experts in the different fields of disease management. At ICPP 2008, 8 out of 56 Sessions dealt with disease management, demonstrating the importance of the subject. The different chapters deal with basic aspects of disease management, mechanisms of action of biological control agents, innovation in fungicide application, exploitation of natural compounds and resistance strategies. Moreover, the management of soil-borne diseases and disease management in organic farming are covered.

Twenty-five chapters are organised in five sections that cover concepts in chemical and biological control, exploitation of natural compounds, control of soil-borne plant diseases, plant breeding and resistance strategies.

We believe that, besides representing a written testimony of ICPP 2008, this book will be useful for all plant pathologists as well as students in advanced courses interested to go in depth into the exciting world of disease management.

We wish to thank all the colleagues who accepted to be part of this book, Zuzana Bernhart and her group at Springer for the technical support and Laura Castellani for her skilful administrative assistance.

Ulrich Gisi
Ilan Chet
M. Lodovica Gullino

Contributors

Husein Ajwa Department of Plant Sciences, University of California, Davis, 1636 East Alisal Street, Salinas, CA 93905, USA, haajwa@ucdavis.edu

Georg F. Backhaus Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Erwin-Baur-Street 27, Quedlinburg 06484, Germany, georg.backhaus@jki.bund.de

Jonathan Banks Piallago, New South Wales 2609, Australia, apples3@bigpond.com

François Barja Laboratoire de Bioénergétique et de Microbiologie, University of Geneva, 10 Ch. Embrouchis, 1254 Jussy-Lullier, Switzerland, francois.barja@bota.unige.ch

Roland Beffa Department of Biochemistry, Bayer CropScience SA, 14-20 rue Pierre Baizet, 69009 Lyon, France, roland.beffa@bayercropscience.com

Emilie Chapelle Institut des Sciences du Végétal, CNRS, Gif-sur-Yvette, France, chapelle@isv.cnrs-gif.fr

Syama Chatterton Department of Biological Sciences, Simon Fraser University, 8888 University Drive, Burnaby, British Columbia V5A 1S6, Canada, michelle_chatterton@sfu.ca

Dan O. Chellemi United States Department of Agriculture, Agricultural Research Service, United States Horticultural Research Laboratory, 2001 South Rock Road, Fort Pierce, FL 34945, USA, Dan.Chellemi@ars.usda.gov

Amélie Cirou Institut des Sciences du Végétal, CNRS, Gif-sur-Yvette, France;Comité Nord Plants de pommes de terre, Bretteville-du-Grand-Caux, France, cirou@isv.cnrs-gif.fr

Ilan Chet Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, 76100 Rehovot, Israel, chet@agri.huji.ac.il

Yves Dessaux Institut des Sciences du Végétal, CNRS, Gif-sur-Yvette, France, dessaux@isv.cnrs-gif.fr

William E. Dolezal Pioneer Hi-Bred International, Johnston, IA, USA, william.dolezal@pioneer.com

Nawal Kishore Dubey Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi 221005, India, nkdubey2@rediffmail.com

Ralf-Udo Ehlers Department for Biotechnology and Biological Control, Institute for Phytopathology, Christian Albrechts-University, Hermann-Rodewald-Street 9, 24118 Kiel, Germany, ehlers@biotec.uni-kiel.de

Denis Faure Institut des Sciences du Végétal, CNRS, Gif-sur-Yvette, France, faure@isv.cnrs-gif.fr

Damien J. Fleetwood AgResearch, Grasslands Research Centre, Private Bag 11008, Palmerston North, New Zealand, damien.fleetwood@agresearch.co.nz

Paul Fraser Centre for Australian Climate and Weather Research, CSIRO Marine and Atmospheric Research, Aspendale, Victoria 3195, Australia, paul.fraser@csiro.au

Stéphanie Gamet Department of Biochemistry, Bayer CropScience SA, 14-20 rue Pierre Baizet, 69009 Lyon, France, stephanie.gamet@bayercropscience.com

Abraham Gamliel Faculty of Agricultural, Food and Environmental Quality Sciences, Rehovot 76100, Israel and ARO, Volcani Center, Bet Dagan 50250, Israel, agamliel@agri.gov.il

Ulrich Gisi Syngenta Crop Protection AG, Schaffhauserstrasse, CH-4332 Stein, Switzerland, ulrich.gisi@syngenta.com

Maria Lodovica Gullino Agroinnova, University of Torino, Via Leonardo da Vinci 44, 10095 Grugliasco (TO), Italy, marialodovica.gullino@unito.it

Guillaume Henry Walloon Center of Industrial Biology, Gembloux Agricultural University, Passage des dépotés, 2, B-5030 Gembloux, Belgium, henry.g@fsagx.ac.be

Imre J. Holb Centre for Agricultural Sciences and Engineering, University of Debrecen, P.O. Box 36, H-4015 Debrecen, Hungary and Plant Protection Institute, Hungarian Academy of Sciences, P.O. Box 102, H-1525 Budapest, Hungary, holb@agr.unideb.hu

Hideo Ishii National Institute for Agro-Environmental Sciences (NIAES), Kannondai 3-1-3, Tsukuba, Ibaraki 305-8604, Japan, hideo@niaes.affrc.go.jp

Richard D. Johnson AgResearch, Grasslands Research Centre, Private Bag 11008, Palmerston North, New Zealand, Richard.Johnson@agresearch.co.nz

Jaacov Katan Faculty of Agricultural, Food and Environmental Quality Sciences, Rehovot 76100, Israel and ARO, Volcani Center, Bet Dagan 50250, Israel, katan@agri.huji.ac.il

Sebastian Kiewnick Plant Protection/Zoology, Research Station ACW, Agroscope Changins-Waedenswil, Schloss, P.O. Box 185, 8820 Waedenswil, Switzerland, sebastian.kiewnick@acw.admin.ch

Jürgen Köhl Plant Research International, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands, jurgen.kohl@wur.nl

Ashok Kumar Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi 221005, India, ashokkumarbhu@gmail.com

Marie-Pascale Latorse Department of Biochemistry, Bayer CropScience SA, 14-20 rue Pierre Baizet, 69009 Lyon, France, marie-pascale.latorse@bayercropscience.com

Xavier Latour Microbiologie du Froid, Université de Rouen, Evreux, France, xavier.latour@univ-rouen.fr

George Lazarovits Agriculture and Agrifood Canada, 1391 Sandford Street, London, Ontario, Canada, George.Lazarovits@AGR.GC.CA

Andy Leadbeater Syngenta Crop Protection AG, Schwarzwaldallee 215, CH-4058, Basel, Switzerland, andy.leadbeater@syngenta.com

Marc-Henri Lebrun Université de Lyon, F-69622, Lyon, France; Université de Lyon 1, Villeurbanne, France; CNRS, UMR; INSA de Lyon, F-69621, Villeurbanne, France; Bayercropscience, F-69263, Lyon, France, Marc-Henri.Lebrun@bayercropscience.com

Shuxian Li United States Department of Agriculture – Agricultural Research Service, Crop Genetics and Production Research Unit, Stoneville, MS 38776, USA, Shuxian.li@ars.usda.gov

Frank J. Louws Department of Plant Pathology, North Carolina State University, 27695 Raleigh, NC, USA, frank_louws@ncsu.edu

Roger D. Magarey Center for Integrated Pest Management, North Carolina State University, 1730 Varsity Drive, Suite 300, Raleigh, NC 27606, USA, Roger_Magarey@ncsu.edu

Scott Mattner Department of Primary Industries, Knoxfield Centre, PMB 15, Ferntree Gully Delivery Centre, Victoria 3156, Australia, scott.mattner@dpi.vic.gov.au

Lucile Mauprivez Department of Biochemistry, Bayer CropScience SA, 14-20 rue Pierre Baizet, 69009 Lyon, France, lucile.mauprivez@bayercropscience.com

Kimberley J. May Institute of Molecular BioSciences and Bio-Protection Research Centre, Massey University, Private Bag 11222, Palmerston North, New Zealand, k.may@massey.ac.nz

Brian B. McSpadden Gardener Department of Plant Pathology-OARDC, The Ohio State University, 1680 Madison Avenue, Wooster, OH 44691, USA, mcspadden-garden.1@osu.edu

Thomas J. Moore Enza Coastal Seeds, Salinas, CA, USA, t.moore@enzasalinas.com

William Ntow University of California, Davis, California, USA, wjntow@ucdavis.edu

Patricia A. Okubara United States Department of Agriculture, Agricultural Research Service, Root Disease and Biological Control Research Unit, Pullman, WA 99164-6430, USA, pokubara@wsu.edu

Marc Ongena Walloon Center of Industrial Biology, Gembloux Agricultural University, Passage des déportés, 2, B-5030 Gembloux, Belgium, ongena.m@fsagx.ac.be

Nicole Orange Microbiologie du Froid, Université de Rouen, Evreux, France, nicole.orange@univ-rouen.fr

Timothy C. Paulitz United States Department of Agriculture, Agricultural Research Service, Root Disease and Biological Control Research Unit, Pullman, WA 99164-6430, USA, paulitz@wsu.edu

Philippe Peret Department of Biochemistry, Bayer CropScience SA, 14-20 rue Pierre Baizet, 69009 Lyon, France, philippe.peret@bayercropscience.com

Ian Porter Department of Primary Industries, Knoxfield Centre, PMB 15, Ferntree Gully Delivery Centre, Victoria 3156, Australia, ian.j.porter@dpi.vic.gov.au

Joeke Postma Plant Research International, P.O. Box 16, 6700 AA Wageningen, The Netherlands, joeke.postma@wur.nl

Zamir K. Punja Department of Biological Sciences, Simon Fraser University, 8888 University Drive, Burnaby, British Columbia V5A 1S6, Canada, punja@sfu.ca

Frédéric Schmitt Department of Biochemistry, Bayer CropScience SA, 14-20 rue Pierre Baizet, 69009 Lyon, France, frederic.schmitt@bayercropscience.com

Kurtis L. Schroeder United States Department of Agriculture, Agricultural Research Service, Root Disease and Biological Control Research Unit, Pullman, WA 99164-6430, USA, kschroeder@wsu.edu

Barry Scott Institute of Molecular BioSciences and Bio-Protection Research Centre, Massey University, Private Bag 11222, Palmerston North, New Zealand, d.b.scott@massey.ac.nz

Ravindra Shukla Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi 221005, India, ravischolarbhu@gmail.com

Priyanka Singh Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi 221005, India, priyabhusingh@gmail.com

Catherine Sirven Department of Biochemistry, Bayer CropScience SA, 14-20 rue Pierre Baizet, 69009 Lyon, France, catherine.sirven@bayercropscience.com

Krishnamurthy Subbarao Department of Plant Pathology, University of California, Davis, 1636 E. Alisal Street, Salinas, CA, USA, kvsubbarao@ucdavis.edu

Daigo Takemoto Institute of Molecular BioSciences and Bio-Protection Research Centre, Massey University, Private Bag 11222, Palmerston North, New Zealand, dtakemo@agr.nagoya-u.ac.jp

Aiko Tanaka Institute of Molecular BioSciences and Bio-Protection Research Centre, Massey University, Private Bag 11222, Palmerston North, New Zealand, aikotana@agr.nagoya-u.ac.jp

Philippe Thonart Walloon Center of Industrial Biology, Gembloux Agricultural University, Passage des dépotés, 2, B-5030 Gembloux, Belgium, thonart.p@fsagx.ac.be

Valérie Toquin Department of Biochemistry, Bayer CropScience SA, 14-20 rue Pierre Baizet, 69009 Lyon, France, valerie.toquin@bayercropscience.com

Shachaf Triky-Dotan Institute of Agricultural Engineering, ARO, The Volcani Center Bet Dagan 50250, Israel, shachaf@volcani.agri.gov.il

Stéphane Uroz Institut des Sciences du Végétal, CNRS, Gif-sur-Yvette, France, uroz@nancy.inra.fr,

Ruth E. Wrenn Institute of Molecular BioSciences and Bio-Protection Research Centre, Massey University, Private Bag 11222, Palmerston North, New Zealand, R.E.Wrenn@massey.ac.nz

Carolyn A. Young The Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK 73401, USA, cayoung@noble.org

Jean-Luc, Zundel Department of Biochemistry, Bayer CropScience SA, 14-20 rue Pierre Baizet, 69009 Lyon, France, jean-luc.zundel@bayercropscience.com

Part I
Concepts in Chemical Control

Chapter 1
The Challenges of Chemical Control of Plant Diseases

Andy Leadbeater and Ulrich Gisi

Abstract Since the first fungicide, sulphur, was used to control powdery mildew on grapes, production of most crops has become dependent on the use of fungicides to avoid disease losses. In the late 1840s the Irish potato famine proved the necessity for chemical intervention to prevent human and economic disaster. Recently it has become increasingly difficult for growers to control crop diseases. Genetic resistance of crops towards diseases has been in many cases short-lived (for example cereal rusts), and GMOs have only limited success for disease control and acceptability. With more intensive cropping, new diseases have arisen which are devastating if not controlled, such as Asian Rust of soybean. In addition, new races and more aggressive pathotypes of diseases have arisen. All these changes require the rapid development of chemical control measures to prevent economic disaster, since reliance on genetic resistance and cultural techniques have been insufficient. Intensive use of chemical control measures has in turn led to its own challenges, including resistance to fungicides. The sustainable use of fungicides to prolong their effectiveness and usefulness to growers is key, and the implementation of resistance management strategies an essential part of this. Only if the long-term effectiveness of fungicides can be ensured will industry invest the money and resources required for their discovery and development, especially considering the high standards of today's registration requirements. The Fungicide Resistance Action Committee (FRAC) and its network play a vital role in the design and support of these strategies.

Keywords Fungicides • Chemicals • Research • Development • Resistance • Legislation

A. Leadbeater (✉) and U. Gisi
Syngenta Crop Protection AG, Schwarzwaldallee 215, CH-4058 Basel, Switzerland
e-mail: andy.leadbeater@syngenta.com

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,*
Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_1,

1.1 The Need for Chemical Control of Plant Pathogens

It has been known for many years that the world population is increasing. Statistics from United Nations (UNFPA, 2006) predict the world population to grow from the current 5 billion to 8.9 billion by 2050, with clear consequences on the quantity of food that will need to be produced to avoid widespread hunger. In addition to the effects of this increase in the number of people, it is clear that diets are changing and will continue to do so. In Asia in particular, increasing wealth has led to a preference for more variety in the diet, and more meat and poultry is now being eaten. As a consequence of the lower energy efficiency of a meat-based diet compared with the direct consumption of grain (rice, wheat, maize, etc.) this dietary change will continue to drive further increases in the global demand for grain crops. Finally, the quite recent demand for biofuels, although today quite small with regard to the use of cereals, is predicted to grow and will also add to the global requirement for grain. Of course, the increasing demand for food has an impact much beyond grain crops and demand will increase for all major crops including legumes, vegetables and fruits.

In the 1960s, on average 1 ha of land was required to grow the crops necessary to feed two people. Nowadays that land has to support double that number and it is estimated that by 2010 the same area will have to feed five people (Syngenta estimation). Some new land may come into agricultural production but it is forecast that currently productive areas will be lost through industrialisation. Already today, more people live in the cities of the world than in the country, which puts even more pressure on agricultural production.

All the above is happening at a time when the world stocks of cereals are at their lowest levels for many years, and as a consequence the world price level of many commodities such as rice and wheat have risen dramatically with very significant consequences in the affordability of food to many millions of people. Put together, it is clear that the world is leading towards a scarcity of food, which has even been referred to as a world food crisis.

The available statistics all add up to a clear situation, with clear consequences for the future – the world needs to grow more food from the available land and with the available water in a sustainable manner. The area of agriculturally productive land is likely to stay stable at around 1.5 billion ha; the population is likely to increase by about 80 million/year. Amongst the many threats to crop production on a worldwide basis is the damage to these crops caused by pests (including fungal pathogens, insects and weeds). Crop losses due to such pests have been calculated to be substantial and crop protection techniques and products have been developed to reduce these losses and make crop productivity as high as possible. Estimates of actual losses in crop production worldwide have been published by Cramer (1967) and Oercke et al. (1994) and recently updated for major food and cash crops for the period 2001–2003 (Oercke, 2006). Whereas weeds are overall the most important pest group in crop production worldwide, the incidence and impact of pathogens is also substantial (Table 1.1). Potential crop losses from pests include losses without

Table 1.1 Estimated loss potential of pathogens and actual losses in six major crops worldwide in 2001–2003 (after Oercke, 2006)

		Crop losses (%)[a] due to			
	Attainable production	Pathogens		Total crop pests	
Crop	(Mt)	Potential	Actual	Potential	Actual
Wheat	785	15.6 (12–20)	10.2 (5–14)	49.8 (44–54)	28.2 (14–40)
Rice	933	13.5 (10–15)	10.8 (7–16)	77.0 (64–80)	37.4 (22–51)
Maize	891	9.4 (8–13)	8.5 (4–14)	68.5 (58–75)	31.2 (18–58)
Potatoes	518	21.2 (20–23)	14.5 (7–24)	74.9 (73–80)	40.3 (24–59)
Soybeans	245	11.0 (7–16)	8.9 (3–16)	60.0 (49–69)	26.3 (11–49)
Cotton	79[b]	8.5 (7–10)	7.2 (5–13)	82.0 (76–85)	28.8 (12–48)

[a]Figures in parentheses indicate variation among 19 regions.

[b]Seed cotton.

physical, biological or chemical crop protection. Actual losses comprise the crop losses sustained despite the crop protection practices employed. Absolute losses vary amongst crops depending upon their ability to compensate for the effects of pathogen attack and tend to be higher under conditions of high productivity and where climatic conditions are favourable to the pathogen. Use of crop protection measures, which include chemical pesticides, obviously has not completely prevented crop losses, but they have significantly contributed to productivity and quality of produce worldwide. In many regions crop protection measures have enabled farmers to increase crop productivity considerably without losing an economically non-acceptable proportion of the crop to pests. Crop yields would be around half their current levels if no crop protection measures were implemented. But even with crop protection, around a third of crop yields are still lost to weeds, diseases and insects. These data show that there are still significant advances in crop protection practices, including chemical disease control, which need to be made in order to maximise worldwide crop production and avoid unnecessary losses. Crop yields will probably have to increase by around 50% on a global level by 2030 to ensure food security. This implies a higher annual rate of yield gain than has been achieved in the major grain crops in recent years and has led to calls for a ‘second green revolution’.

1.2 Options for the Control of Plant Pathogens

To protect crops from diseases there are several crop production and protection options. Organic crop production is one of these options and has been shown to be sustainable. Yields in organic crop production are in general significantly lower than under conventional management. However, these yield differences vary between crops, and to a certain extent also between countries and regions analysed.

For cereals, the range of observed typical yield ratios is quite narrow for most countries, especially in central and Western Europe. Cereal yields in organic production are typically 60–70% of those under conventional management although vegetable yields are often just as high (Offermann and Nieberg, 2000). Few data are available on pasture and grassland yields in organic farming, reported values lie in the range of 70–100% of conventional yields, depending on the intensity of use. Taking organic cereal yields at 60% of conventional systems, this equates to a requirement of 67% more area to produce the same yield. When faced with a growing world population, the need to feed them, and growing urbanisation, this land is unlikely to be available. On the contrary, an increase in intensity of crop production is most likely to be required.

Resistance or tolerance to crop diseases through natural traits or genetically modified crops (GMO) is an area which is under intensive scientific study. Plant breeding for disease tolerance, using classical or marker-assisted selection approaches, is a very important tool in disease management programmes and the use of varieties of plants resistant to particular diseases has proved to be very effective, e.g. stem rust of wheat, rust of dry bean, and *Rhizoctonia* root rot of sugar beet. It must be realised however that the product development time for successful new varieties is similar to that of a new chemical, and in cereals advances take 12 years to bring benefits to the market (AEBC, 2005). Genetically modified crops are well established in several parts of the world, most notably North America and South America, India and China. For tolerance to chemical herbicides and to insects GM crops have been proved to be technically feasible and have been successfully and commercially implemented. The introduction of genetically modified crops has however been more difficult in several other areas of the world (especially Europe) because of consumer and environmental concerns. Examples of tolerance to important crop pathogens have been few however and, to the authors' current knowledge, none have been commercially introduced. Whether disease tolerance is brought about by naturally occurring traits or genetic modification, there is always the doubt about the durability of the disease resistance – plant pathogens have an established history of being able to overcome the host resistance of plants. It is probable that even if disease tolerance becomes successfully implemented in major economic crops such as cereals, potatoes or soybeans these traits will themselves require protecting through the integrated approach of using chemical fungicides in programmes to manage the diseases and avoid the tolerance breaking down.

Biological control methods have been proven to be very effective in certain situations, most notably in the control of several insect pests with natural predators, or with "biopesticides" such as *Bacillus thuringiensis.* Biological control approaches do exist against plant pathogens, based upon organisms such as *Coniotherium minitans*, *Bacillus subtilis* and others, and under the right conditions can be very successful. Challenges to wide scale biological control of plant pathogens in major agricultural crops however include issues with environmental robustness, limitations in production capacity (and therefore availability of product), specialist supply chain requirements such as refrigeration and a certain lack of reliability of control of the

target pathogen. For these reasons, biological control of plant diseases in major agricultural crops has so far only met with limited success.

The control of crop diseases with chemical fungicides has had a successful history for more than a century. They are integral to the production of crops in many countries of the world, resulting in increased yields and farmer income. Economic benefit studies have been carried out which show conclusively that without fungicides for control of plant pathogens, production of some crops would be impossible in parts of the world (Gianessi and Reigner, 2005; Cook and Jenkins, 1988; Cuthbertson and Murchie, 2003). Fungicides have been responsible for ensuring the production of many crops over many years, such as protection of the potato crop against the late blight pathogen *Phytophthora infestans*, the cause of the Irish potato famine in 1846, which led to the deaths of 1.5 million people and the emigration of a similar number of people, mainly to North America. Cooke (1992) wrote that "without the use of fungicides, large scale commercial potato production in Ireland would be impossible".

1.3 The Challenges of Chemical Discovery

According to recent studies (Crop Life International, 2007), a new crop protection product takes around 10 years and approximately US $200 million to be developed (from discovery to first sales). On average around 25%, and as much as 40%, of this cost is invested in researching toxicological risks, environmental fate and impacts. The process of identifying an active ingredient is only the start of the R&D process. For every active ingredient tested, only one in tens or hundreds of thousands actually makes it to the market. This is because there are a number of different obstacles that need to be overcome before a crop protection product is ready to go to market. According to Crop Life International, on average, the ten leading crop protection companies spend approximately 7.5% of sales on research and development, amounting to a Research and Development expenditure of over US $2 billion. This ratio places the plant science industry among the most R&D intensive business sectors.

Fungicides, as is the case for all crop protection chemicals, must be targeted to meet the needs of farmers and growers, to solve the problems which regularly occur to limit the productivity (yields) and quality of produce. They must answer these "biological crop needs" and at the same time must be cost-effective to make their use worthwhile. At the same time the target markets need to be viable to the company inventing and producing the products to justify the large investment of money and resources required to fully develop the products, bring them to the market and support them in the market place. In addition to these requirements, products must meet modern day requirements for human and environmental safety, and be compatible with other crop protection practices, avoiding undesired effects on, for example, beneficial organisms. Fungicide markets change and so do the market potential of crop/disease combinations. For example whilst the worldwide market for the control of powdery mildews has traditionally been targeted by most

agrochemical companies for new product, it has become apparent that on its own this market has become critical in terms of a cost–benefit ratio for industry. In other words, the high level of investment required to bring a new, powdery mildew specific fungicide to the market is becoming difficult to justify against the rather crowded and limited market. On the other hand, new opportunities have opened up and rust control in soybeans in Latin America (a fungicide market estimated at somewhere around US $1 billion) is today an important target for new fungicides. Forecasting these market changes is difficult and challenging and another factor in the complexity of new product invention and development.

So it will be realised that the search for the "ideal" fungicide is complex and difficult. This is because there is a wide combination of properties of the fungicide that need to be considered and met which include:

Biologically Efficient

- High selectivity (on target)
- Fast impact (action)
- Optimal residual effect
- Good plant tolerance
- Low risk of resistance development

User Friendly

- Low acute toxicity
- Low chronic toxicity
- Good formulation characteristics
- Safe packaging
- Easy application method
- Long storage stability

Environmentally Sound

- Low toxicity for non-target organisms
- Fast degradation in the environment
- Low mobility in soil
- No relevant residues in food and fodder
- Low application rate

Economically Viable

- Good cost/profit ratio for the farmer
- Broad use
- Applicability in Integrated Crop and Pest Management
- Innovative product characteristics
- Competitive
- Patentable

To meet all these needs and successfully bring a new active substance to the market is a real challenge to industry. To achieve a high level of potency (activity) against

a target pathogen whilst having minimal or no adverse effect on the crop, on beneficial organisms and on humans and other mammals is extremely difficult. There has been a large effort by academia and industry in recent years to use target site modelling in order to produce "designer molecules" to selectively inhibit fungi at specific target sites and in that way find new modes of action for products and at the same time avoid target sites which are known to be problematic in terms of mammalian toxicology. However, this has proved to be extremely difficult in practice and there have been, to the authors' knowledge, no product which has been successfully discovered and developed utilising these techniques. It also appears that the trend which started a number of years ago of so-called "high throughput screening" and combinatorial chemistry where a huge number of randomly selected chemicals are screened for biological activity against a number of key target diseases has proved not more successful than other approaches. The concept is that according to the laws of averages, the greater the number of chemicals that are screened, the greater the chance of finding interesting biological activity which can then be further optimised. This approach however has proved to be somewhat too untargeted and has been largely abandoned.

The current approach in industry is still very much around optimisation in known chemical classes and modes of action, although at the same time much research goes into the area of discovering new classes of fungicidally active chemistry and hopefully, new modes of action. All companies have their own key "search targets", that is a clearly defined research strategy defining what they want a new product to look like, what its characteristics should be and therefore what the market potential will be. These search targets include elements such as a completely new mode of action against the key diseases, and maybe also multiple sites of action (multisite) or at least a very low risk of resistance occurring. These requirements are easy to define and state – but success in discovering such solutions remains a real challenge. As a consequence, it is a fact that many of the novel fungicides brought to the market recently, as well as several due to be introduced to the market over the next few years, are site specific fungicides, acting against the pathogens at a single binding site in a biochemical pathway. From a product safety point of view this tends to be a good thing, especially if the target pathway is one that does not exist in mammals. However, from the consideration of resistance risk, and consequently the long-term sustainability of the product in the market, this might not be so favourable, depending on the nature of the mode of action, the pathogen and the consequences to the pathogen of the genetic changes needed to adapt to the fungicide.

1.4 The Successes of Industry

The question arises – if it is so difficult and challenging for industry to find and develop new innovative solutions to address the problems faced by farmers in combating crop diseases, how successful has industry actually been? The answer is that in fact industry has been very successful despite the increasing challenges and

hurdles. Considering the history of significant fungicide discoveries and introductions since 1940, the rate of innovation has continued at a rapid rate despite the increasing requirements and escalating costs of research and development (Russell, 2005). During the 20 year period from 1940 to 1960 there were around 13 new fungicides brought to the market, including several that are still on the market today having satisfied modern regulatory requirements (Table 1.2). These include thiram, captan, folpet and dodine; these older products have proved to still provide a valuable contribution to disease management some 60 years after their discovery. In the 10 year period from 1960 to 1970, largely due to the increasing market potential for fungicides and the realisation of the economic importance of controlling plant diseases, the rate of innovation increased, with some 25 new fungicides introduced to the market. These included significant innovations to the market, such as the multisite fungicides mancozeb and chlorothalonil which proved to be very broad spectrum and potent in their activity, whilst carrying a low risk of resistance developing. Such fungicides also remain important in the market today. During this

Table 1.2 Key fungicide introductions from 1940 to the present day (modified from Russell, 2005; sorted chronologically)

Year	Fungicides	Number
1940–1960	Thiram, zineb, nabam, biphenyl, oxine copper, tecnazene, captan, folpet, fentin acetate, fentin hydroxide, anilazine, blasticidin S, maneb, dodine, dicloran	13
1961–1970	Mancozeb, captafol, dithianon, propineb, thiabendazole, chlorothalonil, dichlofluanid, dodemorph, kasugamycin, polyoxins, pyrazophos, ditalimfos, carboxin, oxycarboxin, drazoxolon, tolyfluanide, difenphos, benomyl, fuberidazole, guazatine, dimethirimol, ethirimol, triforine, tridemorph	24
1971–1980	Iprobenfos, thiophanate, thiophanate-methyl, validamycin, benodanil, triadimefon, imazalil, iprodione, bupirimate, fenarimol, nuarimol, buthiobate, vinclozolin, carbendazim, procymidone, cymoxanil, fosetyl-Al, metalaxyl, furalaxyl, triadimenol, prochloraz, ofurace, propamocarb, bitertanol, diclobutrazol, etaconazole, propiconazole, tolclofos-methy, fenpropimorph	29
1981–2000	Benalaxyl, flutolanil, mepronil, pencycuron, cyprofuram, triflumizole, flutriafol, penconazole, flusilazole, diniconazole, oxadixyl, fenpropidin, hexaconazole, cyproconazole, myclobutanil, tebuconazole, pyrifenox, difenoconazole, tetraconazole, fenbuconazole, dimethomorph, fenpiclonil, fludioxonil, epoxiconazole, bromuconazole, pyrimethanil, metconazole, fluquinconazole, triticonazole, fluazinam, azoxystrobin, kresoxim-methyl, metaminostrobin, cyprodinil, mepanipyrim, famoxadone, mefenoxam, quinoxyfen, fenhexamid, fenamidone, trifloxystrobin, cyazofamid, acibenzolar-S-methyl)	42
2001–present	Picoxystrobin, pyraclostrobin, prothioconazole, ethaboxam, zoxamide, fluopicolide, flumorph, benthiavalicarb, iprovalicarb, mandipropamid, boscalid, silthiofam, meptyldinocap, amisulbrom, orysastrobin, metrafenone, ipconazole, proquinazid (plus several known pipeline products)	18+

time significant advances were also made in the control of seed-borne fungal diseases such as smuts and bunts, with fungicides such as carboxin, guazatine and fuberidazole developed for use as seed treatment products (Table 1.2).

From 1970 to 1980, the rate of innovation continued to increase with around 30 new fungicides being introduced. Agriculture was booming in Western Europe and the Americas at this time, government-funded research into crop protection was increasing, the importance of crop diseases and their control was increasingly understood and as a consequence industry stepped up their invention processes to discover totally new chemical classes and modes of action. Key introductions to the market during this time included carbendazim, the first major triazole fungicides (triadimefon, propiconazole), key novel fungicides for the control of downy mildews and late blight such as metalaxyl and cymoxanil, and the amine fungicide fenpropimorph (Table 1.2).

In the following 20 year period from 1980 to 2000, agricultural markets continued to increase at first, but in time over-production of crops such as cereals resulted in a slowing down of the agrochemical market especially in Western Europe (where a large proportion of the fungicide sales were made). At the same time new legislation was introduced concerning the requirements to bring new crop protection products to the market, notably in the USA (Environmental Protection Agency – EPA), and Europe (introduction of European Directive 91/414). At this time, experts forecasted a dramatic decrease in the numbers of innovative new products that would be brought to the market as a consequence of the new legislations. Indeed, for a number of years the rate of introduction of new products slowed dramatically because of delays by regulatory authorities in implementing the new rules and the sheer increases in workloads caused. But over time, in fact the investments made by agrochemical companies continued to increase. As a consequence, over 40 new fungicides were discovered and introduced successfully to the market over that time. These innovations included several significant new triazoles including epoxiconazole, cyproconazole, difenoconazole, as well as novel chemistry with new modes of action such as fludioxonil, pyrimethanil, cyprodinil, acibenzolar-S-methyl, and the ground-breaking new strobilurin fungicides, azoxystrobin and kresoxim-methyl (Table 1.2). The triazole and strobilurin fungicides formed the basis for today's continued successes in these chemistries which consist of a great proportion of fungicide sales around the world.

From the year 2000 up to the present day, industry has continued to invest and has been successful in the discovery of new fungicides. Since the year 2000, many major new introductions have been made to the market including "new generation" triazoles and strobilurins, and chemistries with new modes of action such as metrafenone, mandipropamid, proquinazid and boscalid. This continued rate of new product introductions by Research and Development oriented agrochemical companies is a demonstration of the continued commitment to agriculture by the chemical industry which has resulted in increasing investment by companies into the future, despite rising costs and the threats of declining markets and generic competition. This contrasts strongly with the pharmaceutical industry where, despite increasing R&D expenditure the rate of innovation has fallen dramatically.

1.5 The Importance of Diversity and Sustainability of Chemical Fungicides

The section above has demonstrated that the R&D-based chemical companies have been extremely successful in new product innovation and therefore bringing to the market a rich diversity of solutions for farmers across the globe to use in their efforts to increase agricultural productivity and secure their yields and livelihoods. This chemical diversity has proved to be essential for several reasons, and without the continued research efforts of the leading companies, farmers would be dependent upon aging, less effective products supplied by generic manufacturers with their problems of unsustainability due to outdated product safety, as well as losses of effectiveness due to resistance. Fortunately in today's world this diversity of solutions has so far been maintained.

Innovation and diversity in fungicide research has brought many benefits to agriculture. This includes advances in the safety of products to humans and other organisms as well as the environment. Modern products are well defined, extensively tested and proved to cause minimum risk to operators, consumers and the environment when used according to the manufacturers' recommendations. In addition, use rates (and therefore environmental loads) of fungicides have tended to decrease with some key products: in the case of potato late blight control, mandipropamid or cyazofamid are fully effective at low rates such as 80–150 g active ingredient per hectare, compared with the rates of 2,000–4,000 g active ingredient required for older products. At the same time, LD50's have stayed the same and even increased. Whilst biological activity against target organisms has increased, mammalian toxicity has been significantly reduced in terms of dose applied.

Another example of the importance of having a strong and diverse armoury of chemical fungicides at the disposal of farmers is the case of soybean rust, caused by *Phakopsora pachyrhizi*. This pathogen was first detected in Japan in 1902 and was naturally endemic in several countries in Asia. This is no surprise since soybeans originated in Asia and have a long Japanese history. However, the so-called "Asian Soybean Rust" is rarely a problem in Asia, probably because of a lack of intensity of production of this crop together with the occurrence of cold weather in the winter resulting in a natural slowing of the disease progress. The disease has however spread out of its natural origin to other parts of the world, from Zimbabwe in 1998, to South Africa and Paraguay in 2001, Brazil in 2002, Argentina in 2003 and the USA in 2004–2005. When the disease reached Brazil in 2002 it caused severe epidemics, crop losses and panic amongst farmers in this country. Soybeans are grown very intensively in Brazil with consecutive plantings in neighbouring areas and huge areas of monoculture. Because the winter months remain mild and warm the pathogen is easily able to over-winter and be ready for re-infection the following cropping season. In addition to this, plant breeding efforts had not been aimed at this disease which had not previously been recorded in the country.

The disease reached epidemic proportions in Brazil in 2003 when yield losses (in untreated crops) were 80% or higher. Yield losses were calculated at 4.5 million

tonnes in 2004 (Embrapa Soja) with an economic impact of US $2 billion (including the costs of fungicide treatment). Two major classes of fungicides, the triazoles and strobilurins (QoIs) were found to be highly effective against soybean rust. Mixtures of triazoles and strobilurins provided the best disease control due to the complementary (curative plus long lasting protective) characteristics. It is no exaggeration to say that it is only due to the success of chemical fungicides in controlling this disease that soybean production has been secured in Brazil, and complete crop failure across the country has been avoided. Advances have been made in plant breeding and useful levels of tolerance of varieties to rust have been achieved. The future sustainable strategies for the management of this disease in soybeans must be based around utilising rust tolerant varieties plus an adequate level of chemical fungicides.

Another example of new invasive species may be black stem rust of wheat (*Puccinia graminis*). For several decades the historically enormous problem of wheat stem rust has been "solved" through the use of genetic resistance (CIMMYT, 2005). In Eastern Africa, that resistance has now been overcome by a new physiological race of the pathogen designated as Ug99. With the long distance travel of rust spores in the jet streams and on the clothing of world travellers, it is believed to be only a matter of time until Ug99 reaches across the Saudi Arabian peninsula and into the Middle East, South Asia, and eventually, East Asia and the Americas. The current situation is a wake-up call about the continuing, and potentially devastating, impact that the rusts can have on susceptible varieties. Although the main thrust of work in securing food supplies against this threat in Africa has been by plant breeding, work by industry and other researchers has demonstrated that chemical fungicides available today, such as triazoles, are very effective in controlling this disease.

1.6 The Threat of Fungicide Resistance and Its Management

An important part of the assessment of new (and current) fungicides is resistance risk and resistance management. Fungicide resistance happens when populations of target pathogens arise that are no longer sufficiently sensitive to a fungicide to be controlled adequately in the field (FRAC, 2007a). Resistance generally appears as a response to repeated use of a fungicide or to repeated use of chemically related fungicides expressing a common mechanism of antifungal action. Practical resistance to fungicides has proved to be quite common over the years for a range of fungicide classes. The likelihood of resistance to evolve is a key part of new product evaluation (resistance risk assessment) (FRAC, 2007b). Fungicide resistance is an important consideration for farmers and industry alike, it is a major threat to the sustainability and longevity of a fungicide product in the market. For the farmer it is important to have a guarantee of product effectiveness to protect the crop, and to have effective solutions available. For industry it is important that the significant investment in the research and development of a new product is paid back through a long period of success in the market. So it is in the interests of all that fungicide resistance is managed. To further support the aims of resistance management, the Fungicide Resistance Action

Committee (FRAC, www.FRAC.info) was formed as a group of industry scientific experts to give use recommendations and to study the science of fungicide resistance. As a part of the work of FRAC, new and established fungicides are classified according to their mode of action and resistance risk. This grouping is recognised worldwide as a key basis for designing resistance management strategies.

The latest edition of the FRAC Code List shows there to be an armoury of more than 170 fungicides, grouped in around 50 mode of action groups. This seems to be a fortunate situation in terms of the required diversity to manage resistance problems. It must however be remembered that not all products are registered or available in all countries of the world and of course not all are effective against the same crop/pathogen combinations. Therefore the options to the farmer for their particular situation are often much more limited.

An analysis of the FRAC Code list shows that around 19% of fungicides are currently classified as being of high or high to medium risk of resistance problems arising (Table 1.3). A further 35% are classified as medium risk, with the remaining 46% classified as either low, low to medium or unknown resistance risk. This seems to be a reasonably comfortable situation at first consideration, and the assumption could be made that there is already today almost a luxury situation with regards to diversity and resistance risk. However, when global sales are used as an indicator of the actual use in agriculture of these fungicides (popularity due to effectiveness, benefits, ease of use, etc.) a quite different picture can be seen. According to industry sales figures for 2006, sales of high to medium risk fungicides represents around 65% of the market, with low resistance risk fungicides only representing 22% of sales by value. This is due to the immense success and popularity of the strobilurin (QoI) and triazole (DMI) fungicides which are high and medium resistance risk fungicides, respectively. Resistance management for these groups of fungicides is successfully managed today by the use of combinations of the two key groups with each other and with other fungicides with different modes of action and lower resistance risk. Key amongst these mixture partners are the EBDC fungicides such as mancozeb, and chlorothalonil.

It is for the reasons described above that it remains important for the agricultural industry to respect and practice effective resistance management for fungicides, for innovation and research to continue in the search for novel modes of action, for the

Table 1.3 Resistance risk classification of fungicides according to the FRAC code list 2008[a]

Resistance risk classification	Number of fungicide groups (FRAC code list)	Number of fungicides	Worldwide sales 2006 (US $ × 1,000)[b]
High	4	27	1,951
High to medium	2	6	240
Medium	10	59	2,747
Medium to low	11	36	754
Low	12	25	1,701
Not known	12	17	332
Total	51	170	7,725

[a]Excluding bactericides and unclassified molecules.

[b]Calculated values based on Philips McDougall data.

support of integrated control practices (cultural, varietal, biological, chemical) and for the maintenance in the markets of a safe, effective diversity of fungicide products.

1.7 The Impact of Future Legislation

Increasing legislation globally has had a big impact on the availability to farmers of crop protection chemicals and has dramatically increased the costs to industry of supporting existing products and bringing new ones to the market. Several products have been lost to the market quite justifiably, because of lost effectiveness, very small sales, or unacceptable safety characteristics as judged by modern standards. We all agree that the safety of the food we eat is of the utmost importance – which is why crop protection products are amongst the most highly regulated chemicals. All authorized products go through rigorous independent safety and quality tests before distribution at country level (Backhaus, 2009, this volume). However, the trend of declining numbers of active substances in the market continues and this is most clear in the European Union. Since the implementation in 1991 of the European directive 91/414/EEC concerning the placing of crop protection products on the market in Europe, the crop protection products portfolio in Europe has already been very seriously impacted. According to the European Crop Protection Association (www.ecpa.eu), of the 952 crop protection products that existed previously, 589 have already been eliminated. Of the remaining 268 list three and four compounds still under review at the time of writing this contribution, it is possible that a significant proportion may fail to achieve Annex I inclusion, and for those which do, many uses will no longer be supportable under today's regulatory environment. The directive is currently being revised. Included in the proposed revision is the intention to judge crop protection products on hazard based cut-off criteria rather than the risk-based assessments which are carried out today. If the directive is implemented according to the current proposals it is likely to lead to further and significant attrition of the remaining crop protection products portfolio resulting in the loss of further active ingredients valuable to modern agriculture, with additional impact on the registration of novel products.

There has so far been no Europe-wide impact assessment of the likely consequences to agriculture of the proposed revised directive. However, a study has been carried out by the UK's regulatory authority, the Pesticides Safety Directorate (PSD) who has made an assessment of the impact on crop protection in the UK of the 'cut-off criteria' and substitution provisions in the proposed new Regulation (PSD, 2008). According to this assessment, and depending on the measures actually implemented, it is judged that somewhere between a further 8% and 43% of currently registered fungicides in Europe could become de-registered (7–49% in the UK) with a further 20–64% of the remaining fungicides candidates for substitution (approved for only 5 years and then probably no longer registered). It is judged in this assessment that there would be most probably the loss of key fungicides to agriculture – the Commission proposal may entail the non-approval of the triazole fungicides – or at least some key members of the group. Whilst this would leave a

range of compounds, there may be no fully effective fungicides for the control of the major disease of wheat in the UK, *Septoria tritici*. The non-approval of important triazole compounds would remove the foundation stone of control programmes for this major disease, with potential for 20–30% yield losses. These compounds are also important for the control of many other diseases of wheat which on average reduce yields by at least 20% in the absence of fungicide use. The loss of registration of mancozeb would have considerable significance as this fungicide is of great importance in resistance management strategies as well as for control of *Phytophthora* root and fruit rot in various fruit crops and late blight in potatoes.

The potential impact of the EU proposals has been considered by the industry including FRAC et al. (2008) and concern expressed over the likely impact on resistance management and therefore on sustainable control of crop pests and diseases. As has been indicated above it must be recognized that the continuing availability of a diversity of chemicals is a crucial element in effective resistance management for the world's pesticide portfolio. It is also recognised that control by fungicides needs to be integrated with non-chemical control methods. However, chemicals can only be substituted by non-chemical methods to a limited degree, as control is often poorer, less predictable, and more expensive. The industry supports an Integrated Pest Management (IPM) strategy in which both cultural and chemical elements are used to their full potential. The Resistance Action Committees have recommended that no fewer than 3, and in the case of multi-spray crops (e.g. potatoes, bananas), preferably 5, different modes of action are required per crop/target pest to ensure effective resistance management and sustainable pest, disease and weed management for the future.

1.8 Conclusions

The challenges of feeding a growing world population have a high profile in the international press currently and quite correctly so. These challenges mean that agricultural intensification will need to increase and also solutions will have to be found to resolve the issue of rapidly increasing commodity (food and feed) prices. Despite great technological advances in agricultural production over the past century, the "theoretical yields" of crops are not yet realised, and much more potential still exists for the optimisation of agricultural production. This optimisation will be achieved through further advances in agricultural production systems, including improvements in natural and genetically modified crop traits targeted at improving outputs, and will also continue to be dependent on new innovations in the area of chemical pest and disease control. Despite the increasing costs of new product Research and Development, and increasing legislative requirements, industry continues to invest in fungicide research and has a long and continuing record of success in providing new innovative solutions. Diversity in fungicides with regards to chemistry and mode of action is very important for sustainable crop production. Maintaining the existing supply, and delivering a future supply of new diverse

fungicides is essential to ensure continued and increased crop production, control new threats arising and to manage fungicide resistance. Without such a diversity being available to the farmer in the future to use in integrated production programmes, the ability of the grower to continue in business and to produce sufficient food would be endangered. With this in mind it is important that the maintenance of diversity of products, and the importance of resistance management should be considered as one of the key factors in regulatory legislation.

References

AEBC (2005) What shapes the research agenda? Agriculture and Environment Biotechnology Commission, Plant Breeding Case Study URN05/1084, p 19. http://www.aebc.gov.uk [June 2009]

Backhaus GF (2009) Regulatory aspects in chemical control of fungal diseases: impact on efficient plant protection (this volume)

CIMMYT (2005) Sounding the alarm on global stem rust. An assessment of race Ug99 in Kenya and Ethiopia and the potential for impact in neighbouring regions and beyond. CIMMYT, Mexico, p 26

Cook RJ, Jenkins JEE (1988) The contribution and value of pesticides to disease control in cereals. Clifford BC, Lester E (eds) In: Control of plant diseases: costs and benefits. Blackwell, Oxford, UK, pp 1–12

Cooke LR (1992) Potato blight control in Ireland: a challenging problem. Pestic Outlook 13(4):28–31

Cramer HH (1967) Plant protection and world crop production. Bayer Pflanzenschutz-Nachrichten 20:1–524

Crop Life International (2007) Research and development. Innovation in Support of Sustainable Agriculture. http://www.croplife.org [June 2009]

Cuthbertson AGS, Murchie AK (2003) The impact of fungicides to control apple scab (*Venturia inaequalis*) on predatory mite *Anystis baccarum* and its prey *Aculus schlechtendali* (apple rust mite) in Northern Ireland Bramley Orchards. Crop Prot 22(9):1125–1130

FRAC (2007a) Fungicide resistance in crop pathogens: how can it be managed? FRAC monograph no. 1, 2nd revised edn. Newline Graphics, UK

FRAC (2007b) Fungicide resistance: the assessment of risk. FRAC monograph no. 2, 2nd revised edn. Aimprint, UK

FRAC, IRAC, HRAC (2008) The impact on resistance management and sustainable crop production in Europe. Outlooks Pest Manag 19(4):145–192

Gianessi LP, Reigner N (2005) The value of fungicides in US crop production. Crop Life Foundation, pp 243. http://www.croplifefoundation.org [June 2009]

Oercke E-C (2006) Crop losses to pests. J Agric Sci 144:31–43

Oercke E-C, Dehne H-W, Schönbeck F, Weber A (1994) Crop production and crop protection – estimated losses in major food and cash crops. Elsevier, Amsterdam

Offermann F, Nieberg H (2000) Economic performance of organic farms in Europe. Organic farming in Europe: economics and policy, vol 5. Universität Hohenheim, Germany

PSD (2008) Assessment of the impact on crop protection in the UK of the 'cut-off criteria' and substitution provisions in the proposed Regulation of the European Parliament and of the Council concerning the placing of plant protection products in the market. Pesticides Safety Directorate, pp 46. http://www.pesticides.gov.uk [June 2009]

Russell PE (2005) A century of fungicide evolution. J Agric Sci 143(1):11–25

UNFPA (2006) World population prospects – 2006 revision. UN Population Division. http://www.esa.un.org/unpp/ [June 2009]

Chapter 2
Novel Tools to Identify the Mode of Action of Fungicides as Exemplified with Fluopicolide

Valérie Toquin, François Barja, Catherine Sirven, Stéphanie Gamet, Lucile Mauprivez, Philippe Peret, Marie-Pascale Latorse, Jean-Luc Zundel, Frédéric Schmitt, Marc-Henri Lebrun, and Roland Beffa

Abstract The expanding field of fungal genomics stimulates the development of genome wide functional tools and comparative analyses in plant pathogenic fungi. As a consequence, transcriptomic, proteomic and metabolomic studies coupled with high throughput forward and reverse genetics are now available in a significant number of fungal plant pathogens (e.g. *Ustilago maydis*, *Magnaporthe grisea*, *Fusarium graminearum*, *Botrytis cinerea*). Genomics together with classical biochemical tools and microscopy offer the possibility to accelerate the identification of the biochemical mode of action of novel fungicides. This knowledge is also required to discover efficiently novel antifungal compounds and to characterize and follow efficiently the emergence of resistance. The available genomic tools for plant pathogenic fungi will be reviewed as exemplified with the mode of action of fluopicolide, a novel fungicide active against Oomycetes. Biological studies performed with *Phytophthora infestans* and *Plasmopara viticola* showed that fluopicolide affects the release and motility of zoospores and the germination of cysts, as well as mycelial growth and sporulation. Biochemical studies showed that its mode of action differs from that of known anti-oomycetes compounds. Fluopicolide does not show cross-resistance to commercial fungicide classes such as phenylamides, strobilurins (QoIs) and carboxylic acid amides (CAAs). Cytological studies in *P. infestans* showed that fluopicolide specifically modifies the spatial and cellular distribution of proteins labelled by antibodies specific for

V. Toquin, F. Barja, C. Sirven, S. Gamet, L. Mauprivez, P. Peret, M.-P. Latorse, J.-L. Zundel, F. Schmitt, and R. Beffa (✉)
Department of Biochemistry, Bayer CropScience SA, 14-20 rue Pierre Baizet, 69009 Lyon, France
e-mail: roland.beffa@bayercropscience.com

F. Barja
Laboratoire de Bioénergétique et de Microbiologie, University of Geneva, 10 Ch. Embrouchis, 1254, Jussy-Lullier, Switzerland

M.-H. Lebrun
Université de Lyon, F-69622, Lyon, France, Université de Lyon 1, Villeurbanne, France; CNRS, UMR, INSA de Lyon, F-69621, Villeurbanne, France; Bayercropscience, F-69263, Lyon, France

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,* Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_2,

animal cytoskeleton associated proteins spectrin. Treatments with fluopicolide induced a fast redistribution of spectrin-like protein(s) from the membrane to the cytoplasm in both hyphae and zoospores. Whereas animal spectrin(s) play an important role in membrane stability, they are poorly characterized in fungi and oomycetes. Cytoskeletal proteins such as actins, tubulins, integrins and spectrins provide structural stability to cells as they form a network sustaining the plasma membrane. Fluopicolide may interfere and destabilize this network leading to cell disorganization. This hypothesis is supported by the observation that treatments of zoospores lead to the relocalization of spectrin-like protein(s) into the cytoplasm within a few minutes followed immediately by cell swelling and burst. Preliminary data of gene expression profiling in *P. sojae* treated cells showed a differential expression (up and down regulation) of genes involved in vesicular transport. The link between golgi function, vesicle transport and cellular relocation of spectrin like proteins will be discussed.

Keywords Fungicide • Biochemical mode of action • Novel technologies • Image analysis • Genomics • Oomycetes

2.1 Introduction

The classification of fungicides according to their mode of action and cross resistance pattern became necessary to facilitate resistant management at field level under practical agronomic conditions (Kuck and Gisi, 2007). Fungicides with a novel mode of action and improved performance are needed to comply with the regulatory requests and to overcome existing resistance (Hewitt, 2000). In addition, the identification of the biochemical and molecular target allows the use of modern tools to monitor resistance (Proffer et al., 2006; Leroux et al., 2007; Saito et al., 2009). Despite many studies, the biochemical mode of action of several fungicides remains unclear (Kuck and Gisi, 2007).

In vivo screening of chemical compounds for activity against pathogenic fungi of interest has delivered leads and products used to efficiently protect crops against diseases. Nevertheless, the discovery of novel chemistry is becoming more difficult (Harrison, 1999; Short, 2005; Walsh, 2007). The characterization of hits of known or undesirable modes of action requires a lot of efforts. Finally, the identification of the biochemical target allows to integrate information like affinity, selectivity or structure – activity relationship into lead optimization programmes and can improve the choice of the best lead structures. An integrated approach based on chemistry and genetics requires a well defined phenotype screening, adequate chemical libraries and target identification test methods (Lokey, 2003; Mayer, 2003; Spring, 2003). These methods are currently the step limiting the number of throughput compounds (Lokey, 2003; Burdine and Kodadek, 2004), because no standard methodologies are available, and a case by case approach combining the appropriate technologies has to be used. Since the publication of

the full genome sequence of a phytopathogenic fungus (Dean et al., 2005), about 40 additional fungal pathogens have been sequenced (Soanes et al., 2008). Their appropriate annotation allows to develop high scale bio-informatics and novel approaches, e.g. gene expression profiling, proteomic analyses and functional genomics (De Backer and Van Dijck, 2003; Ihmels et al., 2005; Chengalvala et al., 2007; Ovaa and van Leeuvwen, 2008, Fig. 2.1). The recent development of cellular imaging also strongly contributes to drug characterization by facilitating the study of their effects under the complex physiological environment of cells (Lang et al., 2006; Bullen, 2008). In this contribution, fluopicolide has been chosen to exemplify the coordinated use of various tools for identification of the mode of action of novel fungicides.

Fluopicolide belongs to a new chemical class of fungicides, the acylpicolides exhibiting high activity to a broad spectrum of oomycetes such as *Phytophthora infestans*, *Plasmopora viticola* and various *Pythium* species (Toquin et al., 2007). Fluopicolide does not show cross-resistance to other commercially available fungicide classes such as phenylamides, strobilurins (QoIs) and carboxylic acid amides (CAAs) like dimethomorph, suggesting that fluopicolide has a new mode of action. Already few minutes after application, fluopicolide affects several stages of the life cycle of oomycetes: the release and motility of zoospores, the germination of cystospores, the growth of the mycelium as well as the sporulation. Immunolocalization studies revealed that a cytoskeleton-associated protein called spectrin-like protein, which is only poorly characterized in fungi and oomycetes, was strongly delocalized upon fluopicolide treatment.

Spectrin was first discovered and described in animal cells (Bennett, 1990). The spectrin superfamily is composed of spectrin, α-actinin, dystrophin and utrophin. In protozoa, fungi and oomycetes bioinformatic analyses indicated that the superfamily is represented by the α-actinin protein (Virel and Backman, 2004, 2007). Nevertheless, based on the use of anti-spectrin antibodies, spectrin-like antigens with a higher molecular weight than α-actinin have been also identified in plants (Ryan et al., 2001; Michaud et al., 1991), in yeast (Slaninova et al., 2003), in oomycetes such as *Saprolegnia ferax* (Kaminskyj and Heath, 1995) and in fungi such as *Neurospora crassa* (Degousée et al., 2000). This discrepancy between the bioinformatic data and the immunological studies is further discussed by Cotado-Sampayo et al. (2006). In both plants and fungi, spectrin-like proteins are considered to form a bridge between cytoskeleton and plasma membrane and may play an important role during tip extension and polarity of hypae. Indeed, it was shown in mammalian cells that spectrin plays a crucial role in membrane integrity and dynamics (An et al., 2004; De Matteis and Morrow, 2000). Our results suggest that the protein identified by immunology in *P. infestans* could play a similar role. This hypothesis is supported by the observation that zoospores swell and burst within a few minutes after fluopicolide application, preceded by the delocalization of spectrin-like protein(s). Thus, the delocalization of spectrin-like proteins induced by fluopicolide represents a novel mode of action for fungicides. The possibility of spectrin-like protein(s) to represent a novel biochemical target for a fungicidal compound and its role in the development of oomycetes

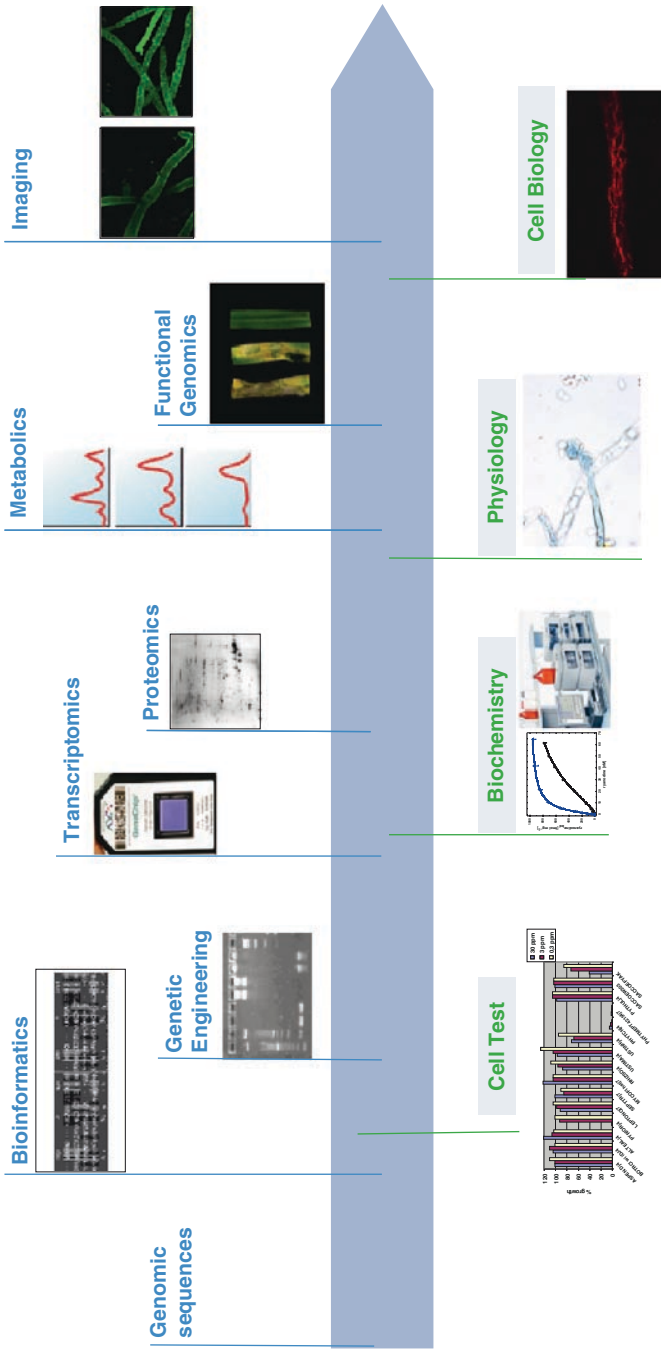


Fig. 2.1 Summary of the main technologies and tools available today to study the mode of action of active chemical classes

will be discussed and linked with bioinformatic analyses and preliminary gene expression studies.

2.2 Results

1. Effect of fluopicolide on zoospores and mycelial growth of *P. infestans*
   The application of fluopicolide induced strong effects on *P. infestans* zoospores. Zoospores stopped swimming within a minute after contact with the compound at concentration as low as 1 μg ml^{-1} resulting in swelling and lysis of zoospores (Fig. 2.2). A total lysis was observed 20 min after treatment. Similar symptoms were observed also with fenamidone, a respiration inhibitor (at same concentrations), and at higher concentrations (30 μg ml^{-1}) with zoxamide, an inhibitor of tubulin polymerization. Fluopicolide also strongly inhibited in vitro mycelium growth of *P. infestans* and other *Phytophthora* species. An 80% growth inhibition was obtained at a concentration as low as 0.1 μg ml^{-1}. In treated mycelium, leakage of cellular content was observed as visualized by staining hyphae with Blue Trypan (Fig. 2.3). This observation suggests that the compound induces perturbation of plasma membranes in mycelium preferentially at the apex of the hyphae.
2. Effect of fluopicolide on cellular distribution of actin and tubulin
   The observed symptoms induced by fluopicolide suggested that the target site could be associated with energy production or function of plasma membrane and cytoskeleton. However, experiments showed that fluopicolide did not affect cellular respiration nor membrane permeability and composition. Moreover it did not affect in vitro tubulin polymerization (bovine brain tubulin) as it is

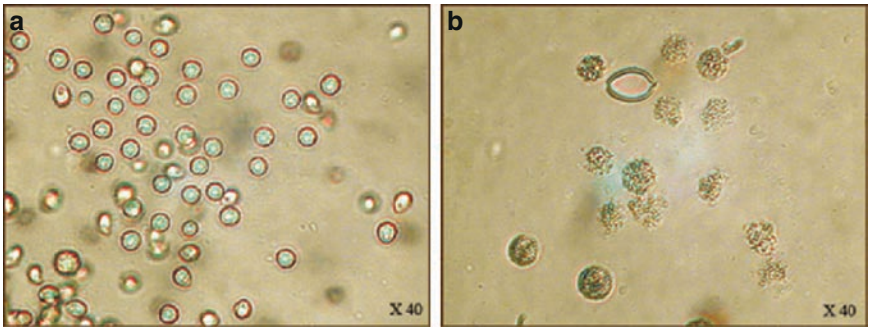


Fig. 2.2 Fluopicolide effect on *P. infestans* zoospores. (**a**) Control represents solvent (DMSO 1%) treated zoospores. (**b**) Fluopicolide treated zoospores at 3 ppm, 10 min after treatment

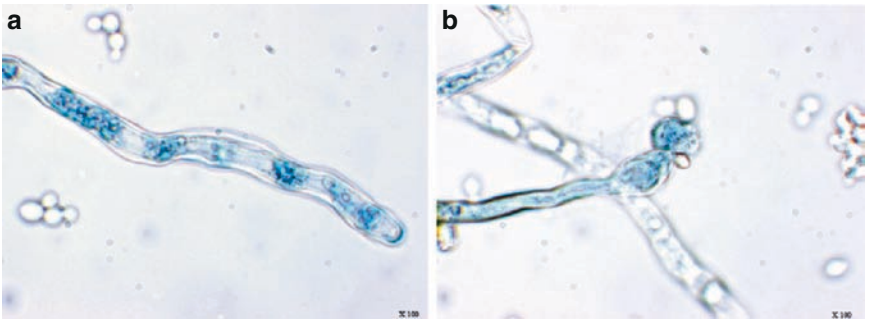


Fig. 2.3 Fluopicolide effect on *P. infestans* hyphae. *P. infestans* mycelium is colored with Blue Trypan. (**a**) Control treated with solvent (DMSO 1%). (**b**) Fluopicolide treated mycelium at 10 ppm, 48 h post-treatment

observed with zoxamide (data not shown). To further analyze a potential effect on the cytoskeleton, fluorescent microscopy was performed to demonstrate effects on the two major cytoskeleton components of the cells, actin and tubulin. Immunostained actin was found to be concentrated mainly in hyphal tips of *P. infestans* where it formed a uniform cap lying essentially in cortical zone of the hyphae (Fig. 2.4). The sub-apical structures of actin were visualized as dots which were consistently spherical in shape, but their number and size varied in different hyphae. Anti-actin immunofluorescence revealed a similar overall pattern of actin distribution in both untreated and fluopicolide treated hyphae of *P. infestans*. Anti-β-tubulin immunofluorescence revealed a dense array of predominately axial and reticulate microtubules in the apical and sub-apical region of hyphae (Fig. 2.4). For the distribution of tubulin no significant difference was found between the untreated and fluopicolide treated hyphae. Similarly, no effects were observed for α-tubulin (data not shown). Therefore, fluopicolide does not affect actin or tubulin.

3. Effect of fluopicolide on the cellular localization of a spectrin-like protein
   Detailed analyses of the effects on the cytoskeleton associated proteins were performed. Spectrin is known to play a crucial role in membrane stability in mammalian cells (Sjöblom et al., 2008) Immunofluorescence studies using antibodies raised against spectrin of chicken erythrocytes were conducted with mycelium of *P. infestans*. The spectrin-like proteins were prominently localized in the peripheral regions (plasma membrane) along the hyphae (Fig. 2.5). In some cases high concentrations of labelling were observed at the tip and the branch initials. Upon fluopicolide treatment, a complete loss of spectrin-like proteins was observed at plasma membrane level. However, they were distributed as spherical spots everywhere in the hyphal cells. A time–course analysis revealed

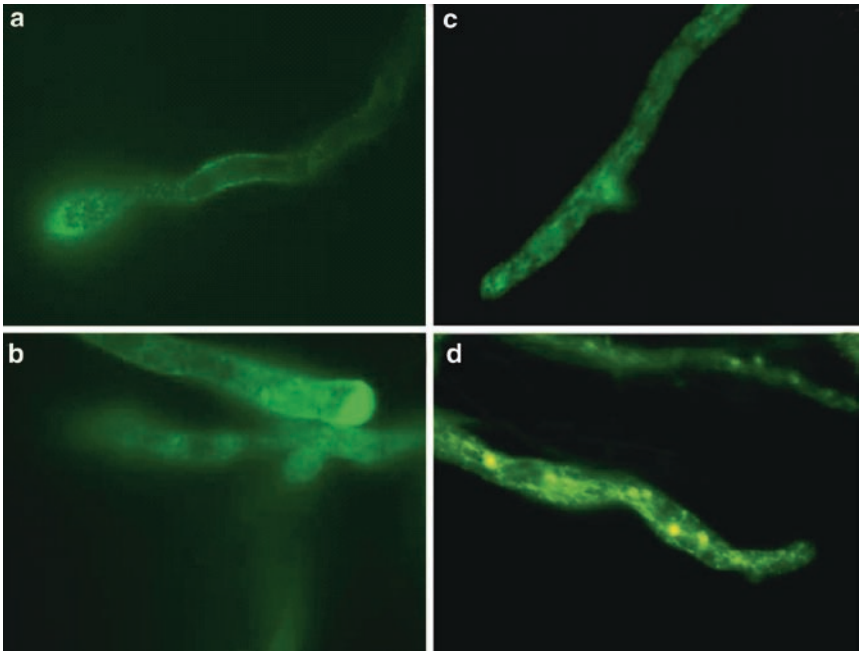


Fig. 2.4 Fluopicolide effect on cellular distribution of cytoskeleton components: tubulin, actin. (**a**, **b**) Immunolocalisation of actin in control and fluopicolide treated hyphae (10 ppm, 24 h post-treatment), respectively. (**c**, **d**) Immunolocalisation of β-tubulin in control and fluopicolide treated hyphae (10 ppm, 24 h post-treatment), respectively

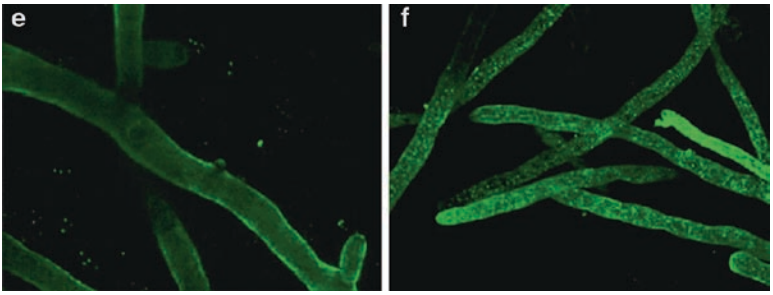


Fig. 2.5 Fluopicolide effect on cellular distribution of spectrin-like protein(s). Immunolocalisation of spectrin-like protein(s) in control (**e**) and fluopicolide treated hyphae (**f**) (10 ppm, 24 h post-treatment)

that delocalisation of spectrin-like proteins occurred very rapidly, as early as 3 min after fluopicolide treatment (Fig. 2.6). In addition, this delocalisation continued with longer exposure of hyphae to fluopicolide. Since fluopicolide application induced swelling and burst of zoospores, the effect on spectrin-like proteins was also investigated in zoospores. A time–course experiment was

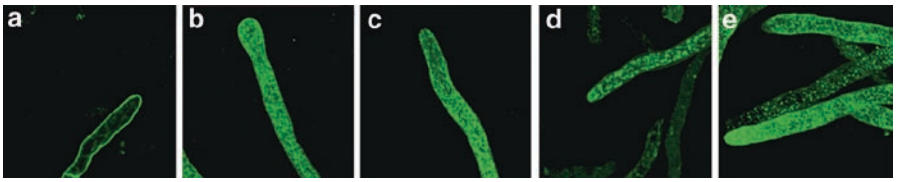


Fig. 2.6 Kinetic of the fluopicolide effect on the distribution of the spectrin-like proteins in hyphae of *P. infestans*. Control cell (**a**), hyphae treated with 10 ppm fluopicolide for 3 min (**b**), 10 min (**c**), 2 h (**d**) and 24 h (**e**)

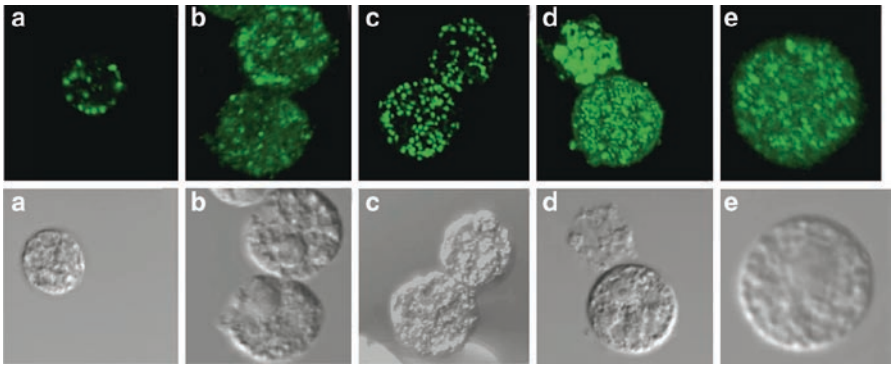


Fig. 2.7 Kinetic of the fluopicolide effect on the distribution of the spectrin-like proteins in zoospores of *P. infestans*. Control cell (**a**), zoospores treated with 3 ppm fluopicolide for 1 min (**b**), 5 min (**c**), 10 min (**d**) and 20 min (**e**)

performed with fluopicolide treatment, i.e. when zoospores were just immobilised (1 min), during swelling (5 and 10 min) and just before cell lysis (20 min). Untreated control samples showed that spectrin-like proteins were predominantly localised as peripheral patches along the plasma membrane and the cell wall (Fig. 2.7). Upon fluopicolide treatment, spectrin-like proteins were redistributed and patches were observed all over the cytoplasm. Interestingly, this effect was extremely fast in zoospores and correlated very well with the first symptom, i.e. zoospore swelling.

In the next set of experiments, the effect of known anti-oomycetes fungicides on spectrin-like proteins was compared to that of fluopicolide over a test period of 3 min to 24 h. None of the tested fungicides (iprovalicarb, fenamidone, dimethomorph, metalaxyl and zoxamide) induced redistribution of spectrin-like proteins in hyphae of *P. infestans* after 2 h of treatment (Fig. 2.8). This observation was confirmed for all the samples analysed until 24 h. Therefore, delocalisation of spectrin-like proteins from the cell periphery into the cytoplasm is specific to fluopicolide.

4. Molecular characterisation of spectrin-like proteins in *P. infestans*
   Spectrin was first discovered and described in red blood cells and proteins related to the erythrocytes spectrins have been detected in different tissues and cells types (Bennett and Gilligan, 1993). Spectrin-like proteins have been found also

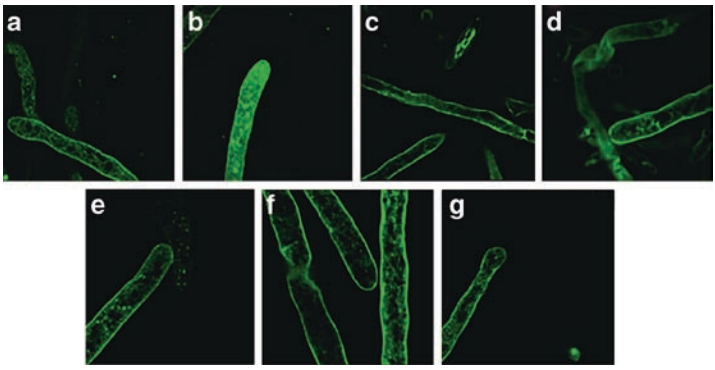


Fig. 2.8 Immunofluorescent localisation of spectrin-like protein(s) in hyphae of *P. infestans* treated with fluopicolide and competitors. Control cell (**a**) or treated with 10 ppm of fluopicolide (**b**), iprovalicarb (**c**), fenamidone (**d**), dimethomorph (**e**), metalaxyl (**f**) and zoxamide (**g**), 2 h post-treatment

in plants and fungi (Michaud et al., 1991; Kaminskyj and Heath, 1995; Degousée et al., 2000; Braun, 2001; Ryan et al., 2001; Slaninova et al., 2003). In both fungi and plants, these proteins were characterized by their cross-reactivity with the anti-chicken a/b spectrin antibody, their size determined on Western-blot and their immunolocalisation to the vicinity of the plasma membrane. To our knowledge, in none of these organisms, amino acid sequences have been identified so far. A rapid homology search using BLAST for spectrin-like proteins in fungi (*M. grisea* and *N. crassa* genome sequences) or in oomycetes (*P. sojae* and *P. ramorum* genome sequences, partial EST sequences of *P. infestans*) was not successful. Hence a search for spectrin domains was initiated. The structure of erythrocytes spectrins is composed of anti-parallel heterodimer of two sub-units α (240 kd) and β (220 kd) characterised by the presence of specific domains: (i) a domain formed by triple-helical repeat of 106–120 amino acids, so-called spectrin repeat (present 4 to over 20 times), (ii) a EF-hand domains, a calcium-binding domain, and (iii) a highly conserved N-terminal domain responsible for binding of actin filaments. The spectrin repeat (SpR) domain was used to start a PFAM analysis (Finn et al., 2006). The SpR domain built only with mammalian representative domains (PF00435) gave no hit in fungal or oomycetes species. To improve the search, a system based on Hidden Markov Models (similar to PFAM analysis), called SMART (a simple modular architecture research tool) was used allowing the identification and the analysis of domain architectures (Schultz et al., 1998). In this system, the motifs are automatically enriched by new sequences coming from world wild databases allowing more diversity. The "seed alignment" of the SMART database (SM00150) was then used to build a new motif for the SpR domain. This approach provided one hit in *P. sojae* corresponding to a protein of around 100 kDa (accession number 137006) corresponding to putative spectrin super-family proteins (α-actinin; Fig. 2.9). This protein contained two SpR domains of 107 and 113 residues. A search with the others domains did nit give better results. In fungi, similar results

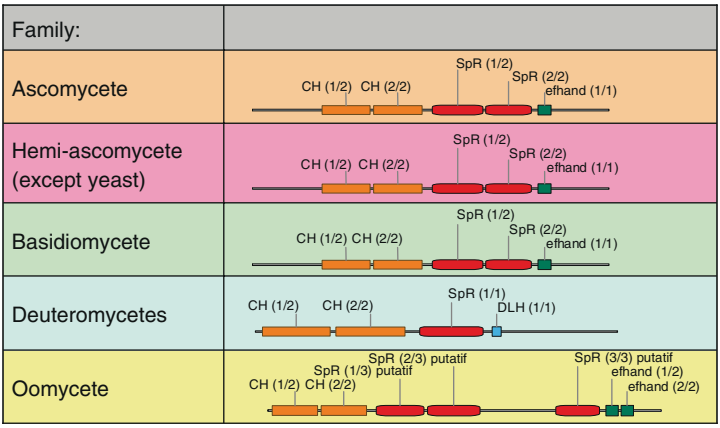


Fig. 2.9 Schematic representation of the α-actinin protein structure determined by bioinformatic analysis in the different groups of fungi and oomycetes

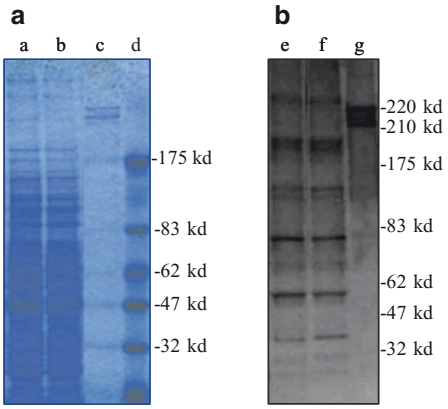


Fig. 2.10 Immunodetection of spectrin-like proteins in cells of *P. infestans*. (**a**) SDS-polyacrylamide gel Blue Comassie-stained for proteins. *Lane a*: total proteins extracted from untreated cells of *P. infestans*. *Lane b*: total proteins extracted from fluopicolide treated cells of *P. infestans* (10 ppm, 24 h). *Lane c*: purified human spectrin as positive control. *Lane d*: molecular weight marker. (**b**) Immunoblot of total proteins showing cross-reactive bands to spectrin, in untreated cells (*lane e*), in fluopicolide treated cells (*lane f*) of *P. infestans* and in the positive control (*lane g*)

were obtained, for example a protein of 113 kDa and 88 kDa can be found for *N. crassa* (NCU06429.1) and *M. grisea* (MG06475.4) respectively, both corresponding to a putative α-actinin.

For further characterisation of the proteins, the presence of spectrin-like proteins was investigated in mycelial extracts of *P. infestans*. Total *P. infestans* proteins were immunolabelled with anti-spectrin antibodies (Fig. 2.10). The immunolabelling of human erythrocytes spectrin was used as a control revealing a doublet of bands

at 220–240 kDa. In a crude extract of total proteins of *P. infestans*, the antibodies reacted with a few proteins of a relative molecular weight lower than the human spectrin. Four bands with a size of 100, 70, 50 and 30 kDa were found. This result might be due to the fact that spectrin has been reported to be very sensitive to proteolytic degradation (Cotado-Sampayo et al., 2006). A similar pattern was obtained in both untreated and treated samples of *P. infestans* mycelia, suggesting that fluocopicolide did not change the content of spectrin-like proteins in the cell. Further characterization of these proteins is ongoing.

2.3 Discussion

Using immunofluorescence, the effect of fluopicolide treatment on the localization of spectrin-like protein(s) was demonstrated in both zoospores and hyphae of *P. infestans*. A fast redistribution of these proteins from the membrane to the cytoplasm was observed after fluopicolide treatment but not after treatment with other anti-oomycete fungicides. Therefore, this effect represents a new mode of action that seems to be specific to fluopicolide.

Spectrin-like proteins are poorly characterized in fungi and, according to our knowledge this report is the first indication of spectrin homologues in *P. infestans*. Spectrin-like proteins described in yeast, *N. crassa* and *S. ferax*, are localized at the cell periphery close to the membrane (Slaninova et al., 2003; Degousée et al., 2000; Kaminskyj and Heath, 1995). The question arises how spectrin-like proteins contribute to the control of fungal growth. In animal cells there are strong indications that spectrin determines the mechanical integrity and plasticity of the plasma membrane. In fungi, spectrin-like proteins are localized in the plasma membrane, in particular at the tip of the growing hyphae supporting membrane stability during elongation. A leakage of cellular content at the apex of hyphae was observed 48 h after fluopicolide treatment suggesting that the membrane was weakened at this point. Because the cell wall is synthesized at the hyphal tip, membrane plasticity is important especially at this place. In addition, in zoospores, which are wall-less cells, membrane stability is particularly important to resist against the turgor pressure controlled by the extra-cellular medium. Spectrin-like proteins could play an important role in maintaining membrane integrity in these cells. This hypothesis is supported by the observation that the zoospore swelled and bursted in a few minutes following fluopicolide application, preceded by the relocalization of spectrin-like protein(s) into the cytoplasm.

To further characterize proteins in *P. infestans* visualized with immunofluorescence microscopy, they were labeled with anti-spectrin antibodies against chicken (the same as used in microscopy) and human providing similar results. Four cross-reactive bands with relative molecular weights lower than expected (100, 70, 50 and 30 kDa instead of 220–240 kDa) were detected. The presence of multiple bands could have resulted from protein degradation. Fragments of similar size were also detected in yeast and in green algae (even detected as doublet at 220–240 kDa) and were

described as spectrin breakdown products (Slaninova et al., 2003; Lorenz et al., 1995). Nevertheless, it should be noted that the spectrin related proteins found by bioinformatics analysis in the oomycete databases corresponded to a protein of 100 kDa (putative α-actinin). Therefore, the 100 kDa band which was detected in Western blot analyses, could match with this putative spectrin related protein. The definition of spectrin is not tight, and the spectrin super family includes proteins with similar structural and functional features. It has been argued that current animal spectrins have evolved from a smaller ancestral protein, α-actinin (Thomas et al., 1997). Thus, the fungal spectrins could derive also from this ancestral protein. In addition, it is supposed that each spectrin repeat domain has likely evolved to perform specialized functions. Even if in some organisms such as *N. crassa* and yeast, spectrin-like proteins of 220–240 kDa were detected by Western-Blot analysis, the corresponding genes have not yet been identified. Based on our results, it is now clear that *P. infestans* does contain potential spectrin-like proteins, but their true relationship to spectrin remains to be further characterized.

Taken together, these data suggest that fungal spectrin-like proteins could share some homology with animal spectrin, epitopes, which is similar in 3D structure, function and cellular localization. Considering the potential role for tip growth in fungi, and their apparent differences in terms of protein sequence, spectrin-like proteins represent a good fungicidal target.

At the present stage of our investigations, it cannot not exclude that spectrin-like delocalization may be the consequence rather than the origin of an effect on other related targets. It was shown that spectrin redistributed to the cytosol and specifically phosphorylated during mitosis in Hela cells (Fowler and Adam, 1992). It was also reported that the interaction of spectrin with actin was controlled by phosphorylation in vitro and in erythrocyte cells (Pinder et al., 1997). In addition, the binding to spectrin domains of tyrosine kinase binding-proteins was demonstrated (Ziemnicka-Kotula et al., 1998). It has been speculated in animal cells that the status of spectrin phosphorylation participates in the determination of its intracellular localisation (Wang et al., 1999). It was also shown that the activity of cytoskeleton associated proteins, including spectrin, is highly regulated by phosphorylation (Manno et al., 1995; Fairbanks et al., 1978). Preliminary data of gene expression profiling using the *P. sojae* affimetrix chip showed that 16 of 49 up-regulated genes were linked with ER/Golgi functions (Fig. 2.11) suggesting that vesicle transport is strongly affected by fluopicolide treatment. These results are of special interest in the view of common factors known to be involved in the regulation of cytoskeleton function and assembly and vesicle transport, in particular the contribution of small GTPases of the Arf family (Myers and Casanova, 2008).

The combination of immunofluorescence analysis, classical biochemistry, bioinformatics, and gene expression profiling experiments has allowed to generate a first insight for describing the cellular functions modified by fluopicolide. Further experiments are on going to better characterize the role of spectrin-like protein(s) in oomycetes development and as a novel target for chemical compounds against oomycetes.

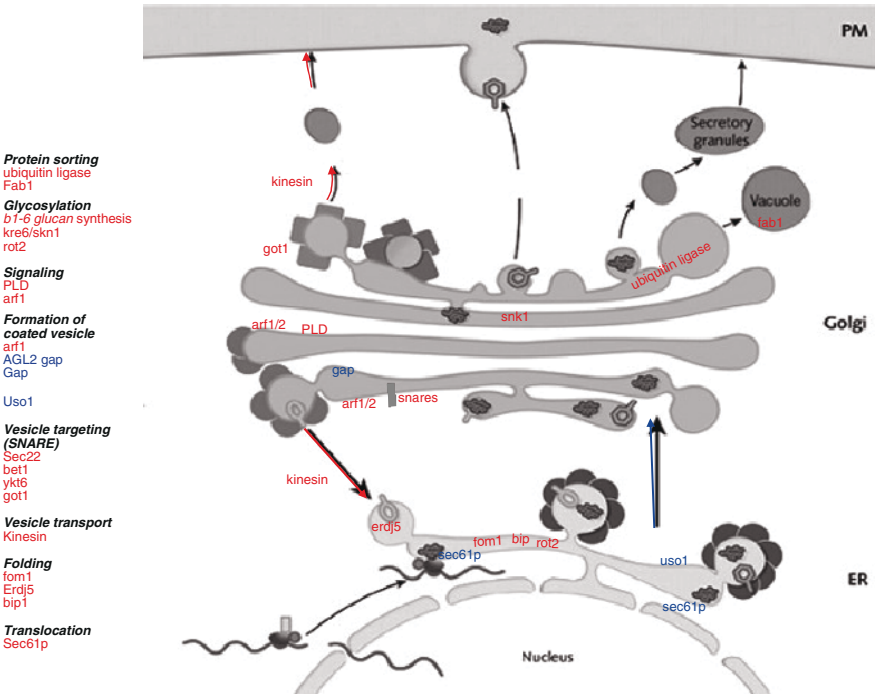


Fig. 2.11 Schematic representation of genes related to endoplamic reticulum and golgi vesicle functions up-regulated (*red*) and down-regulated (*blue*) by a factor of 2 or more after the treatment of *P. sojae* with fluopicolide at 10 μg ml^{-1} during 0.5, 1 and 3 h. Three independent biological replicates were analyzed on Affymetrix chips (PM: plasma membrane; ER: endoplasmic reticulum)

2.4 Materials and Methods

2.4.1 Culture Conditions and Cell Treatment

The isolate of *Phytophthora infestans* was grown in the dark on pea agar medium at 19°C. To study the effects of inhibitors, nonsporulating mycelial mats were obtained from 3-day-old cultures in V8 clarified broth inoculated with 10^5 sporangia/ml. Sporangia were isolated by flooding 8–12-day-old rye agar cultures with water, rubbing off the sporangia with a glass rod, and then separating sporangia from hyphal fragments by passage through 50-μm nylon mesh. Sporangia concentration was adjusted with V8 broth to obtain the final concentration of 10^5 sporangia/ml. Zoospores were obtained by flooding 10 day-old culture plates with 10 ml of cold water. The flooded plates were incubated for 3 h at 4°C to induce zoospore release.

The fungicides were solubilised in DMSO. Fluopicolide solution was added to the mycelium or zoospore cultures to give final concentrations of 10 and 3 μg ml^{-1},

respectively of the fungicide and 1% of DMSO. Controls of mycelia or zoospores contained 1% DMSO.

2.4.2 Antibodies

2.4.2.1 Primary Antibodies

Spectrin was visualized using polyclonal rabbit antibodies raised against chicken (Sigma S-1390) and human (Sigma S-1515) α-β erythrocyte spectrin. For immunoblotting, the antibodies were used at dilution of 1:400. Actin was detected by polyclonal rabbit anti-actin antibodies (Sigma). α and β tubulin were detected using monoclonal antibodies (Sigma). For microscopic examination a 1:30 to 1:50 dilution of antibodies was used.

2.4.2.2 Secondary Antibodies

For immunoblotting, alkaline phosphatase-conjugated anti-rabbit IgG (Sigma) antibodies were used at a dilution of 1:3000. For immunofluorescence, fluorescein-conjugated anti-rabbit antibodies (Sigma) were used at the dilution of 1:50.

2.4.3 Immunofluorescence Microscopy of Tubulins, Actin and Spectrin

Mycelia and zoospores of *P. infestans* treated with DMSO (1%) as control or with fluopicolide were fixed with 3.7% paraformaldehyde in 100 mM phosphate buffer, pH 7, for 30 min at room temperature, rinsed three times with the same buffer. Partial digestion of the cell wall was done by incubating the cells with 5 mg/ml of Novozym (Sigma) for 10 min at room temperature and stopped by rinsing the cells four times with the phosphate buffer. The cells were then permeabilized with 0.1% Triton X-100 in the same buffer for 10 min at room temperature. Triton was removed by washing three times in phosphate buffer pH 7. The distribution of the cytoskeletal proteins was examined using corresponding antibodies. The fixed cells were blocked with phosphate buffer containing 3% BSA at room temperature overnight followed by incubation with α- or β-tubulin monoclonal antibodies (Sigma), diluted to 1:30 and 1:50, respectively, anti-actin antibody (Sigma), diluted to 1:30 and anti-chicken spectrin (Sigma) diluted to 1:50 in 3% BSA phosphate buffer, pH 7, for 2 h at 37°C. Following a rinse in phosphate buffer, the samples were incubated for 1 h at 37°C with Fluorescein Isothiocyanate (FITC) conjugated with corresponding immunoglobulin, diluted to 1:50. After a final rinsed in phosphate buffer, the cells were mounted in p-phenylendiamine-glycerol, with 2.5 μg/ml of 4′, 6-diamidino-2-phenylindole (DAPI). An Orthoplan epiilumination microscope (Ernst Leitz, Wetzlar, Germany)

equipped with fluotar optics and selective filter combinations was used to visualize the FITC fluorescence patterns. The immunofluorescence pictures were taken by Hamamatsu colour chilled 3 CCD camera. The images were developed by RastersOps video capture and treated by Adobe Photoshop 7.0 programme.

2.4.4 Protein Extraction for Western-Blot

The mycelia of *P. infestans* which were untreated or treated with fluopicolide (10 μg ml^{-1}, 30 min treatment) were harvested from liquid media by centrifugation, frozen in liquid nitrogen and ground in a mortar. The frozen powder was suspended in 3 ml of extraction buffer (20 mM MES, 0.1 M NaCl, 5 mM $MgCl_2$, pH 6.5, plus protease inhibitor cocktail (complete Mini from Roche Diagnostics). The crude extract was centrifuged at 14,000 r.p.m for 10 min at 4°C to remove insoluble cell debris. The protein concentration in the supernatants was measured using the method of Bradford (1976), with bovine serum albumin (BSA) as a standard.

2.4.5 Electrophoresis and Immunoblotting

Proteins were separated by SDS-PAGE (SDS-4–12% bis-Tris polyacrylamide pre-cast gel, Bio-Rad) according to Laemmli, 1970. The separated proteins were either stained with Commassie Brilliant Blue (Sigma) or transferred to an immun-blot PVDF membrane (Bio-Rad). Pre-stained standard molecular weight markers were used. After electrophoresis, proteins from unstained gels were electrophoretically transferred to PVDF membrane with the transfer buffer (Invitrogen, NP006). The transfer was carried out in a Bio-Rad electroblotting apparatus equipped with a cooling device, at 50 V overnight. The transferred proteins were stained with Commassie Brillant Blue, distained by washing the membrane in methanol. The PVDF membrane blocking was performed in Tris-buffered saline (TBS) (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10% Western Blocking Reagent (Roche)), for 6 h at room temperature. The membranes were probed overnight at 4ºC with the primary antibodies, then with the secondary antibody for 3 h. After several washes in TBS containing 0.1% Tween 20 and 2 washes in TBS, the antibody-antigen complex was detected by enhanced chemiluminescence on X-ray film.

2.4.6 Microarray Analysis

Quality control measures were applied at each step of both the experimental procedure and the data analysis so that all induced and repressed genes were reproducible and had signals that were within the resolution window of the microarrays hybridization technology.

These controls included the set up of three independent biological replicates with corresponding untreated controls (DMSO) for every treatment time point with fluopicolide.

An essential part of the automatic quality assessment is the selection of appropriate features and background consideration. GCOS (Affymetrix, Santa Clara, CA, USA) was used to generate CEL.files which were obtained after feature extraction and background subtraction considering a zone value plus the smoothing adjustment, as described by Affymetrix (Affymetrix technical notes, 2001).

Furthermore, expressed genes were identified by GCOS generating a present or absent call for genes represented by each probe set on the array, by the use of statistical parameters. A first global normalization adjusted the average intensity of an experimental array to the average intensity of a baseline array by applying a normalization factor of 1.

Microarray data files were then analyzed using Genedata Expressionist (Genedata-Solutions in-silico, Basel, Switzerland). The MAS5 condensing and normalizing algorithm (Lim et al., 2007) was performed with Genedata Expressionist Refiner. Using Genedata Expressionist Analyst, a normalization to the median of intensities, fixed at 300 for each microarray, allowed the global intensity adjustment. The p-value of the condensing algorithm which measured how close the individual probe expression values were to the expression value of the probe set, was set at 0.09 to filter invalid data.

To monitor and assess differential gene expression, the induction or repression ratios of median were calculated with normalized intensities of the signal, with a ratio of at least 2 and a T-test probability value of 0.05.

References

Affymetrix technical notes (2001) Fine tuning your data analysis and new statistical algorithms for monitoring gene expression on GenecChip probe array

An X, Guo X, Sum H, Morrow J, Gratzer W, Mohandas N (2004) Phosophatidylserine binding sites in erythroid spectrin: location and implication for membrane stability. Biochemistry 43:310–315

Bennett V (1990) Spectrin-based membrane skeleton: a multipotential adaptator between plasma membrane and cytoplasm. Physiol Rev 70:1029–1065

Bennett V, Gilligan DM (1993) The spectrin-based membrane skeleton and micron-scale organization of the plasma membrane. Annu Rev Cell Biol 9:27–66

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye binding. Anal Biochem 72:248–254

Braun M (2001) Association of spectrin-like proteins with the actin-organized aggregate of endoplasmic reticulum in the spitzenkörper of gravitropically tip-growing plant cells. Plant Physiol 125:1611–1619

Bullen A (2008) Microscopic imaging techniques for drug discovery. Nat Rev Drug Discov 7:54–67

Burdine L, Kodadek T (2004) Target identification in chemical genetics: the (often) missing link. Chem Biol 11:593–597

Chengalvala M, Chennathukuzhi VM, Johnston DS, Stevis P, Kopf GS (2007) Gene expression profiling and its practice in drug development. Curr Genomics 8:262–270

Cotado-Sampayo M, Ojha M, Ortega-Perez R, Chappuis ML, Barja F (2006) Proteolytic cleavage of a spectrin-related protein by calcium-dependent protease in *Neurospora crassa*. Curr Microbiol 53:311–316

De Backer MD, Van Dijck P (2003) Progress in functional genomics approaches to antifungal drug target discovery. Trends Microbiol 11:470–478

De Matteis MA, Morrow J (2000) Spectin tethers and mesh in the biosynthetic pathway. J Cell Sci 113:2331–2343

Dean R, Talbot NJ et al (2005) The genome sequence of the rice blast fungus *Magnaporthe grisea*. Nature 434:980–986

Degousée N, Gupta GD, Lew RR, Heath IB (2000) A putative spectrin-containing membrane skeleton in hyphal tips of *Neurospora crassa*. Fungal Genet Biol 30:33–44

Fairbanks G, Avruch J, Dino JE, Patel VP (1978) Phosphorylation and dephosphorylation of spectrin. J Supramol Struct 9:97–112

Finn RD, Mistry J, Schuster-Böckler B, Griffiths-Jones S, Hollich V, Lassmann T, Moxon S, Marshall M, Khanna A, Durbin R, Eddy SR, Sonnhammer ELL, Bateman A (2006) Pfam: clans, web tools and services. Nucleic Acids Res Database issue 34:D247–D251

Fowler VM, Adam EJH (1992) Spectrin redistributes to the cytosol and is phosphorylated during mitosis in cultured cells. J Cell Biol 119:1559–1572

Harrison W (1999) Agrochemical research and development in the 21st century: bloom and burst. J Biomol Screen 4:61–65

Hewitt G (2000) New modes of action of fungicides. R Soc Chem 2000:28–32

Ihmels J, Bergmann S, Berman J, Barkai N (2005) Comparative gene expression analysis by a differential clustering approach: application to the *Candida albicans* transcription program. PLoS 1:380–393

Kaminskyj SG, Heath IB (1995) Integrin and spectrin homologues, and cytoplasm-wall adhesion in tip growth. J Cell Sci 108:849–856

Kuck K-H, Gisi U (2007) FRAC mode of action classification and resistance risk of fungicides. In: Krämer W, Schirmer U (eds) Modern crop protection compounds, vol 2. Wiley, VCH. ISBN ISBN 978-3-527-31496-6

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lang P, Yeow K, Nichols A, Scheer A (2006) Cellular imaging and drug discovery. Nat Rev Drug Discov 5:343–356

Leroux P, Albertini C, Gautier A, Gredt M, Walker S (2007) Mutations in the CYP51 gene correlated with changes in sensitivity to sterol 14 alpha-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. Pest Manag Sci 63:688–698

Lim WK, Wang K, Lefebvre C, Califano A (2007) Comparative analysis of microarray normalization procedures: effects on reverse engineering gene networks. Bioinformatics 23:i282–i288

Lokey RS (2003) Forward chemical genetics: progress and obstacles on the path to a new pharmacopoeia. Curr Opin Chem Biol 7:91–96

Lorenz M, Bisikirska B, Hanus-Lorenz B, Strzalka K, Sikorski AF (1995) Proteins reacting with anti-spectrin antibodies are present in *Chlamydomonas* cells. Cell Biol Int 19:83–90

Manno S, Takakuwa Y, Nagao K, Mohandas N (1995) Modulation of erythrocyte membrane mechanical function by β-spectrin phosphorylation and dephosphorylation. J Biol Chem 270:5659–5665

Mayer TU (2003) Chemical genetics: ligand-based discovery of gene function. Nat Rev Genet 1:116–125

Michaud D, Guillet G, Rogers PA, Charest PM (1991) Identification of a 220 kDa membrane-associated plant cell protein immunologically related to human β-spectrin. FEBS Lett 294:77–80

Myers KR, Casanova JE (2008) Regulation of actin cytoskeleton dynamics by Arf-family GTPases. Trends Cell Biol 18:184–192

Ovaa H, van Leeuvwen F (2008) Chemical biology approaches to probe the proteome. ChemBiochem 9:2913–2919

Pinder JC, Bray D, Gratzer WB (1997) Control of interaction of spectrin and actin by phosphorylation. Nature 270:22–29

Proffer TJ, Berardi R, Ma Z, Nugent JE, Ehret GR, McManus PS, Jones AL, Sundin GW (2006) Occurrence, distribution, and polymerase chain reaction-based detection of resistance to sterol demethylation inhibitor fungicides in populations of *Blumeriella jaapii* in Michigan. Phytopathology 96:709–717

Ryan E, Steer M, Dolan L (2001) Immunodetection of spectrin-like proteins in yeasts. Can J Microbiol 49:189–196

Saito S, Suzuki S, Takayanagi T (2009) Nested PCR-RFLP is a high-speed method to detect fungicide-resistant *Botrytis cinerea* at an early growth stage of grapes. Pest Manag Sci 65:197–204

Schultz J, Milpetz F, Bork P, Ponting CP (1998) SMART, a simple modular architecture research tool: identification of signaling domains. Proc Natl Acad Sci U S A 95:5857–5864

Short PL (2005) Growing Agchem R&D. Chem Eng News 83:19–22

Sjöblom B, Salmazo A, Djinovic-Carugo K (2008) Alpha-actinin structure and regulation. Cell Mol Life Sci 65:2688–2701

Slaninova I, Holubarova A, Svoboda A (2003) Cell biology and genetics of root hair formation in *Arabidopsis thaliana*. Protoplasma 215:140–149

Soanes DM, Alam I, Cornell M, Wong HM, Hedeler C, Paton RW, Rattray M, Hubbard SJ, Oliver SG, Talbot NJ (2008) Comparative genome analysis of filamentous fungi reveals gene family expansions associated with fungal pathogenesis. PLoS ONE 3:e2300

Spring DR (2003) Chemical genetics to chemical genomics: small molecules offer big insights. Chem Soc Rev 34:472–482

Thomas GH, Newbern EC, Korte CC, Bales MA, Muse SV, Clark AG, Kiehart DP (1997) Intragenic duplication and divergence in the spectrin superfamily of proteins. Mol Biol Evol 14:1285–1295

Toquin V, Barja F, Sirven C, Beffa R (2007) Fluopicolide, a new anti-oomycetes fungicide with a new mode of action inducing perturbation of a spectrin-like protein. In: Krämer W, Schirmer U (eds) Modern crop protection compounds, vol 2. Wiley, VCH, pp 675–682. ISBN ISBN 978-3-527-31496-6

Virel A, Backman L (2004) Molecular evolution and structure of α-actinin. Mol Biol Evol 21:1024–1031

Virel A, Backman L (2007) A comparative and phylogenetic analysis of the α-actinin rod domain. Mol Biol Evol 24:2254–2265

Walsh TA (2007) The emerging field of chemical genetics: potential applications for pesticide discovery. Pest Manag Sci 63:1165–1167

Wang XY, Ostberg JR, Repasky EA (1999) Effect of fever-like whole-body hyperthermia on lymphocyte spectrin distribution, protein kinase C activity, and uropod formation. J Immunol 162:3378–3387

Ziemnicka-Kotula D, Xu J, Gu H, Potempska A, Kim KS, Jenkins EC, Trenkner E, Kotula L (1998) Identification of a candidate human spectrin Src homology 3 domain-binding protein suggests a general mechanism of association of tyrosine kinases with the spectrin-based membrane skeleton. J Biol Chem 273:13681–13692

Chapter 3
QoI Fungicide Resistance: Current Status and the Problems Associated with DNA-Based Monitoring

Hideo Ishii

Abstract QoI fungicides which inhibit mitochondrial respiration at the ubiquinol oxidation centre (Qo site) of the cytochrome bc1 enzyme complex, are one of the most important class of agricultural fungicides. QoI fungicides generally carry a high risk of pathogen resistance development with resistance occurring in over 30 pathogen species, such as powdery mildews, downy mildews, anthracnose, *Alternaria* spp., scab, and grey mould. Molecular mechanisms of QoI resistance have been intensively studied; a single point mutation which causes an amino acid change in cytochrome *b*, G143A in particular, was described to govern the expression of high resistance. A range of molecular methods including PCR-RFLP, allele-specific PCR, quantitative real-time PCR and pyrosequencing have been developed, enabling the rapid detection and quantification of resistance. However, the status of heteroplasmy in the mitochondrial genome which contains the cytochrome *b* gene can cause instability over time, making it difficult to precisely monitor QoI resistance in some pathogens. The role of the alternative oxidase pathway in QoI resistance is not clear as yet, although this enzyme is very likely involved in resistance development of grey mould in particular. Novel QoI fungicides have been developed, some of which show differential patterns of cross-resistance to pre-existing QoI fungicides. This paper summarizes QoI resistance development over the last decade as well as future research prospects.

Keywords Alternative oxidase • Cucurbit powdery mildew • Cytochrome *b* • Grey mould • Strobilurin fungicides

H. Ishii (✉)
National Institute for Agro-Environmental Sciences (NIAES), Kannondai, 3-1-3, Tsukuba, Ibaraki, 305-8604, Japan
e-mail: hideo@niaes.affrc.go.jp

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,* Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_3,

3.1 QoI Fungicides and Resistance Development in Fungal Pathogens

QoI (strobilurin) fungicides, which inhibit mitochondrial respiration at the ubiquinol oxidation centre (Qo site) of the cytochrome bc1 enzyme complex (complex III), are one of the most important class of agricultural fungicides behind the DMI fungicides. They represented a 15.3% share of total fungicide market value in 2005 (McDougall, 2006). However, QoI fungicides generally carry a high risk of pathogen resistance development with resistance occurring in over 30 pathogen species, such as powdery mildews, downy mildews, anthracnose, *Alternaria* spp., scab, and grey mould (Ishii, 2008; Table 3.1).

3.2 Mechanisms of QoI Resistance

Molecular mechanisms of QoI resistance have been intensively studied (Gisi et al., 2002; Kuck, 2007). A single point mutation which causes an amino acid change in cytochrome *b*, G143A in particular, was described to govern the expression of high resistance (Ishii et al., 2001; Ishii et al., 2007; Table 3.2). The F129L substitution has also been shown to cause a reduction in sensitivity to QoIs (Pasche et al., 2005). Recently, a mutation at position 137 (G137R) was detected at a very low frequency conferred similar low resistance levels as F129L (Sierotzki et al., 2007).

3.3 Molecular Methods for Identifying QoI Resistance and Drawbacks

Based on the mechanisms of QoI resistance, a range of molecular methods have been developed including PCR-RFLP (Polymerase Chain Reaction–Restriction Fragment Length Polymorphism) (Sierotzki et al., 2000), allele-specific PCR

Table 3.1 Recent reports on QoI resistance in the field

Disease	Pathogen	Reference
Barley net blotch	*Pyrenophora teres*	FRAC (2004)
Wheat tan spot	*P. tritici-repentis*	FRAC (2004)
European pear black spot[a]	*Alternaria alternata*	Tanahashi et al. (2006)
Apple powdery mildew	*Podosphaera leucotricha*	Lesemann et al. (2006)
European pear scab	*Venturia pirina*	Pitman et al. (2007)
Chickpea Ascochyta blight	*Ascochyta rabiei*	Wise et al. (2007)
Creeping bentgrass anthracnose	*Colletotrichum cereale*	Young et al. (2007)
Potato black dot	*C. coccodes*	Aqeel et al. (2007)
Grapevine leaf blight[a]	*Pseudocercospora vitis*	Koya et al. (2008)
Tomato leaf mould[a]	*Fulvia fulva*	Watanabe H et al. (pers. comm.)
Almond scab	*Cladosporium carpophilum*	Adaskaveg JE et al. (pers. comm.)

[a]Detected in Japan.

Table 3.2 Deduced amino-acid sequences of cytochrome *b* in field isolates of plant pathogenic fungi

Species	Isolate	Response to QoI[a]	Sequence	
		Codon	129	143
Podosphaera xanthii	K-7-2	S	FLGYGLPYGQMSLW	GAT
	R-2	R	FMGYGLPWGGMSFW	AAT
Pseudoperonospora cubensis	S	S	FMGYVLPWGQMSFW	GAT
	R	R	FMGYVLPWGQMSFW	AAT
Corynespora cassiicola	C6-2	S	FTGYVLPYGQMSLW	GAT
	ST-20S-1	R	FTGYVLPYGQMSLW	AAT
Mycovellosiella nattrassii	T-1	S	FLGYVLPYGQMSLW	GAT
	K-1	R	FLGYGLPYGQMSLW	AAT

[a]S, sensitive; R, resistant. Reprinted from Ishii et al. (2007) Phytopathology 97:1458–1466.

(Ma and Michailides, 2004), quantitative real-time PCR (Fraaije et al., 2005) and pyrosequencing (Stammler et al., 2007), enabling the rapid detection and quantification of resistance. A linear relationship was obtained between the proportions of QoI-resistant isolates (or spores) and mutant alleles of cytochrome *b* gene in *Alternaria* species complex on pistachio (Young et al., 2007) and in *Plasmopara viticola* on grapevine (Toffolatti et al., 2007).

However, the status of heteroplasmy in the mitochondrial genome which encodes the multiple copies of cytochrome *b* gene can cause instability over time, making it difficult to precisely monitor QoI resistance in some pathogens. In 2004, 27 monoconidial isolates of cucumber powdery mildew (*Podosphaera xanthii*) were obtained from four prefectures which were far from each other, and their sensitivity to azoxystrobin was tested by leaf disk assay. All these isolates showed high resistance to azoxystrobin irrespective of the QoI usage history indicating that resistant strains were still widely distributed in Japan. Molecular diagnosis of QoI resistance in these isolates was then carried out by PCR-RFLP. The amplified products of cytochrome *b* gene were treated with the restriction enzyme *Fnu*4HI (=*Ita*I) which recognizes the resistance type sequence 5′-GCNGC-3′ containing the codon 143, followed by electrophoresis on agarose gel. Some of the PCR products derived from QoI-resistant isolates were clearly or partially digested with the enzyme whereas those from other highly resistant isolates were not.

The PCR products were further analyzed by sequencing directly or after cloning. Surprisingly, none of the cloned cytochrome *b* gene from some of the highly resistant isolates showed a mutation at codon 143 but were wild type sequences. Already earlier highly sensitive methods for the detection of mutated cytochrome *b* gene have been developed (Ishii et al., 2007). Using FMBIO, the mutated gene was quantified. It was present at frequencies between 14.7% and 98.3% in some resistant isolates but was much lower in others (below the detection limit of 1%). Recently, it was reported that the mechanism responsible for QoI resistance in *P. fusca* (=*P. xanthii*) was not linked to typical mutations in cytochrome *b* gene (Fernández-Ortuño et al., 2008).

QoI-resistant isolates of grey mould (*Botrytis cinerea*) were detected in commercial citrus orchards (Kansako et al., 2005) and strawberry greenhouses in Japan (Ishii et al., 2006; Fountaine et al., 2007). Results from *in vitro* sensitivity tests using QoI fungicides in a mixture with *n*-propyl gallate, an inhibitor of cyanide-insensitive alternative oxidase (AOX) coincided well with those performed on fungicide-treated cucumber cotyledons inoculated with the pathogen. Interestingly, the mutation at the amino acid position 143 of cytochrome *b* gene, known to be the cause of high QoI resistance in other pathogens, was found only in some but not other isolates. The wild-type sequence was present in the majority of resistant isolates indicating that the proportion of mutated cytochrome *b* gene was very low, but still had a profound effect on QoI resistance. Thus, heteroplasmy of cytochrome *b* gene may be assumed. Therefore, conventional sequence analysis of PCR-amplified cytochrome *b* gene is not appropriate for identification of QoI resistance in grey mould.

3.4 Instability of QoI Resistance

QoI-resistant populations of *Plasmopara viticola* gradually reverted to full sensitivity following consecutive transfers to untreated plants. When transferred on QoI-treated plants, sensitivity decreased resulting in almost full resistance after four generations (Genet et al., 2006). Under practical greenhouse conditions, QoI-resistant populations of cucumber powdery and downy mildew were stable for 1–2 years after QoI fungicides were removed from spray programmes (Ishii et al., 2002). Subsequently, resistant populations of cucumber powdery mildew declined gradually and it became difficult to detect resistant strains after the third year in these greenhouses. However, resistant strains were rapidly recovered when one treatment of azoxystrobin was applied again for the control of Corynespora leaf spot (Ishii et al., 2007).

Isolates of cucumber powdery mildew isolated from bulk conidial mass of lesions were inoculated successively on fungicide-untreated cucumber leaves. After three-and-a-half years, the mutated sequence at the codon 143 of cytochrome *b* gene was no longer detected despite that they still showed high resistance to azoxystrobin. Resistant bulk isolates became sensitive 8 years after the onset of sub-inoculations (Ishii et al., 2008a). Monoconidial isolates which were resistant to azoxystrobin also became QoI-sensitive after 2 years of sub-inoculations in the absence of fungicide selection pressure. The involvement of lower competitiveness of resistant isolates was ruled out in the latter case because all of these monoconidial isolates were sub-inoculated separately.

Lesemann et al. (2006) examined the electropherogram of the cytochrome *b* nucleotide sequence of apple powdery mildew, *Podosphaera leucotricha*. They found two overlapping guanine and cytosine peaks at position 143 in QoI-sensitive isolates, although the guanine peak was prominent indicating that the cytochrome *b* gene of this pathogen was heteroplasmic. Heteroplasmy of cytochrome *b* gene was also detected when QoI-resistant field isolates of *Corynespora cassiicola*, *Mycovellosiella nattrassii*, and *Colletotrichum gloeosporioides* were subcultured

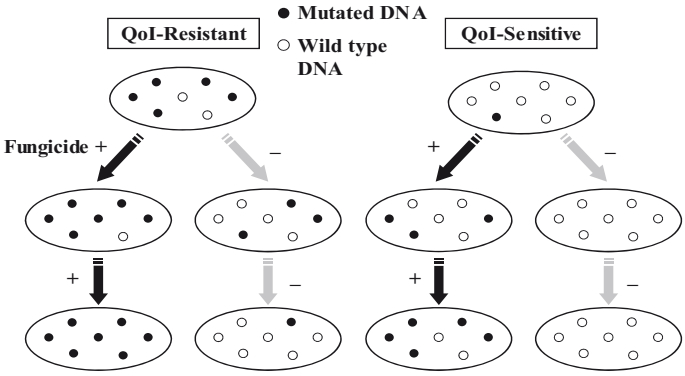


Fig. 3.1 Schematic representation of the effects of presence (+) and absence (–) of QoI fungicide selection pressure on heteroplasmic stages of mitochondrial cytochrome *b* gene (reprinted from Ishii et al., 2007 Phytopathology 97:1458–1466)

on PDA plates in the presence or absence of QoI fungicides (Ishii et al., 2007). The following hypothesis for heteroplasmic stages can be proposed which is supported by experimental and circumstantial evidences (Ishii, 2003; Ishii et al., 2007; Fig. 3.1): (1) in the absence of fungicidal selection pressure the mutated homoplasmic sequences of the cytochrome *b* gene in QoI-resistant strains may gradually revert to wild-type sequences resulting in transient heteroplasmic stages, and (2) the proportion of mutated sequences in heteroplasmic stages rapidly increases by higher copy numbers when receiving strong selection pressure by QoI fungicides.

3.5 Involvement of AOX in QoI Resistance

Ziogas et al. (1997) reported that modulation of AOX may induce QoI resistance but the presence of AOX did not affect sensitivity of *Septoria tritici* in practical situation. In grey mould, the results from *in vitro* sensitivity tests using QoI fungicides in a mixture with *n*-propyl gallate coincided well with those of tests performed on inoculated cucumber cotyledons. Clear correlation between the inhibition of mycelial growth on culture medium and that of lesion development on plants by QoI fungicides was observed also in strawberry anthracnose fungus (*Colletotrichum gloeosporioides*) (H. Ishii et al., 2008 unpublished). Therefore, AOX may also be involved in the expression of QoI resistance in field isolates of these fungi. The role of AOX has been studied also in phytopathogenic fungi such as *Magnaporthe grisea* (Yukioka et al., 1998) and *B. cinerea* (Tamura et al., 1999). In addition, possible interactions between AOX and QoI-target site mutations have been discussed (Avila-Adame and Köller, 2002; Wood and Hollomon, 2003). AOX was induced by reactive oxygen species accumulating in mitochondria when mitochondrial electron transfer was blocked by QoI fungicides. Mitochondrial DNA is generally thought to mutate in a

higher frequency than nuclear DNA. In the presence of QoIs, mutation rates in mitochondrial cytochrome *b* gene might be increased due to elevated levels of reactive oxygen species. Further research will be needed to clarify this possibility.

3.6 New QoI Fungicides

Orysastrobin will become quite popular among rice growers in Japan because the use in seedling boxes will contribute to diminish fungicide applications at later stages in paddy fields through its long-lasting control efficacy against blast and sheath blight. However, it is important to carefully monitor the sensitivity of rice blast fungus to this and other related QoI fungicides such as azoxystrobin and metominostrobin. It is quite possible that the G143A or F129L mutation in the cytochrome *b* gene conferring resistance to QoI fungicides may also be detected in the future in the rice blast fungus (*M. grisea*=*Pyricularia oryzae*) because these mutations caused QoI resistance in *P. grisea* in turf (Vincelli and Dixon, 2002; Kim et al., 2003). A survey on QoI resistance among *M. grisea* isolates from rice was conducted recently in Japan but resistance was not detected (Araki et al., 2005; Stammler et al., 2007). Using site-directed mutagenesis, G143A and F129L mutations were introduced into the plasmids containing cytochrome *b* gene sequence of the rice blast fungus (Wei et al., 2007). Molecular diagnostic methods were further developed for identifying resistance with PCR-RFLP. Recently, PCR-Luminex, a novel system developed for high throughput analysis of single nucleotide polymorphisms (SNPs) was successfully introduced to diagnose MBI-D resistance (inhibitors of scytalone dehydratase in melanin biosynthesis) in the rice blast fungus using specific oligonucleotide probes coupled with fluorescent beads (Ishii et al., 2008b). The PCR-Luminex system was further tested for its potential in simultaneous identification of QoI and MBI-D resistance (Katoh et al., 2008).

A novel QoI fungicide, pyribencarb is under development in Japan. Interestingly, this fungicide shows differential patterns of cross-resistance to pre-existing QoI fungicides (Ishii, 2008) although the same mode of action in complex III of mitochondrial respiration is assumed as with other QoIs (Kataoka et al., 2006). However, pyribencarb exhibits higher control efficacy against QoI-resistant isolates of grey mould than other QoI fungicides (Takagaki et al., 2006). Based on amino-acid sequence analysis, it was suggested that pyribencarb may slightly differ in the binding site of the cytochrome *b* protein pocket from other QoI fungicides (Kataoka et al., 2006).

3.7 Conclusions

The need for chemical control of plant diseases will remain important and the contributions of QoI fungicides won't change for the time being. To maintain or increase their values, it is essential to follow carefully resistance development of

pathogens. DNA-based monitoring of QoI fungicide resistance has severe drawbacks in several pathogens, because heteroplasmy of mutated cytochrome *b* gene can occur. Furthermore, it is equally important to elucidate the mechanisms of QoI resistance using biochemical and cytological approaches because not enough evidence from these fields is available yet. Discovering and developing new QoI fungicides may also be attractive as long as they exhibit a somewhat different cross-resistance pattern compared to pre-existing fungicides.

References

Aqeel AM, Pasche JS, Gudmestad NC (2007) Sensitivity of North American *Colletotrichum coccodes* isolates to azoxystrobin. Phytopathology 97:S159

Araki Y, Sugihara M, Sawada H, Fujimoto H, Masuko M (2005) Monitoring of the sensitivity of *Magnaporthe grisea* to metominostrobin 2001–2003: no emergence of resistant strains and no mutations at codon 143 or 129 of the cytochrome *b* gene. J Pestic Sci 30:203–208

Avila-Adame C, Köller W (2002) Interrelation between alternative respiration and target site mutations in resistance to QoI fungicides. Proc BCPC Conf-Pests Dis 2002:829–834

Fernández-Ortuño D, Torés JA, de Vicente A, Pérez-García A (2008) Field resistance to QoI fungicides in *Podosphaera fusca* is not supported by typical mutations in the mitochondrial cytochrome *b* gene. Pest Manag Sci 64:694–702

Fountaine JM, Takahashi K, Nishimura K, Inada M, Ishii H (2007) Heteroplasmy and its role in QoI resistant field isolates of *Botrytis cinerea*. Jpn J Phytopathol 73:254–255

Fraaije BA, Cools HJ, Fountaine J, Lovell DJ, Motteram J, West JS, Lucas JA (2005) Role of ascospores in further spread of QoI-resistant cytochrome *b* alleles (G143A) in field populations of *Mycosphaerella graminicola*. Phytopathology 95:933–941

FRAC Fungicide Resistance Action Committee (2004) http://www.frac.info/frac/index.htm [26 June 2009]

Genet JL, Jaworska G, Deparis F (2006) Effect of dose rate and mixtures of fungicides on selection for QoI resistance in populations of *Plasmopara viticola*. Pest Manag Sci 62:188–194

Gisi U, Sierotzki H, Cook A, McCaffery A (2002) Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. Pest Manag Sci 58:859–867

Ishii H (2003) Status of strobilurin resistance and related research in Japan. Abstr 8th Intr Congr Plant Pathol:82

Ishii H (2008) Fungicide research in Japan – an overview. In: Dehne HW, Deising U, Gisi U, Kuck KH, Russell P, Lyr H (eds) Modern fungicides and antifungal compounds V. DPG Selbstverlag, Braunschweig, Germany, pp 11–17

Ishii H, Fraaije BA, Sugiyama T, Noguchi K, Nishimura K, Takeda T, Amano T, Hollomon DW (2001) Occurrence and molecular characterization of strobilurin resistance in cucumber powdery mildew and downy mildew. Phytopathology 91:1166–1171

Ishii H, Sugiyama T, Nishimura K, Ishikawa Y (2002) Strobilurin resistance in cucumber pathogens: persistence and molecular diagnosis of resistance. In: Dehne HW, Gisi U, Kuck KH (eds) Modern fungicides and antifungal compounds III. AgroConcept, Bonn, Germany, pp 149–159

Ishii H, Chung WH, Fountaine JM, Tanahashi M, Kansako M, Miyake N (2006) Heteroplasmy in cytochrome *b* genes from cucumber powdery mildew fungus and gray mold: limitations of molecular diagnosis using PCR-RFLP methods. Jpn J Phytopathol 72:274

Ishii H, Yano K, Date H, Furuta A, Sagehashi Y, Yamaguchi T, Sugiyama T, Nishimura K, Hasama W (2007) Molecular characterization and diagnosis of QoI resistance in cucumber and eggplant pathogens. Phytopathology 97:1458–1466

Ishii H, Fountaine J, Nishimura K, Miyamoto T, Ushio S, Uzuhashi S, Kato H (2008a) Characteristics of fungal isolates resistant to QoIs and/or complex II inhibitors and fungicide efficacy against those isolates. Abstr 33rd Ann Meet Pest Sci Soc Jpn:112

Ishii H, Tanoue J, Oshima M, Chung WH, Nishimura K, Yamaguchi J, Nemoto F, So K, Iwama T, Yoshimatsu H, Shimizu M, Kozawa T (2008b) First application of PCR-Luminex system for molecular diagnosis of fungicide resistance and species identification of fungal pathogens. J Gen Plant Pathol 74:409–416

Kansako M, Yoneda Y, Shimadu K, Ishii H (2005) Occurrence of strobilurin-resistant *Botrytis cinerea*, pathogen of citrus gray mold. Jpn J Phytopathol 71:249

Kataoka S, Takagaki M, Nakano T, Kaku K, Fukumoto S, Watanabe S, Kozaki K, Shimizu T (2006) Inhibitory effects of the novel fungicide: pyribencarb on the complex III of the electron transport system in the respiratory chain. Abstr 11th IUPC Intr Cong Pestic Chem:138

Katoh H, Wei CZ, Ishii H (2008) An attempt at simultaneous detection of strobilurin-resistant and MBI-D-resistant isolates of *Magnaporthe grisea* using PCR-Luminex system. Jpn J Phytopathol 74:271

Kim YS, Dixon EW, Vincelli P, Farman ML (2003) Field resistance to strobilurin (QoI) fungicides in *Pyricularia grisea* caused by mutations in the mitochondrial cytochrome *b* gene. Phytopathology 93:891–900

Koya N, Inoue K, Kawaguchi A (2008) Occurrence of strobilurin-resistant isolates of *Pseudocercospora vitis*, grapevine leaf blight fungus. Jpn J Phytopathol 74:73–74

Kuck KH (2007) QoI fungicides: resistance mechanisms and its practical importance. In: Ohkawa H, Miyagawa H, Lee PW (eds) Pesticide chemisty. Crop protection, public health, environmental safety. Wiley-VCH, Weinheim, Germany, pp 275–283

Lesemann SS, Schimpke S, Dunemann F, Deising HB (2006) Mitochondrial heteroplasmy for the cytochrome *b* gene controls the level of strobilurin resistance in the apple powdery mildew fungus *Podosphaera leucotricha* (Ell. & Ev.) E.S. Salmon. J Plant Dis Protect 113:259–266

Ma Z, Michailides TJ (2004) An allele-specific PCR assay for detecting azoxystrobin-resistant *Alternaria* isolates from pistachio in California. J Phytopathol 152:118–121

McDougall P (2006) Phillips McDougall agriservice report. Pathhead, Midlothian, Scotland, UK

Pasche JS, Piche LM, Gudmestad NC (2005) Effect of the F129L mutation in *Alternaria solani* on fungicides affecting mitochondrial respiration. Plant Dis 89:269–278

Pitman TL, Elkins R, Gubler WD (2007) Detection of strobilurin resistant *Venturia pirina* populations in northern California. Phytopathology 97:S93

Sierotzki H, Parisi S, Steinfeld U, Tenzer I, Poirey S, Gisi U (2000) Mode of resistance to respiration inhibitors at the cytochrome bc_1 enzyme complex of *Mycosphaerella fijiensis* field isolates. Pest Manag Sci 56:833–841

Sierotzki H, Frey R, Wullschleger J, Palermo S, Karlin S, Godwin J, Gisi U (2007) Cytochrome *b* gene sequence and structure of *Pyrenophora teres* and *P. tritici-repentis* and implications for QoI resistance. Pest Manag Sci 63:225–233

Stammler G, Itoh M, Hino I, Watanabe A, Kojima K, Motoyoshi M, Koch A, Haden E (2007) Efficacy of orysastrobin against blast and sheath blight in transplanted rice. J Pestic Sci 32:10–15

Takagaki M, Kataoka S, Fukumoto S, Ishii H, Yamaguchi J, Inada M, Kansako M, Nozaki K (2006) The efficacy of the novel fungicide pyribencarb to the several QoI resistant fungal strains. Jpn J Phytopathol 72:274–275

Tamura H, Mizutani A, Yukioka H, Miki N, Ohba K, Masuko M (1999) Effect of the methoxyiminoacetamide fungicide, SSF129, on respiratory activity in *Botrytis cinerea*. Pestic Sci 55:681–686

Tanahashi M, Nakano T, Ishii H, Kodama M, Otani H (2006) Possible occurrence of strobilurin-resistant strains in *Alternaria alternata* causing black spot of European pear. Jpn J Phytopathol 72:275

Toffolatti SL, Serrati L, Sierotzki H, Gisi U, Vercesi A (2007) Assessment of QoI resistance in *Plasmopara viticola* oospores. Pest Manag Sci 63:194–201

Vincelli P, Dixon E (2002) Resistance to QoI (strobilurin-like) fungicides in isolates of *Pyricularia grisea* from perennial ryegrass. Plant Dis 86:235–240

Wei CZ, Nishimura K, Ishii H (2007) Bioassay for strobilurin sensitivity of rice blast fungus in Japan and site-directed mutagenesis of PCR-amplified cytochrome *b* gene fragment leading to the G143A and F129L substitutions. Jpn J Phytopathol 73:38

Wise KA, Bradley CA, Pasche JS, Gudmestad NC (2007) Reduced sensitivity of *Ascochyta rabiei* isolates to azoxystrobin and pyraclostrobin. Phytopathology 97:S124

Wood PM, Hollomon DW (2003) A critical evaluation of the role of alternative oxidase in the performance of strobilurin and related fungicides acting at the Qo site of complex III. Pest Manag Sci 59:499–511

Young J, Tomaso-Peterson M, Wong FP (2007) Screening for azoxystrobin-resistant *Colletotrichum cereale* isolates of the Mid-South. Phytopathology 97:S128

Yukioka H, Inagaki S, Tanaka R, Katoh K, Miki N, Mizutani A, Masuko M (1998) Transcriptional activation of the alternative oxidase gene of the fungus *Magnaporthe grisea* by a respiratory-inhibiting fungicide and hydrogen peroxide. Biochim Biophys Acta 1442:161–169

Ziogas BN, Baldwin BC, Young JE (1997) Alternative respiration: a biochemical mechanism of resistance to azoxystrobin (ICIA 5504) in *Septoria tritici*. Pestic Sci 50:28–34

Chapter 4
Regulatory Aspects in Chemical Control of Fungal Diseases: Impact on Efficient Plant Production

Georg F. Backhaus

Abstract Chemical control measures against plant diseases have a long history. Already in the nineteenth century and even earlier chemicals containing copper, sulphur, or phenolic compounds were used. In the middle of the twentieth century new efficient fungicides against plant pathogens were invented. Since the 1960s an official approval for Plant Protection Products (PPPs) by special authorities was demanded. In Germany, this was the beginning of an obligatory testing of PPPs by authorities. Nowadays PPPs are intensively tested in the frame of the European evaluation system for active substances and in the national registration procedures of products. In addition, many regulations have been set up for the use of registered PPPs with special emphasis to application machinery, user protection, and distances to water bodies, natural habitats or living areas. Current discussions about new proposals for regulations in the European Union lead to suspects that regulations might become more strict in the future. As a consequence, the supply of innovative and efficient fungicides in agriculture may become insufficient. In contrast, other expert groups demand for efficient new PPPs as part of strict quarantine regulations to prevent spread and establishment of dangerous plant pathogens. Private food supply retailers build up their own regulations concerning residue levels. When combining all regulations including those of private organisations, there is reason for concern that efficient control of plant pathogens may be hampered in the future.

Keywords Plant protection products • Pesticide • Regulation • Registration • Fungal diseases

G.F. Backhaus (✉)
Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Erwin-Baur-Str. 27, 06484, Quedlinburg, Germany
e-mail: georg.backhaus@jki.bund.de

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,* Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_4,

4.1 Introduction

Since few years, concerns are expressed that specific regulations in plant protection, which are in preparation in Europe, might strongly influence efficient plant protection against fungal diseases in the future. In this paper, the actual situation in Europe will be described from a plant pathologist's point of view (dating July 2008). The European Union (Commission, Council and Parliament) intensively works on changing the *plant protection council directive 91/414 EEC of 15 July 1991* concerning the placing of plant protection products on the market, which is the basis for the evaluation and registration procedure of plant protection products in the European Union. In addition, the EU is developing a new strategy to further reduce the application of pesticides under practical conditions (so-called *thematic strategy for the sustainable use of pesticides*). This strategy has already been proposed for becoming a framework directive and is under intensive discussion.

On the other hand, important global changes are in progress: the significance of plant production is recognised widely, and prices for agricultural products in the meantime have risen in a way which has not been seen for decades. World population faces growing demands for food, feed and resources. Several developing countries suffer from a growing food and feed deficiency, whereas in developed countries a competition came up between crops for food and feed, crops for energy and crops for renewable basic materials for industry (Tiedemann et al., 2008). In addition, a country like Germany looses 110 ha of arable land every day for infrastructure like roads, housing and industry. Not long ago, the European Union financially supported the set-aside of agricultural land for ecological purposes. Nowadays, the intensification of agricultural land has become important and fallow land is used for agricultural or horticultural productions again.

At the same time, the genetic potential of most crops is still not sufficiently exploited. Experts of the Commonwealth Agricultural Bureau recently estimated for the seven most important crops world wide that about 30–70% of the potential yields may still be lost mostly due to diseases, pests and weeds. However, it is difficult to estimate the losses caused by fungal diseases, because the number of pathogens and the extent of diseases they cause vary widely between crops, climatic conditions and cropping systems. Estimations for annual losses vary between 28% in Europe and almost 50% for Africa. In addition, there are crops like sweet lupines, protein peas and broad beans which are not very competitive in production systems compared to e.g. soybeans, mainly because they are highly susceptible to diseases caused by *Ascochyta* sp. and *Botrytis fabae* in beans, *Colletotrichum lupini* in *Lupinus angustifolius*, and *Sclerotinia sclerotiorum* and *Rhizoctonia* sp. in peas, and also because abiotic stress and low reliability in yield are of concern.

4.2 Chemical Control of Plant Diseases and Public Opinion

When considering the importance of fungal diseases in plants and comparing the known strategies for disease control, it becomes clear that fungicides belong to the most important tools for farmers worldwide to be economically successful.

Despite intensive search for resistant varieties, biological methods against plant diseases and sophisticated crop management tools like computerized forecasting models or threshold values, the use of fungicides is urgently needed to stop or reduce infection frequency and intensity. Due to the biology and genetic variability of pathogens, a bundle of active substances applied in diverse combinations is necessary to control fungal diseases of important crop plants and to avoid the development of resistance against fungicides.

Plant pathologists and farmers are familiar with this situation, however a great part of the largely urbanized society especially in developed wealthy countries is still not aware of:

- The importance of agricultural and horticultural productions for the well-being of humans
- The need to increase the capacity, intensity and efficiency of agricultural and horticultural production on the available area of productive land
- The amount of losses in plant yield and plant products due to fungal diseases and pests
- The risks or dangers which might arise from the worldwide shortage of resources and the competition for food, feed, bio-energy and renewable primary products

The society in developed countries tends to overestimate the potential negative effects resulting from the use of antifungal compounds in agriculture and horticulture and underestimates the risks arising from shortage of tools which are necessary to meet the challenges of plant production for food and feed in the future. Antifungal treatments are important tools of integrated pest management systems.

4.3 Historical Aspects

Opinions and convictions of the society are always reflected in regulatory activities of governments and authorities in democratic countries. In this respect, also the control of fungal diseases, in particular when using chemical products, interferes with regulatory systems as will be exemplified with historical aspects of plant protection and its regulations in Germany. Ever since mankind had to cope with plant diseases caused by pathogenic fungi, chemical means were used as preferred solutions: In ancient China, lime and wood ash were used against harmful organisms. Democritos of Abdera (460–377 b. Chr.) recommended to treat seeds with sap of *Sedum acre* before sowing to prevent plants from soil borne diseases. Plinius senior (79–23 b. Chr.) suggested to use sulphur against mildews. During the European middle age, farmers were sprinkling oil and sulfur against powdery mildews and mould. Glauber (1604–1670) used sodium sulphate and salicylic acid against mycosis on roses or calcium oxide against diseases on grapevine (Mayer, 1959). In 1857 in France and later all over Europe the famous Bordeaux mixture was applied in vineyards to control downy mildew. In addition, also mercury chloride, saltpetre and other antifungal anorganic substances were used. Copper-fungicides are still widely used today, in particular in organic productions. From 1930 onwards the

first organic fungicides such as Tetramethylthiuramdisulfide (Thiram) came to the market (for more information see also Reichardt Chr., 1771; Frank, 1896; Mayer, 1959; Raychaudhuri, 1964).

In Germany, legal regulations in plant protection already started in the beginning of the nineteenth century. Most of these regulations, however, were addressed to garden or property owners to control weeds and certain pests and to prevent their spread. One of the most important political aims at that time was the production of sufficient food and feed for the fast growing human population and the reduction of yield losses due to pests and diseases. The evaluation of plant protection products was only voluntary (Böning, 1972; Drees, 1987). The first regulations for official tests and approvals of plant protection chemicals by state authorities were laid down in the 1960s (plant protection act) after farmers associations demanded an official guarantee for good efficacy of the plant protection products without causing phytotoxicity. Additional pressure was generated by the publication of "Silent Spring" (Carson, 1962) although scientists expressed concerns about side effects of chemicals already at the beginning of the twentieth century (Richter, 1910). Since then increasing attention was paid to the potential health effects of agricultural products to users (farmers) and consumers as well as effects of plant protection products on bystanders, ground water, bees, earthworms, birds, mammals, beneficial organisms, water organisms and diverse environmental compartments. In the last 20 years, the evaluation procedures for plant protection products, in particular the demands by environmental authorities rose significantly.

4.4 Facing the Future

Today, there still is an efficient number of antifungal compounds available for manifold applications and uses in agriculture and horticulture. In addition, modern substances have many advantages compared to the chemicals used in old times. This was supported over decades by intensive research and responsible cooperation through authorities, companies and research institutes. However, in Europe the number of active substances which are allowed to be used in PPPs declined drastically since 2001 from more than 800 to about 250 substances today. This is in largely due to the intensive regulatory system built up in Europe over the last two decades. Four specifications of regulations with impact on treatments against fungal diseases may be differentiated and will be discussed below.

4.4.1 Regulations Concerning the Registration and Placing on the Market of Fungicides

These regulations are most important for the availability of fungicides and have considerable impact on the successful control of fungal diseases of plants, even though they may not become obvious to the farmer:

4.4.1.1 Regulations Concerning the Evaluation of New Active Substances

In Europe, new plant protection substances are evaluated at first by one or two of the EU-member states which carry the overall responsibility. The other member states send their experts in the different expert panels. All requirements are laid down in the appendices of the European plant protection council directive 91/414 EEC. Each criterion requires a certain work and data package. Scientists responsible for human or animal health and for certain compartments of the environment contribute to the requirements with manifold justifications. At the end of the procedure the European commission decides, based on scientific recommendations, whether the substance will be included in a positive list, the Annex 1 of the directive 91/414 EEC. After this process, national evaluations of the commercial product follow and may lead to a final registration and national use permit. This complicated and expensive process takes many years. According to the European definition of the term "plant protection product" also the so called "biologicals", if based on micro-organisms or viruses as active substances, are included in such evaluation procedures, although in some cases fewer or simpler evaluation criteria may be applied (Anonymous, 2001).

4.4.1.2 Regulations Concerning Substances Already in Use

Substances which are already in use have to be re-evaluated every 10 years at the very latest. They again undergo a European evaluation similar to that described above and in addition also a national evaluation of the product. Often new studies are demanded for most criteria which had already been evaluated 10 years ago. When handled carefully and responsibly, such regulations may result in a good compromise between the requirements for ecology, consumer's protection and agricultural/horticultural needs, as the past decade has shown. However, if over-regulation would arise, severe consequences for the successful control of fungal diseases in crop production systems have to be assumed.

In Europe, new regulations for the years to come are already in discussion. The European Union intends to change the existing plant protection council directive 91/414 EEC (Beerbaum, 2008). Directives are not in force until they are transferred to the member states by national legal acts. However, the new proposals will be included in a European regulation which will then be directly in force in the member states. The proposed regulation may contain positive decisions, like registrations oriented to climatic conditions and cropping systems (zones) which may include several European countries instead of single national registration. On the other hand, new and very strict evaluation criteria for pesticides are in discussion including "cut-off criteria", e.g. for so called endocrine disruptors. If an active substance does not meet such criteria, registration may be almost impossible. Other criteria are combined with a risk management programme for the application under conditions of good agricultural practice or integrated pest management. These drawbacks might result in a withdrawal from registration of several key fungicides urgently needed for disease

control in important crops. Preliminary estimations suggested that probably between 10% and 40% of the known active substances of PPPs may disappear. Other estimations talk about a reduction of up to 70%, depending on the quantity and quality of cut-off criteria (Driver and Rush, 2008; IVA, 2008; Kaus, 2008; Mukherjee, 2008; Rickard, 2008). Even though these regulations might enhance legal harmonization between European countries, the consequences will be highly significant for an efficient plant production in the near future.

4.4.1.3 New Legal Regulations and Their Possible Consequences

Although chemical companies may continue to develop new innovative substances, these are not available for farmers until the official evaluation processes may reach positive decisions. This means: farmers, although in competition on the world market, may not participate in innovative technologies early enough. In minor crops and major crops in which fungicide resistant pathogen populations exist new innovative substances are urgently needed. Older active substances are often used for covering minor use gaps. If costs and the duration of registrations are strongly enhanced by additional regulations, companies may loose interest in keeping certain compounds on the market after patents have run out. The more complicated and strict regulations become, the fewer substances will be available for disease control particularly in minor crops. It is an open question whether resistant crop varieties or biological and biotechnical methods may completely replace the application of plant protection substances used so far. Also in organic farming no true alternatives to certain naturally occurring plant protection chemicals such as copper and sulphur are available although it is well known that some substances like copper exerts negative side effects on environmental compartments.

There are specific disease problems such as those caused by invasive species or quarantine diseases which may not be controlled when strict regulations for the registration and use of PPPs exist. A lack of efficient tools hinders the successful eradication or prevention of the spread of invasive and aggressive pathogens. This is also true for diseases, like those caused by *Fusarium* spp., which can lead to produce mycotoxins in crops like cereals, maize, or asparagus.

It is increasingly difficult to discover new substances with antifungal activity which meet all requirements at the same time. Compounds with a high potential against diseases may be abandoned in the screening process of chemical companies because other requirements are not fulfilled such as toxicological, economical and ecological criteria. The new European regulations and evaluation procedures may further downgrade the factor of success in this selection process. In addition, only few companies can nowadays afford research and development for new active ingredients with much less money spent for developing new products. The evaluation procedures require many highly qualified scientists while on the other hand the number of specialists in plant pathology, taxonomy and development of modern plant protection technologies has continuously diminished over the last years. It is time to change this development, e.g. through educational and political measures,

if modern society wants to meet the future challenges for the production of high quality food, feed and bio-energy.

4.4.2 Regulations Concerning the Use and Application of Fungicides

Many specific regulations have been set up for the use and application of registered plant protection products with special regard to application machinery and equipment, users' protection, distance of applications to water bodies, natural habitats and living area. In cases where the ecological risks for a compound are generally not acceptable, the registration may still be possible if the application is accompanied by special risk management regulations. There are general regulations but also substance-specific regulations. One important general regulation laid down in the German plant protection act is as follows: PPPs are allowed to be applied only for agricultural, horticultural and forestry use. This means that in many parts of the country they may not be used, at least not without applying for a special permission, e. g. in parks, on playgrounds, in parking places, on plants along roads, inside cities, on sport fields, on managed grass land with fruit trees and in parts of allotment gardens. Another general regulation says that applications of PPPs can be made only at a certain distance to surface water. On the other hand, this general regulation differs in Germany from state to state. Applications of substances with properties affecting water organisms require the obligation to keep a minimal distance from surface water between 10 and 50 m, depending on the application technique. These regulations may satisfy environmental concerns; on the other hand, they may have epidemiological and economical consequences. The inoculum potential of plant pathogens may remain high in such area, and the spread of new infections may occur from there at any suitable condition. This is especially true for diseases which spread by aerial propagules, but also for some soil borne fungal diseases and quarantine organisms as it may not be possible to eradicate the pathogen unless the entire crop is removed. For emergency cases, specific regulations and exceptions can be granted also for the use of nonregistered substances. In addition, the European Union is developing a new strategy to further reduce the application of pesticides (thematic strategy for the sustainable use of pesticides) which is proposed to become a framework directive.

4.4.3 Regulations Concerning the Prevention of Spread of Dangerous/Invasive Pathogens

With the rapidly growing global trade activities an enhanced spread of alien species from one continent to the other is recorded. In addition, a gradient of pathogen dispersal is observed along changing climatic conditions, in particular

in Europe from south to north or from southeast to northwest. This is not only a result of changing climatic conditions but also of different disease pressure and crop systems and intensity, e.g. the expansion of certain crops like maize. There are intensive international activities, in particular in the frame of IPPC, the International Plant Protection Convention, with more than 120 member states, to create international standards for plant quarantine issues. Most of these standards include guidelines or principles for practical measures which may include eradication programmes. The application of chemical PPPs may be part of such programmes. Guidelines often demand to eradicate pathogens by using a so called "suitable pesticide", while on the other hand such chemicals may not be legally marketed and used. In such cases, the regulations on the use of chemicals and on phytosanitary obligations are conflicting. In the end the only solution for the grower concerned may be to eliminate his crop. This is of particular significance for high value crops like ornamentals, spice and herb plants or for standing plantations like fruit trees, hops or grapevine. It is vital to promote a close cooperation and better understanding between the different authorities which are responsible for quarantine issues on the one hand and for registration of pesticides on the other hand.

4.4.4 Nonofficial Regulations on the Basis of Private Contracts

A very peculiar case of a nonofficial regulation came up during the last few years in Europe. Officially, the residue levels for plant protection products in plants and plant products are assessed on the basis of scientific expert evaluations. Since September 2008, most of the residue levels are harmonized within the EU member states. On the other hand, private food supply retailers recently got under severe pressure by NGOs concerning residue levels in vegetables and fruits, because they do not accept many of the scientific evaluations and results. As a consequence, certain food retailers demand from their suppliers that plants or plant products may contain a maximum of one third of the officially allowed residue levels of a registered plant protection product and not more than three different chemical compounds are identified. With this strategy the retailers hope to better advertise their goods, attract more clients and at the same time avoid the criticism of NGOs and consumers associations. However, a severe drawback of this attitude for vegetable and fruit growers is that waiting periods between the last application of plant protection products and harvesting time will be stretched such far that a reasonable disease control especially in crops with short rotations becomes extremely difficult. As a reaction, some farmers even intensify chemical applications in the beginning of the crop and stop applying towards harvest in order to meet those strict requirements which may be seen in conflict to the principles of integrated pest management.

4.5 Summary

Official and private regulations have been set up for the registration and use of PPPs in Europe. They are partly in conflicting with the obligation to prevent the spread of dangerous and invasive plant pathogens. There is serious concern that regulations in plant protection in Europe already now and more so in the near future affect the availability of suitable means for pathogen control in agriculture and horticulture. Future prospects might lead to severe deficiencies in plant protection for the effective control of fungal pathogens, especially in minor crops.

References

Anonymous (2001) Council directive 2001/36/EG of 16th May 2001. Off J Eur Union L164:1–64

Beerbaum S (2008) Die neue EU-Verordnung über das Inverkehrbringen von Pflanzenschutzmitteln – aktueller Sachstand. Proceedings of 56th German plant protection, Mitt. Julius Kühn-Institut 417:287

Böning K (1972) Zur Geschichte der Polizeiverordnungen über Gifte und giftige Schädlingsbekämpfungsmittel. Nachrichtenbl. Deut. Pflanzenschutzd. 24:104–108

Carson R (1962) Silent spring. Riverside Press, Cambridge, USA 368 pp

Drees H (1987) 50 Jahre Pflanzenschutzgesetz. Gesunde Pflanzen 39(3):120–122

Driver A, Rush T (2008) EU pesticide proposals threaten food production. http://www.farmersguardian.com/story.asp?sectioncode=19&storycode=18488 access: 22.5.2008

Frank AB (1896) Die Krankheiten der Pflanzen – Ein Handbuch für Land- und Forstwirte, Gärtner, Gartenfreunde und Botaniker, 2nd vol. Die pilzparasitären Krankheiten der Pflanzen. Trewendt, Breslau, Germany, 674 pp

IVA, Industrieverband Agrar (2008) Pflanzen ohne Schutz? Brüsseler Pläne gefährden die Zukunft des Pflanzenschutzes. http://www.iva.de/Branche_Verband/br_pflanzenohneschutz.asp access: 05.8.2008

Kaus V (2008) Aktuelle Entwicklungen bei der Novelle der EU-Pflanzenschutzrichtlinie 91/414/EWG. Proceedings of 56th German plant protection conference, Mitt. Julius Kühn-Institut 417:287

Mayer K (1959) 4500 Jahre Pflanzenschutz. Ulmer, Stuttgart, p 45

Mukherjee S (2008) Effective chemicals may be lost. BBC news –science/nature. http://newsvote.bbc.co.uk/mpapps/pagetools/print/news.bbc.co.uk/1/hi/sci/tech/74056 access: 22.5.2008

Oerke E-C (1999) The importance of disease control in modern plant production, pp 11–17. In: Lyr H, Russell PE, Dehne H-W, Sisler HD (eds) Modern fungicides and anti-fungal compounds II. 12th international reinhardsbrunn symposium, Intercept, Andover, UK

Oerke E-C, Dehne HW, Schönbeck F, Weber A (1999) Crop production and crop protection – estimated losses in major food and cash crops. Elsevier, Amsterdam, The Netherlands, p 790

Raychaudhuri SP (1964) Agriculture in ancient India. Indian Council Agricultural Research, New Delhi, pp 84–98

Reichardt Chr (1771) Land- und Gartenschatzes sechster Teil. Worinnen vom Hopfenbaue, wie auch von den vornehmsten Blumen-Gewächsen, deßgleichen von Vertilgung der schädlichen Thiere und Ungeziefer auf den Aeckern und in den Gärten gehandelt worden. 3. Edition, Universitäts-Buchdr. Heinrich Rudolph Nonne, Erfurt, Germany

Richter R (1910) Der neue Obstbau, 2nd edn. Jungborn, Stapelburg/Harz, Germany, p 134

Rickard S (2008) An economic assessment of the impact of proposed restrictions on crop protection substances. Cranfield University School of Management, Research Papers September 2008, 25 pp

Tiedemann A, von Heitefuss R, Feldmann F (2008) Pflanzenproduktion im Wandel – Wandel im Pflanzenschutz. *Spectrum Phytomedizin*. German Phytomedical Society, Braunschweig, Germany, p 164

Part II
Concepts in Biological Control of Plant Pathogens

Chapter 5
The Roles of Cyclic Lipopeptides in the Biocontrol Activity of *Bacillus subtilis*

Marc Ongena, Guillaume Henry, and Philippe Thonart

Abstract *Bacillus subtilis* species are well-known and extensively-used beneficial rhizobacteria for biocontrol of plant diseases. Their interest arises from their ability to generate a broad array of bioactive metabolites among which three families of cyclic lipopeptides (CLPs). These CLPs display a huge diversity of structures and physico-chemical and biological properties which probably account for an important part of the biocontrol potential of the producing strains. Beside their antimicrobial properties, they are also involved in colonization and motility as well as in the systemic stimulation of immune system of the host plant. We summarize here the current knowledge of CLPs activities and focus on the recent findings in the context of biocontrol.

Keywords Surfactin • Iturin • Fengycin • Antibiotic • Biofilm • Induced systemic resistance

5.1 Introduction

Members of the *Bacillus* genus are among the beneficial bacteria mostly exploited as biopesticides to control plant diseases. *Bacillus*-based products represent about half of the commercially available bacterial biocontrol agents (Fravel, 2005). Within the *Bacillus* genus, members of the *B. subtilis* species have the potential to produce a wide array of bioactive compounds among which, cyclic lipopeptides (CLPs) of the surfactin, iturin and fengycin families that have well-recognized

M. Ongena (✉)
Centre Wallon de Biologie Industrielle, Unité de Bioindustries, Gembloux Agricultural University, Passage des déportés, 2, B-5030, Gembloux, Belgium
e-mail: ongena.m@fsagx.ac.be

G. Henry, and P. Thonart
Walloon Center of Industrial Biology, Gembloux Agricultural University, Belgium

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,* Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_5,

potential utilization in biotechnology and biopharmaceutical applications due notably to their surface-active properties (Banat et al., 2000; Singh and Cameotra, 2004). These surfactants may also have different roles in the development and survival of *B. subtilis* strains in their natural habitat. It includes increasing of the surface area and bioavailability of hydrophobic water-insoluble substrates, heavy metal binding, bacterial pathogenesis, quorum sensing, motility and biofilm formation (Mulligan, 2005; Ron and Rosenberg, 2001). Other works have highlighted additional traits that are also very important for the fitness of *Bacillus subtilis* in the rhizosphere and for its efficacy as biocontrol agent. All these biocontrol properties that are reviewed here explain why these three CLPs families focus researchers and companies' attention.

5.2 *Bacillus* CLPs: A Variety of Structures

Bacillus CLPs are synthesized by non ribosomal peptide synthetases or hybrid polyketide synthases/non ribosomal peptide synthetases. These modular proteins are megaenzymes organized in iterative functional units called modules that catalyze the different reactions leading to polyketide or peptide formation (Stein, 2005; Finking and Marahiel, 2004). Such biosynthetic systems lead to a remarkable heterogeneity among the CLPs products which vary in the type and sequence of amino acid residues, the nature of the peptide cyclisation and in the nature, length and branching of the fatty acid chain (Fig. 5.1).

The three CLPs families thus encompass structural variants depending on the genetic background of the strain considered. The variants differ in their peptide sequence due to some amino acid substitutions. Within each variant group, there

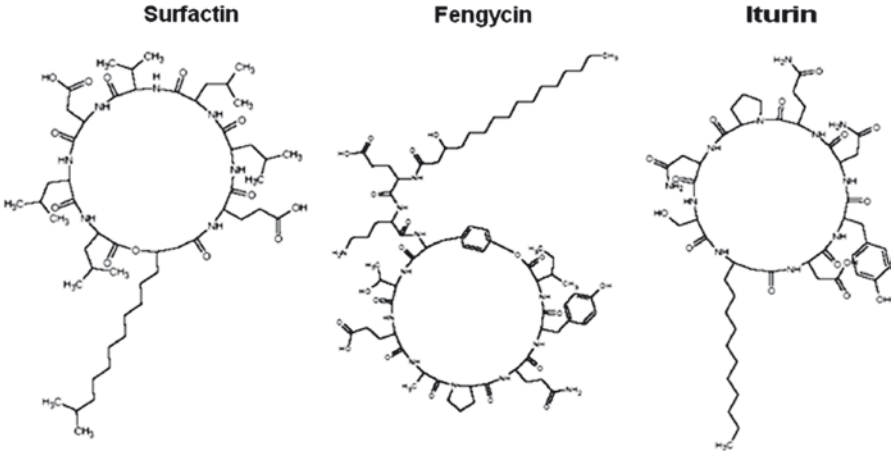


Fig. 5.1 Structures of representative members of the three lipopeptide families synthesized by *Bacillus* species

are several homologues that are co-produced and that differ in the length and isomery of the fatty acid chain. Surfactins are heptapeptides interlinked with a β-hydroxy fatty acid to form a cyclic lactone ring structure. The group of iturins encompasses seven variants including bacillomycins and mycosubtilin. All are heptapeptides linked to a β-amino fatty acid chain with a length from C_{14} to C_{17}. The third family comprises fengycins A and B, which are also called plipastatins. These molecules are lipodecapeptides with an internal lactone ring in the peptidic moiety and with a β-hydroxy fatty acid chain (C_{14}–C_{18}) that can be saturated or unsaturated.

Each *Bacillus* CLP family displays both common and specific activities. Because of their amphiphilic nature, they all may readily associate and tightly anchor into lipid layers (Brasseur et al., 2007; Heerklotz and Seelig, 2007). They can thus interfere with biological membrane integrity in a dose-dependent manner but the susceptibility of biological membranes varies in a specific manner. This could explain the different targets of each family. Besides the antimicrobial activities described below and mainly depending on membrane interactions, surfactins could also interfere with several stages of the immune processes. They indeed display lipopolysaccharide-binding and neutralizing activities (Hwang et al., 2005; Takahashi et al., 2006), antitumor (Kameda et al., 1974; Kim et al., 2007), anti-inflammatory (Hwang et al., 2007) properties and can also inhibit platelet aggregation (Kim et al., 2006). Finally surfactins could inhibit AMPc phosphodiesterase, alkaline phosphatase activities (Bortolato et al., 1997) and either inhibit or enhance phospholipases A2 depending on their origin (Kim et al., 1998).

5.3 *Bacillus* CLPs: A Variety of Biocontrol-Related Activities

5.3.1 Involvement in Root Colonization

The ability of *B. subtilis* to efficiently colonize surfaces of plant roots is a prerequisite for phytostimulation. The first step of translocation on surfaces like roots, the spreading, could be achieved through several ways. The probably most studied is the cell motility in colonies, swarming, which involves differentiation of vegetative cells into hyperflagellated ‘swarmer cells’ that undergo rapid and coordinated population migration across solid surfaces (Fraser and Hughes, 1999). This swarming process allows an easier colony spreading but also an improved antimicrobial resistance. The second step for rhizosphere competence is linked to the capability to form sessile, highly structured and antimicrobial resistant multicellular communities. Microbial populations such as plant-associated bacteria evolve and behave as structured communities called biofilms (on solid surfaces) or pellicles (at air/liquid interfaces) that could adhere to root and on soil particle surfaces (Danhorn and Fuqua, 2007). Such microcolonies are sites for bacteria to communicate with each other (quorum sensing) and to act in a coordinated manner. When confronted with nutrient deprivation, *B. subtilis* cells could, among others, sporulate at highly ordered and surface associated cell clots within the aerial structures projecting from

the surfaces of *B. subtilis* biofilms and pellicles, the "fruiting-bodies" (Branda et al., 2001). Several studies showed that CLPs could be involved at different levels in the complex network linking motility and biofilms/pellicles/fruiting-bodies formation.

So the CLPs surfactin and mycosubtilin have been shown to be implicated in a flagella-independent surface motility of *B. subtilis* (Kinsinger et al., 2003; Leclère et al., 2006). Surfactin is thought to act by the aggregation of the cells into dendrites and by the coordination of their advance throughout the swarm front (Julkowska et al., 2005). This surfactant-induced spreading is most likely due to a reduction of frictions between cells and surface in combination with a surface tension-driven flow. A low surface tension which could be reached with strong surfactants such as surfactin and mycosubtilin was demonstrated to be sufficient to facilitate microbial colonization (Leclère et al., 2006). This could explain why surfactin production is necessary but not sufficient for swarming, in which other factors like genes *swrABC* and *efp* are additionally involved (Kearns et al., 2004). Nonetheless, results obtained with *B. subtilis* A1/3 have shown that surfactin but no other lipopeptides produced by the strain was required for the formation of biofilms and pellicles indicating that surfactins may still serve specific developmental functions (Hofemeister et al., 2004). More conclusively in the view of biocontrol, the production of surfactin was demonstrated to be essential for biofilm formation and colonization of Arabidopsis roots by the strain *B. subtilis* 6051 and that biocontrol exhibited against *P. syringae* is linked to the production of this antibiotic at the root surface (Bais et al., 2004). Finally, genes that mediate production of surfactin (*srfAA* and *sfp*) were shown to be required for the erection of fruiting-bodies (Branda et al., 2001), at least in part by their ability to lower the surface tension of water. Interestingly, the regulation of surfactin biosynthesis is under the control of a complex network that governs cellular differentiation, including quorum sensing, confirming a correlation between *Bacillus* colonization and this CLP (Hamoen et al., 2003).

5.3.2 Involvement in Direct Antagonism

Once established in the phytosphere, *Bacillus* isolates can deploy their antibiotic arsenal and the involvement of CLPs in direct antagonism of phytopathogens is obvious. Surfactins display haemolytic (Dufour et al., 2005), antiviral (Kracht et al., 1999), antimycoplasma and antibacterial (Huang et al., 2008) activities but intriguingly, no marked fungitoxicity. Though they are also strongly haemolytic, iturins display a strong in vitro antifungal action against a large variety of yeast and fungi but only limited antibacterial and no antiviral activities (Hiradate et al., 2002; Yu et al., 2002). Finally fengycins are less haemolytic than iturins and surfactins but retain a strong fungitoxic activity more specifically against filamentous fungi (Vanittanakom et al., 1986) and some bacteriostatic effect against *E. coli* (Huang et al., 2008).

Reports suggesting a role for CLPs in antagonism based on in vitro studies are numerous but there are only few studies that associate biocontrol activity and CLPs by

testing the pure compounds *in planta*, or through their detection and quantification in the microenvironment where the producer strain has been inoculated, or by correlating the biocontrol activity and the use of non-producing or overproducing derivatives. In the case of soil-borne diseases, iturin A produced by *B. subtilis* RB14 was involved in damping-off of tomato (a seedling disease) caused by *Rhizoctonia solani* (Asaka and Shoda, 1996). Overexpression of mycosubtilin in *B. subtilis* ATCC 6633 also led to a significant reduction of seedling infection by *Pythium aphanidermatum* (Leclère et al., 2005). As examples in control of phyllosphere diseases, a contribution of both iturins and fengycins was recently shown in the antagonism of *B. subtilis* toward *Podosphaera fusca* infecting melon leaves (Romero et al., 2007). This was demonstrated by identifying iturins and fengycins as the main antibiotic products excreted by the strains, by showing the strong inhibitory effect of these CLPs on *P. fusca* conidia germination, and by recovering CLPs from bacterial-treated leaves and using CLP-deficient transformants. In the protection of post harvest diseases, the strain *Bacillus subtilis* strain GA1 which efficiently produces CLPs from the three families and notably a wide variety of fengycins, protected wounded apple fruits against gray mold disease caused by *Botrytis cinerea*. The role of fengycins was demonstrated by the very effective disease control provided by treatment of fruits with CLPs-enriched extracts and by in situ detection of fengycins in inhibitory amounts (Touré et al., 2004).

5.3.3 Involvement in Plant Systemic Resistance Elicitation

Another well established way for beneficial *Bacillus* isolates to provide plant protective effect is through the stimulation of the plant immune system. Some non-pathogenic rhizobacteria are indeed able to reduce disease through the stimulation of a priming state in host plant which allows an accelerated activation of defense responses upon pathogen or insect attack, leading to an enhanced resistance against the attacker encountered (Conrath et al., 2006). The list of *Bacillus* strains reported as plant resistance inducers has grown rapidly over the last decade and includes members of the *B. pumilus*, *B. mycoides*, *B. subtilis*, *B. amyloliquefaciens*, *B. pasteurii*, *B. thuringiensis* or *B. cereus* species (Bent, 2005; Kloepper et al., 2004). Much remains to be discovered about the molecular aspects of the *Bacillus*-mediated immunity and until recently, volatile organic compounds were the sole determinants for elicitation identified from *Bacillus* spp. (Ryu et al., 2004). However, we recently demonstrated that surfactins and fengycins also retain such eliciting activity (Fig. 5.2). In bean, pure fengycins and surfactins provided a significant induced protective effect similar to the one induced by living cells of the producing strain. Experiments conducted on bean and tomato showed that overexpression of both surfactin and fengycin biosynthetic genes in the naturally poor producer *B. subtilis* strain 168 was associated with a significant increase in the potential of the derivatives to induce resistance (Ongena et al., 2007).

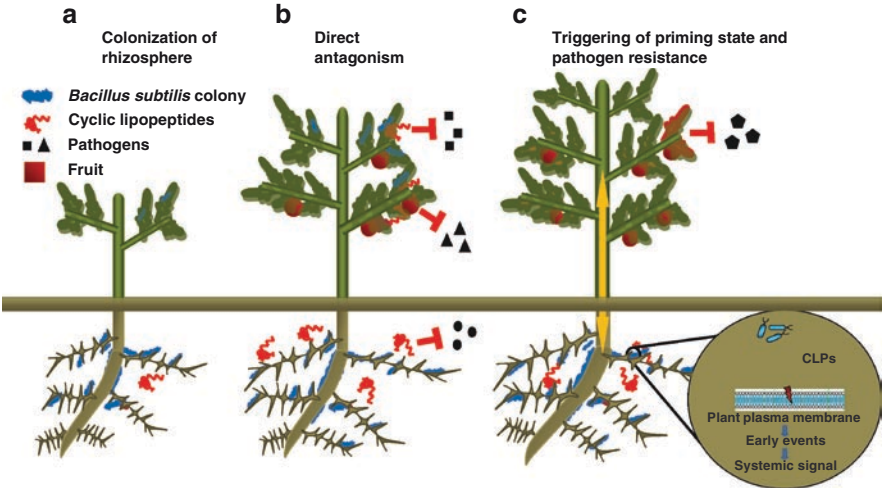


Fig. 5.2 Schematic view of the involvement of cyclic lipopeptides in the biocontrol activity displayed by *Bacillus subtilis*. (**a**) An efficient colonization of the host plant requires the ability to move along root surfaces and to develop colonies in organized structures such as biofilms. These amphiphilic CLPs allow spreading, and biofilm formation. (**b**) The three CLPs families possess specific antimicrobial properties which allow to antagonize various pathogens on different plant organs. (**c**) Surfactin and fengycin families may be perceived at the root level and trigger a systemic signal through the whole plant leading to the establishment of a primed state which allows a rapid activation of defense reactions in case of pathogen attack

5.3.3.1 Defense-Related Events Stimulated by CLPs in Plants

The macroscopic disease reduction induced by CLPs overproducers was associated with defense-related metabolic changes. In tomato, CLPs-overproducing *Bacillus* isolates stimulate two key enzyme activities of the oxylipin pathway (Ongena et al., 2007), a metabolic route initiated by the lipoxygenase enzyme (LOX) and leading to the formation of a wide array of biologically active secondary metabolites (Blée, 2002). A clear accumulation of non-polar antifungal compounds also occurred in the treated plants, suggesting a phytoalexin-inducing activity of *Bacillus* lipopeptides (Adam, 2008). In other systems, major changes in the defense response of plant cells were also observed upon treatment with CLPs. The addition of surfactin to tobacco cell suspension cultures and of fengycin to potato tuber cells led to significant modifications in the pattern of phenolics produced by the elicited cells (Jourdan et al., 2009). This strongly suggests some induction of the phenylpropanoid metabolism, which is stimulated concomitantly with the activation of plant defence reactions against pathogen infection or after other physical ingresses such as wounding (Dixon et al., 2002). Treatment of tobacco cell suspensions with surfactin in the micromolar range also induced some defence-related early events such as phosphorylation- and Ca^{2+}-dependent extracellular alkalinisation and oxidative burst without causing any significant cell death (Jourdan et al., 2009).

5.3.3.2 Relationship Between CLP Type and Plant Defense Inducing Activity

Surfactins are active on bean, tomato and tobacco but not potato. By contrast, fengycins which also stimulates an induced systemic resistance in bean and tomato plants, can elicit a response in potato tuber cells but do not trigger early events in cultured tobacco cells. Semi-purified CLPs extracts from the three families failed to confer any protection in cucumber, suggesting that they are not involved in the resistance triggering process in that plant. Intriguingly, ISR-inducing activity of iturins is not observed upon treatment of tomato plants, potato tuber slices or tobacco cells. It thus appears that the different types of *Bacillus* CLPs retain a specific ability to stimulate different plants suggesting the importance of specific structural features in the peptide moiety for the perception process by host cells.

However, it is not clear whether bacterial CLPs are recognized by plant cells via specific receptors since the reduced activity of surfactin homologues with short fatty acid chains also indicates that CLPs perception is dictated by structural clues in the acyl moiety (Jourdan et al., 2009). CLPs may thus interact via a less specific mechanism based on some destabilization of the lipid bilayer structure. Given their very low phytotoxicity, this perturbation should obviously remain limited but sufficient to induce disturbance or transient channelling in the plasma membrane that can in turn activate a biochemical cascade of molecular events leading to defensive responses.

5.3.4. Ecological Compatibility of CLP Producers

Because of their potential to disturb the integrity of biological membranes, cyclic lipopeptides secreted by *Bacillus* are potentially toxic for auxiliary microflora and higher organisms. For the later, several studies indicate that adverse concentrations should be up to 30 μM (toxicity against animal cell lines) or even much higher for hemolysis or toxicity toward insect larvae and fish (Das and Mukherjee, 2006; Dufour et al., 2005; Vollenbroich et al., 1997). Such concentrations are not biologically relevant in the context of the use of Bacillus inoculates for plant disease control since cLPs amounts produced by cells colonizing the host plant are far much lower (Ongena et al., 2007; Romero et al., 2007). Independent of the inoculum used, *Bacillus* populations in the rhizo/phyllosphere tends to equilibrate after a few weeks to reach a threshold sufficient (10^5–10^7 CFU/g of plant tissue fresh weight) to locally provide their beneficial effect to the host but most probably not sufficient to have a drastic and negative impact on other non-pathogenic communities sharing the environment. It is well exemplified by the two *Bacillus* strains QST-713 and FZB24, respectively registered as the biocontrol products Serenade (Agraquest) and TAEGRO (Novozymes Biologicals Inc.), that fulfill the ecotoxicological requirements. These bacteria co-produce the different lipopeptide families but do not pose a risk to non-target organisms or to the environment (http://www.epa.gov/pesticides/).

5.4 Other *Bacillus* Antibiotics Involved in Biological Control

Bacillus species synthesize numerous antimicrobial compounds with well-established activity in vitro. But only few of these metabolites have been studied for their production in natural field conditions in a way to demonstrate their implication in the biocontrol potential of the producing strains. However some studies have linked an in vitro antimicrobial activity with a direct antagonism of the phytopathogens but not with other ways of biocontrol so far.

Bacillus brevis (*Brevibacillus brevis*) and *Bacillus polymyxa* (*Paenibacillus polymyxa*) produce gramicidin S and polymyxin B peptide antibiotics that strongly inhibited in vitro *Botrytis cinerea* germination, growth and extra-cellular enzyme production but also exhibited under natural field conditions high activity against the *Botrytis* grey mould on strawberry (Haggag, 2008). *B. cereus* UW85 produces two fungistatic antibiotics, zwittermicin A and kanosamine that are suggested to contribute to the suppression of damping-off disease of alfalfa caused by *Phytophthora medicaginis* (Silo-Suh et al., 1994). Zwittermicin A may also control the fruit rot of cucumber (Smith et al., 1993) and suppress other plant diseases (Silo-Suh et al., 1998).

5.5 Conclusion

CLPs join volatile organic compounds (VOC) as *Bacillus* elicitors of the induced systemic resistance phenomenon. But more globally CLPs also constitute a novel class of microbial-associated molecular patterns that can be specifically perceived by plant cells as signals to activate defence mechanisms. This property adds with their numerous others beneficial activities making these compounds and their producing strains interesting weapons as biocontrol agents. From a more global point of view, the efficiency of biological control in field or greenhouse trials can been improved by combining microorganisms with different modes of action (De Boer et al., 2003; Guetsky et al., 2002). This implies that every component of the consortium must be produced separately prior to application and, after inoculation, each of them should efficiently colonize the environment without providing any negative effect on the development of the others. The selection of single strains evolving diverse mechanisms to reduce disease incidence is thus of prime interest. *Bacillus* isolates that co-produce the three LP families should display such a multi-faceted biocontrol activity.

Acknowledgements Work in our laboratory is funded by the Program F.R.F.C. No. 2.4624.06 (National Funds for Scientific Research, FRS-F.N.R.S, Belgium). G. Henry is recipient of a grant from the Formation à la Recherche dans l'Industrie et l'Agriculture (F.R.I.A.). M. Ongena and E. Jourdan are respectively research associate and post-doctoral researcher at the FRS-F.N.R.S.

References

Adam A (2008) Elicitation de la résistance systémique induite chez la tomate et le concombre et activation de la voie de la lipoxygénase par des rhizobactéries non-pathogènes. Ph.D. thesis, University of Liège, Belgium

Asaka O, Shoda M (1996) Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. Appl Environ Microbiol 62:4081–4085

Bais HP, Fall R, Vivanco JM (2004) Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiol 134:307–319

Banat IM, Makkar RS, Cameotra SS (2000) Potential commercial applications of microbial surfactants. Appl Microbiol Biotechnol 53:495–508

Bent E (2006) Induced systemic resistance mediated by Plant Growth-Promoting Rhizobacteria (PGPR) and Fungi (PGPF). In Multigenic and induced systemic resistance in plants. Tuzun, S., and Bent, E. (eds). New-York: Springer, pp. 225–258

Blée E (2002) Impact of phyto-oxylipins in plant defense. Trends Plant Sci 7:315–321

Bortolato M, Besson F, Roux B (1997) Inhibition of alkaline phosphatase by surfactin, a natural chelating lipopeptide from *Bacillus subtilis*. Biotechnol Lett 19:433–435

Branda SS, Gonzalez-Pastor JE, Ben-Yehuda S, Losick R, Kolter R (2001) Fruiting body formation by *Bacillus subtilis*. Proc Natl Acad Sci U S A 98:11621–11626

Brasseur R, Braun N, El Kirat K, Deleu M, Mingeot-Leclercq MP, Dufrene YF (2007) The biologically important surfactin lipopeptide induces nanoripples in supported lipid Bilayers. Langmuir 23:9769–9772

Conrath U, Beckers GJM, Flors V, Garcia-Agustin P, Jakab G, Mauch F, Newman MA, Pieterse CMJ, Poinssot B, Pozo MJ, Pugin A, Schaffrath U, Ton J, Wendehenne D, Zimmerli L, Mauch-Mani B, Prime APG et al (2006) Priming: getting ready for battle. Mol Plant Microbe Interact 19:1062–1071

Danhorn T, Fuqua C (2007) Biofilm formation by plant-associated bacteria. Annu Rev Microbiol 61:401–422

Das K, Mukherjee AK (2006) Assessment of mosquito larvicidal potency of cyclic lipopeptides produced by *Bacillus subtilis* strains. Acta Trop 97:168–173

De Boer M, Born P, Kindt F, Keurentjes JJB, van der Sluis I, van Loon LC, Bakker PAHM (2003) Control of Fusarium wilt of radish by combining *Pseudomonas putida* strains that have different disease-suppressive mechanisms. Phytopathology 93:626–632

Dixon RA, Achnine L, Kota P, Liu CJ, Reddy MSS, Wang LJ (2002) The phenylpropanoid pathway and plant defence – a genomics perspective. Mol Plant Pathol 3:371–390

Dufour S, Deleu M, Nott K, Wathelet B, Thonart P, Paquot M (2005) Hemolytic activity of new linear surfactin analogs in relation to their physico-chemical properties. Biochim Biophys Acta – Gen Subjects 1726:87–95

Finking R, Marahiel MA (2004) Biosynthesis of nonribosomal peptides. Annu Rev Microbiol 58:453–488

Fraser GM, Hughes C (1999) Swarming motility. Curr Opin Microbiol 2:630–635

Fravel DR (2005) Commercialization and implementation of biocontrol. Annu Rev Phytopathol 43:337–359

Guetsky R, Shtienberg D, Elad Y, Fischer E, Dinoor A (2002) Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. Phytopathology 92:976–985

Haggag WM (2008) Isolation of bioactive antibiotic peptides from *Bacillus brevis* and *Bacillus polymyxa* against *Botrytis grey* mould in strawberry. Arch Phytopathol Plant Prot 41:477–491

Hamoen LW, Kausche D, Marahiel MA, van Sinderen D, Venema G, Serror P (2003) The *Bacillus subtilis* transition state regulator AbrB binds to the-35 promoter region of comK. FEMS Microbiol Lett 218:299–304

Heerklotz H, Seelig J (2007) Leakage and lysis of lipid membranes induced by the lipopeptide surfactin. Eur Biophys J Biophys Lett 36:305–314

Hiradate S, Yoshida S, Sugie H, Yada H, Fujii Y (2002) Mulberry anthracnose antagonists (iturins) produced by *Bacillus amyloliquefaciens* RC-2. Phytochemistry 61:693–698

Hofemeister J, Conrad B, Adler B, Hofemeister B, Feesche J, Kucheryava N, Steinborn G, Franke P, Grammel N, Zwintscher A, Leenders F, Hitzeroth G, Vater J (2004) Genetic analysis of the biosynthesis of non-ribosomal peptide- and polyketide-like antibiotics, iron uptake and biofilm formation by *Bacillus subtilis* A1/3. Mol Genet Genomics 272:363–378. Epub

Huang XQ, We ZY, Gao XP, Yang SJ, Cui YH (2008) Optimization of inactivation of endospores of *Bacillus cereus* in milk by surfactin and fengycin using a response surface method. Intl J Pept Res Ther 14:89–95

Hwang MH, Lim JH, Yun HI, Rhee MH, Cho JY, Hsu W, Park SC et al (2005) Surfactin C inhibits the lipopolysaccharide-induced transcription of interleukin-1 beta and inducible nitric oxide synthase and nitric oxide production in murine RAW 264.7 cells. Biotechnol Lett 27:1605–1608

Hwang YH, Park BK, Lim JH, Kim MS, Park SC, Hwang MH, Yun HI (2007) Lipopolysaccharide-binding and neutralizing activities of surfactin C in experimental models of septic shock. Eur J Pharmacol 556:166–171

Jourdan E, Henry G, Duby F, Dommes J, Barthélemy JP, Thonart P, Ongena M (2009) Insights into the defense-related events occurring in plant cells following perception of surfactin-type lipopeptide from *Bacillus subtilis*. Mol Plant-Microbe Interact 22:456–468

Julkowska D, Obuchowski M, Holland IB, Seror SJ (2005) Comparative analysis of the development of swarming communities of *Bacillus subtilis* 168 and a natural wild type: critical effects of surfactin and the composition of the medium. J Bacteriol 187:65–76

Kameda Y, Oira S, Matsui K, Kanatomo S, Hase T (1974) Antitumor activity of *Bacillus natto.* V. Isolation and characterization of surfactin in the culture medium of *Bacillus natto* KMD 2311. Chem Pharm Bull (Tokyo) 22:938–944

Kearns DB, Chu F, Rudner R, Losick R (2004) Genes governing swarming in *Bacillus subtilis* and evidence for a phase variation mechanism controlling surface motility. Mol Microbiol 52:357–369

Kim K, Jung SY, Lee DK, Jung JK, Park JK, Kim DK, Lee CH et al (1998) Suppression of inflammatory responses by surfactin, a selective inhibitor of platelet cytosolic phospholipase A(2). Biochem Pharmacol 55:975–985

Kim SD, Park SK, Cho JY, Park HJ, Lim JH, Yun HI, Park SC, Lee KY, Kim SK, Rhee MH et al (2006) Surfactin C inhibits platelet aggregation. J Pharm Pharmacol 58:867–870

Kim SY, Kim JY, Kim SH, Bae HJ, Yi H, Yoon SH, Koo BS, Kwon M, Cho JY, Lee CE, Hong S (2007) Surfactin from *Bacillus subtilis* displays anti-proliferative effect via apoptosis induction, cell cycle arrest and survival signaling suppression. FEBS Lett 581:865–871

Kinsinger RF, Shirk MC, Fall R (2003) Rapid surface motility in *Bacillus subtilis* is dependent on extracellular surfactin and potassium ion. J Bacteriol 185:5627–5631

Kloepper JW, Ryu CM, Zhang SA (2004) Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology 94:1259–1266

Kracht M, Rokos H, Ozel M, Kowall M, Pauli G, Vater J (1999) Antiviral and hemolytic activities of surfactin isoforms and their methyl ester derivatives. J Antibiot (Tokyo) 52:613–619

Leclère V, Béchet M, Adam A, Guez JS, Wathelet B, Ongena M, Thonart P, Gancel F, Chollet-Imbert M, Jacques P (2005) Mycosubtilin overproduction by *Bacillus subtilis* BBG100 enhances the organism's antagonistic and biocontrol activities. Appl Environ Microbiol 71:4577–4584

Leclère V, Marti R, Bechet M, Fickers P, Jacques P (2006) The lipopeptides mycosubtilin and surfactin enhance spreading of *Bacillus subtilis* strains by their surface-active properties. Arch Microbiol 186:475–483

Mulligan CN (2005) Environmental applications for biosurfactants. Environ Pollut 133:183–198

Ongena M, Adam A, Jourdan E, Paquot M, Brans A, Joris B, Arpigny JL, Thonart P (2007) Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. Environ Microbiol 9:1084–1090

Romero D, de Vicente A, Rakotoaly RH, Dufour SE, Veening JW, Arrebola E, Cazorla FM, Kuipers OP, Paquot M, Perez-Garcia A et al (2007) The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* toward *Podosphaera fusca*. Mol Plant Microbe Interact 20:430–440

Ron EZ, Rosenberg E (2001) Natural roles of biosurfactants. Environ Microbiol 3:229–236

Ryu CM, Farag MA, Hu CH, Reddy MS, Kloepper JW, Pare PW (2004) Bacterial volatiles induce systemic resistance in Arabidopsis. Plant Physiol 134:1017–1026

Silo-Suh LA, Lethbridge BJ, Raffel SJ, He HY, Clardy J, Handelsman J (1994) Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* Uw85. Appl Environ Microbiol 60:2023–2030

Silo-Suh LA, Stabb EV, Raffel SJ, Handelsman J (1998) Target range of Zwittermicin A, an aminopolyol antibiotic from *Bacillus cereus*. Curr Microbiol 37:6–11

Singh P, Cameotra SS (2004) Enhancement of metal bioremediation by use of microbial surfactants. Biochem Biophys Res Commun 319:291–297

Smith KP, Havey MJ, Handelsman J (1993) Suppression of Cottony Leak of Cucumber with *Bacillus cereus* strain Uw85. Plant Dis 77:139–142

Stein T (2005) *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol Microbiol 56:845–857

Takahashi T, Ohno O, Ikeda Y, Sawa R, Homma Y, Igarashi M, Umezawa K et al (2006) Inhibition of lipopolysaccharide activity by a bacterial cyclic lipopeptide surfactin. J Antibiot 59:35–43

Touré Y, Ongena M, Jacques P, Guiro A, Thonart P (2004) Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple. J Appl Microbiol 96:1151–1160

Vanittanakom N, Loeffler W, Koch U, Jung G (1986) Fengycin-a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. J Antibiot (Tokyo) 39:888–901

Vollenbroich D, Ozel M, Vater J, Kamp RM, Pauli G (1997) Mechanism of inactivation of enveloped viruses by the biosurfactant surfactin from *Bacillus subtilis*. Biologicals 25:289–297

Yu GY, Sinclair JB, Hartman GL, Bertagnolli BL, Bertagnolli BL (2002) Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. Soil Biol Biochem 34:955–963

Chapter 6
Biocontrol of Plant Pathogens and Plant Growth Promotion by *Bacillus*

Brian B. McSpadden Gardener

Abstract Numerous *Bacillus* strains have been investigated for their capacities to protect plants from pathogens and stimulate plant growth. Studying the diversity of these bacteria provides clues to the distinctiveness of beneficial strains and raises questions regarding the scale and evolutionary forces that led to the development of biocontrol activities. Soils harbor vast spore banks of *Bacillus*, subsets of which germinate, propagate or go dormant in patches varying size according to the availability of various resources. While the genus as a whole does not seem to be as competitive as some other genera, there do appear to be strains that are truly rhizosphere competent. Those that are will be well positioned to access resources and express activities that can lead to plant disease suppression and/or directly stimulate plant growth. It is now known that many different types of secreted products can affect both pathogens and plants in a variety of ways, all of which might lead to reductions in disease development. A greater understanding of this genus will help to accelerate the development and application of active strains and their products into biopesticidal products that improve crop quality and yields.

Keywords Volatiles • PGPR • Biological control • Biogeography

6.1 Diversity

Over 88 species of *Bacillus* have been cultured from soils (Fritze, 2004), and recent attempts to recover novel isolates have led to the identification of still more species (Heyrman et al., 2004, 2005). Recovery on complex media, such as those containing tryptic soya extract, typically yield multiple isolates of phylogenetically and

B.B. McSpadden Gardener (✉)
Department of Plant Pathology-OARDC, The Ohio State University,
1680 Madison Avenue, Wooster, OH 44691, USA
e-mail: mcspadden-garden.1@osu.edu

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,*
Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_6,

phenotypically similar species related to *B. cereus*, *B.* megaterium, *B. pumilus*, and *B. subtilis* depending on the temperature regime applied to exclude non-spore-forming bacteria. Most distinctive among these morphologically are the *B. mycoides* and *B. pseudomycoides*, which often confound attempts to accurately enumerate the full diversity of cultured populations by virtue of their rapid mycelia-like growth patterns on agar media.

Within the genus genotypic, variation across named species varies considerably. It has long been known that *B. cereus* and *B. thuringiensis* were closely related and quite distinct from *B. subtilis* (Priest, 1993). Recent comparative genomic analyses have confirmed that assessment, showing that the closely related pathogenic species shared less than 40% similarity at the gene level with *B. subtilis* (Anderson et al., 2005). However, the genetic and phenotypic distinctions between *B. amyloliquefaciens* and *B. subtilis* appear to be few, and some authors simply refer to the two as sub-species differing simply in their ability to produce amylase.

Within species, genetic diversity is still quite high. In *B. thuringiensis*, variation in *cry* genes (which encode the crystal proteins that are toxic to various invertebrate species) is well known (Stahly et al., 1992) and the species continues to be investigated as a source of novel biopesticidal ingredients (Masson et al., 1998). In contrast, most plasmids in *B. subtilis* are cryptic and appear to be unusually homologous (Zawadski et al., 1996). Different isolates of *B. subtilis* can be distinguished using a variety of genotypic and phenotypic tests, but biocontrol functions have not been strictly correlated with any of these (Marten et al., 2000). For example, among *B. subtilis* isolates, around 30% of the predicted genes in the type strain, 168, appear to be absent in genomes of other isolates (Earl et al., 2008).

What is the significance of this diversity? Clearly, there is potential for distinct genetic regulons and phenotypes related to biological control and plant growth promotion. However, relatively little is known about the specific genes involved in such functional phenotypes. Initially, it was shown that some cyclic lipopeptides were involved in pathogen suppression (Asaka and Shoda, 1996). Recently, novel polyketide synthases were also shown to be important (Chen et al., 2006). Comparative studies using suppressive–subtractive hybridization revealed a substantial number of gene markers that appeared to be unique to biocontrol strains of *B. subtilis*, including those that were homologous to genes encoding bacillomycin, fengycin, iturin (Joshi and McSpadden Gardener, 2006). However, that same study revealed sequence variation in those genes, as well as a large number of genes of unknown function, was present among different biocontrol strains. Subsequent analyses have revealed that most of the markers recovered map to just a few regions of the genome of FZB42, indicating that they may occur in several operons and be co-regulated. The sheer size of these regions further increases the probability that significant variations in expression occur among closely related biocontrol strains.

It seems likely that variation in gene content and gene sequence in different *Bacillus* species can lead to variation in biocontrol and plant growth promotion activities. From screening studies, it is well known that not all isolates of an individual species, such as *B. subtilis*, express similar capacities for inhibiting pathogens or stimulating plant defense and growth responses. However, the underlying genetic

basis for such natural variation has yet to be fully characterized. Based on analyses of a neutral genetic marker, the 16S gene, we found that the majority of *B. subtilis* with proven biocontrol activity were separated from most other isolates in a phylogenetic analysis of over 60 different sequences. However, most of the strains in that analysis had not been assayed for biocontrol or plant growth promotion activities. So it is unclear whether or not biocontrol strains represent an evolutionarily distinct lineage of strains within the species. Nonetheless, variation in functional gene sequence has been used to identify variation in biocontrol strains. In *B. cereus* the gene for production of and resistance to the antibiotic zwittermicin A was successfully used to identify strains with varying capacities to suppress plant pathogens (Raffel et al., 1996; Stabb et al., 1994). Similar work is now underway, in several laboratories, to screen diverse *B. subtilis* for variation in biocontrol activities based on variations in cyclic lipopeptide synthases and the products that they produce. The degree to which gene content variation is important remains largely unexplored at this time.

6.2 Biogeography

Most *Bacillus* species can survive as saprophytes in soils, which are considered the primary reservoirs of these bacteria, however, most viable cells probably occur as inactive spores at any given time (Nicholson, 2002). Furthermore, multiple species can be recovered as epiphytes and endophytes of plants and animals, as well as foodstuffs and composts derived from them. The rich variety of organic substrates and micro niches present in those environments support a complex milieu of microbial species, so it is not surprising that multiple species often inhabit them.

At the species level, most *Bacillus* and *Paenibacillus* are globally distributed (Priest, 1993). Such widespread occurrence of more defined subspecies of *B. subtilis* and *B. cereus* with the capacity to suppress plant pathogens has also been reported (Pinchuk et al., 2002; Stabb et al., 1994). When we performed terminal restriction fragment length polymorphism (T-RFLP) analyses of these populations, we observed only minor quantitative differences in the relative abundance of different *Bacillus*-like ribotypes occurring at multiple sites throughout Ohio (McSpadden Gardener, 2004). In contrast, highly significant differences were observed in the total and relative abundance of *Bacillus*-like sequences amplified from DNA obtained from bulk soil as compared to crop roots. This is consistent with analyses of whole bacterial communities that showed similar distinctions between soil and rhizosphere communities (Smalla et al., 2001). Culturable counts of *Bacillus* species in bulk soil, which typically range from log 3 to log 6 cells per gram fresh weight, typically exceed those obtained from the rhizosphere (Mahafee and Kloepper, 1997; Halverson et al., 1993). Our data also showed that, at the level of ribotype, *Bacillus* and *Paenibacillus* bacteria are more abundant in bulk soil than in plant tissues.

It seems likely that the abundance and diversity of *Bacillus* subspecies occurs with crop and site, as it does in other biocontrol bacteria (McSpadden Gardener et al., 2005). Indeed, zwittermicin A-producing biocontrol strains of *B. cereus* with differing

membrane composition, as determined by fatty acid methyl ester profiles, were observed to occur in different soils (Raffel et al., 1996) and on different crop genotypes (Simon et al., 2001). And, the structure of *Bacillus* populations have been reported to vary with soil management regime (Garbeva et al., 2003), but the significance of such variation in terms of biocontrol is unknown. Culture-based studies have shown that *Bacillus megaterium* to be one of the most abundant populations of *Bacillus* present in the soybean rhizosphere (Liu and Sinclair, 1992), and *B. pumilus* and *B. subtilis* were reported to be the most abundant bacteria cultured from the phyllosphere of soybeans (Arias et al., 1999). However, variations in culture methods make such estimations of species dominance rather uncertain. Interestingly though, TRFs of the size predicted for those three species were among the most abundant signals observed in our profiles of root- and leaf-inhabiting *Bacillus* populations on soybeans. Such observations indicate that different subsets of *Bacillus* predominate in different soil and plant compartments.

6.3 Activity

The multifactorial basis for biological control and plant growth promotion is at last becoming clear. Antibiotic production has long been the initial basis for selection of biocontrol strains, so it is a foregone conclusion that most field-tested *Bacillus* strains are known to produce one or more potent antifungal or antibacterial metabolites. Indeed, the patents covering Agraquest's flagship products containing AQ713 (a.k.a. strain QST713) specifically note the lipopeptide antibiotics as a source of antifungal and antibacterial activity (Heins et al., 2003). Additionally, polyketide antibiotics are known to be important for pathogen inhibition in *B. cereus* (Raffel et al., 1996) and *B. amyloliquefaciens* (Chen et al., 2006). Because of the general difficulty of obtaining stable transformants in active strains of *Bacillus*, mutation analyses indicating the importance of specific antibiotic-producing genes have been few, but are not unknown (e.g. Asaka and Shoda, 1996; Chen et al., 2006). Because substantial portions of the genome of *Bacillus amyloliquefaciens* strain FZB42 are dedicated to lipopeptide, polyketide, and other antibiotic production (Chen et al., 2007) it seems likely that the secretion of such compounds is important for biological control activity in that strain. What remains to be discerned is the extent to which variations in type and or amount of such antibiotic compounds matter for efficacy in various applications. Additionally, it is now well established that lipopeptides can have multiple activities (Ongena and Jacques, 2008). In addition to their antibiotic activities, various lipopeptides have been shown to be involved in root colonization and biofilm formation and the induction of plant host resistance pathways. It seems likely that all of these activities contribute to the improvement of plant health and growth under different conditions.

Direct antagonism also likely involves a variety of activities beyond the secretion of water-soluble antibiotics. Competition for niche space and nutrients with other chemoheterotrophs will likely also play a role. For example, motile and chemotactic

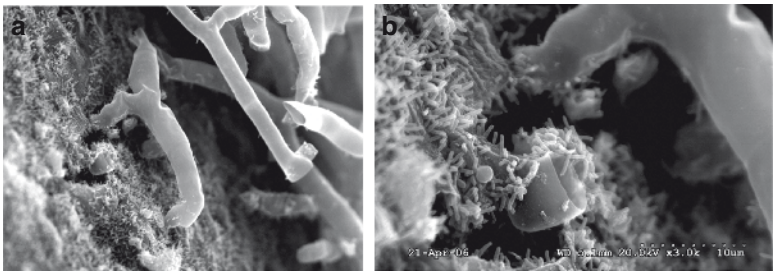


Fig. 6.1 Scanning electron micrograph showing the response of *Bacillus subtilis* MBI600 to root infection by *Rhizoctonia solani* in the rhizosphere of soybean grown under gnotobiotic conditions. Note the high density of biocontrol bacteria (**a**), not seen on uninfected root pieces. And, note the disruption of hyphal integrity, apparently the result of bacterial activity (**b**)

strains of *B. megaterium* have been shown to better colonize roots and suppress *Rhizoctonia solani* better than non-motile derivatives (Zheng and Sinclair, 2000). The dynamic responses of rhizosphere bacteria to the disruption of root structure and rhizosphere chemistry by invading pathogens have not been well characterized. However, electron microscopic analyses have revealed a rapid proliferation of *B. subtilis* on roots infected with *Rhizoctonia solani*, and what appears to be concomitant colonization and disruption of fungal hyphae (Fig. 6.1). A similar pattern of rapid proliferation of fungal antagonists, and subsequent disease suppression, in response to root infection has been reported previously for other biocontrol bacteria (McSpadden Gardener and Weller, 2001). Such data indicate that bacterially-based biocontrol results from an ecological feedback loop set off by disruption of the rhizosphere environment.

Plant host resistance pathways and the related response of plant growth promotion can be induced by select isolates of *B. amyloliquefaciens*, *B. cereus*, *B. mycoides*, *B. pumilus*, *B. sphaericus*, and *B. subtilis* (Bargabus et al., 2003; Kloepper et al., 2004). Priming and induction of resistance pathways can be mediated by a variety of microbially associated molecular patterns (MAMPs), such as those found in flagellin, as well oligosaccharides released during root colonization. And, plant growth promotion is likely to be mediated through several different phytohormones-mediated pathways, depending on how and where the plants are grown (Ryu et al., 2004a, 2004b, 2005). Volatile compounds produced by some *Bacillus* strains can also significantly impact plant growth and development. Ground-breaking work by Ryu et al. (2004a) showed that the volatile compound 2, 3-butanediol can be released by biocontrol and plant growth-promoting strains of *Bacillus* and stimulate growth in *Arabidopsis*. In our laboratory, we have also found that several strains of *B. subtilis* and *B. pumilus* can secrete volatile compounds that can significantly increase carrot and lettuce seed germination and, also, reduce the growth rate of fungal pathogens (S. Maurer, P. Edmiston, and B. McSpadden Gardener, 2008 unpublished results).

The beneficial activities of some *Bacillus* strains can also occur as the result of synergisms with other beneficial microbes. Notably, phytase-producing and phosphate-solubilizing *B. subtilis* strains have been reported to synergistically increase plant nitrogen and phosphate accumulation when co-inoculated with *Glomus intraradices*

(Toro et al., 1997). Other studies have shown that some *Bacillus* strains can increase infection by various mycorrhizae, but such synergies do not always stimulate plant growth (Medina et al., 2003 and references therein). The extent to which biocontrol and plant growth-promoting strains act as mycorrhizae-helper bacteria (MHB), remains largely unexplored. More specifically, legume hosts and their interactions with rhizobia and/or mycorrhizae may be affected by phytohormones produced by various microbial species including *B. subtilis*. Some isolates of *B. subtilis*, *B. cereus*, and *B. thuringiensis* have been shown to stimulate symbioses between *Bradyrhizobium japonicum* and soybeans (Bai et al., 2003; Halverson and Handelsman, 1991). Interestingly, such synergisms appear to be affected by genetic variation in the rhizobia and plant host (Camacho et al., 2001), further reducing the reliability of such synergisms in the broader market. When synergisms do occur, *Bacillus*-mediated increases in nodulation may result from the production of compounds similar to lipo-chitooligosaccharide *nod* factors (Lian et al., 2001).

6.4 Conclusions

Diverse *Bacillus* strains have the potential to significantly impact plant health and yield. Current understanding of the diversity, biogeography, and activities of such strains can be used to enhance the commercial development of biopesticides and inoculants based on them. Several questions arise from the current state of knowledge and the significance of their answers, once discerned, include:

At what scale does measurable genetic diversity mark significant variation in activity? And, to what extent does diversity in the different *Bacillus* species indicate adaptations or neutral genetic drift relative to plant growth and health? Only comprehensive comparative genomic analyses of active and inactive strains will reveal the answers. Those answers will help guide novel strain discovery efforts and establish the ecological and evolutionary significance of biocontrol and plant growth promotion.

What factors limit the abundance of *Bacillus* with biocontrol and plant growth promotion activities in the infection court? The answer to this question may reveal new avenues for enhancing rhizosphere and phyllosphere colonization by active strains. And, what patterns of niche overlap and subspecies succession exist? Better characterization of such niche differentiation could aid in the identification and application of more effective *Bacillus* inoculants for plant disease control. Given the diverse and, likely, dynamic ecology and activities of different *Bacillus* strains, future research will need to focus on determining when and how biocontrol activities are expressed *in situ*.

Does spore germination and biocontrol gene expression occur prior to or just following germination? When are the various biocontrol and plant growth promoting activities expressed during the ontogeny of the crop? Answers to these questions will suggest when and to what degree the various mechanisms and/or strains need to be supplemented to optimize the beneficial activities of inoculant strains.

It will be interesting to discover the extent to which biocontrol mechanisms can be managed to impact plant health and yield under commercial growing conditions. Indeed, *Bacillus*-containing products already play a significant and growing role in plant disease management (Jacobsen et al., 2004; Fravel, 2005). Currently, over $150 million of microbial biopesticides containing *Bacillus thuringiensis* are sold around the world, and other products containing *Bacillus* account for approximately $30 million more in sales (Marrone, 2007). While this is a small portion of the total insecticide and fungicide market, the proportion is expected to grow substantially. Such growth will be driven, in part, by greater awareness of biopesticides as well as increased demand in organic and conventional markets for "green" products. However, substantial growth will only occur if market barriers related to actual and perceived limitations in efficacy can be addressed. With sufficient investment, the means to fully mine the diversity and maximize the efficacy of microbial inoculants and biopesticides based on *Bacillus* can be fully realized in the not too distant future.

References

Anderson I, Sorokin A, Kapatral V, Reznik G, Bhattacharya A, Mikhailova N, Burd H, Joukov V, Kaznadzey D, Walunas T, D'Souza M, Larsen N, Pusch G, Liolios K, Grechkin Y, Lapidus A, Goltsman E, Chu L, Fonstein M, Ehrlich SD, Overbeek R, Kyrpides N, Ivanova N (2005) Comparative genome analysis of *Bacillus cereus* group genomes with *Bacillus subtilis*. FEMS Microbiol Lett 250:175–184

Arias RS, Sagardoy MA, van Vuurde JWL (1999) Spatio-temporal distribution of naturally occurring *Bacillus* spp. and other bacteria on the phylloplane of soybean under field conditions. J Basic Microbiol 39:283–292

Asaka O, Shoda M (1996) Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. Appl Environ Microbiol 62:4081–4085

Bai Y, Zhou X, Smith DL (2003) Enhanced soybean plant growth resulting from coinoculation of *Bacillus* strains with *Bradyrhizobium japonicum*. Crop Sci 43:1774–1781

Bargabus RL, Zidack NK, Sherwood JE, Jacobsen BJ (2003) Characterisation of systemic resistance in sugar beet elicited by a non-pathogenic, phyllosphere-colonizing *Bacillus mycoides*, biological control agent. Physiol Mol Plant Pathol 61:289–298

Camacho M, Santamaria C, Temprano F, Rodriguea-Navarro D, Daza A (2001) Co-inoculation with *Bacillus* sp. CECT 450 improves nodulation in *Phaseolus vulgaris* L. Can J Microbiol 47:1058–1062

Chen XH, Vater J, Piel J, Franke P, Scholz R, Schneider K, Koumoutsi A, Hitzeroth G, Grammel N, Strittmatter AW, Gottschalk G, Suessmuth RD, Borriss R et al (2006) Structural and functional characterization of three polyketide synthase gene clusters in *Bacillus amyloliquefaciens* FZB 42. J Bacteriol 188:4024–4036

Chen XH, Koumoutsi A, Scholz R, Eisenreich A, Schneider K, Heinemeyer I, Morgenstern B, Voss B, Hess WR, Reva O, Junge H, Voigt B, Jungblut PR, Vater J, Suessmuth R, Liesegang H, Strittmatter A, Gottschalk G, Borriss R et al (2007) Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42. Nat Biotechnol 25:1007–1014

Earl AM, Losick R, Kolter R (2008) Ecology and genomics of *Bacillus subtilis*. Trends Microbiol 16:269–275

Fravel D (2005) Commercialization and implementation of biocontrol. Annu Rev Phytopathol 43:337–359

Fritze D (2004) Taxonomy of the genus *Bacillus* and related genera: the aerobic endospore-forming bacteria. Phytopathology 94:1245–1248

Garbeva P, van Veen JA, van Elsa JD (2003) Predominant *Bacillus* spp. in agricultural soil under different management regimes detected via PCR-DGGE. Microb Ecol 45:302–316

Halverson LJ, Handelsman J (1991) Enhancement of soybean nodulation by *Bacillus cereus* UW85 in the field and in a growth chamber. Appl Environ Microbiol 57:2767–2770

Halverson LJ, Clayton MK, Handelsman J (1993) Population biology of *Bacillus cereus* UW85 in the rhizosphere of field-grown soybeans. Soil Biol Biochem 25:485–493

Heins SD, Manker DC, Jimenez DR, McCoy RJ, Marrone PG, Orjala JE (2003) Compositions and methods for controlling plant pests. Official Gazette of the United States Patent and Trademark Office Patents 1275(4)2003

Heyrman J, Vanparys B, Logan Niall A; Balcaen An, Rodriguez-Diaz M, Felske A, De Vos Paul (2004) *Bacillus novalis* sp. nov., *Bacillus vireti* sp. nov., *Bacillus soli* sp. nov., *Bacillus bataviensis* sp. nov. and *Bacillus drentensis* sp. nov., from the Drentse A grasslands. Int J Syst Evol Microbiol 54:47–57

Heyrman J, Rodriguez-Diaz MD, Joke F, Andreas L, Niall A, De Vos P (2005) *Bacillus arenosi* sp nov., *Bacillus arvi* sp nov. and *Bacillus humi* sp nov., isolated from soil. Int J Syst Evol Microbiol 55:111–117

Jacobsen BJ, Zidack NK, Larson BJ (2004) The role of *Bacillus*-based biological control agents in integrated pest management systems: plant diseases. Phytopathology 94:1272–1275

Joshi R, McSpadden Gardener BB (2006) Identification of genes associated with pathogen inhibition in different strains *B. subtilis*. Phytopathology 96:145–154

Kloepper JW, Ryu Choong-Min, Zhang Shouan (2004) Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology 94:1259–1266

Lian B, Prithiviraj B, Souleimanov A, Smith DL (2001) Evidence for the production of chemical compounds analogous to nod factor by the silicate bacterium *Bacillus circulans* GY92. Microbiol Res 156:289–292

Liu ZL, Sinclair JB (1992) Population dynamics of *Bacillus megaterium* strain B153-2-2 in the rhizosphere of soybean. Phytopathology 82:1297–1301

Mahafee WF, Kloepper JW (1997) Temporal changes in the bacterial communities of soil, rhizosphere, and endorhiza associated with field grown cucumber (*Cucumis sativus* L.). Microb Ecol 34:210–223

Marrone PG (2007) Barriers to adoption of biological control agents and biological pesticides. In: CAB reviews: perspectives in agriculture, veterinary science, nutrition and natural resources. CABI: published online, ISSN 1749-8848, 2, No. 051

Marten P, Smalla K, Berg G (2000) Genotypic and phenotypic differentiation of an antifungal biocontrol strain belonging to *Bacillus subtilis*. J Appl Microbiol 89:463–471

Masson L, Erlandson M, Puzstai-Carey M, Brousseau R, Juarez-Perez V, Frutos R (1998) A holistic approach for determining the entomopathogenic potential of *Bacillus thuringiensis* strains. Appl Environ Microbiol 64:4782–4788

McSpadden Gardener B (2004) Ecology of *Bacillus* and Paenibacillus spp. in agricultural systems. Phytopathology 94:1252–1258

McSpadden Gardener B, Weller D (2001) Changes in populations of rhizosphere bacteria associated with take-all disease of wheat. Appl and Environ Microbiol 67:4414–4425

McSpadden Gardener B, Gutierrez L, Joshi R, Edema R, Lutton E (2005) Distribution and biocontrol potential of phlD+ pseudomonads in corn and soybean fields. Phytopathology 95:715–724

Medina A, Probanza A, Gutierrez Manero FJ, Azcon R (2003) Interactions of arbuscular-mycorrhizal fungi and *Bacillus* strains and their effects on plant growth, microbial rhizosphere activity and fungal biomass. Appl Soil Ecol 22:15–28

Nicholson WL (2002) Roles of *Bacillus* endospores in the environment. Cell Mol Life Sci 59:410–416

Ongena M, Jacques P (2008) *Bacillus* lipopeptides versatile weapon for plant disease control. Trends in microbiology 16:115–125

Pinchuk IV, Bressollier P, Sorokulova IB, Verneuil B, Urdaci MC (2002) Amicoumacin antibiotic production and genetic diversity of *Bacillus subtilis* strains isolated from different habitats. Res Microbiol 153:269–276

Priest F (1993) Systematics and ecology of *Bacillus*. In: *Bacillus subtilis* and other Gram-positive bacteria, biochemistry, physiology, and molecular genetics. American Society for Microbiology Press, Washington, DC, pp 3–16

Raffel SJ, Stabb EV, Milner JL, Handelsman J (1996) Genotypic and phenotypic analysis of zwittermicin A-producing strains of *Bacillus cereus*. Microbiology 142:3425–3436

Ryu CM, Farag MA, Hu CH, Reddy MS, Kloepper JW, Pare PW (2004a) Bacterial volatiles induce systemic resistance in *Arabidopsis*. Plant Physiol (Rockville) 134:1017–1026

Ryu CM, Murphy JF, Mysore KS, Kloepper JW (2004b) Plant growth-promoting rhizobacteria systemically protect *Arabidopsis thaliana* against Cucumber mosaic virus by a salicylic acid and NPR1-independent and jasmonic acid-dependent signaling pathway. Plant J 39:381–392

Ryu CM, Hu CH, Locy RD, Kloepper JW (2005) Study of mechanisms for plant growth promotion elicited by rhizobacteria in *Arabidopsis thaliana*. Plant Soil 268:285–292

Simon HM, Smith KP, Dodsworth JA, Buenthner B, Handelsman J, Goodman RM (2001) Influence of tomato genotype on growth of inoculated and indigenous bacteria in the spermosphere. Appl Environ Microbiol 67:514–520

Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heuer H, Berg G et al (2001) Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant dependent enrichment and seasonal shifts revealed. Appl Environ Microbiol 67:4742–4751

Stabb EV, Jacobson LM, Handelsman J (1994) Zwittermicin A-producing strains of *Bacillus cereus* from diverse soils. Appl Environ Microbiol 60:4404–4412

Stahly DP, Andrews RE, Yousten AA (1992) The genus *Bacillus* – insect pathogens. In: The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications, 2nd edn. Springer, pp 1697–1745

Toro M, Azcon R, Barea JM (1997) Improvement of arbuscular mycorrhiza development by inoculation of soil with phosphate-solubilizing rhizobacteria to improve rock phosphate bioavailability and nutrient cycling. Appl Environ Microbiol 63:4408–4412

Zawadski P, Riley MA, Cohan FM (1996) Homology of nearly all plasmids infecting three *Bacillus* species. J Bacteriol 178:191–198

Zheng XY, Sinclair JB (2000) The effects of traits of Bacillus megaterium on seed and root colonization and their correlation with the suppression of Rhizoctonia root rot of soybean. Biocontrol 45:223–243

Chapter 7
Importance of Multitrophic Interactions for Successful Biocontrol of Plant Parasitic Nematodes with *Paecilomyces lilacinus* Strain 251

Sebastian Kiewnick

Abstract The facultative egg-pathogenic fungus *Paecilomyces lilacinus* is the most widely tested biocontrol agent for control of plant parasitic nematodes. The commercial strain 251 (PL251) is available in several countries and has demonstrated efficacy in reducing root-knot, cyst and free-living nematodes on a range of crops. To better understand the multitrophic interactions between PL251 and host-plants, target nematodes and other soil antagonists, ecological studies were conducted. The interactions of PL251 with host- or non-host plants, nematodes as well as mutualistic fungal endophytes and mycorrhiza were investigated to determine their importance for biological efficacy and potential unwanted side effects. It could be shown that the efficacy of PL251 to control nematodes is not linked to the presence of the nematode or the host plant. Furthermore, some plants seemed to provide unsuitable conditions in their rhizosphere. Unlike other nematophagous fungi, rhizosphere competence is not a key factor for the efficacy of PL251, which makes it a control option in production systems where nematode infestation is severe and highly susceptible crops are grown. In none of the studies conducted did the application of PL251 result in adverse effects on mutualistic fungal endophytes, mycorhizas, fungal antagonists or entomopathogenic nematodes. Co-application of PL251 with rhizosphere-competent antagonists always increased biocontrol efficacy against root-knot nematodes, but synergism was not observed.

Keywords Root-knot nematodes • Rhizosphere competence • Integrated control

S. Kiewnick (✉)
Plant Protection/Zoology, Research Station ACW, Agroscope Changins-Waedenswil, Schloss, P.O. Box 185, 8820, Waedenswil, Switzerland
e-mail: sebastian.kiewnick@acw.admin.ch

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,* Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_7,

7.1 Introduction

Root-knot nematodes (*Meloidogyne* spp.) cause high levels of economic loss in a multitude of agricultural crops worldwide, especially in tropical and sub-tropical agriculture (Sikora and Fernandez, 2005). The concerns on environmental side effects associated with chemical control and the phase out of methyl bromide have spurred research into nematode control alternatives (Nico et al., 2004). The use of antagonistic fungi as an alternative for root-knot nematode control was demonstrated by Sharon et al. (2001) who controlled *Meloidogyne javanica* on tomato with *Trichoderma harzianum*. Kerry and Hidalgo-Diaz (2004) developed a management system for control of root-knot nematodes in organic vegetable production based on *Pochonia chlamydosporia*. Furthermore, Jaffee (2000) demonstrated that the trapping fungi *Hirsutella rhossiliensis* and *Arthrobotrys haptotyla* were effective in reducing *Heterodera schachtii* and *M. javanica* populations.

The egg pathogenic fungus *Paecilomyces lilacinus* (Thom) Samson is the most widely tested biocontrol agent for the control of plant-parasitic nematodes (Atkins et al., 2005; Kiewnick and Sikora, 2006a). Efficient control of plant parasitic nematodes by this fungus such as *M. incognita* and *M. javanica* has been demonstrated (Lara et al., 1996; Siddiqui et al., 2000). Cannayane and Sivakumar (2001) reviewed the biocontrol efficacy of *P. lilacinus* and reported successful control of the potato cyst nematode *Globodera rostochiensis* and root-knot nematodes. However, biocontrol efficacy under glasshouse and field conditions was often inconsistent when *P. lilacinus* was applied to soil using various organic materials such as oil cakes and leaf residues as carrier (Kerry and Evans, 1996; Cannayane and Sivakumar, 2001). Additionally, the effects by these amendments were often difficult to reproduce because changes in the substrate used for the production of biocontrol agents can significantly affect their efficacy (Jenkins et al., 1998). Furthermore, the amounts of product needed to achieve sufficient control reached several tons per ha (Gomes Carneiro and Cayrol, 1991).

The commercial *P. lilacinus* strain 251 (PL251, AGAL; #89/030550) is available for control of nematode pests in several countries (Atkins et al., 2005; Kiewnick and Sikora, 2006a). It is registered with the US Environmental Protection Agency as a biological nematicide under the trade name MeloCon® WG (Environmental Protection Agency, 2005). Recently, PL251 has been included as active substance into Annex I of the European Directive 91/414 EEC (Anonymous, 2008). The efficacy of PL251 has been demonstrated using earlier versions of commercial formulations (Holland et al., 2003). However, advancements in solid state fermentation and formulation technology, a pre-requisite for successful commercialization of biocontrol products (Kiewnick, 2007a), required a re-evaluation of the efficacy of a new, highly concentrated biocontrol preparation. As the production process of fermentation and formulation can significantly alter control activity levels (Jenkins et al., 1998), their effects have to be evaluated at all stages of production (Jones and Burges, 1998; Kiewnick, 2001).

Various mechanisms of action have been investigated to determine the mode of action responsible for the biological activity of *P. lilacinus* against plant-parasitic nematodes.

It was found that direct infection of sedentary stages during nematode development, in particular the egg stage, is the main mode of action. Furthermore, the production of leucinotoxins, chitinases, proteases and acetic acid by *P. lilacinus* has been associated with the infection process (Djian et al., 1991; Khan et al., 2003a, 2004; Park et al., 2004). As the key mode of action for PL251, the production of the enzymes chitinase and protease was identified. Khan et al. (2004) demonstrated that protease and chitinase produced by PL251 caused changes in the eggshell layers of *M. javanica* resulting in significantly reduced hatch of juveniles. Furthermore, Holland et al. (1999) demonstrated that PL251 was able to directly penetrate all sedentary stages, including eggs and females of *M. javanica* after appressoria formation. However, PL251 does not produce leucinotoxins (Khan et al., 2003b), a group of toxins which has been associated with Australian *P. lilacinus* isolates showing biocontrol activity against *M. javanica* (Park et al., 2004).

7.2 Biocontrol Efficacy

7.2.1 Dose Response and Optimized Efficacy

Biocontrol efficacy is critical for the success of the commercialization of antagonistic microorganisms (Kiewnick, 2007a). Evaluation of product efficacy under diverse practical conditions, in particular field studies, can help to identify its limits and a compatibility profile with other crop protection inputs. As the initial step to determine efficacy of a novel formulation of PL251, dose–response relationships between the concentration of antagonist applied and the reduction of plant damage by *M. incognita* were established (Kiewnick and Sikora, 2006a). These studies are an important part in understanding the multiple interactions in biocontrol systems. Reports by Montesinos and Bonaterra (1996) and others (Smith et al., 1997; Larkin and Fravel, 1999) described the importance of pathogen inoculum density, antagonist concentration, host susceptibility to the pathogen and the proportion of a pathogen to exist within a refuge for the biocontrol dose–response relationships. These models may help in understanding factors affecting biocontrol interactions and can be useful in predicting biocontrol efficiency under varying conditions (Larkin and Fravel, 1999). For PL251, growth chamber experiments revealed a significant dose–response relationship between the concentration of conidia applied to soil 6 days before transplanting tomatoes and efficacy to control the root-knot nematode *M. incognita* (Kiewnick and Sikora, 2006a). Using a logistic model, the effective concentration (EC_{50}) values obtained for the parameters gall index, egg masses per root and reproduction ranged from 8.10×10^5 to 1.40×10^6 and 1.29×10^6 to 9.88×10^5 CFU/g soil when PL251 was applied without or with a novel glucose formulation, respectively (Kiewnick and Sikora, 2006a). These data confirmed other reports concerning the density of *P. lilacinus* needed in soil for the control of *Meloidogyne* spp. (Gomes Carneiro and Cayrol, 1991). The dose–response experiments also showed that PL251 provided good control at an inoculum density which was higher than the commonly

observed levels in infested fields (Sikora and Fernandez, 2005). Similar results were obtained against the northern root-knot nematode *M. hapla* where biocontrol efficacy reached up to 90% (Kiewnick and Sikora, 2006b). In these experiments, repeated applications were used as studies on optimizing the biocontrol efficacy of PL251 towards *M. incognita* had revealed that a pre-planting soil treatment combined with a seedling drench and a post-planting soil drench significantly increased the biological efficacy (Kiewnick and Sikora, 2004). The pre-planting soil treatment with PL251 was also shown to reduce *Radopholus similis* penetration rates in Banana roots (Kiewnick and Sikora, 2006c). Furthermore, Mendoza et al. (2007) demonstrated that repeated applications of PL251 reduced the reproduction rate of *R. similis* on Banana by up to 95%. Additionally, studies on the efficacy of PL251 against the sugar beet cyst nematode *Heterodera schachtii* revealed a significant dose–response relationship between a pre-planting soil treatment and the percentage of infected eggs as well as inactivated second stage juveniles (Kiewnick et al., 2004b).

7.2.2 Field Efficacy

Based on the data obtained from greenhouse experiments, the importance to maintain a sufficient density by repeated application was evident (Kiewnick and Sikora, 2006a, b). Field experiments conducted by Schenk (2004) showed a good efficacy of PL251 to control root-knot nematodes on tomato and cucumber when applied as pre-plant soil drench combined with a seedling drench and at six weeks post planting. In a field experiment in Greece in 2004, conducted following EPPO guidelines, PL251 was applied as BIOACT® WG for the control of root-knot nematodes on tomato in comparison to a chemical nematicide (Kiewnick, 2006). PL251 was applied as a soil treatment 2 weeks before planting and transplants received an additional drench, 24 h before transplanting. PL251 was applied again as a soil drench 7 and 14 weeks after planting. The chemical nematicide was re-applied 4 weeks after transplanting. The results demonstrated that PL251 achieved a reduction in root damage and an increase in fruit yield, not different from the chemical control (Kiewnick, 2006). Tomato fruit yield increase for PL251 and the chemical nematicide was up to 132% and 139%, respectively. Therefore, it was demonstrated that with repeated applications PL251 was able to provide significant nematode control under field conditions. Recently, PL251 has also been tested with encouraging results in a range of cropping systems to fully evaluate its potential as biological nematicide (Rich and Johnson, 2008; Anastasiadis et al., 2008).

7.3 Factors Affecting Persistence and Biocontrol Efficacy

In general, the success of a biocontrol product is related to the persistence of the antagonist after application (Thomas et al., 1997). Therefore, persistence is desirable, because it can improve efficiency. The importance of biotic and abiotic factors for

the persistence of fungal biocontrol agents has been investigated intensively. For multiple biocontrol systems, the significance of temperature and moisture (Studdert and Kaya, 1990), soil type (Kessler et al., 2004), application and formulation (Inyang et al., 2000), plant species (Kouassi et al., 2003) and the host plant (Mauchline et al., 2002) was demonstrated. Understanding the ecology of a biocontrol agent and the knowledge of the critical factors affecting its persistence is important to improve application strategies (Bidochka, 2001).

In dose–response experiments with PL251, an average decrease in CFU/g soil of 55% over time was found (Kiewnick and Sikora, 2006a). This confirmed previous studies where CFU numbers of PL251 in soil started to drastically decline soon after application (Kiewnick et al., 2003, 2004a). However, a rapid decline in CFU numbers is typical for *P. lilacinus* as it is applied at very high concentrations which cannot be maintained for long periods in soil (Hewlett et al., 1988). To fully exploit the potential for control of plant parasitic nematodes, the importance of biotic and abiotic factors for the persistence of PL251 was investigated.

7.3.1 Temperature and Substrate Type

Studies conducted under controlled environmental conditions identified temperature as an important factor for persistence of PL251 (Kiewnick et al., 2005a; Rumbos, 2005). PL251 population density was negatively correlated with time and the decrease was significantly stronger at higher (28°C) versus lower temperatures (10–15°C). Studdert and Kaya (1990) reported similar results for *Beauveria bassiana* where numbers in soil decreased steadily as temperatures increased from 10°C to 50°C. Soil texture exerted a pronounced effect on the persistence of PL251 with the sand content being strongly negatively correlated with the decline rates, whereas silt, clay and organic matter content showed a positive correlation (Rumbos, 2005). Furthermore, studies on the persistence of PL251 in soil under control conditions revealed significantly higher persistence in silty loam and clay soils, whereas a stronger decline in CFU numbers was observed when field soil was diluted with 75% sand or more (Rumbos et al., 2008). Conversely, the addition of organic matter to the substrate increased the persistence of PL251 indicating a positive saprophytic response to increased nutrient availability. Soil pH is not an important factor for biocontrol efficacy as PL251 is capable of growing at a wide pH range from 2 to 10 with an optimum at pH 6.5 (Rumbos, 2005).

7.3.2 Host Plant Species and Target Nematode

The host plant species did not alter the decline in population density in rhizosphere bulk soil (Rumbos and Kiewnick, 2006). When the population density of PL251 in the bulk rhizosphere soil was compared to the density in the inner rhizosphere soil,

four of 10 host plants showed an increase in survival rates by 4–68% (Kiewnick et al., 2005a; Rumbos, 2005). As plants can affect the microbial community by the root exudates that they release into the rhizosphere, the increase in survival of PL251 was probably due to a higher nutrient availability. In the presence of root-knot nematodes and the host plant, persistence of PL251 was not different from soil only (Kiewnick et al., 2004a) confirming that plant species is not the primary factor affecting the persistence of PL251. However, in all experiments PL251 numbers declined linearly and proliferation in rhizosphere soil was never found indicating that rhizosphere competence is not one of the key factors responsible for successful biocontrol.

7.3.3 Persistence Under Field Conditions

The potential of PL251 to persist and establish in the environment was evaluated in a sugar beet field after broadcast application of the commercial product BIOACT® WG (Kiewnick et al., 2005b). The product was applied at a rate of 4 kg and 12 kg per ha in 2003 and 2004, respectively, and incorporated into the soil prior to planting sugar beets. For both years it was demonstrated that the initial density of PL251 after application was significantly lower than predicted and the spatial distribution was very heterogeneous. In 2003, the density of PL251 decreased more than 90% at 90 days after application and the fungus was no longer detected after 120 days. In 2004, PL251 was detected in low numbers 200 days past application. The exponential decline rate of PL251 in field soil was independent from the initial density and the population dynamics of *H. schachtii*. Furthermore, PL251 did not alter the level of parasitism of *H. schachtii* eggs by indigenous soil fungi, demonstrating a low potential to establish. Further monitoring PL251 in this field using nested PCR revealed the presence in treated but also the untreated areas (Kiewnick, 2007b). However, the density of PL251 was significantly lower compared to the background level of other filamentous fungi demonstrating the lack of establishment under field conditions.

7.4 Importance of Rhizosphere Competence for Biocontrol

7.4.1 Interaction Between Inoculum Density and Antagonist Dose

A concentration of 1×10^6 CFU/g soil was believed to be critical for successful biocontrol with PL251. However, as field trials had revealed a discrepancy between amount of product applied and the actual density of PL251 found in soil it was suspected that a lower dose of PL251 would suffice when nematode inoculum densities were at levels closer to naturally occurring infestations.

Therefore, efficacy of varying doses of PL251 at different *M. incognita* inoculum densities was investigated. Conversely to previous findings, no dose–response relationships could be established (Kiewnick et al., 2006b). Biocontrol efficacy was greatest at low inoculum densities and decreased with higher application rates. At higher inoculum densities, a higher product rate resulted in higher nematode damage on tomato. As the main mode of action by the pre-planting treatment with PL251 is the reduction of the nematode inoculum in soil, reduced efficacy was due to the lowering of the inoculum level to an optimum for nematode development and root damage. However, at nematode inoculum densities naturally occurring under field conditions, rates as low as 2×10^5 CFU/g soil showed a high efficacy. In contrast to these findings, other biocontrol systems showed different responses to antagonist dose and pathogen inoculum density interaction experiments. Smith et al. (1997) reported the significant effect of the host plant on the dose–response relationship between *Pythium* damping-off and *Bacillus cereus* UW38. Larkin and Fravel (1999) characterized the differences in the inoculum density relationships for three *Fusarium oxysporum* biocontrol isolates. In contrast to *P. chlamydosporia* var. *catenulata*, a colonizer of the rhizosphere and egg masses of *Meloidogyne* spp. (Atkins et al., 2003), the reduction of the nematode inoculum in soil by PL251 is the main mode of action to reduce damage to roots and the reproduction of the nematode.

7.4.2 Interaction with Root-Knot Nematodes

Investigations on the colonization of galls or root-knot nematode egg masses by PL251 revealed no correlation between biocontrol efficacy and the presence of the fungus (Kiewnick, 2006, 2007b). To further understand interaction of PL251 with root-knot nematodes, confocal laser scanning microscopy, fluorescence microscopy and scanning electron microscopy was used to detect colonization of the gelatinous matrix protecting *M. incognita* egg masses (Kiewnick et al., 2006a). However, PL251 was not able to colonize the gelatinous matrix. The role of the gelatinous matrix in the suppression of microorganisms antagonistic to root-knot nematodes was also demonstrated by Orion et al. (2001) who reported the antimicrobial properties of this substance.

To further investigate the interaction between PL251 and *Meloidogyne* egg masses, real-time PCR quantification was performed using TaqManPCR primers and probes, which were designed from the ITS region of the fungal genome (Atkins et al., 2005). To generate a standard curve for real-time PCR quantification, 10 egg masses were taken from roots of a tomato plant infected with *M. incognita* and inoculated with 1×10^7 viable conidia of PL251. Total DNA was extracted from egg masses with a plant DNA extraction kit (Mo-Bio) and serial dilutions of the template DNA were prepared from 1×10^5, 1×10^4 to a final dilution of 10 CFU per egg mass. Real-time PCR was performed with the automated ABI Prism 7,500 sequence detector (Applied Biosystems) according to Atkins et al. (2005). With the standard curve derived from the serial dilution of PL251 from egg masses it was

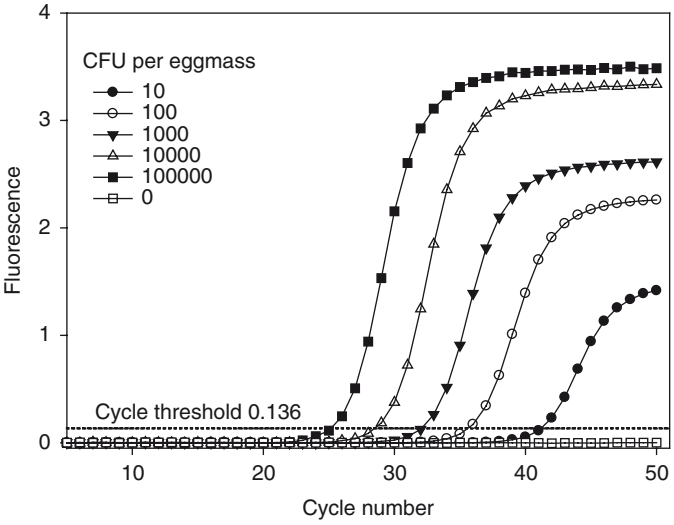


Fig. 7.1 Real-time amplification plot of *Paecilomyces lilacinus* strain 251 ITS DNA from serially diluted template DNA extracted from *Meloidogyne incognita* egg masses inoculated with 10^7 conidia

possible to detect as low as 10 CFU (Fig. 7.1). Preliminary data from the antagonist dose and nematode inoculum density interaction experiments (Kiewnick et al., 2006b) indicate that there is no correlation between biocontrol efficacy and egg mass colonization by PL251. However, further research is needed to fully elucidate the role of egg mass colonization for biocontrol efficacy of PL251.

7.4.3 Interaction with Other Antagonists

As biocontrol agents have to be compatible with integrated pest management strategies, the interaction between antagonists with different modes of action and PL251 was evaluated. Combination of the arbuscular mycorrhizal fungus (AMF) *Glomus intraradices* with PL251 resulted in increased reduction of galling in comparison to single treatments and AMF establishment was not affected (Rumbos et al., 2006). Conversely, Diedhiou et al. (2003) demonstrated that combining AMF with the mutualistic endophytic *Fusarium oxysporum* strain Fo162 did not result in increased biocontrol activity towards *M. incognita* on tomato. They concluded that competition for space and nutrients of the two antagonists reduced the chance for additive or synergistic effects on biocontrol activity. For control of *R. similis* on Banana, combining *F. oxysporum* strain Fo162 with PL251 resulted in biocontrol efficacy, superior to single treatments (Kiewnick, 2007b). As one mode of action for the endophytic *F. oxysporum* strain Fo162 is the reduction of juvenile penetration

(Hallmann and Sikora, 1994), this repellent effect might have increased the exposure of the nematode inoculum to PL251 in the soil, leading to improved biocontrol efficacy. Furthermore, Rumbos (2005) showed a low competitiveness of PL251 against biocontrol fungi such as *Chlonostachys rosae* and *Trichoderma* spp. indicating a high compatibility. Finally, studies on the compatibility with entomopathogenic nematodes revealed that although being a nematode antagonist, PL251 did not affect survival or efficacy of *Steinernema feltiae, Heterorhabditis bacteriophora* and *H. megidis* when co-applied to soil (Rumbos et al., 2007).

7.5 Concluding Remarks

The information and knowledge gained during the development of the biological nematicide based on the egg pathogenic fungus *P. lilacinus* strain 251 has helped to understand (in part) the multitrophic interactions and their importance for biocontrol success. As demonstrated, PL251 can play a valuable role in integrated management systems to reduce damage by plant parasitic nematodes. This is particularly important considering the increasing restrictions in use and availability of chemical control measures. There are a range of factors limiting the use of biological nematicides in general. Compatibility of biocontrol agents with production systems, chemicals or other inputs such as fertilizer, the nematode species to be controlled, and the host plant itself can reduce the biocontrol efficacy. As a consequence, the combination of antagonists with multiple modes of actions attacking different developmental stages of the target nematode and applied at different growth phases of the host plant seem a potential approach to overcome the limitations in biological control of nematodes. Therefore, more research is needed to fully understand the interaction between antagonists, nematodes and the host plant, and identify factors further enhancing biocontrol efficacy.

References

Anastasiadis IA, Giannakou IO, Prophetou-Athanasiadou GSR (2008) The combined effect of the application of a biocontrol agent *Paecilomyces lilacinus*, with various practices for the control of root-knot nematodes. Crop Prot 27:352–361

Anonymous (2008) Commission Directive 2008/44/EC of 4 April 2008 amending Couincil Directive 91/414/EEC to include benthiavalicarb, boscalid, carvone, fluoxastrobin, *Paecilomyces lilacinus* and prothioconazole as active substances. Off J Eur Union L94:13–15

Atkins SD, Hidalgo-Diaz L, Kaslisz H, Mauchline TH, Hirsch PR, Kerry BR (2003) Development of a new management strategy for the control of root-knot nematodes (*Meloidogyne* spp.) in organic vegetable production. Pest Manag Sci 59:183–189

Atkins SD, Clark IM, Pande S, Hirsch PR, Kerry BR (2005) The use of real-time PCR and species-specific primers for the identification and monitoring of *Paecilomyces lilacinus*. FEMS Microbiol Ecol 51:257–264

Bidochka MJ (2001) Monitoring the fate of biocontrol fungi. In: Butt TM, Jackson CW, Magan N (eds) Fungi as biocontrol agents: progress, problems and potential. CABI, Oxon, UK

Cannayane I, Sivakumar CV (2001) Nematode egg – parasitic fungus 1: *Paecilomyces lilacinus* – a review. Agric Rev 22:79–86

Diedhiou PM, Hallmann J, Oerke EC, Dehne HW (2003) Effects of arbuscular mycorrhizal fungi and a non-pathogenic *Fusarium oxysporum* on *Meloidogyne incognita* infestation of tomato. Mycorrhiza 13:199–204

Djian C, Pijarowski L, Ponchet M, Arpin N, Favre-Bonvin J (1991) Acetic acid: a selective nematicidal metabolite from culture filtrates of *Paecilomyces lilacinus* (Thom) Samson and *Trichoderma longibrachiatum* Rifai. Nematologica 37:101–112

Environmental Protection Agency (2005) *Paecilomyces lilacinus* strain 251: exemption from the requirement of a tolerance. Fed Regist 70:19278–19283

Gomes Carneiro RMD, Cayrol JC (1991) Relationship between inoculum density of the nematophagous fungus *Paecilomyces lilacinus* and the control of *Meloidogyne arenaria* on tomato. Rev Nématol 14:629–634

Hallmann J, Sikora RA (1994) Occurrence of plant parasitic nematodes and non-pathogenic species of *Fusarium* in tomato plants in Kenya and their role as mutualistic synergists for biological control of root-knot nematodes. Int J Pest Manage 40:321–325

Hewlett TE, Dickson DW, Mitchell DJ, Kannwischer-Mitchell ME (1988) Evaluation of *Paecilomyces lilacinus* as a biocontrol agent of *Meloidogyne javanica* on Tobacco. J Nematol 20:578–584

Holland RJ, Williams KL, Khan A (1999) Infection of *Meloidogyne javanica* by *Paecilomyces lilacinus*. Nematology 1:131–139

Holland RJ, Williams KL, Nevalainen HKM (2003) *Paecilomyces lilacinus* strain Bioact251 is not a plant endophyte. Australas. Plant Path 32:473–478

Inyang EN, McCartney HA, Oyejola B, Ibrahim L, Pye BJ, Archer SA, Butt TM (2000) Effect of formulation, application and rain on the persistence of the entomogenous fungus *Metarhizium anisopliae* on oilseed rape. Mycol Res 104:653–661

Jaffee BA (2000) Augmentation of soil with the nematophagous fungi *Hirsutella rhossiliensis* and *Arthrobotrys haptotyla*. Phytopathology 90:498–504

Jenkins NE, Heviefo G, Langewald J, Cherry AJ, Lomer CJ (1998) Development of mass production technology for aerial conidia for use as mycopesticides. Biocontr News Inf 19:21N–31N

Jones KA, Burges HD (1998) Technology of formulation and application. In: Burges HD (ed) Formulation of microbial biopesticides: beneficial microorganisms, nematodes and seed treatments. Kluwer, Dordrecht, Boston, London

Kerry BR, Evans K (1996) New strategies for the management of plant parasitic nematodes. In: Hall R (ed) Principles and practice of managing soilborne plant pathogens. APS, St. Paul, Minnesota

Kerry BR, Hidalgo-Diaz L (2004) Application of *Pochonia chlamydosporia* in the integrated control of root-knot nematodes on organically grown vegetable crops in Cuba. IOBC/wprs Bull 27:123–126

Kessler P, Enkerli J, Schweizer C, Keller S (2004) Survival of *Beauveria brongniartii* in the soil after application as a biocontrol agent against the European cockchafer *Melolontha melolontha*. Biocontrol 49:563–581

Khan A, Williams KL, Molloy MP, Nevalainen HKM (2003a) Purification and characterization of a serine protease and chitinases from *Paecilomyces lilacinus* and detection of chitinase activity on 2D gels. Protein Expres Purif 32:210–220

Khan A, Williams KL, Nevalainen HKM (2003b) Testing the nematophagous biological control strain *Paecilomyces lilacinus* 251 for paecilotoxin production. FEMS Microbiol Lett 227:107–111

Khan A, Williams KL, Nevalainen HKM (2004) Effects of *Paecilomyces lilacinus* protease and chitinase on the eggshell structures and hatching of *Meloidogyne javanica* juveniles. Biol Control 31:346–352

Kiewnick S (2001) Advanced fermentation and formulation technologies for fungal antagonists. IOBC/wprs Bull 24:77–79

Kiewnick S (2006) Multitrophic interactions of *Paecilomyces lilacinus* strain 251 in the rhizosphere of host and non-host plants. IOBC/wprs Bull 29:53–61
Kiewnick S (2007a) Practicalities of developing and registering microbial biological control agents. CAB Reviews 2:1–11
Kiewnick S (2007b) Importance of multitrophic interactions for the efficacy of *Paecilomyces lilacinus* strain 251 to control root-knot nematodes. J Nematol 39:72
Kiewnick S, Sikora RA (2004) Optimizing the biological control of plant-parasitic nematodes with *Paecilomyces lilacinus* strain 251. Phytopathology 94:51
Kiewnick S, Sikora RA (2006a) Biological control of the root-knot nematode *Meloidogyne incognita* by *Paecilomyces lilacinus* strain 251. Biol Control 38:179–187
Kiewnick S, Sikora RA (2006b) Evaluation of *Paecilomyces lilacinus* strain 251 for the biological control of the northern root-knot nematode *Meloidogyne hapla* Chitwood. Nematology 8:69–78
Kiewnick S, Sikora RA (2006c) New strategies for the biological control of the burrowing nematode *Radopholus similis* (Cobb) Thorne on banana. In: Hallmann J, Niere B (eds) Recent advances in Nematology. Mitteilungen der BBA, Berlin, Germany
Kiewnick S, Rumbos C, Sikora RA (2003) Risk assessment of biopesticides: monitoring fungal biocontrol agents in the environment. Phytopathology 93:45
Kiewnick S, Rumbos C, Sikora RA (2004a) Risk assessment of fungal biocontrol agents. IOBC/wprs Bull 27:137–143
Kiewnick S, Schmitz A, Sikora RA (2004b) Biological control of the sugar beet cyst nematode *Heterodera schachtii* with *Paecilomyces lilacinus* strain 251. J Nematol 36:327
Kiewnick S, Rumbos C, Sikora RA (2005a) Population dynamics of *Paecilomyces lilacinus* strain 251 formulated as WG in the rhizosphere of host and non target plants. In: Hartmann A, Schmid M, Wenzel W, Hinsinger Ph. (eds) Rhizosphere 2004 – perspectives and challenges – a tribute to Lorenz Hiltner. ISSN0721-1, Neuherberg, Germany
Kiewnick S, Rumbos C, Sikora RA (2005b) Risk assessment of biological control products. Ges Pfl 57:163–166
Kiewnick S, Lauritzen AJ, Eilenberg J (2006a) Investigations on the colonization of root-knot nematode galls and egg masses the antagonistic fungus *Paecilomyces lilacinus* strain 251. Phytopathology 96:60
Kiewnick S, Neumann S, Sikora RA (2006b) Importance of nematode inoculum density and antagonist dose for biocontrol efficacy of *Paecilomyces lilacinus* strain 251. Phytopathology 96:60
Kouassi M, Coderre D, Todorova SI (2003) Effect of plant type on persistence of *Beauveria bassiana*. Biocontrol Sci Technol 13:415–427
Lara J, Acosta N, Betancourt C, Vincente N, Rodríguez R (1996) Biological control of *Meloidogyne incognita* in tomato in Puerto Rico. Nematropica 26:143–152
Larkin RP, Fravel DR (1999) Mechanisms of action and dose–response relationships governing biological control of Fusarium wilt of tomato by non-pathogenic *Fusarium* spp. Phytopathology 89:1152–1161
Mauchline TH, Kerry BR, Hirsch PR (2002) Quantification in soil and the rhizosphere of the nematophagous fungus *Verticillium chlamydosporium* by competitive PCR and comparison with selective plating. Appl Environ Microbiol 68:1846–1853
Mendoza A, Sikora RA, Kiewnick S (2007) Influence of *Paecilomyces lilacinus* strain 251 on the biological control of the burrowing nematode *Radopholus similis* in Banana. Nematropica 37:203–213
Montesinos E, Bonaterra A (1996) Dose–response models in biological control of plant pathogens: an empirical verification. Phytopathology 86:464–472
Nico AI, Jiménez-Díaz RM, Castillo P (2004) Control of root-knot nematodes by composted agro-industrial wastes in potting mixtures. Crop Prot 23:581–587
Orion D, Kritzman G, Meyer SLF, Erbe EF, Chitwood DJ (2001) A role of the gelatinous matrix in the resistance of root-knot nematode (*Meloidogyne* spp.) eggs to microorganisms. J Nematol 33:203–207

Park JO, Hargreaves JR, McConville EJ, Stirling GR, Ghisalberti EL, Sivasithamparam K (2004) Production of leucinostatins and nematicidal activity of Australian isolates of *Paecilomyces lilacinus* (Thom) Samson. Lett Appl Microbiol 38:271–276

Rich JR, Johnson T (2008) NemOut for management of root-knot nematode in peanut, 2007. Plant Disease Management Report 2:NO43

Rumbos C (2005) Ecological studies on *Paecilomyces lilacinus* strain 251 and their importance for the biological control of plant parasitic-nematodes and environmental risk assessment. Cuvillier Verlag Göttingen, ISBN 3-86537-697-5

Rumbos C, Kiewnick S (2006) Effect of plant species on persistence of *Paecilomyces lilacinus* strain 251 and on root colonization by the fungus. Plant Soil 28:25–31

Rumbos C, Reimann S, Kiewnick S, Sikora RA (2006) Interactions of *Paecilomyces lilacinus* strain 251 with the mycorrhizal fungus *Glomus intraradices*: implications for *Meloidogyne incognita* control on tomato. Biocontr Sci Techn 16:981–986

Rumbos C, Mendoza A, Kiewnick S, Sikora RA (2007) Effect of *Paecilomyces lilacinus* strain 251 on the survival and virulence of entomopathogenic nematodes under laboratory conditions. Nematol Mediterranea 35:103–107

Rumbos C, Mendoza A, Sikora RA, Kiewnick S (2008) Persistence of the nematophagous fungus *Paecilomyces lilacinus* strain 251 in soil under controlled conditions. Biocontr Sci Techn 18:1041–1050

Schenk S (2004) Control of nematodes in Tomato with *Paecilomyces lilacinus* strain 251. Hawaii Agricultural Research Center. Veg Rep 5:1–5

Sharon E, Bar-Eyal M, Chet I, Herrera-Estrella A, Kleifeld O, Spiegel Y (2001) Biological control of the root-knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*. Phytopathology 91:687–693

Siddiqui IA, Qureshi SA, Sultana V, Ehteshamul-Haque S, Ghaffar A (2000) Biological control of root-rot disease complex of tomato. Plant Soil 227:163–169

Sikora RA, Fernandez E (2005) Nematode parasites of vegetables. In: Luc M, Sikora RA, Bridge J (eds) Plant-parasitic nematodes in subtropical and tropical agriculture, 2nd edn. CABI, Wallingford, UK

Smith KP, Handelsman J, Goodman RM (1997) Modelling dose–response relationships in biological control: Partitioning host responses to the pathogen and the biocontrol agent. Phytopathology 87:720–729

Studdert JP, Kaya HK (1990) Effect of water potential, temperature, and clay-coating on survival of *Beauveria bassiana* conidia in a loam and peat soil. J Inv Pathol 55:417–427

Thomas MB, Wood SN, Langewald J, Lomer CJ (1997) Persistence of *Metarhizium flavoride* and consequences for biological control of grasshoppers and locusts. Pestic Sci 49:47–55

Chapter 8
Interactions Between *Clonostachys rosea* f. *catenulata, Fusarium oxysporum* and Cucumber Roots Leading to Biological Control of Fusarium Root and Stem Rot

Syama Chatterton and Zamir K. Punja

Abstract *Clonostachys rosea* f. *catenulata* (*Gliocladium catenulatum*) strain J1446 (formulated as Prestop WP) suppressed Fusarium root and stem rot caused by *Fusarium oxysporum* f. sp. *radicis-cucumerinum* on greenhouse cucumber plants. In culture, *C. rosea* produced chitinase and β-1,3-glucanase enzymes on chitin or laminarin as a sole carbon source, respectively, and caused localized degradation of *Fusarium* hyphae. These enzymes were also induced by growth on *Fusarium* mycelial fragments and homogenized cucumber roots in vitro. Cucumber root extracts from *C. rosea*-colonized plants had significantly higher levels of glucanase at 7 days post-application compared to untreated controls. Reverse-transcription polymerase chain reaction using primers designed to amplify a β-1,3-glucanase gene confirmed *C. rosea* glucanase expression on roots. Following transformation of the biocontrol agent with *Agrobacterium tumefaciens* strain AGL-1 containing the hygromycin resistance (*hph*) and β-glucuronidase (*uidA*) genes, blue-stained mycelia could be seen growing on the surface and within epidermal and cortical cells of roots, stems and shoots 3 weeks after treatment. Application of *C. rosea* preceding inoculation with *Fusarium* significantly reduced pathogen populations on roots compared to plants inoculated with *Fusarium* alone, while densities of the biocontrol agent appeared to increase in the presence of the pathogen. Colonization of infection sites by *C. rosea* in the root zone is one of the mechanisms by which pathogen development and disease incidence is reduced.

Keywords *Clonostachys rosea* f. *catenulata* • *Gliocladium catenulatum* • *Fusarium oxysporum* f. sp. *radicis-cucumerinum* • *Cucumis sativus* • Biological control • GUS transformation • Colonization • Competition • Mycoparasitism

S. Chatterton and Z.K. Punja (✉)
Department of Biological Sciences, Simon Fraser University, 8888 University Drive, Burnaby British Columbia, Canada V5A 1S6
e-mail: punja@sfu.ca

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,* Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_8,

8.1 Introduction

Fusarium root and stem rot of greenhouse cucumber caused by *F. oxysporum* f. sp. *radicis-cucumerinum* Vakalounakis causes severe crop losses in cucumber growing regions throughout Canada, and the pathogen has been reported to occur in greenhouses worldwide (Rose et al., 2003). Initial infection occurs through the roots and the fungus subsequently spreads to the crown and stem tissues, where mycelia and spore masses are produced that result in the characteristic symptoms of stem rot (Punja and Parker, 2000). Plants generally do not exhibit disease symptoms until after periods of stress brought about by fruit development or extremes of environmental conditions, such as high temperatures (Punja and Parker, 2000). Current strategies aimed at managing Fusarium root and stem rot include the use of pathogen-free seed or transplants, the planting of resistant or tolerant cultivars (Rose and Punja, 2004), and maintenance of optimal root health through manipulation of environmental conditions (Rose et al., 2003). Beyond this, however, there are few disease control options, as there are no fungicides registered for use on cucumber to control Fusarium root and stem rot in Canada (Rose et al., 2003).

The difficulty in controlling diseases caused by *Fusarium* species in greenhouses in general has stimulated research into developing biological control strategies. A number of fungi and bacteria have been identified that can suppress diseases caused by *Fusarium oxysporum* (Paulitz and Belanger, 2001), but few are registered for commercial use in Canada. Recent studies on the use of biological control agents to reduce root and stem rot have shown that *Clonostachys rosea* f. *catenulata* reduced seedling mortality and was the best of three commercially available fungal biocontrol agents tested in growth room trials (Rose et al., 2003). The fungus *Clonostachys rosea* f. *catenulata* Schroers, Samuels, Seifert & Gams strain J1446 [syn. *Gliocladium catenulatum* Gilman & Abbott; teleomorph *Bionectria ochroleuca* (Schw.) Schroers & Samuels (Schroers, 2001; Schroers et al., 1999)], is a commercially formulated biocontrol agent (Prestop WP and Prestop Mix, Verdera Oy, Finland) with broad-spectrum activity against plant pathogens. Primastop (Prestop Mix) has been registered in the United States since 1998, PrestopWP has been provisionally registered in Europe since 2001 (Lahdenpera and Korteniemi, 2005), and PrestopWP has recently gained registration approval in Canada (Health Canada, 2009).

Clonostachys rosea also reduced damping-off caused by *Pythium ultimum* and *Rhizoctonia solani* on ornamental bedding plants (McQuilken et al., 2001) and by *P. aphanidermatum* on cucumber (Punja and Yip, 2003). Damping-off on ginseng seedlings caused by a complex of soilborne pathogens was also reduced by applications of *C. rosea* f. *catenulata* (Rahman and Punja, 2007). The biocontrol agent has also shown efficacy in reducing anthracnose development caused by *Colletotrichum acutatum* Simmonds when applied to blueberry blossoms and developing fruit (Verma et al., 2006). The fungus was also effective in controlling grey could caused by *Botrytis cinerea* on tomato stems in semi-commercial greenhouses (Utkhede and Mathur, 2002) and on strawberries under field conditions (Lahdenpera and Korteniemi, 2005). These results indicate that *C. rosea* f. *catenulata* has broad-spectrum activity against many fungi infecting different crop species.

The mechanisms of action of *C. rosea* f. *catenulata* that result in disease suppression are not well understood. Parasitism of several fungal plant pathogens in vitro, including *Rhizoctonia solani*, *Pythium ultimum*, *Botrytis cinerea* Pers.: Fr., and *Sclerotinia sclerotiorum* (Lib.) de Bary has been reported (Huang, 1978; McQuilken et al., 2001; Simay, 1988; Turhan, 1990). Production of extracellular lytic enzymes by this biocontrol fungus in culture has been reported (Lahdenpera and Korteniemi, 2005) but the ability of *C. rosea* to parasitize hyphae of *F. oxysporum*, produce enzymes, and reduce pathogen growth *in situ* had not been previously investigated. *C. rosea* f. *catenulata* survived in peat-based growing media for up to 28 days after application and was observed colonizing cucumber roots 5 weeks after application, suggesting it has the ability to grow in the rhizosphere (McQuilken et al., 2001). Therefore, it has been postulated that the rhizosphere competence of this fungus coupled with its mycoparasitic ability may contribute to biocontrol efficacy (Rose et al., 2003; Punja and Yip, 2003). However, the extent of colonization of plant roots by *C. rosea* f. *catenulata*, both externally and internally, and its effect on pathogen populations and disease development had not been previously investigated. Therefore, we developed a GUS-marked strain of *C. rosea* f. *catenulata* to study colonization of roots and spread to other parts of the plant, to determine the potential for endophytic colonization, and to assess the effect of Prestop application on the development of *F. oxysporum* on cucumber roots.

8.2 Mycoparasitic Behavior of *C. rosea* Against *Fusarium*

To determine whether *C. rosea* displays mycoparasitic behaviour against *F. oxysporum* f. sp. *radicis-cucumerinum*, the two fungi were grown in dual culture and samples from the zone of hyphal interaction were processed and viewed under a scanning electron microscope. There was evidence of contact of *C. rosea* hyphae with *Fusarium*, with likely penetration points observed along the pathogen's hyphae (Fig. 8.1). Hyphal strands of *C. rosea* often grew in parallel along the hyphae of *Fusarium*, but lysis of *Fusarium* hyphae was not observed. However, when excised cucumber roots were placed on water agar between *C. rosea* and *Fusarium* cultures, the hyphae of the two fungi were rarely seen growing together. In areas of the root segments colonized by both fungi, there was evidence of contact of *Fusarium* hyphae by *C. rosea* similar to that observed in dual cultures, but penetration was not observed. Evidence of direct interactions between an antagonist and a pathogen in the rhizosphere is difficult to obtain (Whipps, 2001). In contrast, on excised cucumber roots, *C. rosea* hyphae were observed to coil around the hyphae of *Pythium*, producing short branches that surrounded the host hyphae (Fig. 8.1) and collapse and dissolution of *Pythium* cells was observed at later stages of parasitism. This growth behavior of *C. rosea* hyphae was not observed on colonized cucumber roots in the absence of the pathogen, indicating that this mycoparasitic behavior was induced by the presence of fungal hyphae. Similarly, hyphal branching and formation of specialized structures by *T. atroviride* on colonized cucumber roots appeared to be an active response to the presence of a fungal host (Lu et al., 2004).

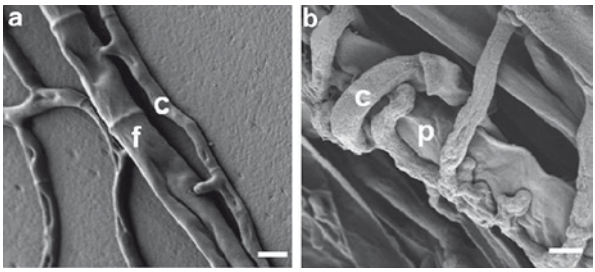


Fig. 8.1 Scanning electron micrographs of the interactions between *C. rosea* (*c*) and *Fusarium oxysporum* (*f*) in dual culture on water agar (**a**), or between *C. rosea* and *Pythium* (*p*) on excised cucumber roots on water agar (**b**). (**a**) Contact of *C. rosea* hyphae with *Fusarium* scale. The hyphae of *Fusarium* are much larger than that of *C. rosea*; scale bar = 30 μm; (**b**) hyphae of *C. rosea* branching and coiling around *P. aphanidermatum* on a cucumber root; scale bar = 30 μm (Chatterton and Punja (2009) Can J Microbiol 55:256–267)

8.2.1 Cell-Wall Degrading Enzyme Production

Secretion of extracellular enzymes capable of lysing cell walls of pathogenic fungi is important in the mycoparasitic process and these enzymes are well characterized in many biocontrol agents, especially *T. harzianum* (Viterbo et al., 2002b; Whipps, 2001). Purified endo-β-1,3-glucanase and endochitinases from *T. harzianum* inhibited spore germination and had lytic activity against the cell walls of a number of plant pathogenic fungi, including *Botrytis cinerea*, *Rhizoctonia solani*, *Sclerotium rolfsii* and *F. oxysporum* f. sp. *melonis* (Lorito et al., 1994; Viterbo et al., 2001), providing evidence for their involvement in mycoparasitism. Characterization of hydrolytic enzyme activity has not been previously reported in *C. rosea*. We observed that enzymes capable of degrading chitin and β-1,3-glucan, both major cell wall components in *Fusarium* (Schoffelmeer et al., 1999), were produced by *C. rosea* when it was cultured on chitin or laminarin as the sole carbon source, respectively. Production of chitinase increased steadily and peaked at 14 days (30 chitinase units, CHU) after which time the levels remained constant (Fig. 8.2). When grown on minimal salts medium only, chitinase activity was not detected. Production of β-1,3-glucanase peaked at 3 days when grown on laminarin

respectively. Chitinase and β-1,3-glucanase activities are expressed as micromoles of N-acetylglucosamine (CHU) or as micromoles of glucose (GU) per milligram of protein per hour, respectively. Values are the combined means from two independent trials. *Vertical bars* indicate standard error of the mean (n = 6). (**c**) Northern blot analysis of a β-1,3-glucanase gene from *C. rosea.* The fungus was grown as a shake culture in MSM supplemented with 0.2% cell wall fragments of *Fusarium* (Fo) or *Pythium* (Pa), homogenized cucumber roots (R), 2% glucose (G2) or 0.1% glucose (G0.1). Approximately 5 μg of total RNA, extracted after 3 days or 7 days of growth, was electrophoresed on a formaldehyde gel, blotted, and hybridized to a radiolabelled probe designed from a 750 bp fragment of a β-1,3-glucanase gene isolated from *C. rosea* (*top panel*). The *bottom panel* shows ethidium bromide-stained rRNA (Chatterton and Punja (2009) Can J Microbiol 55:256–267)

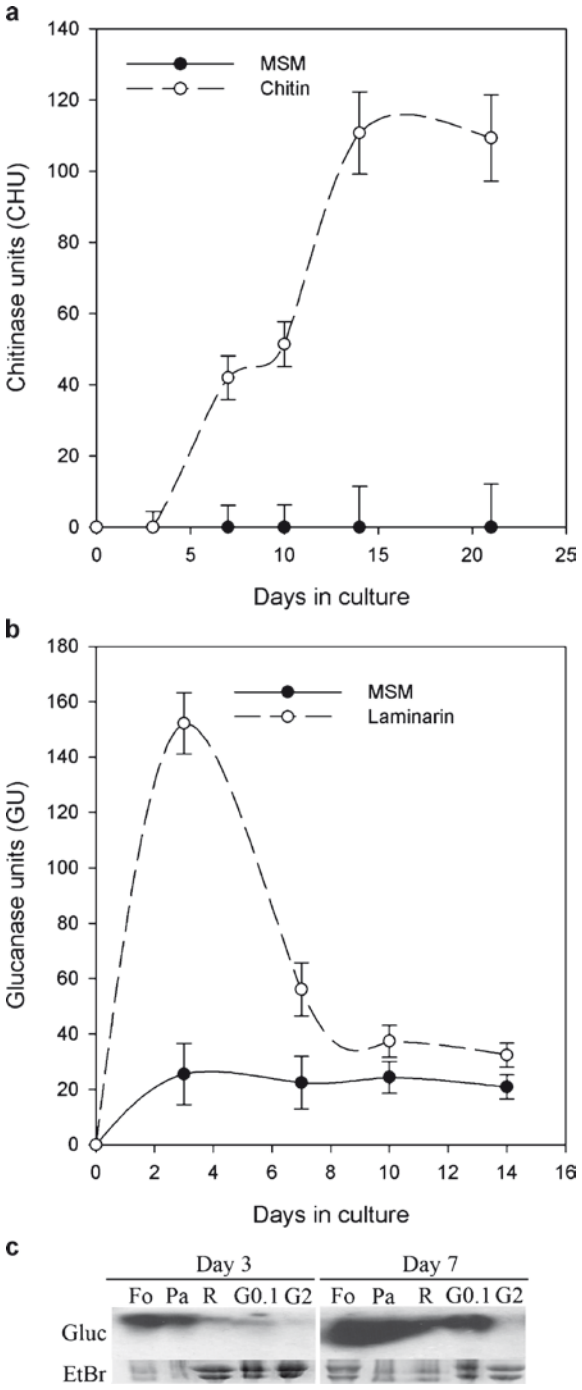


Fig. 8.2 Time course of (**a**) chitinase and (**b**) β-1,3-glucanase production by *C. rosea* on minimal salt medium (no carbon source) and on medium containing chitin or laminarin as a carbon source,

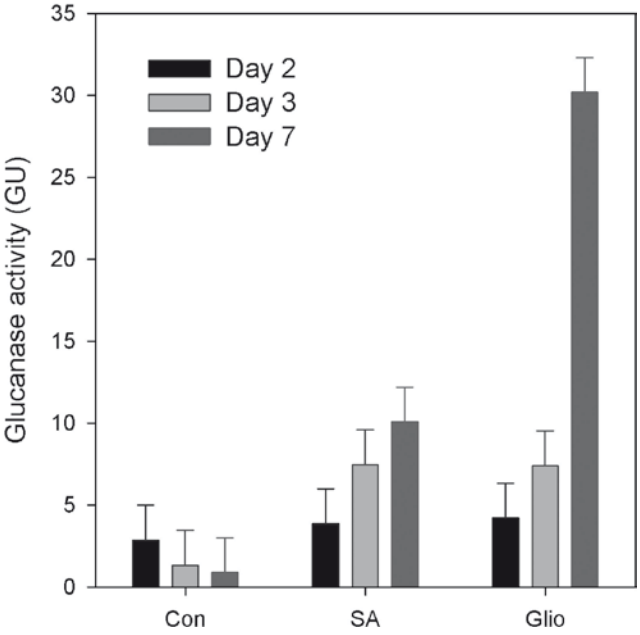


Fig. 8.3 Glucanase activity in roots of 10-day old cucumber seedlings at 2, 3 and 7 days following inoculation with *C. rosea* (Glio), treatment with salicylic acid (SA) or water (control). Enzyme activity is expressed as micromoles of glucose (GU) per milligram of protein per hour, respectively. Values represent the combined mean from two independent trials. *Vertical bars* indicate standard error of the mean (n = 10) (Chatterton and Punja (2009) Can J Microbiol 55:256–267)

and was highest at 150 glucanase units (GU) than at any other time period (Fig. 8.2). Glucanase levels were lower after 7 days in culture and remained steady at around 40 GU for the duration of the experiment. When grown on minimal salts medium only, *C. rosea* produced detectable levels of glucanase over the time period assayed. Both enzymes reduced the germination of conidia and growth of *Fusarium* in vitro. Production of both enzymes was repressed by high levels of glucose and was induced by fungal cell wall extracts, cucumber roots, or by polymers such as laminarin and chitin. Northern analysis of glucanase mRNA confirmed that expression of glucanase mRNA was induced by pathogen cell walls and low levels of glucose (Fig. 8.2).

To further investigate the expression of β-1,3-glucanase by *C. rosea* on colonized cucumber roots, plants were grown in Magenta boxes and roots were inoculated with the biocontrol agent. Roots were harvested 7 days after application of *C. rosea* and protein or total RNA was extracted. β-1,3-Glucanase activities in the protein extracts from the root were assayed, and results showed that there was a marked increase in glucanase activity in the roots 7 days after treatment with *C. rosea* (Fig. 8.3). The protein extract was resolved on an SDS-PAGE gel and stained for glucanase activity. The glucanase isoform patterns indicated that the glucanase

activity was of fungal origin. This was further supported by detection of fungal glucanase mRNA expression on the colonized cucumber roots. First-strand cDNA was synthesized from total RNA and was used as the template in PCR amplification using primers that were designed to amplify a 750 bp amplicon from the partial *Glu1* mRNA sequence for *C. rosea* in the GenBank database (accession number DQ975304). The PCR product from the above reaction was used as the template in a second PCR amplification using primers designed to amplify a 220 bp band from the internal sequence of the *Glu1* fragment. PCR from cDNA synthesized from total RNA from cucumber plants colonized by *C. rosea* produced an amplicon of approximately 250 bp, which was absent in control plants not treated with *C. rosea*, confirming the expression of the fungal glucanase on cucumber roots. Under conditions of carbon starvation and reduced growth, many fungi can actively secrete high levels of hydrolytic enzymes (Tweddell et al., 1994; Ramot et al., 2000; Viterbo et al., 2002a). For example, *T. harzianum* retained its ability to produce glucanase in the presence of easily fermented carbon sources, such as those found in plant exudates (Thrane et al., 2000).

8.3 Biological Control Activity and Survival of *C. rosea*

The long-term biological control activity of *C. rosea* was tested in growth room trials by applying a suspension of Prestop WP to cucumber seeds planted into rockwool blocks. Plants were inoculated with *Fusarium* by application of conidia to the roots of the cucumber plants 30 days later. Treatment with Prestop prior to *Fusarium* inoculations resulted in a significantly lower disease severity index (DSI) compared to cucumber plants treated with *Fusarium* alone, with Prestop-treated plants displaying an average of 60–70% reduction in mortality (Fig. 8.4). Furthermore, *C. rosea* was isolated from cucumber roots for at least 60 days at levels above 1×10^5 CFU/g root fresh weight and was also recovered from the crown region of cucumber plants and from the rockwool blocks at 60 days post-application (Fig. 8.4). Lower levels of *C. rosea* were recovered from the nutrient solution inside the plastic bags. Plating of root segments also indicated that almost 100% of root sections were colonized by *C. rosea*, even after 60 days. Thus, *C. rosea* provided protection against Fusarium root and stem rot for a period of up to 60 days following a single application to the growing medium at seeding, even when pathogen challenge occurred 30 days following application of the biocontrol agent.

Scanning electron microscopic studies further revealed the ability of *C. rosea* to sporulate abundantly on the root surface, as cucumber roots were extensively colonized by *C. rosea* hyphae within 7 days after application and formed a dense network over the root surface. Hyphae were found intertwined near root hairs and were closely associated with the junction of epidermal cells (Fig. 8.5). On roots treated with both *C. rosea* and *Fusarium*, colonization by *C. rosea* was clearly present on the root surface (Fig. 8.5), but there was no visible hyphal interaction between the two fungi even when they were observed growing in close proximity

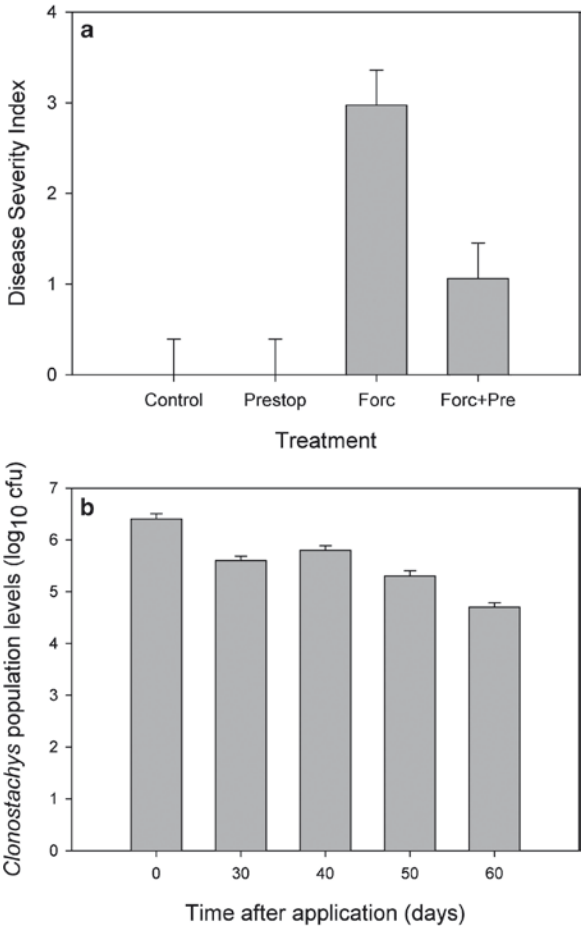


Fig. 8.4 (**a**) Disease severity index (DSI) of cucumber plants treated with Prestop WP (containing *C. rosea* f. *catenulata*) 30 days before inoculation with *F. oxysporum* f. sp. *radicis-cucumerinum*. Plant height and mortality was measured 30 days after pathogen inoculation and used to calculate the DSI. Values represent the combined DSI from three independent growth room trials. (**b**) Population densities, expressed as $\log_{(10)}$ colony forming units per gram root fresh weight, of *C. rosea* f. *catenulata* associated with cucumber roots over a 60 day period. Prestop WP was applied to rockwool blocks at seeding at an initial concentration of 1×10^7 CFU/ml. Values represent the combined means from two independent trials. *Vertical bars* indicate standard error of the mean (Chatterton et al. (2008) Biol Control 46:267–278)

to one another (Fig. 8.5). However, there was less evidence of the presence of *Fusarium* hyphae on plants treated with *C. rosea* compared to plants inoculated with *Fusarium* alone. This study was conducted in a closed-system, in sealed sterile Magenta boxes, which may have overestimated the spread and extent of colonization by *C. rosea*.

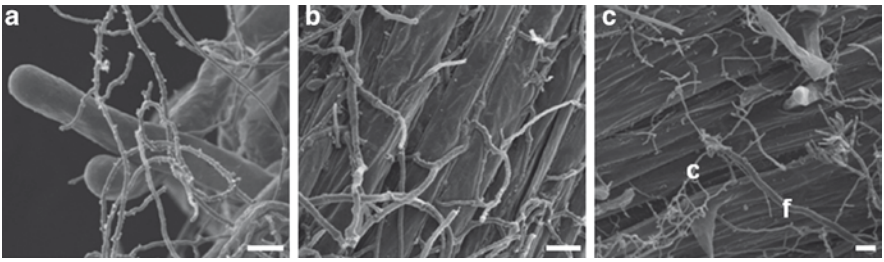


Fig. 8.5 Scanning electron micrographs of cucumber roots 7 days after inoculation with *C. rosea* f. *catenulata* (**a**, **b**), or *C. rosea* f. *catenulata* followed 3 days later by *F. oxysporum* (**c**). (**a**) Colonization of root hair zone; scale bar = 100 µm; (**b**) extensive colonization of the root surface; scale bar = 100 µm; (**c**) hyphal contact between *C. rosea* f. *catenulata* (*g*) and *F. oxysporum* (*f*) on the root surface; scale bar = 100 µm (Chatterton et al. (2008) Biol Control 46:267–278)

8.3.1 Colonization of Cucumber Plants by C. rosea

To facilitate further study of the colonization behaviour and distribution on greenhouse cucumber plants, *C. rosea* was transformed with the GUS marker gene using an *Agrobacterium*-mediated transformation system. Cucumber seeds were treated with the GUS-transformed strain (CrA1) and planted into sterile Magenta boxes or into rockwool blocks. In Magenta boxes, colonization of the seed coat was visible within 24 h, while colonization of the developing cotyledons and the main root was evident within 3 days after seed germination (Fig. 8.6). In rockwool blocks, colonization of the roots occurred primarily at the root tip and along the junction of lateral root emergence, with discontinuous colonization along the surface of the mature root zone after 14 days. The ability of *C. rosea* to spread to newly developing roots and maintain populations on mature roots from a single-seed application indicates that this biocontrol agent displays rhizosphere competence. This rhizosphere competence likely aids in protecting the germinating seed and emerging radical from damping-off pathogens, such as *Pythium* spp. and *Rhizoctonia solani* (McQuilken et al., 2001; Punja and Yip, 2003). Seed treatment or application of *C. rosea* to rockwool blocks resulted in colonization of above-ground plant parts, with hyphae of *C. rosea* visible on the crown area, shoot meristem and emerging true leaves of 14-day old plants (Fig. 8.6). On the stem, *C. rosea* was found to be associated externally with trichomes and formed a network of hyphae over the epidermis. This indicates that this isolate can spread to aerial parts of plants after seed treatment and can survive on plant surfaces under experimental conditions. Strains of *C. rosea* that colonize leaf surfaces have been shown to be effective biocontrol agents of seed-borne diseases of cereals (Lubeck et al., 2002) and against *B. cinerea* on strawberry and raspberry leaves (Sutton et al., 1997). However, whether sufficient population levels of *C. rosea* can be achieved on the phylloplane, after application to the roots, for effective disease suppression of foliar pathogens on cucumbers has yet to be determined.

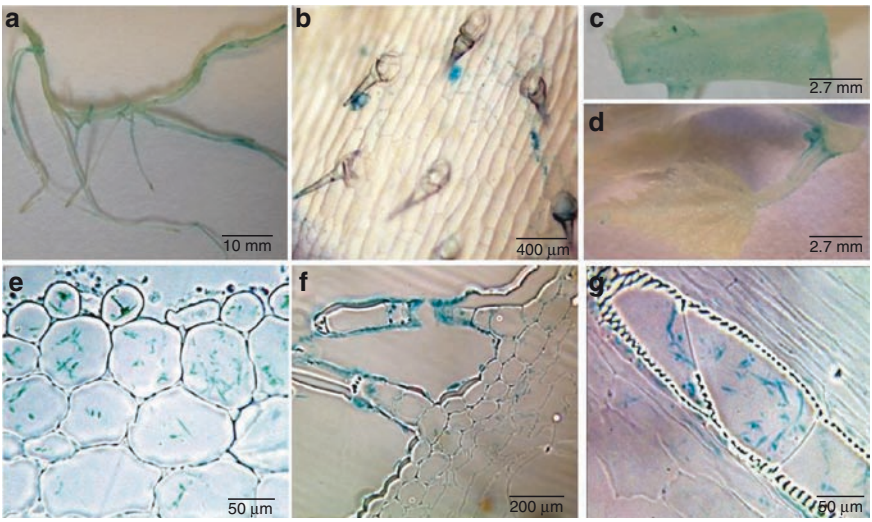


Fig. 8.6 Colonization pattern of cucumber plants by *C. rosea* f. *catenulata* strain J1446 expressing the GUS gene. (**a**) Colonization of roots, (**b**, **c**) stem, (**d**) meristem and true leaves by *C. rosea* f. *catenulata* 14 days after seed treatment in Magenta boxes. (**a**) Colonization of roots was visible at junctions of lateral roots and at root tips, while hyphae were found associated with (**b**) trichomes on the stem surface. (**e**–**g**) Light micrographs showing internal colonization by *C. rosea* f. *catenulata*. (**e**) Colonization of the epidermal and cortical cells in roots of 3-week old plants grown in rockwool blocks. (**f**) Hyphae on the surface of the stem with ingress into trichomes and cortical cells and (**g**) xylem vascular elements of plants grown in Magenta boxes (Chatterton et al. (2008) Biol Control 46:267–278)

The ability of *C. rosea* to colonize cucumber plants internally was determined by the visualization of blue-stained hyphae in cells from epidermal and cortical regions of roots and stems inoculated with the GUS-marked strain (Fig. 8.6). Hyphae were also observed in the xylem vessels of cucumber stems in plants growing in nutrient solution in Magenta boxes (Fig. 8.6). Colonization of the epidermal layer of young true leaves was observed on plants grown both in Magenta boxes and rockwool blocks. This behaviour is consistent with that of rhizosphere competent fungi, such as *Trichoderma harzianum*, which colonize root surfaces but can penetrate the epidermis and outer cell layers of the cortex (Yedidia et al., 2000). As well, similar responses have been reported for endophytic growth of fungi in roots which can be inter- or intracellular, leading to systemic colonization, characteristics which were observed for *C. rosea* (Schulz and Boyle, 2005). Therefore, our observations suggest that *C. rosea* displays the colonization capabilities of an incidental opportunist, since it has the potential to grow endophytically but likely spends most of its life cycle as a saprophyte. Many root colonizing fungal biocontrol agents can induce plant defence reactions that limit their growth inside plants while protecting the plant from pathogen attack (Harman, 2006). For example, when *Trichoderma* hyphae penetrate the roots, the fungus produces a series of bioactive molecules that induces walling off and stimulates plant biochemical pathways that limit the growth

of the hyphae to a small area, with the interaction resulting in both localized and systemic resistance to subsequent pathogen infection (Yedidia et al., 2000; Harman, 2006). Whether or not *C. rosea* can also induce defence responses in cucumber plants remains to be determined.

8.3.2 *Survival of F. oxysporum on Cucumber Roots in the Presence of C. rosea*

Application of *C. rosea* to the rockwool blocks before inoculation with *Fusarium* resulted in a significant decrease in pathogens population on the roots and crown when compared to plants inoculated with *Fusarium* only, regardless of the initial pathogen inoculum concentration (Fig. 8.7). At a *Fusarium* concentration of 10^4 conidia/ml, treatment with *C. rosea* reduced pathogen levels on the roots to nil. These findings indicated that *Fusarium* survival was reduced in the presence of *C. rosea*, suggesting that the biocontrol agent can effectively exclude *Fusarium* from colonizing the roots. These results were confirmed using scanning electron microscopy, where mycelia of *Fusarium* were rarely observed at sites colonized by the biocontrol agent and infrequently found on colonized roots compared to roots that had been treated with *Fusarium* only. Pre-emptive colonization of the niches

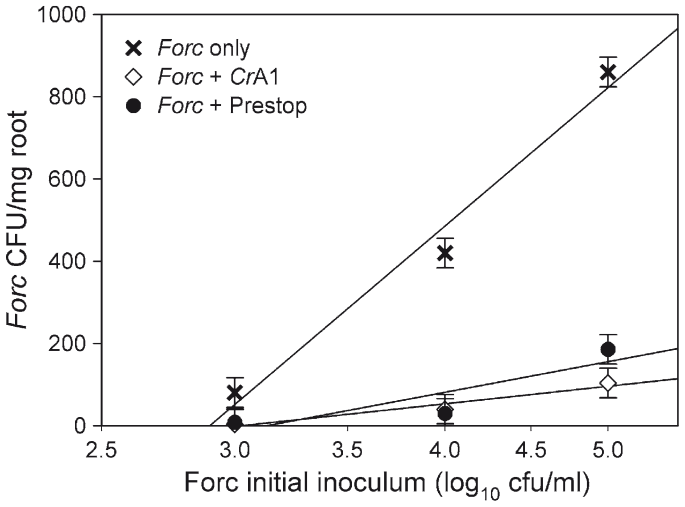


Fig. 8.7 Population size of *F. oxysporum* in the roots of cucumber plants treated with *C. rosea* f. *catenulata* (Prestop WP at the recommended rate or conidia) to the rockwool blocks prior to inoculation with *Fusarium* at an initial inoculum level of either 10^4, 10^5, or 10^6 conidia/ml. Plants were sampled 2 weeks after inoculation with *Fusarium*. Population levels were determined by plating supernatant from ground tissues onto a selective medium for *Fusarium*. The means and standard error were obtained from 10 replicates per treatment (Chatterton et al. (2008) Biol Control 46:267–278)

by *C. rosea* that are often favoured for infection by *Fusarium*, such as root hairs and cellular junctions along the main root (Lagopodi et al., 2002), likely reduced the infection sites available for *Fusarium*. Since Fusarium root and stem rot develops when primary infections occur early in the growing season (Punja and Parker, 2000), protection through pre-emptive colonization by *C. rosea* likely results in long-term protection against this disease.

8.4 Conclusions

Despite the absence of direct penetration of *Fusarium* hyphae by *C. rosea* on cucumber roots, it is possible that glucanase and chitinase levels can inhibit growth of *Fusarium* when the two interacting fungi are in close proximity to one another. This mechanism, termed hyphal interference, can occur through the action of diffusible metabolites, despite a physical separation between the interacting organisms (Thrane et al., 1997). Rhizosphere competence is also strongly related to biocontrol efficacy in mycoparasitic isolates of *Trichoderma* spp. (Thrane et al., 1997; Whipps, 2001). Therefore, production of antifungal β-1,3-glucanases by *C. rosea* in the rhizosphere would help create an environment inhibitory to growth and colonization of *Fusarium*, prior to the introduction of pathogen inoculum (pre-emptive colonization). This is supported by the significant biocontrol efficacy of *C. rosea* when applied 24 h to 3 days prior to pathogen inoculation (McQuilken et al., 2001; Punja and Yip, 2003; Rose et al., 2003), and by the findings that the density of *Fusarium* propagules on cucumber root and crown tissues was significantly reduced in the presence of *C. rosea*. Investigations into the potential production of volatile and nonvolatile antibiotics by *C. rosea* indicate that these were absent in culture. The extensive root colonization ability of this fungus, coupled with its ability to rapidly produce glucanase in vivo, likely are major contributors to its efficacy as a biocontrol agent against *F. oxysporum*. Investigations are currently underway to determine the environmental factors that affect the colonization capability of *C. rosea* on cucumber roots and the role of induced systemic resistance in the biocontrol efficacy of *C. rosea*. The growth and spread of *C. rosea* in the phylloplane is also being examined to gain further information on the efficacy of *C. rosea* against foliar pathogens such as *B. cinerea* and *C. acutatum*.

References

Chatterton S, Jayaraj J, Punja ZK (2008) Colonization of cucumber plants by the biocontrol fungus *Clonostachys rosea*. Biol Control 46:267–278

Chatterton S, Punja ZK (2009) Chitinase and b-1,3-glucanase enzyme production by the mycoparasite *Clonostachys rosea* f. *catenulata* against fungal plant pathogens. Can J Microbiol 55:356–367

Harman GE (2006) Overview of mechanisms and uses of *Trichoderma* spp. Phytopathology 96:190–194

Health Canada Pest Management Regulatory Agency PMRA. (2009) Registration decision: *Gliocladium catenulatum* strain J1446. RD2009–04

Huang HC (1978) *Gliocladium catenulatum*: hyperparasite of *Sclerotinia sclerotiorum* and *Fusarium* species. Can J Bot 56:2243–2256

Lagopodi AL, Ram AFJ, Lamers GEM, Punt PJ, Van den Hondel CAMJJ, Lugtenberg BJJ, Bloemberg GV (2002) Novel aspects of tomato root colonization and infection by *Fusarium oxysporum* f. sp. *radicis-lycopersici* revealed by confocal laser scanning microscopic analysis using the green fluorescent protein as a marker. Mol Plant-Microbe Interact 15:172–179

Lahdenpera M-L, Korteniemi M (2005) Application of microbial fungicides on horticultural crops: a case study with *Gliocladium catenulatum* J1446. In: Sorvari, S, Toldi, O (eds) EBA 2005. Proceedings 1st international conference on plant microbe interactions: endophytes and biocontrol agents, pp 80–89

Lorito M, Hayes CK, Di Pietro A, Woo SL, Harman GE (1994) Purification, characterization and synergistic activity of a glucan 1,3-ß-glucosidase and an N-acetylglucosaminidase from Trichoderma harzianum. Phytopathology 84:398–405

Lu Z, Tombolini R, Woo S, Zeilinger S, Lorito M, Jansson JK (2004) In vivo study of *Trichoderma*–pathogen–plant interactions, using constitutive and inducible green fluorescent protein reporter systems. Appl Environ Microbiol 70:3073–3081

Lubeck M, Knudsen IMB, Jensen B, Thrane U, Janvier C, Jensen DF (2002) GUS and GFP transformation of the biocontrol strain *Clonostachys rosea* IK726 and the use of these marker genes in ecological studies. Mycol Res 106:815–826

McQuilken MP, Gemmel J, Lahdenpera MA-L (2001) *Gliocladium catenulatum* as a potential biological control agent of damping-off in bedding plants. J Phytopathol 149:171–178

Paulitz TC, Belanger RR (2001) Biological control in greenhouse systems. Annu Rev Phytopath 39:103–133

Punja ZK, Parker M (2000) Development of fusarium root and stem rot, a new disease on greenhouse cucumber in British Columbia, caused by *Fusarium oxysporum* f. sp. *radicis-cucumerinum*. Can J Plant Pathol 22:349–363

Punja ZK, Yip R (2003) Biological control of damping-off and root rot caused by *Pythium aphanidermatum* on greenhouse cucumbers. Can J Plant Pathol 25:411–417

Rahman M, Punja ZK (2007) Biological control of damping-off on American ginseng (*Panax quinquefolius*) by *Clonostachys rosea* f. *catenulata* (= *Gliocladium catenulatum*). Can J Plant Pathol 29:203–207

Ramot O, Cohen-Kupiec R, Chet I (2000) Regulation of β-1,3-glucanase by carbon starvation in the mycoparasite *Trichoderma harzianum*. Mycol Res 104:415–420

Rose S, Punja ZK (2004) Greenhouse cucumber cultivars differ in susceptibility to fusarium root and stem rot. HortTechnology 14:240–242

Rose S, Parker M, Punja ZK (2003) Efficacy of biological and chemical treatments for control of Fusarium root and stem rot on greenhouse cucumbers. Plant Dis 87:1462–1470

Schoffelmeer EAM, Klis FM, Sietsma JH, Cornelissen BJC (1999) The cell wall of *Fusarium oxysporum*. Fungal Genet Biol 27:275–282

Schroers H-J (2001) A monograph of *Bionectria* (*Ascomycota*, *Hypocreales*, *Bionectriaceae*) and its *Clonostachys* anamorphs. Stud Mycol 46:1–214

Schroers H-J, Samuels GJ, Seifert KA, Gams W (1999) Classification of the mycoparasite *Gliocladium roseum* in *Clonostachys* as *C. rosea*, its relationship to *Bionectria ochroleuca*, and notes on other *Gliocladium*-like fungi. Mycologia 91:365–385

Schulz B, Boyle C (2005) The endophytic continuum. Mycol Res 109:661–686

Simay EI (1988) Occurrence of hyperparasitism of *Botrytis cinerea* Pers. by *Gliocladium catenulatum* Gilman et Abbott. Acta Phytopathol Entomol Hungarica 23:133–135

Sutton JC, Li D-W, Peng G, Yu H, Zhang P, Valdebeneito-Sanhueza RM (1997) *Gliocladium roseum*: a versatile adversary of *Botrytis cinerea* in crops. Plant Dis 81:316–328

Thrane C, Tronsmo A, Funck Jensen D (1997) Endo-1,3-β-glucanase and cellulose from *Trichoderma harzianum*: purification and partial characterization, induction of and biological activity against plant pathogenic *Pythium* spp. Eur J Plant Pathol 103:331–344

Thrane C, Funck Jensen D, Tronsmo A (2000) Substrate colonization, strain competition, enzyme production in vitro, and biocontrol of *Pythium ultimum* by *Trichoderma* spp. isolates P1 and T3. Eur J Plant Pathol 106:215–225

Turhan G (1990) Further hyperparasites of *Rhizoctonia solani* Kühn as promising candidates for biological control. J Plant Dis Prot 97:208–215

Tweddell RJ, Jabaji-Hare SH, Charest PM (1994) Production of chitinases and β-1,3-glucanases by *Stachybotrys elegans*, a mycoparasite of *Rhizoctonia solani*. Appl Environ Microbiol 60:489–495

Utkhede RS, Mathur S (2002) Biological control of stem canker of greenhouse tomatoes caused by *Botrytis cinerea*. Can J Microbiol 48:550–554

Verma N, MacDonald L, Punja ZK (2006) Inoculum prevalence, host infection and biological control of *Colletotrichum acutatum*: causal agent of blueberry anthracnose in British Columbia. Plant Pathol 55:442–450

Viterbo A, Haran S, Friesem D, Ramot O, Chet I (2001) Antifungal activity of a novel endochitinase gene (chit36) from *Trichoderma harzianum* Rifai TM. FEMS Microbiol Lett 200:169–174

Viterbo A, Montero M, Ramot O, Friesem D, Monte E, Llobell A, Chet I (2002a) Expression regulation of the endochitinase chit36 from *Trichoderma asperellum* (*T. harzianum* T-203). Curr Genet 42:114–122

Viterbo A, Ramot O, Chernin L, Chet I (2002b) Significance of lytic enzymes from *Trichoderma* spp. in the biocontrol of fungal plant pathogens. Antonie van Leeuwnhoek 81:549–556

Whipps JM (2001) Microbial interactions and biocontrol in the rhizosphere. J Exp Bot 52:487–511

Yedidia I, Benhamou N, Kapulnik Y, Chet I (2000) Induction and accumulation of PR proteins activity during early stages of root colonization by the mycoparasite *Trichoderma harzianum* strain T-203. Plant Physiol Biochem 38:863–873

Chapter 9
Screening of Biocontrol Agents for Control of Foliar Diseases

Jürgen Köhl

Abstract Candidate antagonists for the development of biocontrol agents have to fulfill many criteria. The criterion often investigated first in detail is the antagonistic potential of candidates against the target pathogen. However, candidates must also have high ecological competence, must be suitable for an economically feasible production and must be safe in use. Consequently, a broad range of criteria must be tested to fulfill the key factors for success of a biocontrol product. Assays are needed to test such major criteria in simple and inexpensive high throughput systems to exclude candidates in an early stage which may show strong antagonisms but do not fulfill other major criteria for a successful commercialization. The case of a screening program aimed at the biological control of apple scab caused by *Venturia inaequalis* is presented and discussed.

Keywords Biological control • Foliar diseases • Screening • Selection criteria

9.1 Introduction

Fungal leaf diseases can potentially be controlled by antagonists preventing infections or reducing the formation of primary or secondary inoculum. Antagonism often is based on nutrient competition, antibiotic production or hyperparasitism. For the choice of a control strategy and the selection of suitable antagonists, the whole life cycle of the pathogen should be considered. Potential antagonists may be found on the host plant interfering with the pathogen during its pathogenic stage, as well as on crop residues. The antagonistic efficacy of candidates can be evaluated in bio-assays under controlled conditions and has to be confirmed under field conditions. For the development of a commercial biocontrol agent for

J. Köhl (✉)
Plant Research International, Droevendaalsesteeg 1, 6708 PB, Wageningen, The Netherlands
e-mail: jurgen.kohl@wur.nl

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,*
Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_9,

use against foliar diseases many selection criteria besides antagonistic efficacy have to be considered in such screening programs. Important ecological criteria are the adaptation of antagonist candidates to the specific environmental characteristics of the phyllosphere. Important economical criteria are amongst others the possibility of inexpensive production of antagonist inocula and a long shelf life of formulated biocontrol agents. Considering legal registration of biocontrol agents, important criteria are possible toxin production by the antagonist candidates and other potential health risks for users and consumers.

9.2 Selection Criteria for Antagonists

9.2.1 Epidemics of Foliar Diseases and Mechanisms of Antagonists

Antagonists potentially can interact with pathogens during three different stages of epidemics. As with fungicides antagonists can interfere with the pathogens during spore germination and infection. Pre-requisite for such applications is an antagonist which is present on the leaf surface in sufficient densities whenever conditions favor infections. Potential interaction periods are short during the few hours of an infection process, so that successful biocontrol depends on rapid mechanisms. Antagonists may also interfere during a second epidemiological stage. After infection, pathogens sporulate so that the disease is spread through the crop. Antagonists reducing sporulation will slow down the epidemic within the crop. Examples are the use of *Sporothrix flocculosa* against powdery mildew of roses as a biotrophic pathogen (Bélanger et al., 1994) or of *Ulocladium atrum* against onion leaf spot caused by *Botrytis* spp. as necrotrophic pathogens (Köhl et al., 2003). Advantage of this biocontrol strategy is the long potential time period for interactions of days or even weeks compared to the few hours during infection processes. A third epidemiological stage during which biocontrol successfully has been used is the long-term survival during periods of pathogens. An example is the use of *Coniothyrium minitans* against sclerotia of *Sclerotinia sclerotiorum* to control the disease in fields where susceptible crops such as rapeseed are grown in rotation. The antagonists has been formulated and marketed as Contans® by the German company Prophyta Biologischer Pflanzenschutz GmbH, Poel/Malchow.

The development of new biocontrol agents should start with a thorough evaluation of the epidemiology of the targeted disease. After identification of vulnerable epidemiological stages suitable antagonists must be screened which fit to the ecological niche, e.g. green leaves before infection, necrotic leaf parts after infection by necrotrophic pathogens or crop residues harboring survival structures of the pathogen. Consequently, screening assays for selection of antagonists must be specifically developed for the envisaged application and should not rely on simple general assays such as dual cultures on artificial media.

Several mechanisms, operating alone or in concert, are involved in antagonistic interactions in the phyllosphere. Nutrient competition, antibiosis and mycoparasitism are the major mechanisms. Additional mechanisms such as induced resistance, production of biosurfactants, interference with pathogen-related enzymes, and undoubtedly a number of still unknown mechanisms may complete the microbial arsenal, reviewed by Elad (1996).

Nutrient competition is involved in all situations in which the antagonist consumes nutrients which otherwise may be utilized by the pathogen. Large exogenous nutrient sources such as pollen and aphid honeydew (Fokkema et al., 1983; Dik et al., 1991) can rapidly be removed by phyllosphere yeasts, thus preventing infections by foliar pathogens depending or stimulated by such nutrients. The extent to which this reduces infection may vary with the infection strategy of the pathogen involved.

Production of antibiotic secondary metabolites is common for many microorganisms. The effect of antibiotics shows much similarity with the use of fungicides. The presence of antibiotics on the leaf surface produced by antagonists may ensure efficient control of pathogens also during the characteristically short time periods during the infection process, since disorganization of cytoplasmic structures of host cells can be observed after short interaction times (Bélanger et al., 1995). Two factors limit the utilization of antibiotics. Firstly, antibiotics must continuously be present on the surface to protect the leaf from new infections. However, antibiotics are generally not stable under field conditions for long periods, as shown for *Chaetomium globosum*, an antagonist applied against *V. inaequalis* in apple (Boudreau and Andrews, 1987). Secondly, the pathogen may build-up resistance against the antibiotic, e.g. *Botrytis cinerea* developed resistance against two antibiotics produced by *Bacillus subtilis* (Li and Leifert, 1994).

Enzymes degrading fungal cell walls such as chitinases and beta-glucanases are commonly produced by hyperparasites (Elad et al., 1982). Parasitism depends on close contact between antagonist and host, on the secretion of enzymes and on the active growth of the hyperparasite into the host. These processes need time so that it is unlikely that infection structures of pathogens can be parasitized and killed rapidly enough to prevent penetration of the host plant. Mycoparasitism is often aimed at reduced sporulation and spread of biotrophic pathogens already established on the host, e.g. *Ampelomyces quisqualis* or *Verticillium lecanii* parasitizing on powdery mildews of cucumber or roses (Philipp and Crüger, 1979; Verhaar et al., 1993).

9.2.2 Ecological Competence for Phyllosphere Colonization

Development of both pathogen and antagonist in the phyllosphere is determined by several abiotic factors such as availability of nutrients, temperature, water availability, UV radiation and the deposition of agrochemicals. Detailed information on the microbial ecology of the phyllosphere can be found in the proceedings of several symposia (Preece and Dickinson, 1971; Dickinson and Preece, 1976;

Blakeman, 1981; Fokkema and van den Heuvel, 1986; Andrews and Hirano, 1992; Morris et al., 1996).

On healthy plant surfaces, important nutrient sources are mainly deposits. Leachates from the host plant may form a nutritional background (Tukey, 1970) but play no important role as long as cell membranes are intact (Schönherr and Baur, 1996). Exogenous nutrient deposits on the leaf may consist of pollen grains, flower remains, insect honeydew and organic and inorganic dust. Pollen grains deposited on the leaf surface leaching high amounts of easily degradable nutrients such as amino acids and sugars, are an important nutrient source in the phyllosphere (Fokkema, 1971).

Water availability and temperature in the boundary layer of green leaves (Burrage, 1971) and in necrotic leaf tissues determine microbial growth. Rapid fluctuations in water availability and temperature, e.g. on hot and dry summer days with cool and dewy nights, are characteristics of these niches and are main factors limiting the development of microbial populations. Microbial colonizers of leaf surfaces or of necrotic leaf tissue must have the capacity to survive during dry periods (Diem, 1971) and to utilize the limited leaf wetness periods for rapid colonization of the substrate. Therefore, short lag times for mycelial regrowth (Park, 1982) or cell multiplication of single cell organisms are a prerequisite for successful colonization.

UV radiation is another detrimental factor for microbial colonization of leaf surfaces. Exposure of spores on leaf surfaces to direct UV radiation reduces their longevity (Rotem et al., 1985). Protection against UV radiation during the pre-colonization stage is often achieved by pigmentation or cell clustering of the spores. After having invaded plant tissues, fungal mycelium is protected from the detrimental effect of sunlight (Rotem and Aust, 1991).

Various agrochemicals are applied to canopies of many crops. This may include fungicides, insecticides, herbicides, fertilizers and growth regulators. Microbial phyllosphere communities including introduced antagonists may be affected by agrochemicals. Such possible interference with phyllosphere communities will be much stronger than for soil communities since leaves mostly are directly targeted by applications of chemicals and are less buffered than soil environments.

The ecological competence of antagonists is a key selection factor for biocontrol agents. Antagonist inoculum must survive during unfavorable conditions. Mechanisms to increase survival are the production of slime, protecting cells from drying, or pigmentation, protecting from detrimental effects of UV radiation (Dickinson, 1976). Antagonists must reach high growth rates under favorable conditions during leaf wetness at moderate temperatures and with sufficient nutrient supply as well as under marginal conditions e.g. at low temperatures or at low water potentials. Pathogens and their potential antagonists can markedly differ in their activity at low water potentials (Pfender et al., 1991; Köhl et al., 1992) and low temperatures (Köhl and Schlösser, 1989). Particularly for above-ground applications, antagonists must be adapted to rapid changes between unfavorable and favorable conditions with short lag-times for regrowth e.g. to profit from short periods of leaf wetness. Köhl and Molhoek (2001) studied the effect of interrupted wetness periods on

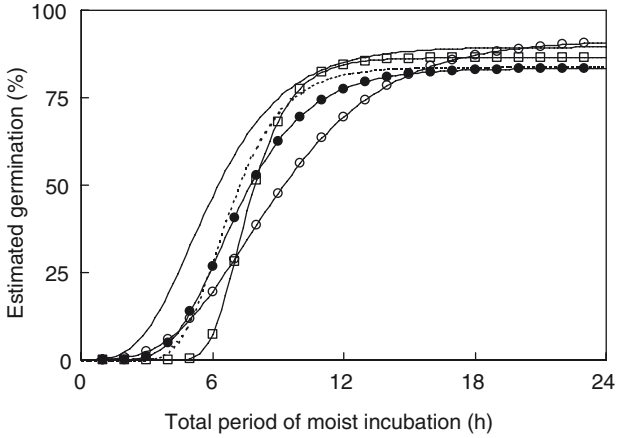


Fig. 9.1 Estimated germination percentage of conidia of *Ulocladium atrum* incubated on agar slides under moist conditions (−1 MPa) interrupted by a dry period at −10 MPa during the initial stage of germination. Initial period of moist incubation was 0 (—), 2 (…), 4 (□), 6 (○), or 8 h (●). Sum of wetness hours of initial moist period and after dry period was 24 h. Gompertz curves were fitted to data obtained from repeated experiments (from Köhl and Molhoek 2001)

conidia of the fungal antagonist *Ulocladium atrum.* The germination process stopped during dry periods (−10 MPa) but continued after re-wetting without any significant time-lag and the final level of the percentage of germinated conidia was similar for all treatments (Fig. 9.1). It can be assumed that germinating spores of fungi from other environments, e.g. from soil, are more vulnerable to such rapid changes of water availability and germ tubes may die during dry periods (Diem, 1971).

A simple experiment demonstrated the resistance of conidia of *U. atrum* against UV irradiation and high temperatures. Conidial suspensions were sprayed on a field-grown onion crop at 11:30 or 22:00 at a summer day with bright sunshine and temperatures above 30°C. Leaves were sampled directly after spray application and 2 and 3 days after application. During the experiment conidia did not germinate in the field due to the dry conditions. After incubation of sampled leaves in moist chambers more than 80% of the spores germinated regardless application time or sampling date. The results indicate that for this antagonist an application late at a day close to the onset of dew formation as often recommended for foliar applications of antagonists is not necessary. This will give much more flexibility to the end-users of a biocontrol product.

Such knowledge about the autecology of candidate antagonists helps to restrict the number of isolates that have to be tested under more practical and complex conditions. However, their synecology in the natural systems is the criterion that finally will determine their competitive ability under the practical conditions (Andrews, 1990).

9.2.3 Economical Features

Commercial companies will invest in development, registration and marketing of biocontrol products only if economical conditions are sound and guarantee a return of the investments. Key factors for an economical success are, amongst others, market size, competing products on the market, efficacy under a broad range of environmental conditions, ease in handling for the end user, production, formulation and storage costs, costs for registration on the different markets in different countries, and possibilities for protecting the intellectual properties through patenting or other means. Although biocontrol industries strongly depend on these key factors, screening programs for new biocontrol agents often focus primarily on the antagonistic interactions between candidate antagonists and a target pathogen. It can be assumed that many antagonists with promising efficacy against a pathogen do not fulfill all other major criteria for development of a biocontrol product, e.g. spores production may be low or may depend on specific growing conditions different from those in the available production systems. Scientific publications on screening programs considering broadly also such very different economical features are rare. However, for progress on the market for biological control products, such a simultaneous testing of the various key factors will be essential. Wherever possible, scientists should collaborate with the biocontrol industries, and develop and apply selection criteria for new antagonists, considering the various economical features also during the first screening steps.

9.3 A Case of a Screening Program: Development of a Biocontrol Agent for Apple Scab Control

A screening program was carried out from 2004–2007 with the objective to develop a biocontrol agent for scab control in organic apple production. The work was part of the EU-funded project REPCO (www.rep-co.nl).

Apple scab caused by *V. inaequalis* is worldwide the major disease in apple production (MacHardy, 1996). *V. inaequalis* can infect leaves and fruits during the entire growing period resulting in losses in yield and reduced fruit quality. The pathogen overwinters in fallen leaves as saprophyte. Ascospores produced in spring are the primary inoculum initiating the summer epidemic. After primary infections of leaves, the pathogen behaves as biotroph and produces conidia during several infection-sporulation cycles.

Control of the disease currently depends on frequent application of fungicides. In organic farming, scab control largely depends on applications of copper or sulphur fungicides. Permitted amounts of copper will be reduced stepwise during the following years (European Commission, 1991) to avoid environmental risks. The development of novel antagonists for biological control of apple scab may offer alternative options for disease control. Earlier research on biological control

of *V. inaequalis* mainly focused on the overwintering stage of the pathogen in fallen leaves (Carisse et al., 2000). Only few reports described preliminary work on the possible use of antagonists during scab epidemics in summer (Burr et al., 1996; Fiss et al., 2000). Sporulating colonies of *V. inaequalis* may harbor antagonistic microorganisms which may affect the sporulation capacity of the pathogen.

Objectives of our study reported here were (1) to build-up a collection of microorganisms obtained from *V. inaequalis* colonies on apple leaves, (2) to select possible antagonists suppressing sporulation of the pathogen, and (3) to test selected antagonists under field conditions. More detailed information on the experiments can be found in Köhl et al. (2009).

9.3.1 Building-Up a Collection of Candidate Antagonists: Sampling and Isolation

All candidate antagonists included in the screening program originated from the ecological niche of interest. Scab infected leaves were collected during late summer 2004 after a severe increase of the scab epidemics had been observed in orchards during September. In total, 216 leaf samples were collected in various parts of The Netherlands (82 samples), Belgium (18 samples), northwest Germany (11 samples) and central Germany (105 samples). Most samples originated from old standard trees (without any further cropping management), e.g. planted along secondary roads. Samples were also collected in organically managed orchards, abandoned orchards, or orchards with integrated management.

Leaves with sporulating colonies of *V. inaequalis* on leaf parts not yet necrotic were placed in moist chambers and incubated for 3 days at 20°C. Developing mycelium was isolated on agar and pure cultures were made from fungal colonies different from *V. inaequalis*. It was possible to isolate several hundreds of fungi different from *V. inaequalis* from the sampled scab colonies. Based on colony characteristics, a high diversity between isolates was observed.

9.3.2 Pre-screening: Considering Mass Production, Ecological Competence and Safety

A rapid throughput system was developed for a first check of candidate antagonists regarding their ecological characteristics, potential risks and economical feasibility for the development of a biocontrol product. Fungi belonging to the genera *Aspergillus*, *Penicillium* or *Fusarium* were discarded because of the potential of various species within these genera to produce mycotoxins. From the remaining fungal isolates, 148 isolates were cultured on nutrient media and the spore production was determined. Isolates producing less than 1×10^5 spores per plate were discarded because of a mass production of such isolates is considered not to be cost effective.

Subsequently, isolates growing at 5°C and at low water activity were selected because they were considered to be cold tolerant and drought tolerant. These characteristics are pre-requisites for successful colonization of the phyllosphere. Isolates growing at 36°C were discarded because such isolates may demand special risk studies during a registration procedure.

From the 148 fungal isolates, 17 isolates showed poor sporulation on agar. These isolates were excluded from further screening because spore production may not be economically feasible. From the remaining 131 isolates, spores of 14 isolates produced colonies at 36°C. These isolates were excluded from further screening because of their potential risk for human health. All isolates tested produced colonies at 5°C and can be considered as cold tolerant. Only 16 isolates out of 136 did not produce a colony at −10 MPa and were excluded from further screening because they may have low drought tolerance. In summary, from 148 isolates tested, 46 were excluded from further screening for efficacy against *V. inaequalis* in bio-assays.

9.3.3 Antagonistic Efficacy: Screening on Seedlings

The potential of candidate antagonists to suppress the conidia production of *V. inaequalis* on infected leaves was tested on young apple seedlings. Seedlings were sprayed with conidial suspensions of *V. inaequalis* (1×10^5 ml^{-1}) until run-off and placed in a moist chamber consisting of a plastic tray closed by transparent plastic top. After 2 days incubation at 15°C with diffuse light, the tops were removed from the trays and seedlings further incubated for 5 days at 85% RH, 15°C and 16 h light per day. Thereafter, *V. inaequalis*-inoculated seedlings were sprayed with antagonist suspensions (containing 1×10^6 spores or cells mL^{-1}) or water (containing 0.01% Tween 80) as control. Two seedlings were used for each replicate of each treatment. The sets of two inoculated seedlings were placed in a polyethylene tent in a block design with six blocks (replicates) and complete randomization within blocks. Seedlings were grown for 9 to 12 days at 15°C, with 16 h light per day at 138 $\mu E\ s^{-1}\ m^{-2}$. From both seedlings of each replicate, the youngest five true leaves were carefully removed, put into Duran bottles (100 mL) containing 35 mL of tap water with 0.01% Tween 80. Bottles were shaken with a flask shaker and the concentration of conidia of *V. inaequalis* was determined for each suspension with the aid of a haemocytometer. Leaf surfaces were measured with an area meter.

Fourteen experiments on seedlings were carried out. Figure 9.2 presents the results of one of the experiment as example. Most of the 63 candidate isolates tested on seedlings did not statistically significantly reduce conidiation of *V. inaequalis*. Four isolates caused a significant reduction of *V. inaequalis* sporulation on leaves which could be repeated in subsequent independent experiments. However, efficacies of these antagonists varied between 18% and 80% in the various experiments in which they were tested and reduction of conidiation in some cases was not statistically significant. A few more isolates showed a strong statistically significant

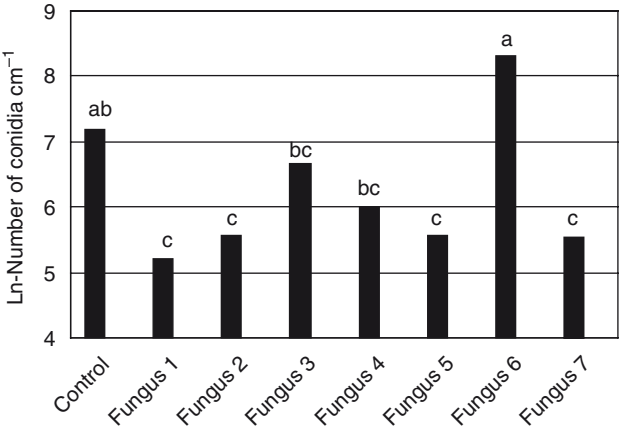


Fig. 9.2 Effect of fungal isolates on production of conidia of *Venturia inaequalis* on apple seedlings grown under controlled conditions at 15°C. Seedlings were inoculated with conidia of *V. inaequalis*. After 7 days, seedlings were treated with water (control) or spore suspensions of fungal candidate antagonists. Columns with the same letter do not differ statistically significantly (LSD-test; $\alpha = 0.05$)

antagonistic effect in one experiment but such effects could not be repeated. In no case significant enhancements of conidiation of *V. inaequalis* after application of candidate isolates was observed.

Screening bio-assays on apple seedlings were laborious and costly because high numbers of seedlings had to be produced and climate facilities were needed for a long time period. Furthermore, the assessment of conidiation showed huge variations since sporulation intensity is the result of the complex interactions between host conditions, environmental conditions and the applied candidate antagonists. Nevertheless a few superior antagonists could be identified. Since the design of the screening assay was close to orchard conditions, we assumed that such selected antagonists also can perform well under orchard conditions. The mechanisms of action may be different for each antagonist and most likely a combination of several modes-of-action may be involved. To allow the detection of antagonists with a broad possible range of modes of action is another reason for testing candidates in a situation very close to field conditions even if assays are complex and time consuming. We avoided any pre-screening for antagonism in more simple systems as in the frequently used dual cultures on agar plates. In such a simplified screening system toxin-producers are preferably selected and antagonists exploiting different modes-of-action may be excluded from further evaluation during the first screening step. Another disadvantage of the exclusive selection of toxin-producing antagonists is the potential of pathogens to build up resistance against toxins (Li and Leifert, 1994). Furthermore, more toxicological studies may be needed during registration of biocontrol products based on toxin-producing antagonists and may thus affect economic feasibility of such products.

9.3.4 Suitability at Industrial Scales: Production and Formulation

Promising isolates were assessed in small scale Solid-State Fermentation (SSF) for their suitability for large scaled biotechnological production processes by Prophyta Biologischer Pflanzenschutz GmbH, Malchow/Poel, Germany (U. Eiben, pers. communication). Several fermentation conditions regarding media and incubation conditions were evaluated (data not presented). Only antagonists which passed this screening step were tested in subsequent experiments in the orchard. Final experiments were carried out to develop prototype protocols for mass production, down streaming and formulation for the selected antagonist H39. Fermentation was based on the Prophyta laboratory scaled SSF system and formulated products were applied in the orchard experiments.

9.3.5 Orchard Experiments: Towards Practical Use

Several rows of var. Jonagold within an organically managed orchard at Applied Plant Research, Randwijk, The Netherlands, were pruned during spring and summer 2006 and 2007 so that trees produced new shoots with young leaves highly susceptible for *V. inaequalis*. To stimulate the development of new shoots also the majority of young fruits was removed. A series of eight experiments was carried out in 2006, each on a different set of trees. For each experiment two to six trees, depending on the number of newly produced shoots per tree, were chosen for each of six blocks (replicates). Within each block, the different treatments were randomly distributed. Shoots were labeled with colored metal rings so that the two youngest leaves fully expanded at the day of the first treatment could later be distinguished from the other leaves of the shoot. Treatments consisted of spraying tap water containing 0.01% Tween 80 as control, or suspensions of freshly produced spores of four different antagonists. Separate treatments were carried out with fermenter-produced spores of the antagonist H39 formulated as dry powder and resuspended in tap water containing 0.01% Tween 80. Spray applications were done using a compressed air-driven knapsack sprayer at 250 kPa until run-off. The different experiments were carried out in the period between June 22 and September 28, 2006. Experiments started with the first treatment 1 to 3 days after an infection period for *V. inaequalis* had been predicted according to the Mills table based on leaf wetness duration and temperature. During all experiments, subsequent treatments were carried out at 3 to 4 day intervals. Leaves were sampled 3 to 5 weeks after the first treatment. In each experiment, the two youngest leaves fully expanded at the beginning at the experiment together with the two next younger leaves (expanded during the course of the experiments) were pooled for three shoots belonging to the same replicate so that a sample consisted of 12 leaves. Leaves were shaken in bottles in water containing 0.01% Tween 80 with a flask shaker.

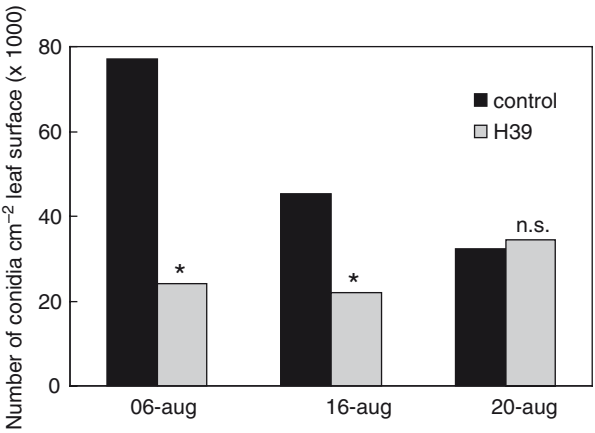


Fig. 9.3 Conidia production of *Venturia inaequalis* on apple leaves treated twice per week with *Cladosporium cladosporioides* H39 (approximately 2×10^6 viable spores mL^{-1}) in an organically managed orchard. Statistically significant effects of antagonist treatments in comparison with the control treatment are indicated by '*' separately per sampling date; one-sided unprotected LSD-test ($\alpha = 0.05$); n.s.: no significant difference

From the obtained suspensions, the concentration of conidia of *V. inaequalis* was determined with the aid of a haemocytometer. The leaf surface of all leaves per sample was measured with an area meter.

In 2007, a similar experiment was carried but the same trees were treated during a longer period of 8 weeks and leaves were repeatedly sampled from such trees.

In the series of eight experiments carried out in 2006, only the pilot-formulated spore product of the antagonist *Cladosporium cladosporioides* H39 reduced significantly pathogen sporulation by 35% to 55%. Treatments with other antagonist did not reduce sporulation of *V. inaequalis*. In 2007, apple trees were treated with formulated *C. cladosporioides* H39 with similar results. A significant reduction by 51% to 69% was found for two assessment dates (Fig. 9.3). However, at a later assessment date no difference between treated and untreated trees was found. This may be due to a reduced quality of the available antagonist product formulated following a first pilot protocol during the field experiment which had an insufficient shelf life.

9.4 Conclusions

During the screening program a few key factors were considered regarding ecological competence, antagonistic efficacy, and cost effectiveness and safety in industrial use. Out of 148 tested candidate antagonists only one, *Cladosporium cladosporioides* H39, fulfilled all criteria. The antagonist has been effective in reducing sporulation of *V. inaequalis* under orchard conditions. Furthermore, the results of the pre-screening

indicate that it is cold and drought tolerant and results of experiments on spore production in solid state fermentation show that mass production is economically feasible. These results have been obtained in a stepwise selection approach.

More selection criteria should be included in future screening programs. Such criteria could include testing fungicide tolerance regarding fungicides commonly applied in practice in the targeted crops. Other criteria could be UV tolerance and resistance against rapidly changing water potentials. Simple assays are needed to test various economically important characteristics of potential candidates for biocontrol products in high-throughput systems so that substantial numbers of candidates can be pre-screened efficiently and cost effectively. Such efforts can only be realized by close collaborations of biocontrol industries together with plant pathologists and microbial ecologists.

Acknowledgements The projects BIOSPORSUPPRESS (Project No CT96-1898) on biocontrol of *Botrytis* and REPCO (Project No 501452) on biocontrol of apple scab were funded by the European Commission and the Dutch Ministry of Agriculture, Nature and Food quality.

References

Andrews JH (1990) Biological control in the phyllosphere: Realistic goal or false hope? Can J Plant Pathol 12:300–307

Andrews JH, Hirano SS (1992) Microbial ecology of leaves. Springer, New York

Bélanger RR, Labbé C, Jarvis WR (1994) Commercial scale control of powdery mildew with a fungal antagonist. Plant Dis 78:420–424

Bélanger RR, Dufour N, Caron J, Benhamou N (1995) Chronological events associated with the antagonistic properties of *Trichoderma harzianum* against *Botrytis cinerea*: Indirect evidence for sequential role of antibiosis and parasitism. Biocontrol Sci Technol 5:41–53

Blakeman JP (1981) Microbial ecology of the phylloplane. Academic, London

Boudreau MA, Andrews JH (1987) Factors influencing antagonism of *Chaetomium globosum* to *Venturia inaequalis*: A case study in failed biocontrol. Phytopathology 77:1470–1475

Burr TJ, Matteson MC, Smith CA, Corral-Garcia MR, Huang T-C (1996) Effectiveness of bacteria and yeasts from apple orchards as biological control agents of apple scab. Biol Control 6:151–157

Burrage SW (1971) The micro-climate at the leaf surface. In: Preece TF, Dickinson CH (eds) Ecology of leaf surface micro-organisms. Academic, London, pp 91–101

Carisse O, Philion V, Rolland D, Bernier J (2000) Effect of fall application of fungal antagonists on spring ascospore production of the apple scab pathogen, *Venturia inaequalis*. Phytopathology 90:31–37

Dickinson CH (1976) Fungi on the aerial surfaces of higher plants. In: Dickinson CH, Preece TF (eds) Microbiology of aerial plant surfaces. Academic, London, pp 293–324

Dickinson CH, Preece TF (1976) Microbiology of aerial plant surfaces. Academic, London

Diem HG (1971) Effect of low humidity on the survival of germinated spores commonly found in the phyllosphere. In: Preece TF, Dickinson CH (eds) Ecology of leaf surface micro-organisms. Academic, London, pp 211–219

Dik AJ, Fokkema NJ, van Pelt JA (1991) Consumption of aphid honeydew, a wheat yield reducing factor, by phyllosphere yeasts under field conditions. Neth J Plant Pathol 97:209–232

Elad Y (1996) Mechanisms involved in the biological control of *Botrytis cinerea* incited diseases. Eur J Plant Pathology 102:719–732

Elad Y, Chet I, Henis Y (1982) Degradation of plant pathogenic fungi by *Trichoderma harzianum*. Can J Microbiol 28:719–725

European Commission (1991) Council regulation (EEC) No 2092/91 of 24 June 1991 on organic production of agricultural products and indications referring thereto on agricultural products and foodstuffs. Off J Eur Union L 198:22.7.1991, pp. 1–89

Fiss M, Kucheryava N, Schönherr J, Kollar A, Arnold G, Auling G (2000) Isolation and characterization of epiphytic fungi from the phyllosphere of apple as potential biocontrol agents against apple scab *Venturia inaequalis*. Z PflKrankh PflSchutz 107:1–11

Fokkema NJ, Riphagen I, Poot RJ, Jong de C (1983) Aphid honeydew, a potential stimulant of *Cochliobolus sativus* and *Septoria nodorum* and the competitive role of saprophytic microflora. Trans Br Mycol Soc 81:355–363

Fokkema NJ (1971) The effect of pollen in the phyllosphere of rye on colonization by saprophytic fungi and on infection by *Helminthosporium sativum* and other leaf pathogens. Neth J Plant Pathol 77 Suppl 1:1–60

Fokkema NJ, van den Heuvel J (1986) Microbiology of the phyllosphere. Cambridge University Press, Cambridge

Köhl J, Molhoek WML (2001) Effect of water potential on conidial germination and antagonism of *Ulocladium atrum* against *Botrytis cinerea*. Phytopathology 91:485–491

Köhl J, Schlösser E (1989) Decay of sclerotia of *Botrytis cinerea* by *Trichoderma* spp. at low temperatures. J. Phytopathol 125:320–326

Köhl J, Krijger MC, Kessel GJT (1992) Drought tolerance of *Botrytis squamosa*, *B. aclada* and potential antagonist. In: Verhoeff K, Malathrakis NE, Williamson W (eds) Recent advances in *Botrytis* research. Pudoc Scientific, Wageningen, pp 206–210

Köhl J, Molhoek WML, Goossen-van de Geijn HM, Lombaers-van der Plas CH (2003) Potential of *Ulocladium atrum* for biocontrol of onion leaf spot through suppression of sporulation of *Botrytis* spp. BioControl 48:349–359

Köhl J, Molhoek WML, Groenenboom-de Haas BH, Goossen-van de Geijn HM (2009) Selection and orchard testing of antagonists suppressing conidia production of the apple scab pathogen *Venturia inaequalis* 123:401–414

Li H, Leifert C (1994) Development of resistance in *Botryotinia fuckeliana* (de Bary) Whetzel against the biological control agent *Bacillus subtilis* CL27. Z PflKrankh PflSchutz 101:414–418

MacHardy WE (1996) Apple scab: biology, epidemiology, and management. APS, St. Paul, MN

Morris C, Nicot P, Nguyen-The C (1996) Aerial plant surface microbiology. Plenum, New York

Park D (1982) Phylloplane fungi: Tolerance of hyphal tips to drying. Trans Br Mycol Soc 79:174–178

Pfender WF, Sharma U, Zhang W (1991) Effect of water potential on microbial antagonism to *Pyrenophora tritici-repentis* in wheat residue. Mycol Res 95:308–314

Philipp WD, Crüger G (1979) Parasitismus von *Ampelomyces quisqualis* auf Echten Mehltaupilzen an Gurken und anderen Gemüsearten. Z Pflanzenkr Pflanzensch 86:129–142

Preece TF, Dickinson CH (1971) Ecology of leaf surface micro-organisms. Academic, London

Rotem J, Aust HJ (1991) The effect of ultraviolet and solar radiation and temperature on survival of fungal propagules. J. Phytopathol 133:76–84

Rotem J, Wooding B, Aylor DE (1985) The role of solar radiation, especially ultraviolet, in the mortality of fungal spores. Phytopathology 75:510–514

Schönherr J, Baur P (1996) Cuticle permeability studies: A model for estimating leaching of plant metabolites to leaf surfaces. In: Morris C, Nicot P, Nguyen-The C (eds) Aerial plant surface microbiology. Plenum, New York, pp 1–23

Tukey HB Jr (1970) The leaching of substances from plants. Annu Rev Plant Phys 21:305–324

Verhaar MA, van Strien PAC, Hijwegen T (1993) Biological control of cucumber powdery mildew (*Sphaerotheca fuliginea*) by *Verticillium lecanii* and *Sporothrix* cf. *flocculosa*. In: Fokkema NJ, Köhl J, Elad Y (eds) Biological control of foliar and postharvest diseases IOBC/WPRS Bulletin 16(11), pp 79–81

Chapter 10
Quorum Sensing as a Target for Novel Biocontrol Strategies Directed at *Pectobacterium*

Amélie Cirou, Stéphane Uroz, Emilie Chapelle, Xavier Latour, Nicole Orange, Denis Faure, and Yves Dessaux

Abstract Members of the species *Pectobacterium carotovorum* and *P. atrosepticum* are pathogenic bacteria that are responsible for tissue maceration on various host plants. Pathogenicity essentially relies upon the production of plant cell wall degradation enzymes, the synthesis of which is regulated in a bacterial cell density dependent fashion, a process called quorum sensing (QS). This process involves key low molecular weight signal molecules belonging to the acyl homoserine lactone class. This paper reports on the various strategies that have been developed to prevent the expression of the pathogenicity determinants in *Pectobacterium*. These could target signal production, accumulation or sensing. Two approaches yield promising results in the *Pectobacterium* pathosystem: a biocontrol approach based on the isolation of strains able to degrade the QS signal, and a biostimulant approach based on amendment the plant environment with compounds favoring the growth of microbial consortia able to degrade the acyl homoserine lactones. The possibility to combine these two approaches is discussed.

Keywords Quorum-sensing • *Pectobacterium* • Ecological engineering • Biological control • Biostimulation

A. Cirou, S. Uroz, E. Chapelle, D. Faure, and Y. Dessaux (✉)
Institut des Sciences du Végétal, CNRS, Gif-sur-Yvette, France
e-mail: dessaux@isv.cnrs-gif.fr

A. Cirou
Comité Nord Plants de pommes de terre, Bretteville-du-Grand-Caux, France

X. Latour and N. Orange
Microbiologie du Froid, Université de Rouen, Evreux, France

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,*
Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_10,

10.1 Introduction

Various bacterial systems regulating gene transcription exist. One of these couples the expression (or the repression) of gene(s) to the bacterial cell density. This regulatory mechanism is known as quorum sensing (QS). It involves the production by the bacteria of diffusible signal molecules, the concentration of which increases as cell density does. Once a threshold concentration of the signal is reached, i.e. a threshold cell density (or quorum), the presence of the signal is sensed by the bacteria that emitted it, and the QS regulated gene(s) are expressed or repressed (recent reviews: Keller and Surette, 2006; Smith et al., 2006; Williams et al., 2007). In several cases, one of the genes regulated by QS encodes the signal synthase. The sensing of the signal therefore further activates the transcription of this gene, leading to an increased amount of signal produced that permits the perception of the signal by the relevant bacterial population (Whitehead et al., 2001). This feature allows the bacteria to synchronize the expression of the QS-regulated function(s). Because QS relies upon the emission and sensing of signal molecules, the diffusion of these signals in the bacterial environment will be a key element affecting their perception by the population. To a certain extend, QS may also be viewed as a process that permits bacteria to sense how "open" is their environment. To account for this phenomenon, the term "diffusion sensing" has been proposed (Hense et al., 2007).

QS signals are diverse and belong to several molecule families. These include cyclic peptides in Gram positive bacteria, and often fatty acid derivatives in Gram negative bacteria, such as 3 hydroxy palmitic acid methyl ester, 2 methyl dodecenoic acid, or N-acyl homoserine lactones (N-AHSLs) (reviews: Von Bodman et al., 2003; Lyon and Novick, 2004; Williams et al., 2007). All these signals are sensed at a very low concentration that ranges from pico to micromolar. The AI-2 (autoinducer 2) signal (Schauder et al., 2001; Xavier and Bassler, 2003) constitutes a remarkable exception in terms of structure and specificity. Though wide spread in the bacterial world, the QS specificity of this last signal remains debated as it may be convincingly described as a metabolic signal or an interspecies one, as much as a QS one (Winzer et al., 2002; Wang et al., 2004; Diggle et al., 2007; Turovskiy et al., 2007). Amongst the bona fide QS molecules, N-AHSLs are the most common ones in Gram negative bacteria. They are synthesized in a one-step process most often by a LuxI-like synthase (Schaefer et al., 1996). Their presence is sensed via binding to a LuxR-like sensor protein. Upon binding, the LuxR-like receptor conformation changes; it dimerizes and activates (or sometimes represses) the transcription of QS-regulated genes (Engebrecht et al., 1983; Hanzelka and Greenberg, 1995). The designation of these two key proteins refers to the luminescence phenotype of *Photobacterium fischeri* (formerly *Vibrio fischeri*), the first bacterial species in which QS-regulated genes (namely the *lux* genes) were found.

N-AHSL signals exhibit some structural changes from one bacterial species to the other. The length and saturation of the acyl side chain and the substitution at the carbon 3 of this chain, permit a specific recognition of the signal by the bacterial

population that emitted it. This specificity is most often partial as cross-communication may exist. This characteristic has been exploited to develop biosensor bacteria, unable to produce their own N-AHSLs but still responding to exogenous ones via the production of a detectable phenotype such as light emission, production of a pigment, ice nucleating protein, or beta-galactosidase activity (e.g. McClean et al., 1997; Cha et al., 1998).

In bacteria, the QS-regulated functions are very diverse. Aside from the prototypic light emission process detected in *P. fischeri*, QS-regulated functions include plasmid transfer in the alpha-proteobacteria *Agrobacterium* and *Rhizobium*, production of antifungal and bactericidal compounds in various species of the genus *Pseudomonas*, production of pigments by *Chromobacterium* and *Serratia* species, biofilm maintenance in various microbial species, and virulence towards the plant or animal hosts in both Gram negative (*Xanthomonas*, *Ralstonia*, *Pectobacterium*) and Gram positive (*Staphylococcus*) species (a nonlimitative list; Whitehead et al., 2001; Von Bodman et al., 2003; Williamson et al., 2006; White and Winans, 2007; Williams et al., 2007). Overall, QS-regulated functions appear to be involved in the interaction of the bacteria with their environment, and often with the biotic environment.

10.2 QS-Regulated Functions in *Pectobacterium* spp.

Pectobacterium atrosepticum and *P. carotovorum* are plant pathogenic bacteria responsible for diseases characterized by a maceration of the tissues, such as the black leg disease of potato, or the soft rot disease of carrot or melon (Toth and Birch, 2005). The pathogens are of major commercial importance as they are responsible for ca. 5% to 7% loss of the potato crop in Eastern Europe and in some Asian countries. The cost of production loss averaged yearly ca. 40 million euros in France and an estimated 200 million euros in Europe. The maceration results from the production by the bacteria of a set of enzymes such as cellulase, pectate lyases, pectine methyl esterase, the activities of which disrupt the pectocellulose wall of the plant cells (Toth and Birch, 2005). Additional virulence factors are the harpins, which can be regarded as toxic peptides. They are directly "injected" from the bacteria into the plant cell via a type three secretion system determined by the *hrp* genes (Grant et al., 2006).

Production of virulence factors in *Pectobacterium* (maceration enzymes and harpin) are dually controlled by both gluconic acids resulting from the cell wall degradation and by an N-AHSL-dependent QS system that relies upon 3-oxo hexanoyl-N-homoserine lactone (3O,C6-HSL) or octanoyl-homoserine lactone (C8-HSL) as the main signals (Whitehead et al., 2001; Von Bodman et al., 2003; Barnard and Salmond, 2007). The regulation of these functions involves a complex process that also relies upon peptide binding by small RNA. Briefly, at low cell density, the presence of N-AHSL is not sensed in the environment. Under those conditions, a regulatory protein, RsmA, is synthesized. This protein binds the limited amount of the transcripts of the genes encoding plant cell wall degradation

enzymes (PCWDE) that are made, favoring degradation of mRNA. Consequently, no PCWDE are synthesized. At high cell density, the N-AHSLs are sensed by the QS sensor ExpR, the activation of which blocks the expression of *rsmA*. Some PCWDE are synthesized. The activity of these enzymes led to the production of oligomeric compounds that are strong inducers of the expression of both the PCWDE genes and the *rsmB* gene. The expression of this last gene is also dependent upon the global regulatory GacA/GacS system. The *rsmB* gene encodes a RNA that binds efficiently RsmA (Barnard and Salmond, 2007; Faure and Dessaux, 2007). This positive feedback loop leads to the production of high amounts of PCWDEs and eventually to the rapid maceration of the plant tissues.

In nature, the disease evolves according a two-phase pattern. First *Pectobacterium* multiplies in the plant environment without producing harpins or maceration enzymes. Once a threshold cell density is reached, production of enzymes and harpin is activated leading to a rapid maceration of plant tissues (Smadja et al., 2004; Liu et al., 2008). The biological rationale underlying this phenomenon is related to the plant ability to induce strong defense reactions upon sensing of compounds that arise from the partial degradation of the cell walls (Davis and Hahlbrock, 1987). By turning off the synthesis of both harpin and the maceration enzymes at low cell density, *Pectobacterium* can proliferate in the plant environment without triggering the defense reactions, until it reaches a concentration that allows a massive production of maceration enzymes, lethal to the plant.

Aside from the synthesis of pathogenicity determinants, the QS regulatory system controls the production of the antibiotic carbapenem in *Pectobacterium*. The synthesis of this beta lactam molecule only occurs at high cell density, hence in the infected plant tissues. The production of the antibiotic most likely protects macerated tissues, a valuable source of nutrients, from being colonized by opportunistic bacteria (Whitehead et al., 2002). Interestingly, carbapenem antibiotics are generally highly resistant to beta-lactamase activities (review: Keam, 2008).

10.3 An Overview of Quorum Quenching Strategies

Taking in account the above data, several authors have proposed to target the QS regulation to develop innovative approaches to fight plant, animal or human pathogens that rely upon this mechanism to control the expression of pathogenicity determinants (Dong et al., 2007; Sperandio, 2007). Three elements of the QS regulation could be targets of quorum quenching (QQ) strategies. The first one is the N-AHSL synthase, the activity of which could be drastically reduced by inhibitors. The natural enzyme substrates are S-adenosylmethionine (SAM) and a fatty acid residue loaded on an acyl carrier protein (ACP). The SAM analogs butyryl-S-adenosylmethionine and L/D-S-adenosylhomocysteine are capable of limiting efficiently in vitro the synthesis of the QS signal (Parsek et al., 1999). However, due to the key metabolic role played by SAM, these analogs may exhibit deleterious effect on the bacterial cells but also on the eukaryotic host, limiting the therapeutical value of this approach.

The second target is the LuxR-like receptor protein. The approach consists in preventing the receptor from binding the QS signal, hence impeding QS-regulation to occur. Additionally, because N-AHSLs are involved in the conformation and stabilization of the receptor (Qin et al., 2000), competition may lead to an increased degradation of this protein. This phenomenon occurs in nature as several plant compounds are capable of interfering with QS-regulated functions. Thus extracts from *Allium sativum* (garlic), *Bucida buceras* (Florida "black olive tree"), *Callistemon viminalis* (weeping bottlebrush), *Coronilla varia* (Crown Vetch), *Fragaria vesca* (Woodland Strawberry), *Glycina max* (soybean), *Lycopersicum esculentum* (tomato), *Medicago* sp., *Oryza sativa* (rice), *Pisum sativum* (pea) and vanilla contain strong QS inhibitors (Teplitski et al., 2000; Fray, 2002; Gao et al., 2003; Choo et al., 2006; Rasmussen and Givskov, 2006). Most active compounds are unknown. However L-canavanine was identified in *Medicago sativa* as one of the QS inhibitors. This molecule is also produced by other legume plants (Keshavan et al., 2005). The main class of QS inhibitors remains the furanones, first identified in the red algae *Delisea pulchra* (Rasmussen et al., 2000). They prevent the N-AHSL signals from binding the LuxR-like receptor and indeed affect its stability, reducing its half-life (Manefield et al., 2002). Being often toxic to eukaryotic cells, furanones and their chemical derivatives are used with some success in boat paintings to prevent biofouling.

The third approach aims at preventing the accumulation of the QS signal in the bacterial environment. Abiotic factors such as pH and temperature affect N-AHSL stability (Yates et al., 2002) but these parameters are not easily exploitable in the frame of therapeutical or crop protection approaches. Accumulation of N-AHSLs can also be impacted by the presence of enzymes, the activity of which will degrade these signals molecules (Dong and Zhang, 2005; Turovskiy et al., 2007). To date, N-AHSLs can be degraded by two main enzyme classes, the lactonases that convert N-AHSLs to N-acyl homoserine derivatives not recognized as a QS signal, and the amidohydrolases or acylases, that yield homoserine lactone and a fatty acid residue.

Lactonases capable to hydrolyze N-AHSLs have been identified in various microorganisms such as Gram positive bacteria (e.g. *Arthrobacter*, *Bacillus*, *Rhodococcus*) and Gram negative bacteria (e.g. *Agrobacterium*, *Klebsiella*, *Mesorhizobium*) (Faure and Dessaux, 2007). Some have also been identified in eukaryotes such as mammalian cells (bovine, goat, horse, human, mouse and rabbit) (Xu et al., 2003; Chun et al., 2004; Yang et al., 2005) and fungi (mycorrhizal and non mycorrhizal fungi) (Uroz and Heinonsalo, 2008). As a rule, they generally hydrolyze N-AHSLs irrespectively of the length of the acyl side chain and substitution at carbon 3. N-AHSL amidohydrolases (acylases) were first evidenced in *Variovorax paradoxus* (Leadbetter and Greenberg, 2000), and detected later in bacteria belonging to various genera *Comamonas*, *Pseudomonas*, *Ralstonia*, *Rhodococcus*, *Shewanella* and *Streptomyces* (Faure and Dessaux, 2007). They generally hydrolyze long chain N-AHSLs, some enzymes being totally inactive on short chain N-AHSLs, such as butyroyl-homoserine lactone or hexanoyl-homoserine lactone.

A last and minor class of enzymes affecting N-AHSL signal molecules are oxidases and reductases. N-AHSL degradative activity in *Bacillus megaterium* was

identified as a P450 monooxygenase, an enzyme previously reported to oxidize fatty acids and N-fatty acyl amino acids (Chowdhary et al., 2007). In *Rhodococcus erythropolis*, an enzymatic activity reducing 3-oxo N-AHSL to 3-hydroxy N-AHSL was reported (Uroz et al., 2005). Though this activity cannot be regarded as a degradative one per se, the resulting hydroxyl derivative may not be recognize as a QS signal anymore by the bacterial population that emitted it.

From the above, micro-organisms appear as a valuable source of enzymes and genetic determinants involved in N-AHSL degradation. Early attempts to use these resources have led to the production of genetically engineered potato plants producing a N-AHSL lactonase isolated from *Bacillus* sp. Remarkably, these plants were resistant to *Pectobacterium* infection (Dong et al., 2001).

10.4 Validity of the Biocontrol Approach

The genetic engineering strategy described above, though valid, may not be easily implemented in Europe, considering the strong public and politic reservations towards plant genetic engineering. Another approach, more acceptable to the public, and equally satisfactory scientifically, consisted in isolating bacteria that naturally degrade N-AHSL and assay them for their ability to quench the pathogenicity of *Pectobacterium*. Uroz et al. (2003) enriched a bacterial consortium isolated from bare soil and rhizosphere for strains capable to grow in a minimal medium supplemented with hexanoyl-homoserine lactone (C6-HSL) as sole C source. Out of 50 isolates that constituted the enriched consortium, 6 strains demonstrated an active capability to degrade not only C6-HSL but various N-AHSL. Two isolates exhibited very versatile degradation ability: one was *Comamonas* sp. strain D1 (Uroz et al., 2007), the other one *Rhodococcus erythropolis* strain W2. Both strains were capable to quench pathogenicity in *Pectobacterium* sp. efficiently when co-inoculated on various potato host plants (Uroz et al., 2003). Remarkably, strain W2 retains a strong quenching ability even when co-inoculated at a 1 to 10 ratio with the pathogen, respectively. This elevated quenching ability may be attributed to the occurrence of a triple metabolic pathway with N-AHSLs as substrates. Strain W2 indeed harbors a lactonase and an amidohydrolase activity, plus another enzymatic activity that reduces 3-oxo N-AHSL to 3-hydroxy N-AHSL (Uroz et al., 2005; Park et al., 2006). The genetic determinants responsible for the two last activities are not known. However, a gene encoding a peptide analogous to phosphotriesterase was cloned from *R. erythropolis* strain W2 and shown to encode the lactonase activity (Uroz et al., 2008).

Comparable studies were undertaken on microbial populations originating from various soil or rhizosphere origins. They lead to the isolation of microbes having N-AHSL degrading activity some of them being of potential interest to develop biocontrol strategy. Remarkably, a recent study suggests that N-AHSL degradative functions are not limited to the culturable bacteria. Using a metagenomic approach, Riaz et al. (2008) demonstrated the existence of a lactonase-encoding determinant originating from an acidobacterial isolate that is, as most of these bacteria, a frequent

soil inhabitant and often a yet-unculturable organism (Lee et al., 2008). The cloned determinants expressed in *Pectobacterium atrosepticum* quenched pathogenicity on the host plant potato and prevented the expression of the harpin as judged from the loss of the ability to induce a hypersensitive response on the non-host plant tobacco (Riaz et al., 2008).

Though only at a laboratory stage, the possibility exists to use plants as potential quenchers of *Pectobacterium* pathogenicity. Clover and *Lotus* sp. exhibit a strong ability to degrade NAHL molecules (Delalande et al., 2005). Recent data suggest that this ability may be common amongst legume plants and related to a lactonase-like activity that converts a broad range of N-AHSLs to the corresponding N-acyl-homoserines (Chapelle et al., personal communication).

10.5 The Biostimulant Approach: An Example of Innovative Ecological Engineering

As most biocontrol strategies, the above QQ approach relies upon the biological properties of single strains to quench the pathogenic traits of *Pectobacterium* and its fitness in the plant environment. The biostimulant approach differs from the previous one as it relies upon the biological properties of a microbial consortium, the fitness of which is favored by amendment with a biostimulant compound. To favor the development of a microbial consortium capable of quenching the expression of the QS regulated pathogenicity determinant of *Pectobacterium*, Cirou et al. (2007) have investigated whether N-AHSL analogues could be used to select microbial consortia with an increased ability to degrade N-AHSL. Bacteria obtained from rhizospheric soil samples, cultivated in the presence of either gamma-caprolactone (GCL), 6 caprolactone (6CL) or 4 heptanolide (HTN) exhibited an increased N-AHSL degradation capability, while those cultivated in the presence of mannitol or a series of other analogues did not.

Upon co-inoculation of the consortia with a *P. atrosepticum* strain, the GCL- and HTN-selected consortia were able to quench the pathogenicity of this strain whereas the 6CL-seleted consortium was not. Used as a biostimulant in hydroponic cultures of potato plants, GCL, a non toxic compound used as flavoring agent in the food industry, was found to promote the growth of N-AHSL degrading bacteria up to 28 days post amendment. This result is very encouraging with respect to the possible use of a biostimulant amendment to limit the pathogenicity of *Pectobacterium* in such cultivation systems.

The GCL- and HTN-selected consortia mostly consisted in strains belonging to the *Delftia* and *Rhodococcus* genera (Cirou et al., 2007). Remarkably, a member of this last genus was also identified in the biocontrol approach described above as both a bacteria degrading a broad range of NAHL and as a valuable quencher. Rhodococcal metabolism is generally regarded as very active within the bacterial world. These features prompted experiments that aimed at coupling the biocontrol approach and the biostimulant approach. They involved the biostimulant GCL and

Rhodococcus strain R138 isolated from hydroponic culture of potato and able to inactivate QS signal molecule and to quench *Pectobacterium* pathogenicity. When strain R138 was inoculated in hydroponic cultures of potato not amended with GCL, a 1% weak increase of the R138 population was observed 14 days post inoculation. This increase was consistent with that of the whole bacterial population. When strain R138 was inoculated in hydroponic cultures of potato plants amended with GCL, a 32% increase of the R138 population was observed 14 days post treatment (Cirou et al., personnal communication). GCL treatment therefore stimulates the growth of the inoculated *Rhodococcus* strain, a result that opens a path towards coupled biocontrol/biostimulant strategies. Last and not least, 40% (day 28 post treatment) to 55% (day 14 post treatment) of the bacteria stimulated by the addition of 0.4 gL^{-1} GCL to hydroponic cultures of potato plants were capable of degrading both NAHL and GCL. This feature is of great agronomical interest because the application of the GCL biostimulant in the plant environment selects bacteria with the dual ability to quench the pathogenicity of *Pectobacterium* and eliminate the amending molecule that therefore appears as fully biodegradable.

Acknowledgments AC was supported by a Ph.D. fellowship from Comité Nord Plants de pommes de terre. SU and EC were supported by Ph.D. fellowships from French ministère de l'enseignement supérieur et de la recherche. XL and NO were supported by grants from Université de Rouen. This work was also made possible by grants from CNRS to DF and YD.

References

Barnard AM, Salmond GP (2007) Quorum sensing in *Erwinia* species. Anal Bioanal Chem 387:415–423

Cha C, Gao P, Chen YC, Shaw PD, Farrand SK (1998) Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. Mol Plant Microbe Interact 11:1119–1129

Choo JH, Rukayadi Y, Hwang JK (2006) Inhibition of bacterial quorum sensing by vanilla extract. Lett Appl Microbiol 42:637–641

Chowdhary PK, Keshavan N, Nguyen HQ, Peterson JA, González JE, Haines DC (2007) *Bacillus megaterium* CYP102A1 oxidation of acyl homoserine lactones and acyl homoserines. Biochemistry 46:14429–14437

Chun CK, Ozer EA, Welsh MJ, Zabner J, Greenberg EP (2004) Inactivation of a *Pseudomonas aeruginosa* quorum-sensing signal by human airway epithelia. Proc Natl Acad Sci USA 101:3587–3590

Cirou A, Diallo S, Kurt C, Latour X, Faure D (2007) Growth promotion of quorum-quenching bacteria in the rhizosphere of *Solanum tuberosum*. Environ Microbiol 9:1511–1522

Davis KR, Hahlbrock K (1987) Induction of defense responses in cultured parsley cells by plant cell wall fragments. Plant Physiol 84:1286–1290

Delalande L, Faure D, Raffoux A, Uroz S, D'Angelo-Picard C, Elasri M, Carlier A, Berruyer R, Petit A, Williams P, Dessaux Y (2005) N-hexanoyl-L-homoserine lactone, a mediator of bacterial quorum-sensing regulation, exhibits plant-dependent stability and may be inactivated by germinating *Lotus corniculatus* seedlings. EMS Microbiol Ecol 52:13–20

Diggle SP, Gardner A, West SA, Griffin AS (2007) Evolutionary theory of bacterial quorum sensing: when is a signal not a signal? Philos Trans R Soc Lond B Biol Sci 362:1241–1249

Dong YH, Zhang LH (2005) Quorum sensing and quorum-quenching enzymes. J Microbiol 43:101–109

Dong YH, Wang LH, Xu JL, Zhang HB, Zhang XF, Zhang LH (2001) Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. Nature 411:813–817

Dong YH, Wang LY, Zhang LH (2007) Quorum-quenching microbial infections: mechanisms and implications. Philos Trans R Soc Lond B Biol Sci 362:1201–1211

Engebrecht J, Nealson KH, Silverman M (1983) Bacterial bioluminescence: isolation and genetic analysis of the functions from *Vibrio fischeri*. Cell 32:773–781

Faure D, Dessaux Y (2007) Novel biocontrol strategies directed at *Pectobacterium carotovorum*. Eur J Plant Pathol 119:353–365

Fray RG (2002) Altering plant-microbe interaction through artificially manipulating bacterial quorum sensing. Ann Bot (Lond) 89:245–253

Gao M, Teplitski M, Robinson JB, Bauer WD (2003) Production of substances by *Medicago truncatula* that affect bacterial quorum sensing. Mol Plant Microbe Interact 16:827–834

Grant SR, Fisher EJ, Chang JH, Mole BM, Dangl JL (2006) Subterfuge and manipulation: type III effector proteins of phytopathogenic bacteria. Annu Rev Microbiol 60:425–449

Hanzelka BL, Greenberg EP (1995) Evidence that the N-terminal region of the *Vibrio fischeri* LuxR protein constitutes an autoinducer-binding domain. J Bacteriol 177:815–817

Hense BA, Kuttler C, Müller J, Rothballer M, Hartmann A, Kreft JU (2007) Does efficiency sensing unify diffusion and quorum sensing? Nat Rev Microbiol 5:230–239

Keam SJ (2008) Doripenem: a review of its use in the treatment of bacterial infections. Drugs 68:2021–2057

Keller L, Surette MG (2006) Communication in bacteria: an ecological and evolutionary perspective. Nat Rev Microbiol 4:249–258

Keshavan ND, Chowdhary PK, Haines DC, González JE (2005) L-Canavanine made by *Medicago sativa* interferes with quorum sensing in *Sinorhizobium meliloti*. J Bacteriol 187:8427–8436

Leadbetter JR, Greenberg EP (2000) Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus*. J Bacteriol 182:6921–6926

Lee SH, Ka JO, Cho JC (2008) Members of the phylum *Acidobacteria* are dominant and metabolically active in rhizosphere soil. FEMS Microbiol Lett 285:263–269

Liu H, Coulthurst SJ, Pritchard L, Hedley PE, Ravensdale M, Humphris S, Burr T, Takle G, Brurberg MB, Birch PR, Salmond GP, Toth IK (2008) Quorum sensing coordinates brute force and stealth modes of infection in the plant pathogen *Pectobacterium atrosepticum*. PLoS Pathog 4:e1000093

Lyon GJ, Novick RP (2004) Peptide signaling in *Staphylococcus aureus* and other Gram-positive bacteria. Peptides 25:1389–1403

Manefield M, Rasmussen TB, Henzter M, Andersen JB, Steinberg P, Kjelleberg S, Givskov M (2002) Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover. Microbiology 148:1119–1127

McClean KH, Winson MK, Fish L, Taylor A, Chhabra SR, Camara M, Daykin M, Lamb JH, Swift S, Bycroft BW, Stewart GS, Williams P (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. Microbiology 143:3703–3711

Park SY, Hwang BJ, Shin MH, Kim JA, Kim HK, Lee JK (2006) N-acylhomoserine lactonase producing *Rhodococcus* spp with different AHL-degrading activities. FEMS Microbiol Lett 261:102–108

Parsek MR, Val DL, Hanzelka BL, Cronan JE Jr, Greenberg EP (1999) Acyl homoserine-lactone quorum-sensing signal generation. Proc Natl Acad Sci USA 96:4360–4365

Qin Y, Luo ZQ, Smyth AJ, Gao P, Von Bodman SB, Farrand SK (2000) Quorum-sensing signal binding results in dimerization of TraR and its release from membranes into the cytoplasm. EMBO J 19:5212–5221

Rasmussen TB, Givskov M (2006) Quorum sensing inhibitors: a bargain of effects. Microbiology 152:895–904

Rasmussen TB, Manefield M, Andersen JB, Eberl L, Anthoni U, Christophersen C, Steinberg P, Kjelleberg S, Givskov M (2000) How *Delisea pulchra* furanones affect quorum sensing and swarming motility in *Serratia liquefaciens* MG1. Microbiology 12:3237–3244

Riaz K, Elmerich C, Moreira D, Raffoux A, Dessaux Y, Faure D (2008) A metagenomic analysis of soil bacteria extends the diversity of quorum-quenching lactonases. Environ Microbiol 10:560–570

Schaefer AL, Val DL, Hanzelka BL, Cronan JE Jr, Greenberg EP (1996) Generation of cell-to-cell signals in quorum sensing: acyl homoserine lactone synthase activity of a purified *Vibrio fischeri* LuxI protein. Proc Natl Acad Sci USA 93:9505–9509

Schauder S, Shokat K, Surette MG, Bassler BL (2001) The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. Mol Microbiol 41:463–476

Smadja B, Latour X, Faure D, Chevalier S, Dessaux Y, Orange N (2004) Involvement of N-acylhomoserine lactones throughout plant infection by *Erwinia carotovora* subsp. *atroseptica Pectobacterium atrosepticum*. Mol Plant Microbe Interact 17:1269–1278

Smith D, Wang JH, Swatton JE, Davenport P, Price B, Mikkelsen H, Stickland H, Nishikawa K, Gardiol N, Spring DR, Welch M (2006) Variations on a theme: diverse N-acyl homoserine lactone-mediated quorum sensing mechanisms in gram-negative bacteria. Sci Prog 89:167–211

Sperandio V (2007) Novel approaches to bacterial infection therapy by interfering with bacteria-to-bacteria signaling. Expert Rev AntiInfect Ther 5:271–276

Teplitski M, Robinson JB, Bauer WD (2000) Plants secrete substances that mimic bacterial N-acyl homoserine lactone signal activities and affect population density-dependent behaviors in associated bacteria. Mol Plant Microbe Interact 13:637–648

Toth IK, Birch PR (2005) Rotting softly and stealthily. Curr Opin Plant Biol 8:424–429

Turovskiy Y, Kashtanov D, Paskhover B, Chikindas ML (2007) Quorum sensing: fact, fiction, and everything in between. Adv Appl Microbiol 62:191–234

Uroz S, Heinonsalo J (2008) Degradation of N-acyl homoserine lactone quorum sensing signal molecules by forest root-associated fungi. FEMS Microbiol Ecol 65:271–278

Uroz S, D'Angelo-Picard C, Carlier A, Elasri M, Sicot C, Petit A, Oger P, Faure D, Dessaux Y (2003) Novel bacteria degrading N-acylhomoserine lactones and their use as quenchers of quorum-sensing-regulated functions of plant-pathogenic bacteria. Microbiology 149:1981–1989

Uroz S, Chhabra SR, Cámara M, Williams P, Oger P, Dessaux Y (2005) N-Acylhomoserine lactone quorum-sensing molecules are modified and degraded by *Rhodococcus erythropolis* W2 by both amidolytic and novel oxidoreductase activities. Microbiology 151:3313–3322

Uroz S, Oger P, Chhabra SR, Cámara M, Williams P, Dessaux Y (2007) N-acyl homoserine lactones are degraded via an amidolytic activity in *Comamonas* sp. strain D1. Arch Microbiol 187:249–256

Uroz S, Oger PM, Chapelle E, Adeline MT, Faure D, Dessaux Y (2008) A *Rhodococcus qsdA*-encoded enzyme defines a novel class of large-spectrum quorum-quenching lactonases. Appl Environ Microbiol 74:1357–1366

Von Bodman SB, Bauer WD, Coplin DL (2003) Quorum sensing in plant-pathogenic bacteria. Annu Rev Phytopathol 41:455–482

Wang LH, He Y, Gao Y, Wu JE, Dong YH, He C, Wang SX, Weng LX, Xu JL, Tay L, Fang RX, Zhang LH (2004) A bacterial cell–cell communication signal with cross-kingdom structural analogues. Mol Microbiol 51:903–912

White CE, Winans SC (2007) Cell cell communication in the plant pathogen *Agrobacterium tumefaciens*. Philos Trans R Soc Lond B Biol Sci 362:1135–1148

Whitehead NA, Barnard AM, Slater H, Simpson NJ, Salmond GP (2001) Quorum-sensing in Gram-negative bacteria. FEMS Microbiol Rev 25:365–404

Whitehead NA, Byers JT, Commander P, Corbett MJ, Coulthurst SJ, Everson L, Harris AK, Pemberton CL, Simpson NJ, Slater H, Smith DS, Welch M, Williamson N, Salmond GP (2002) The regulation of virulence in phytopathogenic *Erwinia* species: quorum sensing, antibiotics and ecological considerations. Antonie Van Leeuwenhoek 81:223–231

Williams P, Winzer K, Chan WC, Cámara M (2007) Look who's talking: communication and quorum sensing in the bacterial world. Philos Trans R Soc Lond B Biol Sci 362:1119–1134

Williamson NR, Fineran PC, Leeper FJ, Salmond GP (2006) The biosynthesis and regulation of bacterial prodiginines. Nat Rev Microbiol 4:887–899

Winzer K, Hardie KR, Williams P (2002) Bacterial cell-to-cell communication: sorry can't talk now – gone to lunch!. Curr Opin Microbiol 5:216–222

Xavier KB, Bassler BL (2003) LuxS quorum sensing: more than just a numbers game. Curr Opin Microbiol 6:191–197

Xu F, Byun T, Deussen HJ, Duke KR (2003) Degradation of N-acylhomoserine lactones, the bacterial quorum-sensing molecules, by acylase. J Biotechnol 101:89–96

Yang F, Wang LH, Wang J, Dong YH, Hu JY, Zhang LH (2005) Quorum quenching enzyme activity is widely conserved in the sera of mammalian species. FEBS Lett 579:3713–3717

Yates EA, Philipp B, Buckley C, Atkinson S, Chhabra SR, Sockett RE, Goldner M, Dessaux Y, Cámara M, Smith H, Williams P (2002) N-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. Infect Immun 70:5635–5646

Chapter 11
The Status of Biological Control of Plant Diseases in Soilless Cultivation

Joeke Postma

Abstract Avoidance of plant diseases has been a major driver for the development of soilless cultivation systems. Nevertheless, diseases still occur in these systems and the need for additional control measures exist. Traditionally, control has relied on the use of chemical fungicides but environmental pressure to reduce chemical usage in the environment, and fewer active ingredients registered for use, has stimulated the development of biological methods of disease control. One approach has been to utilise microbial inoculants as straight replacements for chemical pesticides and some commercial products are now available. Sufficient root colonization and activity are key issues for effective biocontrol. Another approach has been to create growing systems with improved suppressiveness towards plant diseases. The challenge is to combine the available strategies into environmentally and economically sound soilless plant production systems with low risks for pathogen outbreaks. Soilless systems have the potential of creating a balance between a pathogen-free start and a suppressive microflora.

Keywords Biological control • Disease-suppressive substrate • Hydroponics • Recirculated nutrient solution • Microbial populations • Rhizosphere • Root pathogens

11.1 Introduction

A major reason to shift from the use of soil to soilless cultivation systems was the proliferation of soil-borne diseases in intensively cultivated greenhouses with a narrow crop rotation (Raviv and Lieth, 2008). Initially, methyl bromide and other soil fumigants have been used to tackle soil-borne disease problems. However, these fumigants have large environmental drawbacks and human safety risks. A phase-out of the use of methyl bromide in agriculture was assigned in the Montréal Protocol for 2005.

J. Postma (✉)
Plant Research International, P.O. Box 16, 6700, AA Wageningen, The Netherlands
e-mail: joeke.postma@wur.nl

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,*
Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_11,

Soilless cultivation systems have proven to be a successful alternative to the use of these chemical fumigants. In addition, advantages of soilless systems are a higher production, energy conservation, better control of growth, and independence of soil quality (Van Os, 1999).

In general, the strategy in greenhouse horticulture has been to keep the growing systems as "clean" as possible, by using pathogen-free propagation material, new substrates, by applying disinfection techniques and other sanitation techniques. Due to the use of clean substrates, soil-borne pathogens such as *Fusarium oxysporum*, *Verticillium dahliae*, nematodes and many others which can survive in the deeper soil-layers, can be circumvented. However, during the entire period of crop growth, contamination with pathogens can never be excluded, since they can be brought in via the water supply, by air, insects, or inadvertently, by the grower directly.

Soilless production systems often exhibit substrates with more free water, and the nutrient solution may be recirculated. Zoospore-producing organisms, such as *Pythium* spp. and *Phytophthora* spp., are well adapted to life in liquids, and pose a serious threat in soilless systems (Stanghellini and Rasmussen, 1994). Zoospores actively swim to their hosts so that infection can occur within minutes. Multiplication of these pathogens is explosive under favourable conditions.

Chemical control of plant pathogens is a robust and effective method to control pests and diseases, but it has environmental and human safety risks. For the safety of the consumers, a period of several days should elapse between the application of a pesticide and harvesting a food crop. In greenhouse crops, where vegetables and flowers are often harvested daily, re-entry time regulations can be a serious practical disadvantage. The ability to apply chemical crop protection agents depends on regulation by the authorities and differs by country. In general, the number of registered chemical crop protection agents is more limited for greenhouse crops than for the large field crops, since registration costs are high for the relatively small greenhouse market (Paulitz and Bélanger, 2001).

The risks of root-borne diseases can be reduced by various non-chemical strategies, such as the use of (partial) resistant varieties, sanitation, cultural practices, and biological control. In greenhouse systems climate and water regime can be managed, which offer possibilities to control diseases. A unique feature of soilless systems is the possibility of starting a production cycle completely free of pathogens, and the eradication of pathogens present in the recirculated irrigation water with various disinfestation techniques. Sanitary practices to avoid pathogen infestation should be combined with preventive measures to make crops in closed systems less susceptible to pathogen outbreaks, either by stimulating the suppressiveness of the system, or by applying biological control agents.

11.2 Microbial Populations in Closed Soilless Systems

Due to the use of new or sterilized substrates, soilless systems in general start with a 'microbiological vacuum', lacking a diverse and competitive microflora. A fundamental difference with soil is that many soilless systems do not contain a substantial

organic fraction. Organic matter in soil is an important source of nutrients for microorganisms. Soil is a microbiologically rich substrate, generally containing 10^7–10^9 colony-forming units (CFU) of bacteria and 10^4–10^6 culturable fungal propagules per gram of agricultural soil (Alexander, 1977).

Soilless systems are generally not sterile, but they contain distinctly lower levels of microorganisms compared to soil. Bacterial plate counts showed that 10^5–10^7 CFU of bacteria are present per mL nutrient solution depending on the crop type, age and type of soilless system (Postma et al., 2000, 2003; Koohakan et al., 2004; Calvo-Bado et al., 2006). Soilless systems without organic components are a relatively poor growing medium for microorganisms at the start of a crop due to the lack of organic matter, but once plants grow in the system, exudates from the roots, or even soughed root material itself, provide organic substrates on which the microorganisms can grow. A substantial part of the nutrients used by the microflora is derived from plant roots, resulting in high numbers of microorganisms on the surface of plant roots (rhizoplane) (Fig. 11.1). Exudates released by the roots consist of organic compounds such as short-chain organic acids, carbohydrates, mucilage and lysates. In addition, dead cell material is accumulating with time. All the released organic material was estimated to account for 15–20% of the total carbon fixed by the plant (Bolton et al., 1992). The nature of plant-derived compounds is dependent on plant species, growth conditions, rooting medium, the stage of plant development and plant root health.

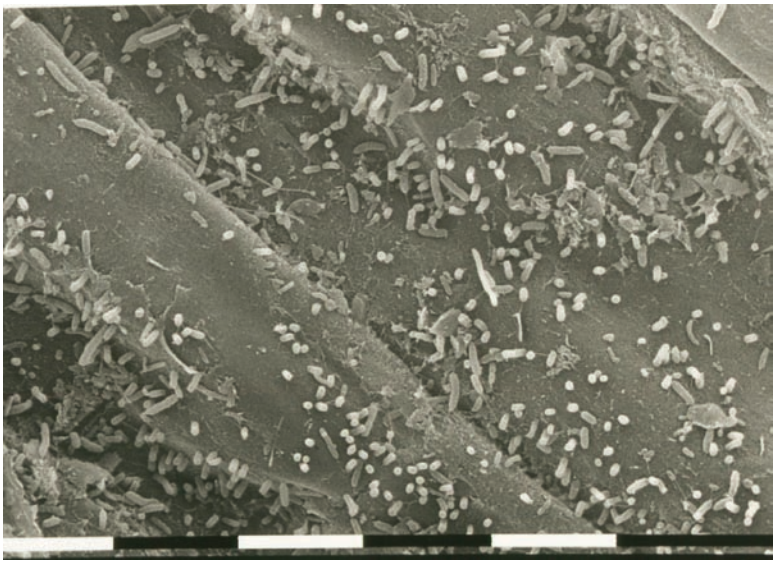


Fig. 11.1 Bacterial population on a 3-week-old cucumber root grown in rockwool visualized by scanning electron microscopy (SEM) (bar = 10 μm) (photo by Anke Clerkx, Plant Research International)

The following examples illustrate that soilless systems exhibit a quickly increasing microflora, but start with a low level of microorganisms:

1. Numbers of bacteria in the solution of a tomato (*Lycopersicon esculentum* Mill.) crop were 10^3 CFU per mL before crop growth, and 10^6 CFU per mL 20 h after planting (Berkelmann, 1992).
2. Moist rockwool with young cucumber plants contained 10^6, compared with up to 6×10^7 CFU bacteria per gram moist rockwool in an older crop (Postma et al., 2000).
3. The numbers of fungi (including *Trichoderma* spp.) are very low at the start of a crop in soilless systems: often less than 10^2 CFU per gram moist rockwool are present (Postma et al., 2000, 2005; Koohakan et al., 2004). Numbers increased up to 10^4 CFU per gram moist rockwool in 4 weeks.

That root derived material is of great importance for microbial populations present in soilless systems is also reflected in the higher numbers of microorganisms which are detected in the slab and the drainage water from the slabs, compared to the nutrient solution which had not yet passed the plant roots (Van Os et al., 2004b). This illustrates that the rhizosphere has a significant effect in the soilless system, and the importance of the exudates and other plant-root-derived materials as influencing factor (nutrient source) for the microflora.

While the numbers of microorganisms in soilless systems are typically low at the start of a crop, so is the diversity. Diversity of bacterial populations in rockwool increased strongly between the first few weeks when cucumber plants were grown on it (Table 11.1) (Postma et al., 2000). Number of bands in the PCR–DGGE (polymerase chain reaction–denaturing gradient gel electrophoresis) profile reflect the dominant bacterial species in the community. This increase in community diversity finds place in the first weeks after planting; bacterial communities in a tomato crop did not change anymore after 6 weeks of plant growth (Calvo-Bado et al., 2006). Thus, the establishment of the microflora probably occurs within the first weeks of a crop, and later treatments do not result in distinct influences on the dominant species which are represented in the PCR–DGGE profile.

Table 11.1 Number of bands in PCR–DGGE profiles of bacterial populations in rockwool systems at different periods after sowing or planting cucumbers (mean numbers of bands of three or four replicates)

	Weeks after sowing				
Substrate system	1	2	3	4	Reference
New rockwool block	1	5	10	14	Postma et al. (2000)
Sterilized re-used rockwool block	6	11	11	15	Postma et al. (2000)
Sterilized re-used rockwool block	14	18	20	23	Postma et al. (2005)
	Weeks after planting				
	0	1	3		
Nutrient solution from a production system on rockwool slabs	13	20	27		Postma et al. (2008)

Microorganisms that occur at the start of a crop in a soilless growing system are probably mainly those that can survive in water, air, seed and plant material, and on dry surfaces. Plant-specific microorganisms and those adapted to the conditions in soilless systems should be introduced during crop growth or with the plant material. In agricultural soil systems, the diversity of the microflora and the presence of several plant-associated microorganisms, such as *Rhizobium*, mycorrhizal fungi, plant growth promoting bacteria, are important characteristics of a soil (Alexander, 1977). In soilless systems, however, these plant-associated organisms are most likely not present in new or sterilized substrate. Little in depth research is done on the development of the microbial populations during crop growth; that is, which species are present at various growth stages in these growing systems.

11.3 Disease-Suppressive Substrate

Whereas in soil and organic media the existence and the potential of a suppressive microflora towards root pathogens is generally accepted (Alabouvette et al., 1979; Weller et al., 2002; Termorshuizen et al., 2006), suppressiveness in soilless systems has only recently been demonstrated.

11.3.1 Biotic Factors in Substrate

Suppression of *P. aphanidermatum* disease in cucumber in re-used rockwool was proven to be the result of the microflora present in this substrate, since suppressiveness of sterilized rockwool was recovered after its recolonization with the original microflora (Fig. 11.2). The indigenous microflora of re-used rockwool slabs caused reproducible suppression of Pythium crown and root rot in cucumber. Without exception, all rockwool slabs of different batches without Pythium symptoms in the previous crop were suppressive (Postma et al., 2000, 2005). The suppressiveness in this rockwool system correlated with bacterial diversity as well as the number of filamentous actinomycetes (mainly Streptomycetes) in these experiments. However, growers should not re-use rockwool slabs with distinct Pythium symptoms in the previous crop, because these slabs often resulted in high disease percentages in the experiments.

Also the nutrient solution of a hydroponic system can become suppressive. This was discovered by Berkelmann (1992). The solution from a 9-week-old tomato crop inhibited mycelial growth of *F. oxysporum* f.sp. *lycopersici* very strongly in an in vitro test. This was due to the living microflora, since heat or filter sterilization destroyed the inhibition completely. A fresh nutrient solution, which had not been in contact with the crop, was only weakly suppressive. It is not precisely known at what time after planting this suppressiveness in the system develops and which factors are influencing this development of suppressiveness.

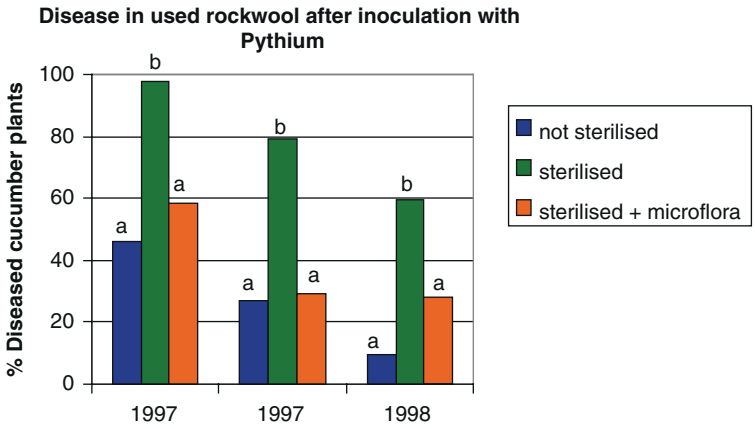


Fig. 11.2 Suppressiveness of *Pythium aphanidermatum* in non-sterilized re-used rockwool slabs, as well as in sterilized and thereafter recolonized rockwool slabs. Sterilized rockwool slabs exhibit high percentages of diseased plants

The composition of microflora in root samples of two soilless substrates has been shown to differ in tomato production (Khalil and Alsanius 2001). Lower numbers of total aerobic bacteria and pseudomonads were present in peat than in rockwool slabs, while the numbers of filamentous actinomycetes and fungi were higher. Similarly Koohakan et al. (2004) found lower numbers of pseudomonads, higher numbers of fungi, but similar numbers of bacteria in the nutrient solution, as well as on tomato roots, grown in coconut fibre versus rockwool. However, it is not known if these differences in composition of the microflora are reflected in differences in disease suppressiveness. More recent research showed that suppressive peats towards *Pythium sylvaticum* contained higher frequencies of certain bacterial groups, i.e. the *Rhizobium-Agrobacterium* group and *Acidobacteria* (Hunter et al., 2006). Moreover, two specific PCR–DGGE bands correlating with *Basidiomycetes* yeast genera occurred in the suppressive samples (Hunter et al., 2006).

In other cases, physical and chemical properties of a substrate might influence disease suppressiveness. Using a substrate with low water content, such as perlite instead of rockwool, is also a strategy to avoid the development of Pythium in a crop (Van Der Gaag and Wever, 2005). This suppressiveness is probably due to a lower level of available free water for the transport of Pythium zoospores or to an altered root morphology rather than to the presence of a suppressive microflora.

11.3.2 Recirculation of Nutrient Solution

Soilless production systems afford the possibility to save water and nutrients, as well as to avoid pollution of ground and surface water by the excess of fertilizers

in the drain water, by re-using the irrigation water. Systems which re-use all effluent from the root zone are called 'closed systems'. One of the main barriers to recirculation of irrigation water, however, is the presence of plant disease causing organisms in the drainage water. Failure to manage such organisms emanating from even one infected plant will subject the entire crop to significant risks. Already in 1996, an overview was given on the *Fusarium*, *Pythium* and *Phytophthora* species that could spread through the growing system by recirculation of the irrigation water (Rattink, 1996).

In two independent studies, however, it was suggested that a disease-suppressive microflora was build up in a closed system due to recirculation of the nutrient solution. The first report was about suppressiveness of *Phytophthora cryptogea* in a tomato production system (McPherson, 1998); a disease suppressive potential was generated within the recirculating hydroponic solution and this prevented expression of disease symptoms when compared with an equivalent open system. The suppressiveness was lost if the nutrient solution was not recycled anymore; that is the system was changed into run-to-waste. This intriguing phenomenon provoke detailed research on the microbial communities present in these systems (Calvo-Bado et al., 2006). However, no single organism or group of organisms could be identified as responsible for the pathogen suppression in these systems. These results indicate that the development of suppression depends upon the establishment of an active, non-specific microflora.

Also suppressiveness towards *Pythium* sp. in tomato was found in a closed recirculating rockwool system as well as in a closed recirculating nutrient film technique (NFT) system when compared to an open rockwool system (Tu et al., 1999). Both closed systems exhibited higher numbers of bacteria compared to the open system. However, no further research to prove the biological mode of action was performed.

11.3.3 Disinfestation of Recirculated Nutrient Solution

Over the years, various methods and technologies have been developed for disinfestation of the recirculated nutrient solution. These technologies can be grouped by the following approaches: filtration, heat treatment, oxidation (e.g. hydrogen peroxide, ozone), electromagnetic radiation (e.g. UV), active carbon adsorption and copper ionization (Ehret et al., 2001; Postma et al. 2008).

Slow sand filters (SSFs) have had specific attention, since they are effective in removing pathogens such as *Pythium* and *Phytophthora* species, they are relatively cheap and robust, and their mode of action removing pathogens, depends largely upon the development of an active microflora. SSFs always need time before they become highly effective. Several studies have been performed to enhance and accelerate this process (Wohanka et al., 1999). The addition of proper microorganisms, which can be obtained from already effective filters, can enhance this process. The biological activation of a filter unit was very significantly

enhanced by the addition of three *Pseudomonas putida* and two *Bacillus cereus* strains (Déniel et al., 2004). The bacteria-amended filter effectively eliminated *F. oxysporum* after 1 month, whereas the non-inoculated filter needed 6 months to become effective.

Interestingly, these SSFs have been suggested to contribute to disease suppressiveness. It was hypothesized that slow sand filtration, which do not remove the entire beneficial microflora from the recirculated nutrient solution, would result in higher disease suppressiveness in the growing system compared to an almost complete sterilization method such as UV (Van Os et al., 2004a). Experiments in the EU-project MIOPRODIS could not prove this hypothesis: a decrease of the disease suppressiveness due to the disinfestation treatments was never detected. Probably, the plant-driven microflora is not disturbed. Measurements showed that the microflora in the nutrient solution around the roots was changed only little or not at all by the disinfection method. This is explained by the fact that only part of the solution, and, as a consequence, also part of the microflora is removed from the substrate around the roots.

11.4 Biological Control Agents

Biological control agents (BCAs) can play an important role in suppressing root pathogens in soilless systems. Biocontrol agents are those products that control plant pathogens or pests or reduce their amount or their effect by one or more organisms other than man (i.e. viruses, bacteria, fungi, insects). Antagonists can be active through several mechanisms, such as (myco)parasitism, antibiosis is or other inhibitory substances, competition for nutrients or space or induced resistance (Whipps, 2001).

11.4.1 Advantages of Soilless Systems for BCAs

Compared to a field soil, where it is difficult to introduce biocontrol agents in sufficient concentrations at lower parts of the roots, the limited volume of the matrix around the roots in soilless systems facilitates introduction of the antagonist in the root environment. In general, soilless systems allow a good interaction among host, pathogen and antagonist. Also, the establishment of antagonists is easier in soilless systems with a new substrate having an unbalanced microflora than in a microbiologically buffered system such as soil with a tremendous competition with the microorganisms already present (Paulitz and Bélanger, 2001). A third advantage of greenhouse systems for a successful introduction of antagonists is the regulated climate with a more uniform temperature (Paulitz and Bélanger, 2001).

11.4.2 Biocontrol with Biosurfactants

A biocontrol mechanism more recently discovered is the application of microorganisms that produce biosurfactants which cause lysis of zoospores (Stanghellini and Miller, 1997). This is an interesting mechanism for soilless systems with high water retention capacity that, as a consequence, have problems with many different zoospore producing pathogens. The first successful demonstration of this mechanism in a greenhouse crop was with *Pseudomonas aeruginosa* producing rhamnolipids. This strain could control *Phytophthora capsici* in pepper grown on rockwool blocks (Stanghellini and Miller, 1997); olive oil was added as a nutrient source for selective growth of the rhamnolipid producing strain and improving the biocontrol effect. Other *Pseudomonas* species producing different surfactants have been selected during the subsequent years. *P. fluorescens* strain SS101 provided significant protection against root rot caused by *Pythium intermedium* in hyacinth bulbs grown in soil (De Souza et al., 2003). Rhamnolipid formulations and other surfactants, as well as *Pseudomonas* spp. producing surfactants, could control *Phytophthora nicotianae* in tomato grown in rockwool (De Jonghe, 2006; De Jonghe et al., 2007).

11.4.3 Root Colonization as Key Factor for Biocontrol

In soilless substrate, a flow of nutrient solution and diffusion are transporting root exudates more easily away from the roots as compared to soil, due to the free water which is present. The microorganisms rapidly metabolize the available carbon leaking from the roots. Attachment to the surface of the root could be an important strategy of microorganisms to compete for nutrients in soilless systems. A promising selection criterion therefore was hypothesized to be the selection for root colonizing ability. This strategy was followed by Kamilova et al. (2005); a sequential inoculation procedure followed by sequential isolation of bacteria from the root tip was performed. *Pseudomonas putida* strain PCL1760 was obtained by this procedure, thus being an excellent root colonizer. This strain did not show any antibiosis, but could effectively control *Fusarium oxysporum* f.sp. *radicis-lycopersici* in root rot in tomato assays (Validov et al., 2007) as well as in a certified greenhouse with plants up to 31 days (Validov, 2007).

11.4.4 Combined Applications of BCAs with Stimulatory Compounds

A new type of bacterium, effective against *Pythium aphanidermatum* in soilless systems, was isolated from cucumber roots grown in suppressive rockwool. This bacterium, i.e. *Lysobacter enzymogenes* strain 3.1T8, produced antibiotics inhibiting

Table 11.2 Biological control efficacy and root colonization of *Lysobacter enzymogenes* 3.1T8 in combination with different concentrations of chitosan in a 32-days-old cucumber crop. All treatments were inoculated with the same concentration of *Pythium aphanidermatum*

BCA treatment	Diseased plants (%) Experiment 1	Diseased plants (%) Experiment 2	Lysobacter (cells/g root)[b] Experiment 1	Lysobacter (cells/g root)[b] Experiment 2
–	90	80	$\leq 10^5$	$\leq 10^5$
0.1 chitosan[a]	100	–	–	–
Lysobacter + 0.1 chitosan	11	–	$1 \times 10^9/1 \times 10^9$	–
Lysobacter + 0.03 chitosan	0	15	$2 \times 10^8/1 \times 10^9$	$4 \times 10^7/8 \times 10^8$
Lysobacter + 0.01 chitosan	15	65	$3 \times 10^6/5 \times 10^8$	$1 \times 10^7/4 \times 10^8$
Lysobacter + 0.001 chitosan	–	40	–	$3 \times 10^7/2 \times 10^8$
Lysobacter	–	55	–	$1 \times 10^7/1 \times 10^8$

[a]Dissolved chitosan; dosage in g per plant.
[b]Lysobacter was detected with a strain-specific qPCR on respectively young and old roots; detection limit is ~10^5 cells/g root.

mycelial growth, lytic enzymes, as well as a surfactant causing zoospore collapse (Folman et al., 2003). However, the surfactant was not involved in controlling root rot symptoms (Folman, 2003). The strain could effectively control root rot in a small hydroponic system with plants up to 16 days old (Folman et al., 2004). However, in larger systems with 10 plants on rockwool blocks and 30 L recirculated nutrient solution with cucumber plants up to 35 days, application of *L. enzymogenes* alone was not effective. For effective biocontrol of *P. aphanidermatum* a combined application of the biocontrol strain with chitosan was needed (Postma et al., 2009). Due to the chitosan, which likely served as a nutrient source, numbers of the inoculant colonizing the roots increased and the percentage of diseased plants decreased (Table 11.2). Also in other studies, beneficial BCAs have been combined with natural compounds such as chitin (Sid Ahmed et al., 2003) or chitosan (Benhamou et al., 1998) to improve their effect.

11.4.5 Hurdles

A large variety of antagonists of root pathogens have been tested in several greenhouse cropping systems. However, only a limited numbers of antagonists became available as commercial product. Some examples of registered BCA products against root-borne (fungal) pathogens which can be applied in greenhouse systems are belonging to the following species: *Trichoderma harzianum, Gliocladium virens, G. cathenulatum*, *Coniothyrium minitans, Pseudomonas chlororaphis, Streptomyces griseoviridis* and *Bacillus subtilis* (Fravel, 2005).

Although soilless systems are less diverse in microbiota than agricultural soils, and have a more stable climate, the development and application of BCAs is still not simple and many problems have to be overcome.

1. In several cases, BCAs were originally isolated from soil or from a crop different from the one to which it is applied, and as a consequence they may not be fully adapted to soilless systems.
2. For effective biocontrol, the activities of the antagonist and pathogen should be synchronized in time and in space. The influence of the location of the antagonist in relation to the location of the pathogen is illustrated by the failure of suppression of Fusarium wilt in carnation in a recirculation system, when the antagonist was added on top of the rockwool blocks, while the pathogen was introduced via the nutrient solution (Rattink and Postma, 1996). However, when introduced via the nutrient solution where the pathogen was located, the antagonist proved to be extremely effective.
3. Survival of the BCA after drying and storage, so called shelf life, is crucial. In general, it is more difficult to make a storable product of non-sporulating organisms.
4. For commercial application of biocontrol agents, the need for legislation of these products is an economical barrier. To get a BCA registered, safety for the user, the consumer and the environment, as well as biocontrol efficacy need to be proven. This registration procedure is relatively expensive for products which can only be applied within a relatively small market.

Some of the above mentioned hurdles can be overcome for example by selecting endophytic microorganisms that act as a BCA (Hallmann et al., 1997). Endophytic microorganisms are less dependent on the cropping system and environmental conditions. They have the advantage of being protected from environmental stresses and might be able to protect the plant in a soilless system from propagation material throughout the crop cycle. Non-pathogenic *Fusarium* species which are present in plants can, for example, protect carnation (*Dianthus caryophyllus* L.) against wilt disease caused by *F. oxysporum* (Postma and Luttikholt, 1996), or tomato against the plant-parasitic nematode *Meloidogyne incognita* (Hallmann and Sikora, 1994). An example with bacterial endophytes is the endophytic colonization of tomato tissue with a *Pseudomonas* strain that inhibits the wilting by *V. dahliae* (Sharma and Nowak, 1998).

Biological control has the potential to solve problems with root diseases in soilless systems. However, there is a need for better products, and more knowledge on the optimal inoculation procedures as well as environmental conditions under which the biocontrol agent will be active. There is a need for organisms which are better adapted to soilless systems and synchronized to the pathogens occurring in these systems.

11.5 Conclusions

Several examples of soilless systems being suppressive towards certain root diseases prove the existence of beneficial microorganisms in soilless systems. The question arises, how can suppressiveness be used as a tool and perhaps be increased?

In soil, the occurrence of disease suppression is fully accepted and has led to the isolation of antagonistic species. In soilless systems it is not yet known, which species or properties within the microflora are causing disease suppression. Hopefully, antagonists that are better adapted to soilless systems will be found by analysing the microbial composition of suppressive soilless systems.

For practical applications, it is important to understand how growers or soilless substrate suppliers can enhance the abundance or activity of a suppressive microflora. The increase of the suppressiveness of a soilless system is most likely by introducing well-adapted antagonistic organisms at the start of the crop or by influencing the microflora through the crop itself or its growing conditions. Good root colonization of the introduced microorganism is probably a crucial property for efficient biological control. If soilless systems can be created with a disease suppressiveness comparable to natural soils, they will combine the advantages of soil systems and soilless substrates, that is a microbiologically well-buffered culture system, as well as the possibility to have a pathogen free start by renewing the substrate. The exploitation of the indigenous microflora to suppress diseases in soilless systems might be a new trend, which is breaking with the 'sterility' concept commonly used in soilless systems.

References

Alabouvette C, Rouxel F, Louvet J (1979) Characteristics of *Fusarium* wilt-suppressive soils and prospects for their utilization in biological control. In: Schippers B, Gams W (eds) Soil-borne plant pathogens. Academic, New York, pp 165–182

Alexander M (1977) Introduction to soil microbiology, 2nd edn. Wiley, New York, p 467

Benhamou N, Kloepper JW, Tuzun S (1998) Induction of resistance against *Fusarium* wilt of tomato by combination of chitosan with an endophytic bacterial strain: ultrastructure and cytochemistry of host response. Planta 204:153–168

Berkelmann B (1992) Characterisierung der Bakterienflora und des antagonistischen Potentials in der zirkulierenden Nährlösung einer Tomatenkultur (*Lysopersicon esculatum* MILL) in Steinwolle. Geisenheimer Berichte Band 10, p 119

Bolton HJ, Fredrickson JK, Elliot LF (1992) Microbial ecology of the rhizosphere. In: Metting FB (ed) Soil microbial ecology: application in agriculture and environmental management. Marcel Dekker, New York, pp 27–63

Calvo-Bado LA, Petch G, Parsons NR, Morgan JAW, Pettitt TR, Whipps JM (2006) Microbial community responses associated with the development of oomycete plant pathogens on tomato roots in soilless growing systems. J Appl Microbiol 100:1194–1207

De Jonghe K (2006) Control of *Phytophthora* spp. by means of (bio)surfactant-producing *Pseudomonas* spp. Ph.D. Thesis, University Gent, Belgium, pp 87–114

De Jonghe K, Hermans D, Höfte M (2007) Efficacy of alcohol alkoxylate surfactants differing in the molecular structure of the hydrophilic portion to control *Phytophthora nicotianae* in tomato substrate culture. Crop Prot 26:1524–1531

Déniel F, Rey P, Chérif M, Guillou A, Tirilly Y (2004) Indigenous bacteria with antagonistic and plant-growth-promoting activities improve slow-filtration efficiency in soilless cultivation. Canadian Journal of Microbiology 50:499–508

De Souza JT, De Boer M, De Waard P, Van Beek TA, Raaijmakers JM (2003) Biochemical, genetic, and zoosporicidal properties of cyclic lipopeptide surfactants produced by *Pseudomonas fluorescens*. Appl Environ Microbiol 69:7161–7172

Ehret DL, Alsanius B, Wohanka W, Menzies JG, Utkhede R (2001) Disinfestation of recirculating nutrient solutions in greenhouse horticulture. Agronomie 21:323–339

Folman LB (2003) Biological control of *Pythium aphanidermatum* in soilless systems: selection of biocontrol agents and modes of action. Ph.D. Thesis, University of Leiden, The Netherlands, pp 123–143

Folman LB, Postma J, Van Veen JA (2003) Characterisation of *Lysobacter enzymogenes* (Christensen and Cook 1978) strain 3.1T8, a powerful antagonist of fungal diseases of cucumber. Microbiol Res 158:107–115

Folman LB, De Klein MJEM, Postma J, van Veen JA (2004) Production of antifungal compounds by *Lysobacter enzymogenes* isolate 3.1T8 under different conditions in relation to its efficacy as a biocontrol agent of *Pythium aphanidermatum* in cucumber. Biol Control 31:145–154

Fravel DR (2005) Commercialization and implementation of biocontrol. Annual Review of Phytopathology 43:337–359

Hallmann J, Sikora RA (1994) Influence of *Fusarium oxysporum*, a mutualistic fungal endophyte, on *Meloidogyne incognita* infection of tomato. Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz 101:475–481

Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW (1997) Bacterial endophytes in agricultural crops. Can J Microbiol 43:895–914

Hunter PJ, Petch GM, Calvo-Bado LA, Pettitt TR, Parsons NR, Morgan JAW, Whipps JM (2006) Differences in microbial activity and microbial populations of peat associated with suppression of damping-off disease caused by *Pythium sylvaticum*. Appl Environ Microbiol 72:6452–6460

Kamilova F, Validov S, Azarova T, Mulders I, Lugtenberg B (2005) Enrichment for enhanced competitive plant root tip colonizers selects for a new class of biocontrol bacteria. Environ Microbiol 7:1809–1817

Khalil S, Alsanius BW (2001) Dynamics of the indigenous microflora inhabiting the root zone and the nutrient solution of tomato in a commercial closed greenhouse system. Gartenbauwissenschaft 66:188–198

Koohakan P, Ikeda H, Jeanaksorn T, Tojo M, Kusakari SI, Okada K, Sato S (2004) Evaluation of the indigenous microorganisms in soilless culture: occurrence and quantitative characteristics in the different growing systems. Scientia Horticulturae 101:179–188

McPherson G.M (1998) Root diseases in hydroponics - their control by disinfection and evidence for suppression in closed systems. In: Proceedings of 7th international congress of plant pathology, Edinburgh, pp 3.8.1S

Paulitz TC, Bélanger RR (2001) Biological control in greenhouse systems. Annu Rev Phytopathol. 103–133

Postma J, Luttikholt AJG (1996) Colonization of carnation stems by a nonpathogenic isolate of *Fusarium oxysporum* and its effect on *Fusarium oxysporum* f.sp. *dianthi*. Can J Botani 74:1841–1851

Postma J, Willemsen-De Klein MJEIM, Van Elsas JD (2000) Effect of the indigenous microflora on the development of root and crown rot caused by *Pythium aphanidermatum* in cucumber grown on rockwool. Phytopathology 90:125–133

Postma J, Alsanius BW, Whipps JM, Wohanka W (2003) La microflora nei sistemi di coltivazione fuori suolo. Informatore Fitopathligico 3:35–39

Postma J, Geraats BPJ, Pastoor R, Van Elsas JD (2005) Characterization of the microbial community involved in the suppression of *Pythium aphanidermatum* in cucumber grown on rockwool. Phytopathology 95:808–818

Postma JV, Os EA, Bonants PJM (2008) Microbial and phytopathological aspects in closed growing systems. In: Raviv M, Lieth JH (eds) Soilless culture: theory and practice. Elsevier, Amsterdam, pp 425–457

Postma J, Stevens LH, Wiegers GL, Davelaar E, Nijhuis EH (2009) Biological control of Pythium aphanidermatum in cucumber with a combined application of Lysobacter enzymogenes strain 3.1T8 and chitosan. Biological Control 48:301–309

Rattink H (1996) Root pathogens in modern cultural systems: assessment of risks and suggestions for integrated control. IOBC wprs Bullet 19:1–10

Rattink H, Postma J (1996) Biological control of fusarium wilt in carnations on a recirculation system by a nonpathogenic *Fusarium oxysporum* isolate. Med. Fac. Landbouww. Univ. Gent 61/2b, pp 491–498

Raviv M, Lieth JH (2008) Significance of soilless culture in agriculture. In: Raviv M, Lieth JH (eds) Soilless culture: theory and practice. Elsevier, Amsterdam, pp 1–11

Sharma VK, Nowak J (1998) Enhancement of verticillium wilt resistance in tomato transplants by *in vitro* co-culture of seedlings with a plant growth promoting rhizobacterium (*Pseudomonas* sp. strain PsJN). Can J Microbiol 44:528–536

Sid Ahmed A, Ezziyyani M, Pérez Sánchez C, Candela ME (2003) Effect of chitin on biological control activity of *Bacillus* spp. and *Trichoderma harzianum* against root rot disease in pepper (*Capsicum annuum*) plants. Eur J Plant Pathol 109:633–637

Stanghellini ME, Miller RM (1997) Biosurfactants; their identity and potential efficacy in the biological control of zoosporic plant pathogens. Plant Dis 81:19–27

Stanghellini ME, Rasmussen SL (1994) Hydroponics: a solution for zoosporic pathogens. Plant Dis 78:1129–1138

Termorshuizen AJ, van Rijn E, van der Gaag DJ, Alabouvette C, Chen Y, Lagerlof J, Malandrakis AA, Paplomatas EJ, Ramert B, Ryckeboer J, Steinberg C, Zmora-Nahum S (2006) Suppressiveness of 18 composts against 7 pathosystems: variability in pathogen response. Soil Biol Biochem 38:2461–2477

Tu JC, Papadopoulos AP, Hao X, Zheng J (1999) The relationship of a pythium root rot and rhizosphere microorganisms in a closed circulating and an open system in stone wool culture of tomato. Acta Horticulturae 481:577–583

Validov S (2007) Biocontrol of tomato foot and root rot by *Pseudomonas* bacteria in stonewool. Ph.D. Thesis, University Leiden, The Netherlands, pp 123–134

Validov S, Kamilova F, Qi S, Stephan D, Wang JJ, Makarova N, Lugtenberg B (2007) Selection of bacteria able to control *Fusarium oxysporum* f.sp. *radicis-lycopersici* in stonewool substrate. J Appl Microbiol 102:461–471

Van Der Gaag DJ, Wever G (2005) Conduciveness of different soilless growing media to Pythium root and crown rot of cucumber under near-commercial conditions. Eur J Plant Pathol 112:31–41

Van Os EA (1999) Closed soilless growing systems: a sustainable solution for Dutch greenhouse horticulture. Water Sci Technol 39:105–112

Van Os EA, Postma J, Bruins M, Willemsen-de Klein MJEIM (2004a) Investigations on crop developments and microbial suppressiveness of *Pythium aphanidermatum* after different disinfection treatments of the circulating nutrient solution. Acta Horticulturae 644:563–570

Van Os EA, Postma J, Pettitt TR, Wohanka W (2004b) Microbial optimisation in soilless cultivation: a replacement for methyl bromide. Acta Horticulturae 635:47–58

Weller DM, Raaijmakers JM, McSpadden Gardener BB, Thomashow LS (2002) Microbial populations responsible for specific soil suppressiveness to plant pathogens. Ann Rev Phytopathol 40:309–348

Whipps JM (2001) Microbial interactions and biocontrol in the rhizosphere. J Exp Botany 52:487–511

Wohanka W, Lüdtke H, Ahlers H, Lübke M (1999) Optimization of slow filtration as a means for disinfecting nutrient solutions. Acta Horticulturae 481:539–544

Chapter 12
REBECA – EU-Policy Support Action to Review Regulation of Biological Control Agents

Ralf-Udo Ehlers

Abstract The potential of biological control is not well exploited in Europe. One reason is the stringent regulation policy for biological control agents (BCAs), which, to a large degree, follow rules implemented for registration of synthetic chemical pesticides. Regulation of BCAs is expensive and time-consuming often surpassing 8 years. Knowledge on safety is limited often resulting in exaggerated registration requirements. The REBECA Action made several proposals how to overcome these problems. Details are reported for innovative procedures on the regulation of microbial BCAs and general proposals are discussed how regulation can be accelerated using more balanced and adapted procedures.

Keywords Biological control • EU policy support action • REBECA • Registration • Regulation policy

12.1 Significance of Biological Control

Biological control products have 2–3% of the world-wide annual turnover of plant protection products. This is still a small share of the total market. However, double-digit growth rates of the biocontrol markets and extension of the demand for food products from organic farming are indications that the significance will increase and that biological control will play a major role in plant protection in the future (Frost and Sullivan, 2001).

Several factors have contributed to the growing success of biocontrol:

- Many of the so-called "old" chemicals have been banded.
- A number of chemical plant protection products have lost efficacy and industry was not able to keep pace with the development of pesticide resistance.

R.-U. Ehlers (✉)
Department for Biotechnology and Biological Control, Institute for Phytopathology, Christian-Albrechts-University, Hermann-Rodewald-Str. 9, 24118, Kiel, Germany
e-mail: ehlers@biotec.uni-kiel.de

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,* Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_12,

- Safety requirements have become more stringent and the development of innovative products often is an insurmountable obstacle.
- Retailers of vegetables demand residue-free produce or at least tolerate levels which are much beyond what governments allow.

Major progress was made in biocontrol of insect pests with beneficial nematodes, mites and insects. Their use started in Northern Europe in the 1970s and is now rapidly expanding to Mediterranean horticulture. More than 90 species of invertebrates are presently on the EPPO list of widely used and commercialized biological control agents. Almost all companies with activities in this area are SMEs (small and medium-sized enterprises). The success was possible, because trade barriers disappeared with the introduction of the EU Common Market (Euro currency and no borders) and courier services improved transport logistics to guarantee delivery of beneficials within a day. But the driving force was that most European governments refrained from introduction of regulation for these organisms. Thus innovation

Table 12.1 Microbial control agents (including Granulose Virus) listed on Annex 1 of the Directive 91/414/EEC (CEC, 1991) until May 2008

Microbial control agent	Product name	Company	Use
Ampelomyces quisqualis	AQ10	Intrachem, I	Powdery mildew
Bacillus subtilis	Serenade	Agraquest, USA	Fungal control
Bacillus thuringiensis	Several	Several	Lepidoptera, Chrysomelidae, Nematocera
Beauveria bassiana	Naturalis	Intrachem, I	White flies
	Botanigard	Laverlam, COL	Homoptera, Thrips
Coniothyrium minitans	Contans	Prophyta, DE	*Sclerotinia sclerotiorum*
Cydia pomonella NPV	Madex	Andermatt, CH	Apple codling moth
	Granupom	Probis, DE	Apple codling moth
	Carpovirusine	Arysta, F	Apple codling moth
Gliocladium catenulatum	Prestop	Verdera, FIN	Fungal control
Lecanicillium muscarium	Mycotal	Koppert, NL	Homopteran insects
Metarhizium anisopliae	Granmet	Agrifuture, I	Insect pathogen
Paecilomyces fumosoroseus	Preferal	Biobest, BE	Homopteran insects
Paecilomyces lilacinus	Bioact	Prophyta, DE	Plant nematodes
Phlebiopsis gigantea	Rotstop	Verdera, FIN	*Heterobasidium anosum*
Pseudomonas chlororaphis	Ceral, Cedomon	Bioagri, SE	Seed treatment fungi
Pythium oligandrum	Polyversum	Biopreparaty, CZ	Fungal control
Streptomyces griseoviridis	Mycostop	Verdera	Fungal control
Trichoderma atroviride	Binap	Binap Bioinovation, SE	Fungal control
Trichoderma harzianum	Trianum	Koppert, NL	Fungal control
Trichoderma polysporum	Binap	Binap Bioinovation, SE	Fungal control
Trichoderma gamsii	Several	Several	Fungal control
Verticillium dahliae alboatrum	Dutch Trig	BTL Bomendienst, NL	Dutch elm disease

could immediately be introduced into practice and SMEs received a quick return from their R&D investments. One example for the success of the use of beneficial invertebrates is the company Koppert (The Netherlands), which was started in the late 1960s selling predatory mites and today has more than 500 employees with an annual turnover surpassing 50 million Euros.

Whereas the use of invertebrate biological control agents has solved many problems in greenhouse horticulture, the use of microbial plant protection products to control insects and plant diseases still is in its infancy. Few products are on the market and can substitute chemical control products. Table 12.1 summarizes those micro-organisms which are registered and listed on Annex 1 of the Directive 91/414 EEC (CEC, 1991). Many of these have not yet received national authorization and some will not be registered as products because markets are considered to be too small to justify costs related with the registration at national level.

Biodiversity in micro-organisms is tremendous and we have only started to understand the antagonistic and symbiotic role of bacteria and fungi established in the plants rhizosphere. However, European agriculture cannot well exploit the potential mainly because registration of these organisms is too costly, time-consuming and complicated.

12.2 Regulation of Biological Control Agents

Plant protection products (PPPs) can be harmful to humans and the environment. For this reason their risks need to be evaluated and active ingredients must be authorised according to Directive 91/414/EEC prior to commercial use. Authorisation for use is only given if unacceptable negative effects to humans and the environment can be excluded. Registration of PPPs based on botanicals, semiochemicals and micro-organisms also follows rules originally developed for the risk assessment of synthetic chemical compounds. The data requirements for micro-organisms have been adapted twice and improvements have facilitated the registration process. But progress has been small and the EU published the following call for proposals: "Despite considerable research efforts on BCAs the number of such products on the market in Europe is currently still extremely low. BCA cannot be treated like synthetic chemicals and need different approaches for registration purposes". After 15 years of disappointing results with registration of biocontrol agents following Dir. 91/414 the need for a review of regulation procedures for BCAs was realized.

The current situation for registration of BCAs is as follows:

- Considering the market potential, costs are too high (between 0.5 and 2.5 million Euros).
- The market size often cannot support costs, consequently few products are available.
- BCA registration takes too long, sometimes surpassing 9 years for Annex 1 inclusion.
- Major obstacle is the following member state authorisation (additional 2 years).
- Countries vary in interpretation of guidelines.

- Mutual recognition not well implemented.
- Guidelines/requirements not set up for BCAs.
- With a lack of knowledge and experience regulation adopts the precautionary principle.
- Efficacy trials more difficult and costly.
- Regulation authorities and SMEs often have limited knowledge about risks of BCAs.
- Registration is a blackbox, this cannot attract venture capital and investment.
- Registration is a major barrier of entry for SMEs.

Due to the nature of the Biological Control Agents (BCAs), they usually pose less risk to users, consumers and the environment than synthetic chemical compounds. In risk analysis the major hazard is the loss of human lives. To my knowledge, never in the past has there been reported a loss of human lives due to the use of BCAs. But more than 300,000 fatalities per year are reported due to the misuse of synthetic PPPs (WHO, 1990). The environmental damages caused by biological control, if any, are of much less magnitude than hazards related with control measures based on the use of synthetic pesticides. Several specific traits qualify BCAs as low risk agents compared to synthetic compounds. For instance, their mode of action is usually much more specific compared to synthetic PPPs and consequently their environmental impact is limited. As they originate from nature they are bio-degradable. Compounds thus do not persist in the environment. Organisms are part of the ecosystem and most micro-organisms are cosmopolitans.

The REBECA community has identified the regulatory requirements for BCAs as one of the major hurdles preventing the access of further of these generally safer PPPs to the market. Alternative measures are therefore urgently needed which

- Are better adapted to carry out risk assessments for the specific risks of BCAs
- Are more efficient for assessment of the real risks of BCAs
- Reduce costs related with the registration process
- Accelerate the registration process

12.3 History of Regulation of Plant Protection Products

In Europe, PPP regulation was introduced in the 1960s. On initiative of the chemical industry, governments gave authorisation exclusively for those pesticides, for which evidence for their efficacy was provided. Environmental aspects were considered in the 1970s in response to concerns about accumulation of the organochlorine insecticide DDT in the food chain. Since then PPPs posing unacceptable risks have been banned and/or substituted and chemical industry adapted to the increasing standards by monitoring safety aspects already at an early stage of product development. The history of regulation has been a process of replacement of one chemical group by another, which often exhibited another set of problems. This process was accompanied by the development of more and more stringent rules taking into

account scientific reports of damage caused by synthetic compounds and anticipated risks of new compounds. Governments responded to reports of damage with the development of new rules to ensure that similar impacts will not occur with new compounds.

This policy is necessary for hazardous synthetic compounds. However, relatively safe BCAs might need less stringent regulation requirements. Politicians deciding on new rules focus on hazards reported for synthetic compounds. Little knowledge of biological control is among decision makers and they hardly ever recognized that when they introduce new legislations for synthetic compounds they restrict BCAs at the same time. Without relation to the hazards reported for synthetic PPPs, BCAs suffer from the increasing regimentation.

The REBECA consortium therefore proposes to:

- Acknowledge the lower risk of BCAs in the development of new rules
- Consider the possibilities to separate legislation of BCAs from synthetic compounds
- Develop more flexible risk assessment procedures
- Produce definitions for low risk products including BCAs
- Introduce fast track systems for low risk BCAs

Since the introduction of regulation, registration requirements and guidance documents had always been developed in consultation with multinational agrochemical companies. Other than regulation of synthetic compounds, regulations for biological plant protection products have not evolved within such a process:

- Regulation of biological PPPs was not a gradual evolution involving industry.
- Regulation was not based on scientific reports of damages.
- BCAs have no evolution of regulatory rules.
- Adapted and more balanced approaches existing in some member states were even rolled back with the introduction of Dir. 91/414 as a consequence of better harmonisation.

Compared to chemical industry, the participation of biocontrol industry in definition of regulatory rules was minor. One reason certainly was the rudimentary representation and low organisation status of the comparatively young biocontrol industry. Another was the low level of knowledge and experience available in these companies and also on the side of regulation authorities. Weighing the economic importance of biocontrol during the time of implementation of Dir. 91/414, one can understand why little emphasis was given to specify regulation for BCAs. However, this situation has now changed. Growers and consumers demand safe PPPs and biological control can provide them. A better adapted regulation procedure would help to reduce restrictions and ease the market access of environmentally sound biocontrol PPPs.

In view of the history of regulation of BCAs, the REBECA consortium proposes to

- Intensify the dialogue between all stakeholders to improve regulation requirement
- Critically review the existing regulatory practice
- Develop new and innovative strategies for BCA regulation
- Consider more adapted regulatory measures according to the real risks of BCAs

In case of little knowledge about hazards regulation follows the precautionary principle to avoid damage. The precautionary principle is the basis of European risk management and is also applied for biological control agents. However, within the EU Commission the interpretation of the precautionary principle is less treating the principle like a dogma but more as the beginning of a serious analysis of how to approach risks within the authorities dealing with risk assessment and management. The Commission published a communication on the precautionary principle (CEC, 2000a) outlining the Commission's approach to use the principle and establishing guidelines for application. The Commission clearly states "that recourse to the precautionary principle presupposes that potentially dangerous effects … have been identified and that scientific evaluation does not allow the risk to be determined with sufficient certainty".

Risks related with the use of BCAs have been described and in many cases their dimension has been scientifically assessed. The RAFBCA project (QLK1-CT-2001-01391) worked on fungal antagonists, identifying potential risks and concluding on their dimension and probability of occurrence. Together with scientific publications, the results gathered and summarized by the REBECA Action (www.rebeca-net.de: Safety information) or the biopesticide fact sheets provided by the Environmental Protection Agency (EPA) in the USA much information is now available to conclude that regulation of BCAs can be based on scientific evidence and that we do not need to apply the precautionary principle.

Reviewing the Commission's view on the precautionary principles, REBECA proposes to:

- Treat BCAs in a non-discriminative way
- Considering their lower risk compared to synthetic compounds
- Take into consideration experience and available data from comparative use
- Re-examine measures based on new scientific results on the safety of BCAs

In order to avoid unnecessary over-regulation and costs, REBECA proposes to:

- Analyse costs and benefits prior to introduction of new regulation demands
- Take into account trade-off effects of regulation
- Minimize trade-off effects and maximize efficiency of regulation
- Develop cost-effective procedures and accelerate the registration process

12.4 Stakeholders and Conflicting Interests

Conflicts about how risks should be regulated or the implementation of costly regulation procedures are often a result of wrong information policy by a small fraction of interest groups. To avoid limited information it is important to ensure the integration of all stakeholders into the process of decision finding. Decision finding costs might be higher in the beginning, however, on the long term, a consensus will save

resources as all stakeholders support the result. Stakeholders in the area of regulation of BCAs are

- Scientists developing biological control products and strategies
- Governments implementing legislation
- Regulators dealing with the dossiers and transferring regulation into practice
- Environmentalists organised in NGOs
- The retail sector and consumers of agricultural products
- Farmers
- Chemical industry
- Biological control industry

The different stakeholders have overlapping and/or conflicting interests. Conflicting interests exist between some environmentalists and biocontrol industry when aspects of non-target effects are discussed. On the other hand, some environmentalists prefer less dramatic impacts of BCAs over broad-spectrum insecticides.

During the REBECA Action it became apparent that some enterprises supported the introduction of regulation or continuation of high level registration procedures. These enterprises have registration departments with experienced personal and know how to handle registration. As intellectual property can often not be protected in the area of biological control, registration is a measure to keep competitors off the market.

If governments and regulators implement regulation a conflict situation can evolve with industry, should the later stakeholder be severely affected in its marketing activities. The dimension of this conflicting situation is also defined by the character of the regulation authority. In the USA, for instance, the EPA has a profound interest in the promotion of biological control. This resulted in significantly more biological control products registered and a quicker market excess in the USA than in the EU. In Europe regulation is in the hand of authorities in charge of agricultural, environmental and health affairs. All three sectors have conflicting interests. Consensus finding is time-consuming. Often personnel dealing with files and monographs are experts in reviewing information on synthetic chemistries. If BCAs are regulated by these agencies, which lack background information on the risks of BCAs, the process of consensus finding will be particularly long and expensive and will result in exaggeration of risks. Lack of knowledge is resulting in overestimation of risks. The microbial biological control sector in the EU has significantly suffered from implementation of registration requirements following the rules developed for synthetic agrochemicals. Many potential products based on microbials are not submitted for EU registration due to costly data requirements. In order to prevent bureaucratic hurdles and unnecessary consensus finding costs, attempts should be made to get regulation of BCAs into the hands of experts. Should European policy makers seriously want to promote the further introduction of biological control strategies, then they should take measures to equip authorities with more and with experienced personnel and include outside expert knowledge.

They even might want to waive fees and support the data production necessary for the risk assessment. The REBECA consortium proposes to:

- Reduce consensus finding costs
- Equip registration authorities with skilled personnel
- Consider expert knowledge in the regulation process
- Not allow abuse of registration system to protect markets
- Waive fees for registration of BCAs
- Support production of safety data

12.5 Discrepancy Between Political Agenda and Support for Biocontrol Concepts

Biological control is often not yet recognized as a viable alternative to conventional PPPs. This makes policy approaches difficult to succeed. Some examples will describe the situation.

The European and National Parliaments are not aware of the benefits of biocontrol. Although their concepts and policy strategies want to avoid the use of chemical PPPs, the legislation is actually not promoting the use of biological control agents. Since the reduction of the use of chemical compounds is part of the EU Common Agriculture Policy (CEC, 2002), many national parliaments have introduced pesticide reduction programmes. However, hardly any of these programmes support the use of BCAs. Another example are NGOS which support the reduction of synthetic compounds. When REBECA asked NGOs to participate, members of Greenpeace and PAN (Pesticide Action Network) confessed that they had no expertise in the risks related with the use of BCAs.

Positive examples for support of biological concepts also exist. The Spanish government is currently supporting the introduction of BCAs into greenhouse vegetable production with 1,000 €/ha in order to overcome problems with resistance against synthetic compounds, illegal use of non-registered products and residues in vegetable and fruit products. As a consequence the introduction of biological control agents in greenhouse production jumped from approximately 200 ha in 2005 to more than 10,000 ha in 2008. Introduction of biological concepts needs policy support. Should more funding go into the biocontrol sector, there would certainly be more products on the market and agriculture ecosystems would be safer environments with increasing biodiversity.

Also, introduction of biological controls would bring immense socio-economic benefits to the society. Benefits from controlling diseases and pests would still account, but negative externalities related to the production and use of synthetic compounds would disappear. The current regulatory system for BCAs in the EU has severe trade-off effects. These contribute in several key areas to the EU not meeting their stated policy objectives, which are:

– Pesticide reduction programmes
– Increasing proportion of organic production

– Safer food with less pesticide residues
– Safer and more diverse environment
– More jobs
– More SMEs

These goals can easily be reached should European societies consider serious support of R&D into biological control and further reduction of registration hurdles. Evaluating the results of the REBECA Action one aspect has become obvious: With increasing knowledge on the risks of BCAs the requirements are reduced and regulators are more confident to give waivers on certain non-relevant data requirements. The success in dissemination of results on the safety of BCAs during the REBECA Action was much based on the outcome of the EU-supported R&D projects ERBIC (on invertebrate BCAs) and RAFBCA (on fungal BCAs). In order to further reduce the registration requirements more data on their safety is desirable.

Many applicants during the REBECA Action complained about lack of guidelines. In many cases guidelines are not applicable. Research into the development of methods and models including the drafting of guidelines for human risk assessment of microbial BCAs are urgently needed. It is comparably cheap to develop biocontrol strategies compared to the development of a synthetic PPP. But as markets are limited due to the specificity of most biocontrol agents, the return on investment is lower. Thus it might be reasonable for European societies to support the introduction of BCAs and promote the integration of biocontrol into IPM strategies by giving support to the production of safety data.

The REBECA consortium proposes to:

- Support knowledge transfer on BCA concepts to the farmers (from lab to farmer to fork)
- Support development of risk assessment guidelines
- Support closure of knowledge gaps on risks related with the use of BCAs
- Take into account BCAs as potential substitutes for synthetic compounds
- Focus on introduction of BCAs in reduction programmes
- Support farmers during introduction of BCAs into IPM systems

12.6 Proposal to Simplify Registration of Microbial Biological Control Agents

12.6.1 Information Check List for Pre-submission Meetings

Based on the currently available experience with the use of MBCAs and scientific information on their risks we can conclude that registered and most other MBCAs pose little risk for humans, non-target organisms and the environment. In order to simplify the registration procedure it is therefore recommended to summarize the available data and to discuss relevant data requirements in a pre-submission meeting

with the Rapporteur MS (member state) prior to submission of the dossier. The decision on the relevant data to be provided shall be based on the following information, which can be derived from the applicant's data and/or published literature:

- Identification and taxonomic position of the MBCA
- Natural distribution of the species in particular on food and feed and in agriculture environments
- Modes of action and host range
- Toxicity data
- Metabolites produced by the MBCA
- Intended use of the product (target organisms)
- Formulation of the product
- Site and method of application
- Health and medical reports
- Absence from the list provided in Dir. 2000/54 EC (CEC, 2000b) concerning worker's protection from micro-organisms
- Maximum growth temperature
- List of available effective antibiotics

Data provided shall be the basis for a decision on the provision of additional data in the dossier and the definition of waivers. Should no relevant potential risks be identified from this information, no further information should be required on metabolites, toxicology and non-target effects. For the following risk assessment of the MBCA it is essential to refer to the above listed data, which is basically contained in Section 12.1 according to the OECD format (OECD, 2004). These data might be sufficient to estimate a risk index as proposed in the REBECA deliverable 28 (www.rebeca-net.de) to identify low risk products.

12.6.2 Comments on Data Requirements

Data requirements listed under OECD Series on Pesticides 23 (OECD, 2004) and 2001/36/EC (CEC, 2001) and methods to assess pathogenicity, infectivity and toxicology have been discussed several times within the action. The requirements are complex and extensive. It was discussed whether more adapted approaches to test pathogenicity, infectivity and toxicity might produce better data with less effort. However, it becomes very clear in an early stage of this action that the development of such proposals is hindered so far by a significant lack on validated risk assessment methods for microbials and knowledge gaps on natural exposition of humans and other non-target organisms. This is hampering an adequate risk assessment of microbial plant protection products. Therefore, REBECA demands the initiation of a research programme by the EU with the aim to develop more appropriate risk assessment methods for microbials and to reduce whole animal testing and costs.

12.6.3 Human Infectivity

Humans are regularly exposed to a wide range of micro-organisms and the human community is spending a lot of resources to identify pathogens. Therefore, human pathogens are well described and documented in the relevant literature and databases (Möllby, 1998). On the basis of this knowledge microbes are categorised into 4 risk groups (Directive 2000/54 EC). The Directive is aiming at protection of workers against risks to their health and safety, including the prevention of such risks, arising or likely to arise from exposure to biological agents at work. If a biological agent is included in risk group 1, it is unlikely to cause human diseases. In that case no special measures are required according to the Directive to prevent or reduce the risk of exposure to such an organism (article 4, clause 1). Only general principles of good occupational safety and hygiene should be followed. All micro-organisms used in registered plant protection products to date are not listed in the risk groups 2–4.

In Dir. 2000/54 only organisms categorized into the groups 2–4 are listed. This means, "In line with the scope of the Directive, only agents, which are known to infect humans are to be included in the classified list. Animal and plant pathogens which are known not to affect man are excluded". However, not explicitly listing the group 1 organisms is a drawback of this Directive, because not listed micro-organisms might be not categorized at all so far. In contrast Germany lists as well the group 1 organisms in the so called technical advises for biological substances (Bundesministerium für Arbeit und Sozialordnung, 2002, 2005). In all EU member states adaptations of Dir. 2000/54 EC exist. A quite similar categorisation of micro-organisms as used in the EU is used by the WHO Biosafety Manual (WHO, 2003) and many non-European countries. It can be concluded that the risk for infection of humans is very well known for many micro-organisms and that the EU and the member states already made a decision concerning this risk regarding the exposure of workers. This classification should also be applied to micro-organisms used in plant protection products.

REBECA experts concluded that more emphasis should be given to the clinical findings and published reports on adverse effects of the species of an MBCA during the risk assessment procedure. A correct identification of the micro-organism by the applicant and in the literature will be an indispensable prerequisite. It was questioned whether the classification of a micro-organism into group 1 delivers at least the rationale to waive the risk assessment requirements regarding extensive infectivity studies of the micro-organism or in other words to waive the clearance investigations in the Tier I assessment.

Despite the group 1 classification further key indicators for the human (mammalian) safety of MBCAs are:

- No growth at temperatures >35°C
- No clinical reports and indications in relevant scientific literature or databases
- Data on susceptibility of MBCA to antibiotics

The potential of nosocomial infections of immune-compromised patients by MBCAs was discussed. These infections are a result of treatment in a hospital or a

healthcare service unit, but secondary to the patient's original condition. Nosocomial infections are alarming as antibiotic resistance has widely spread. Data on the susceptibility of the MBCA to common antibiotics can minimize the risk of nosocomial infections. Reports on infections of immune suppressed patients, however, should not hamper registration of a micro-organism for use in PPP since contact of immune-suppressed patients to PPP should be avoided in any case.

Currently the infectivity is assessed by so called clearance investigations. In this investigation the clearance of the micro-organisms from the inner organs of rats or mice is assessed after an intra-tracheal instillation. This method has several disadvantages and alternative assays should be investigated (for more information see REBECA deliverable 11). REBECA proposes that if all the following criteria are fulfilled, the data requirements for clearance or respectively infectiveness (Dir. 2001/36 EC point 5.2.2) should be waived:

1. No (or few) clinical reports and indications in relevant scientific literature or databases. A low number in most cases is a wrong identification or an indication for an opportunistic infection. This can be assessed from the data provided with the record.
2. Point 1 criteria should be cross-checked with Directive 2000/54 EC or equivalent member state documents.
3. Data on susceptibility of MBCA to antibiotics, indicating that the strain is susceptible to several available compounds.
4. Data on distribution and occurrence of species, which underpin the regular exposure of humans to the micro-organism in question (e.g. common on food and feed, common on food and feed plants foliage or roots, common in the soil, etc.).

In other words, if humans are already regularly exposed to the micro-organism and no relevant clinical reports exist (risk group 1), the risks of infections is negligible. In addition, if the micro-organism is susceptible to antibiotics, putative infections can be cured.

Proposals were made also for

- Genetic stability
- Sensitisation
- Risk assessment of metabolites
- Assessment of metabolites in the environment
- Fate and behaviour in the environment
- Eco-toxicological studies and effects on non-target organisms

Details are summarized within deliverable 10 of the REBECA report to the EU Commission and can be downloaded from the webpage (www.rebeca-net.de).

12.7 General Improvement of Regulatory System

REBECA developed in meetings with representatives from regulation, industry and science general proposals how the current system can be improved. This process was started in 2006 and continued in 2007. Proposals were developed regarding:

- Introduce pre-submission meetings between applicant and authorities
- Improved communication between regulators and applicants
- Improved communication between regulators in different member states
- Produce better adapted guidance documents
- Reduce or waive fees and provide financial support to SMEs
- Introduce generic approaches in risk assessment
- Stick to strict and short timelines
- Develop specific data requirements for each group of BCAs
- Reduce requirements for efficacy
- Centralize regulation authorities
- Introduce mutual recognition in climatic zones
- Include BCAs into low risk or basic substances
- Increase transparency in regulation process

Strategies for the implementation of the proposals have been developed. A number of partners need to be involved in the implementation process. REBECA identified these partners and described measures to obtain implementation.

The main partners are:

- The European Commission, DG SANCO
- The European Food Safety Authority (EFSA)
- All of the EU member states (politicians as well as regulatory authorities)
- OECD-BioPesticide Steering Group
- BCA Industry/applicants and IBMA (Int. Biological Manufacturers Association)
- Academia
- Grower and consumer organisations (national as well as international)

12.8 Conclusion

This contribution tried to summarize the major achievements of the REBECA Action, which was terminated in January 2008. Further information is available on the internet page of REBECA (www.rebeca-net.de).

Politicians, consumers and farmers are about to realize that antagonist biodiversity and biological control provide a huge potential to improve agricultural production processes. We cannot afford to not use this potential in the future. In order to increase food safety and develop integrated and sustainable strategies for plant protection, which are safe to the consumer, producer and the environment we need to promote biological control. In order to reach these goals *we need less, rather than more registration requirements*. We need to increase our knowledge on safety aspects as well as the mode of action and biology of BCAs. We should also try to adapt the regulation system to the nature of biological control systems. We are not dealing with chemistry but with living systems which are much more complex and interacting. Often not one BCA provides success but a combination of two or more. Should European authorities not be able to include experience of long term safe use, experience of other research fields and published results, we will not adequately

handle the real risks of BCAs but continue to build hurdles for implementation of BCAs. The national registration authorities and in particular the European offices should start to take biological control serious and cooperate to improve the system. REBECA was a starting point. We will need more networking and input. If all stakeholders will pick up the thread we will be able to develop more adapted, better balanced and innovative regulation strategies to make European food produce safer and maintain or even increase the biodiversity of our agriculture ecosystems.

Acknowledgements Thanks are due to the European Commission for financial support of the REBECA Action and to all colleagues, who contributed to the success of the action, in particular, Anita Fjelsted, Rüdiger Hauschild, Hermann Strasser, Olaf Strauch, Bernhard Speiser and Ulf Heilig.

References

Bundesministerium für Arbeit und Sozialordnung (2002) Einstufung von Pilzen in Risikogruppen. Technische Regeln für Biologische Arbeitsstoffe (TRBA 460). Bundesarbeitsblatt 2002–2010, p 78 (http://www.umwelt-online.de/regelwerk/t_regeln/trba/trba400/460_ges.htm)

Bundesministerium für Arbeit und Sozialordnung (2005) Einstufung von Bakterien (Bacteria) und Archaebakterien (Archea) in Risikogruppen. Technische Regeln für Biologische Arbeitsstoffe (TRBA 466). Bundesarbeitsblatt 2005–2007, p 33 (http://www.umwelt-online.de/regelwerk/t_regeln/trba/trba400/466_ges.htm)

CEC (1991) Commission of the European Communities, Council Directive 1991/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market. Off J Eur Union. Luxembourg (http://euro-lex.europa.eu)

CEC (2000a) Commission of the European Communities, Communication of the Commission on the precautionary principle. Brussels COM (2000) 1 (http://ec.europa.eu/dgs/health_consumer/library/pub/pub07_en.pdf)

CEC (2000b) Commission of the European Communities, Council Directive 2000/54/EEC Directive of the European Parliament and of the Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work. Off J Eur Union. Luxembourg (http://euro-lex.europa.eu)

CEC (2001) Commission of the European Communities, Commission Directive 2001/36/EC of 16 May 2001 amending Council Directive 91/414/EEC concerning the placing of plant protection products on the market. Off J Eur Union. Luxembourg (www.euro-lex.europa.eu)

CEC (2002) Commission of the European Communities, towards a thematic strategy of the sustainable use of pesticides. Communication from the Commission to the Council, the European Parliament and the Economic and Social Committee. Brussel, CO (http://europa.eu/scadplus/leg/en/lvb/l21288.htm)

Frost & Sullivan (2001) European biopesticides market (www.frost.com)

Möllby R (1998) Pathogenic properties of micro-organisms – human hazard identification. In: Proceedings of "microbiological plant protection products – workshop on the scientific basis for risk assessment". KEMI, Stockholm, Sweden, 26–28, October 1998, pp 49–56

OECD (2004) OECD Guidance for Industry Data Submissions for Microbial Pest Control Products and their Microbial Pest Control Agents (Dossier Guidance for Microbials), Series on Pesticides No. 23 (http://www.oecd.org/dataoecd/60/6/30919600.pdf)

RAFBCA Risk Assessment of Fungal Biological Control Agents, EC-Project QLK1-CT – 2001-01391, November 1 2001–September 30, 2004 (www.rafbca.com)

REBECA Regulation of Biological Control Agents, EC-Project SSPE-CT-2005-022709, January 1, 2006–December 31, 2007 (www.rebeca-net.de)

WHO (1990) Public health impact of pesticides used in agriculture. World Health Organization/ United Nations Environment Programme. Geneva
WHO (2003) Laboratory Biosafety Manual. WHO/CDS/CSR/LYO/2003.4. Geneva 2003
Pesticide Action Network International (http://www.pan-international.org)
US Environmental Protection Agency, Biopesticide Active Ingredient Fact Sheets (http://www.epa.gov/oppbppd1/biopesticides/ingredients/index.htm)

Chapter 13
Fungal Disease Management in Organic Apple Orchards: Epidemiological Aspects and Management Approaches

Imre J. Holb

Abstract Environmental considerations are becoming increasingly important and, as a consequence, interest has turned from conventional to organic fruit production, including apple. In this production system, synthetic products are banned e.g. in plant protection and nutrient supply, only natural products are permitted according to organic production standards. As a result, disease control is less effective in organic apple orchards than in conventional ones with the consequence that epidemics of key diseases are likely to be more serious in such a system. The majority of sprays in organic apple production are consumed for apple scab control. Therefore, improvement of fungal disease management in organic apple production is largely dependent on apple scab control. This review will provide novel epidemiological aspects and season-long management options against apple scab in organic orchards. This will include several topics (i) risks of early scab epidemics in organic apple orchards initiated by sexual and asexual forms of fungi, (ii) specific features of apple scab epidemics in organic orchards, (iii) possible control strategies to reduce primary inoculum sources, (iv) appropriateness of various sanitation practices against scab in organic production, and (v) some aspects of efficacy and phytotoxicity of approved fungicidal products against scab in organic orchards. Based on the above examples, a theoretical and practical decision-making approach for apple scab and future trends for organic disease management will be provided for organic orchards based on mechanical, agro-technical and chemical control options.

Keywords Apple scab • Calcium-polysulphide • Copper • Decision support system • Disease control • Disease warning • Epidemiology • Overwintered conidia • Pruning • Sustainable agriculture

I.J. Holb (✉)
Centre for Agricultural Sciences and Engineering, University of Debrecen,
P.O. Box 36, H-4015, Debrecen, Hungary

and
Plant Protection Institute, Hungarian Academy of Sciences,
P.O. Box 102, H-1525, Budapest, Hungary
e-mail: holb@agr.unideb.hu

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,*
Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_13,

13.1 Introduction

Environmental considerations are becoming increasingly important and, as a consequence, interest has turned from conventional to organic fruit production (Sansavini, 1990, 1997; Sansavini and Wollesen, 1992; Bellon et al., 2001; Reganold et al., 2001; Ferron and Deguine, 2005; Lancon et al., 2007). By now, the rules and some tools for fungal disease management are well-defined and most of them are successfully implemented for organic production of apple (Anonymous, 1989, 2000; Weibel and Häseli, 2003). Disease management practices in organic apple production differ markedly from those in conventional production. Synthetic products are banned in organic apple production. In organic apple growing, only natural products such as compost, suspendable rock powder, sulphur and copper compounds, fungicidal and botanical soaps, traps and biological methods are permitted against fungal diseases according to IFOAM (International Federation of Organic Agriculture Movements) standards (Anonymous, 2000), while many synthetic pesticides can be used in conventional apple production. Via the application of these management options, disease management may be less effective in organic apple production than in conventional production, with the consequence that production risks are likely to be higher in such systems.

Organic production has its origins in Germany starting at the beginning of the twentieth century (Vogt, 2000), though its worldwide establishment and regulation started in 1977 when the first IFOAM congress was held in Sissach, Switzerland (Weibel, 2002). Industrialised organic apple production started only in the late 1980s in Europe (Weibel and Häseli, 2003). Presently, the organic apple production area is still small (a few thousand hectares in Europe) compared to integrated production, but it is continuously growing year by year. The major problem of fungal disease management in organic apple production is the lack of effective fungicides or natural products against the most damaging apple diseases such as apple scab. Therefore, organic apple growers have to rely strongly on integration of direct and indirect non-chemical control options, which often result in 15–50% yield loss caused by fungal diseases (Weibel, 2002; Holb, 2005a, 2008).

Fungicides comprise the greater proportion of pesticides applied to apple orchards (Bower et al., 1993; Penrose, 1995) and the largest proportion of sprays are applied to control apple scab, caused by *Venturia inaequalis* (Cooke) G. Wint (MacHardy, 1996; MacHardy et al., 2001; Holb, 2005a). Scab management may require 6–16 treatments per season, depending on weather conditions and disease pressure (Köller et al., 1991; Van der Scheer, 1992; Beresford and Manktelow, 1994; MacHardy, 1996; MacHardy et al., 2001; Vincent et al., 2004; Holb et al., 2005b, 2006), which represent up to 90% of the annual fungicide cost in apple production. Thus, scab control has a fundamental effect on the whole fungal disease management. As apple scab consumes the majority of sprays also in organic apple production, our aims were to investigate the epidemiological aspects of scab inoculum sources in organic apple production and then to use this knowledge into improvements of disease management.

13.2 Justifying Asexual Survival Features of Apple Scab and Its Control in Organic Apple Orchards

A generally accepted biological feature of apple scab is that the sources of primary infections are the ascocarps overwintering on fallen leaves (Keitt and Jones, 1926; Keitt, 1936). In a recent study, it was justified that early spring symptoms appear earlier in organic apple orchards than in the integrated or conventional ones (Holb, 2000). Moreover, these scab symptoms appear as early as green tip stage in spring, indicating that infection can only occur at the time when buds are still closed. However, ascospores of ascocarps from overwintered fallen leaves are not able to physically infect at that time yet. This phenomenon indicated an asexual survival strategy of apple scab in organic orchards that is essentially different from the well-established survival strategy of sexual ascocarps in integrated or conventional orchards. The general concept was based on the notion that the resistance of the asexual forms (such as conidia, mycelium) to environmental exposition is so low, that their role in overwintering cannot be of great importance in commercial apple scab management (Cook, 1974; Hill, 1975; Becker et al., 1992). However, the in vivo and in vitro experiments in organic orchards proved that the asexual form of the fungus (conidia) can overwinter under the protection of budscales in such amounts which can cause scab epidemics in early spring (Holb et al., 2004a). A part of these results was that the overwintering of conidia could be verified only in those European regions where winters are mild (e.g. The Netherlands), but not under Central European conditions where winter is usually cold. As the role of the asexual (conidial) form in early epidemics was significant (Holb et al., 2004a), it became obvious that effective management options are needed to be developed against overwintered conidia. In order to solve this, first, it was necessary to replace the time-consuming laboratory work of conidial detection and viability examination with a more simple method that can be applied in the practice; second, it was necessary to determine a threshold level above which control is needed; third, new environmentally-benign management options had to be developed; and finally, these promising new management options had to be tested in commercial orchards. Holb et al. (2004a) developed a so-called "bud-pressing" method for a simple examination of conidia overwintering under the protection of budscales. This new method was simpler and quicker than the earlier ones (Becker et al., 1992), and it can be recommended for plant protection advisers for detecting the overwintered conidia before bud break (Holb et al., 2004a). Meanwhile a study of Holb et al. (2005a) including 18 integrated and organic orchards demonstrated that a threshold level of 40% autumn scab incidence could be measured in the previous autumn for a significant amount of infection caused by overwintered conidia in the following spring (Fig. 13.1). This value was a cornerstone in forecasting the early spring epidemics of overwintered conidia (Holb et al., 2005a).

Two practically-applicable, environmentally-benign management options: – (i) copper spray in early spring at bud break and (ii) winter pruning – were developed

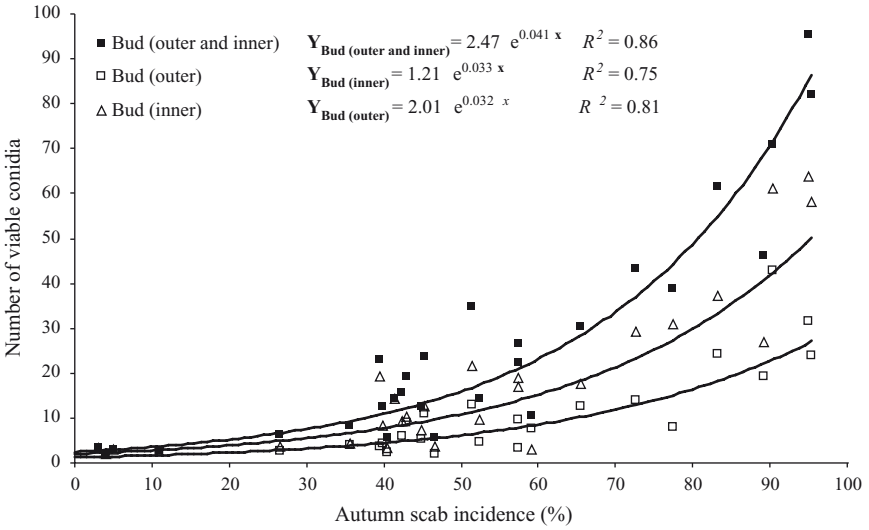


Fig. 13.1 Relationships between autumn scab incidence in the previous year and the number of viable overwintered conidia of Venturia inaequalis associated with buds and shoots before bud break. Presented data were combined for 2000 and 2001 (The Netherlands) (after Holb et al. (2005a))

against conidial epidemics in spring in orchards exceeding the threshold level of 40% autumn scab incidence (Holb et al., 2004a, 2005a; Holb, 2005a,b).

The study of Holb et al. (2004a) demonstrated the efficacy of copper spray treatment against conidial epidemics in early spring as follows (Fig. 13.2). In their study, there was an ascosporic inoculum source at 0 m and the other part of the orchard was covered with plastic foil, so the amount of symptoms was decreasing with distance. In the experiment, there were an untreated block (which represented symptoms of ascosporic and overwintered conidial infections) and a copper-treated block (which represented the symptoms caused by ascospores as the overwintered conidia were killed by copper). It can be seen in the figure that there was a gap between symptoms on untreated trees and copper-treated trees. This gap is the amount of symptoms caused by overwintered conidia, which were killed by copper (Fig. 13.2). Early spring copper spray against overwintering conidia was recommended when autumn scab incidence is between 40% and 60%.

The study of Holb (2005a) demonstrated the efficacy of winter pruning treatment against conidial epidemics in early spring as follows (Fig. 13.3). In his study, the upper third of the shoots was cut in a weakly and a strongly pruned treatment on a susceptible (‘Mutsu’), a moderately susceptible (‘Idared’) and a resistant cultivar (‘Prima’). Results showed that strong winter pruning reduced scab incidence significantly on the scab susceptible cultivars in April due to the removal of those shoots which were infected with overwintered conidia (Fig. 13.3).

Further experiments revealed that the combinations of copper spray and winter pruning have a synergistic effect and they can be successfully applied against

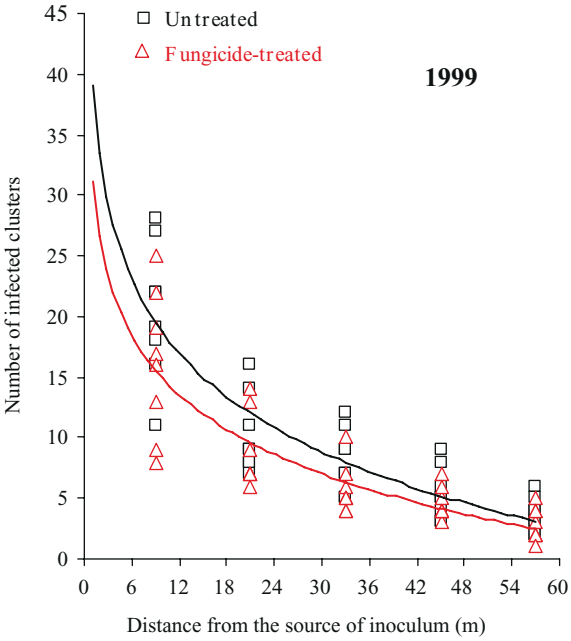


Fig. 13.2 Efficacy of copper spray treatment against overwintering conidia of apple scab. Mean numbers of infected clusters for five replicates of untreated and fungicide-treated experiments in the spring of 1999 (Randwijk, The Netherlands) (after Holb et al. (2004a))

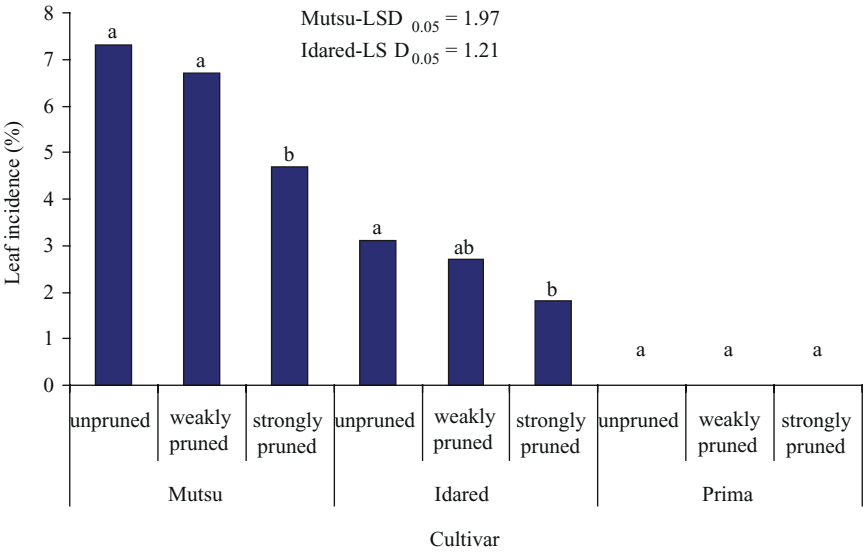


Fig. 13.3 Efficacy of winter pruning method against overwintering conidia of apple scab. Effect of three pruning treatments (unpruned, weakly pruned, and strongly pruned) on leaf incidence of apple scab for three apple cultivars differing in their susceptibility to apple scab in the second week of April in an organic apple orchard (Eperjeske, Hungary, 2002). LSD is least significant difference (after Holb (2005a))

overwintering conidia even above the level of 60% autumn scab incidence (Holb et al., 2005a; Holb, 2005a,b). In further research, Holb (2005a) proved that pruning reduces not only the number of conidia overwintering between the budscales, but it also has a diminishing effect on the summer scab epidemics in organic production.

In overwintering conidia research, a further new question arose that how and when the conidia get between the tightly-closed budscales. Preliminary investigations of Holb et al. (2005a) hypothesized that the bud formation processes of the preceding season should be studied. Further experiments revealed that the budscales are so loosely connected during the summer bud formation that conidia could become entrapped between the budscales through the effects of wind or rainfall water, and then, they can successfully overwinter and cause symptoms on the inner bud tissues next spring. In addition, a positive correlation was revealed in summer and autumn between cumulative leaf incidence and the number of conidia entrapped between budscales (Holb et al., 2005a). These results indicated that a novel scab management strategy is needed already in the preceding summer, which can slow down the formation of summer epidemics in organic orchards. However, the biological background necessary for developing these control methods was missing, as characteristics of summer scab epidemics were not accurately defined in organic management systems. Meanwhile, copper, the most effective fungicide in organic production, was banned for plant protection use due to environmental and human health considerations (Holb and Heijne, 2001). Consequently, the organic apple growers were left without an effective fungicidal compound in the most critical periods of apple scab control in spring and summer; in addition, the early spring copper spray treatment developed against overwintering conidia also had to be modified. So, further scab management research had two parallel directions (i) study of the epidemic features of summer scab epidemics and (ii) possibilities of copper replacement in the organic management system.

13.3 Characteristics of Scab Epidemics in Summer

A summer scab epidemic study of Holb et al. (2003a, 2005b) demonstrated that the number of scab symptoms continuously increased until mid-August in organic apple orchards (Fig. 13.4) and disease measures showed significant incidence-severity relationships described by linear and exponential functions (Table 13.1).

The rate of scab development reduced only after mid-August, after bud closure. Bud closure coincided with the end of shoot growth; and consequently, with the final date for conidial entrapment between budscales (Holb et al., 2005a). Authors concluded that this date, Y_{75} plays an essential role in management decisions (see Chapter 13.6). Holb et al. (2005b) also demonstrated that summer epidemic features of apple scab can be demonstrated with disease variables derived from mathematical functions fitted to disease progress curves. Authors tested several non-linear function models and the three parameter logistic function fitted the best. Three

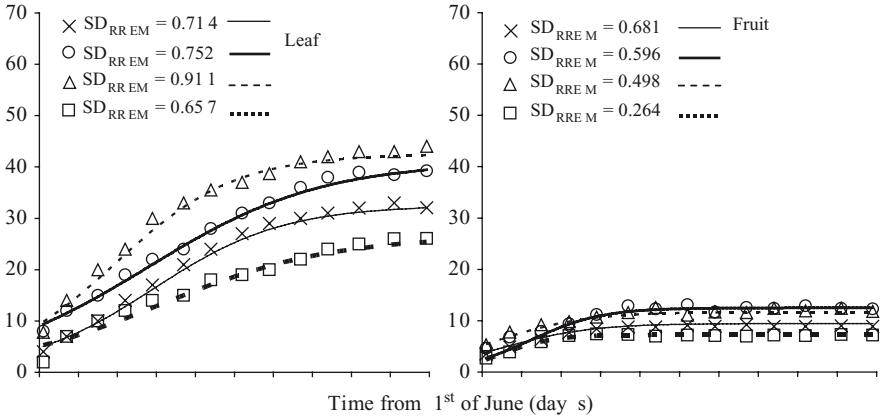


Fig. 13.4 Samples for demonstrating leaf and fruit incidences in organic-sprayed treatment class in Hungary: × 2000; ○ 2001; △ 2002; □ 2003. SD_{RREM} represents the standard deviation of the residual obtained from the fitted random effects model for each fitted three-parameter logistic curve. Symbols of ×, ○, △, and □ represent mean values of the 14 incidence data in a year. Different lines represent the fitted curves of the three-parameter logistic function to the yearly 25 × 14 data set. Results of a *t* test for the three parameter estimates of the logistic function were P > 0.001 in all cases, therefore, they were not shown separately for each curve (after Holb et al. (2005b))

Table 13.1 Samples for demonstrating practically meaningful relationships between the measurements of apple scab obtained by regression analyses in organic apple orchards (Randwijk, The Netherlands, 1998–1999). Analysed incidence and severity measurements were I_s = shoot incidence, S_s = shoot severity, I_l = leaf incidence, S_l = leaf severity, I_f = fruit incidence, S_f = fruit severity
(after Holb et al. (2003a))

Model	R^2 [a]
$S_s = 0.885e^{0.034 \cdot (Is)}$	0.72
$S_l = 0.138e^{0.039 \cdot (Is)}$	0.55
$S_l = 0.649e^{0.056 \cdot (Il)}$	0.88
$S_f = 0.001e^{0.064 \cdot (Is)}$	0.61
$S_f = 0.001e^{0.077 \cdot (Il)}$	0.81
$S_f = 0.001e^{0.181 \cdot (If)}$	0.82
$I_l = 1.1e^{0.036 \cdot (Is)}$	0.82
$I_f = 0.513e^{0.036 \cdot (Is)}$	0.92
$I_f = 0.402 \cdot (I_l) + 1.966$	0.92

[a] Coefficient of determination.

disease variables: (i) area under the disease progress curve (*AUDPC*), (ii) leaf incidence and severity (%) estimated on day 75, the day when shoot growth effectively stopped (Y_{75}) and (iii) 'absolute' rate parameter (Θ) were the most suitable for the

Table 13.2 Samples for demonstrating the selection of the most important parameters for characterization of summer scab epidemics. Factor loadings calculated from Principal Axis Factor analysis (Varimax rotation) using apple scab progress parameters of incidence in The Netherlands and Hungary (after Holb et al. (2005b))

	The Netherlands 1998/2001		Hungary 2000/2003	
Factor	1	2	1	2
Incidence parameters[a]				
Y_s	0.321; 0.345[c]	−0.035; −0.123	0.322; 0.358	−0.068; −0.156
Y_f	**0.352; 0.428**[d]	0.065; 0.156	**0.351; 0.439**	0.078; 0.178
Y_{sf}[b]	0.211; 0.246	**−0.745; −0.884**	0.235; 0.295	**−0.352; −0.411**
Y_{40}	**0.354; 0.411**	0.093; 0.122	**0.353; 0.418**	0.143; 0.223
Y_{75}	**0.354; 0.409**	0.002; 0.089	**0.355; 0.422**	0.005; 0.067
β	−0.267; −0.329	0.022; 0.111	−0.288; −0.326	0.045; 0.107
θ	**0.351; 0.443**	−0.089; −0.158	**0.351; 0.438**	−0.123; −0.145
M	−0.235; −0.311	**0.521; 0.657**	−0.169; −0.217	**0.602; 0.698**
$AUDPC_S$	**0.355–0.432**	0.017; 0.098	**0.367; 0.447**	0.047; 0.138
Variance	7.1–8.2	0.6–1.1	6.5–7.7	0.8–1.2
Explained variance (%)	79–87	5–13	72–84	8–16

[a] Y_s and Y_f are disease incidence on the day of the first assessment and final disease incidence, respectively (%); Y_{40} or Y_{75} is an estimate of fruit incidence on day 40 and of leaf incidence on day 75(%); β is the relative rate parameter (day^{-1}); θ is the 'absolute' rate parameter ($\beta \cdot Y_f/4$) (% day^{-1}); M is the inflection point, measured in days; standardised area under the disease progress curve ($AUDPC_S$) estimated directly from the data with the midpoint rule for area estimation and divided by T_t, the duration of the epidemic in days from 1 June until 15 September (%-days day^{-1}).

[b] Y_{sf} = final leaf or fruit severity; Y_{if}= final leaf and fruit incidence.

[c] Mimimum (left) and maximum (right) factor loadings (in absolute values) obtained from separated factor analyses for fruit and leaf incidences or severities for each treatment class and year.

[d] Values in bold are the highest factor loadings, which are above the critical value (0.35) suggested by Kranz (1968).

characterization of the epidemic in organic production selected by correlation and factor analyses (Table 13.2; Holb et al., 2005b).

13.4 Management Options for Delaying Scab Epidemics

Summer scab epidemic studies in organic production showed that the epidemic starts at such a high level in the beginning of the summer, that inoculum reduction in the spring can have a determining role in the successful disease control (Holb, 2000; Holb et al., 2003a, 2005b). Based on this, evaluations of orchard sanitation practices against apple scab were started in organic apple orchards. Sanitation methods for reducing the primary inoculum sources (ploughing of leaves, leaf collection, leaf shredding, urea application) have already been studied earlier in conventional and integrated management systems (MacHardy, 1996; Sutton et al., 2000). These studies pointed out that only those sanitation practices which reduce the scab inoculum pressure by 50% can be applied successfully in commercial apple orchards. Studies of MacHardy (1996) and Sutton et al. (2000) proved that leaf shredding satisfied this criterion. However, organic orchards with high disease pressure required a higher efficacy of sanitation practices against apple scab which also have an impact on summer scab development.

Holb et al. (2004a,b) designed an experiment based on the principle that the scab inoculum sources overwintering in infected leaf litter should be removed as completely as possible for an efficient reduction of spring scab epidemics. For this purpose, overwintered leaves were collected with a flail mower, then the orchard floor was covered with a plastic foil, which served as a barrier between the air and the inoculum on the floor. With this procedure, authors were able to reduce sexual inoculum sources by 100%; however, airborne ascospores from the neighbouring orchards could enter, against which this elimination method offered no protection. Aylor (1998) demonstrated that long range transport of ascospores of *V. inaequalis* can be over 5 km and Holb et al. (2004b) demonstrated that maximum spreading distance of ascospores – which can result in an infection – is 70 m within an orchard. Accordingly, one can suppose that sanitation methods can be applied successfully in organic orchards if we take into consideration the 70 m ascopore spread within orchards and no long-range transportation of ascospores occurs. Although leaf collection combined with foil cover of the orchard had an excellent efficacy, it cannot be applied in the practice due to high material and labor costs (Holb et al., 2004a,b). So, a cheaper procedure was needed which can be applied in organic production and can be performed simultaneously with other tasks of fruit production management. Holb (2006a, 2007b) demonstrated results on efficacy of several sanitation practices on apple scab comparing integrated and organic orchards. The author's objectives were to determine how foil cover, leaf collection, leaf shredding, burying, mulching, and lime sulphur treatments and their combinations reduce the aerial ascospore concentration and the number of scab symptoms. Results showed that leaf collection alone or in combination with mulching resulted in higher than 50% reduction of the disease

pressure, which was higher than the efficacy of leaf shredding. Holb (2007b) also demonstrated how these sanitation methods can be incorporated into the general production technology. For instance, a leaf collector adapter, which is commercially available to most tractors can be used during general orchard management practices and/or a combination of leaf collector adapter with disc-tilling can increase scab reduction efficacy from 65% to 75%. Holb (2006a, 2007b) evaluated leaf removal as currently the best sanitation option against apple scab in commercial organic apple orchards. In addition, collection of leaves can be supplemented by composting and the compost then can be used as natural manure for the nutrient supply of the organic orchard (Holb, 2006a). This recommendation would have a great priority in organic orchards where only pesticide-free farmyard manure or compost can be used as an orchard nutritional supply (Anonymous, 1989).

13.5 Studies for Copper Replacement

The basis of the fungicide treatment developed against overwintering conidia was copper (Holb et al., 2004a), which is considered the most effective active ingredient permitted in organic production. After the banning of copper, copper replacement had to be solved on a scientific basis and a viable alternative should be offered to growers. Testing of several active ingredients approved in organic production showed that 2% lime sulphur (with the active ingredient Ca-polysulphide) in combination with 2–4% elemental sulphur showed a similar efficacy against apple scab to that of copper fungicides (Holb and Heijne, 2001). In the course of the experiment, a post-infection activity of Ca-polysulphide against *V. inaequalis* was also shown (Fig. 13.5). A field study verified that Ca-polysulphide has a 15–25 h

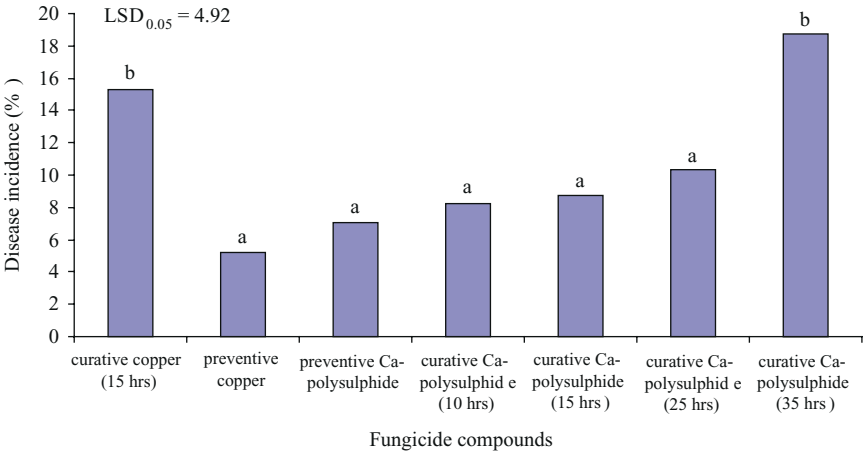


Fig. 13.5 Post-infection efficacy of Ca-polysulphide against apple scab in comparison with preventive application of copper and Ca-polysulphide compounds. LSD is least significant difference (after Holb et al. (2003b))

post-infection activity (Holb et al., 2003b) which was re-justified later in a laboratory study (Montag et al., 2005).

Application of Ca-polysulphide in 25 h post-infection schedule raised the option of using a scab warning system in organic apple orchards. This was a new highlight in organic disease management as previously only preventive fungicides of low efficacy could be applied in organic production. A field study of Holb et al. (2003b) showed that post-infection application of lime sulphur according to a computer-based scab warning system resulted in a more accurate timing of the fungicide applications and reduced the number of sprays against the pathogen.

13.6 Newly Developed Scab Management Decision Support System in Organic Apple Production

Previous research on scab epidemiology verified that several epidemiological characteristics are different in organic apple production compared to integrated and/or conventional production systems (Sections 13.2 and 13.3). Moreover, it became obvious that the results described above (e.g. the role of overwintering conidia in epidemics and their control options, the role of pruning, spreading of spores within an orchard, application of new sanitation methods, copper replacement, scab forecasting based on the post-infection activity of lime sulphur) modified the management decisions, which called for a re-thinking of system analysis and a separation of organic disease management from integrated and/or conventional production. According to this, a new simulation management decision model was developed specifically for organic apple production (Holb et al., 2005b; Holb, 2006b) as described below.

The new features of the decision support model were based on the research detailed in Sections 13.2–13.4 (e.g. results on overwintering conidia, pruning, spore dispersal, sanitation methods and Ca-polysulphide application). The scheme of the model developed by Holb et al. (2005b) is illustrated in Fig. 13.6, in which three disease variables (*AUDPC*, Θ, and Y_{75}) were proposed for organic orchards. The time of Y_{75} indicates the time until conidia could be entrapped inside budscales. However, a considerable amount of conidia can overwinter if scab incidence in the previous autumn is above 40%. In addition, the risk of early scab epidemics is highly dependent upon *AUDPCs* and Θ values of the preceding summer. Therefore, three parameters should be incorporated into a current scab warning system for organic disease management: (a) Y_{75} as the time for bud closure for cv. Jonagold; (b) previous year autumn scab incidence (40%) as minimum threshold criterion for overwintering conidia; and (c) minimum values of *AUDPCs* and Θ for calculating the present year epidemic intensity until the tree reaches day Y_{75} (Fig. 13.6). The effect of spray application can be modelled based on the above factors in order to suppress conidial entrapment as much as possible. If it is successful and until mid-October the orchard has a lower level of scab incidence than 40%, then no further control is needed against overwintering

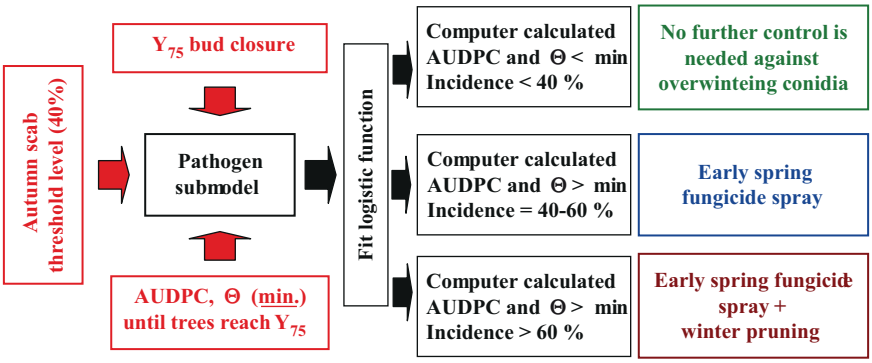


Fig. 13.6 Possible implications of the most important disease parameters (AUDPC – Area Under the Disease Progress Curve; theta (Θ) – the absolute rate of disease progress; Y_{75} – the time for bud closure for cultivar 'Jonagold') in a decision support model for apple scab management in the organic production system (after Holb et al. (2005b))

conidia. However, if autumn scab incidence reaches 40% until mid-October and *AUDPCs* and Θ are higher than the minimum, then an additional copper spray should be applied at bud burst next spring. In some countries where copper is banned, it may be replaced by lime sulphur (Holb and Heijne, 2001; Holb et al., 2003b). Probably after the 60% autumn scab level, an additional control practice (winter pruning at dormant bud stage) is also advisable (Holb et al., 2004a, 2005a) (Fig. 13.6). Moreover, in such a case, infection arising from overwintered leaves is also high; therefore, sanitation procedures should also be followed according to the study of MacHardy et al. (1993).

Based on the model, a practical scab management strategy could be developed which integrates several chemical and non-chemical control tools. However, Holb et al. (2003a) revealed that the Zadoks threshold theory for timing final sprays was not applicable on scab susceptible cultivars in organic production, so the scab management strategy specific for organic production could only be developed if further studies were performed on cultivars with different scab susceptibility. In order to consider this, a study of Holb (2008) combined potential management options (fungicide treatments, sanitation methods – leaf removal, winter pruning) using different starting and end dates of chemical spray applications on cultivars with different scab susceptibility. Results of the study showed that the decision support model of Holb et al. (2005b) can only be used in such scab management strategies which apply cultivars with low and moderate susceptibility to scab. (1) On cultivars with low scab susceptibility, the management strategy based on the decision support model can be successfully applied if the fungicide treatments are applied from pink bud until mid-July. With this strategy the 1% fruit incidence at harvest can be maintained. (2) On cultivars with moderate scab susceptibility, the management strategy based on forecasting can be successful if fungicide treatments start from tight cluster and end at mid-August under Hungarian climate conditions (Holb, 2007a, 2008).

13.7 Conclusions and Future Trends

Evaluations of the above described scab management strategies showed that overall sprays against diseases could be reduced with 15–25% in organic apple orchards. However, there are still several efficacy problems in fungal disease management strategies in organic apple orchards, so further improvements are needed for integrating chemical, cultural and biological control methods in a much more effective way. An urgent task is to develop effective non-chemical control options that are practically feasible and can be incorporated easily into the orchard management practices of organic apple production. These new technological elements must result in acceptable yield and fruit quality parameters. Until these tasks are achieved, an essential change in the current status of organic apple production cannot be expected.

Acknowledgements The author thanks Dr. B. Heijne, Prof. Dr. M. J. Jeger, J. Holb and F. Abonyi for their excellent scientificúcooperation. This research was partly supported by grants of the Hungarian Scientific Research Fund and the NKTH-OM-00227/2008 as well as by a János Bolyai Research Fellowship awarded to I. J. Holb.

References

Anonymous (1989) Basic standards for organic agriculture. Tholey-Theley, New York, USA

Anonymous (2000) IFOAM basic standards for organic production and processing. Tholey-Theley, New York, USA

Aylor DE (1998) The aerobiology of apple scab. Plant Dis 82:838–849

Becker CM, Burr TJ, Smith CA (1992) Overwintering of conidia of *Venturia inaequalis* in apple buds in New York orchards. Plant Dis 76:121–126

Bellon S, Lescourret F, Calmet JP (2001) Characterisation of apple orchard management systems in a French Mediterranean Vulnerable Zone. Agronomie 21:200–213

Beresford RM, Manktelow DWL (1994) Economics of reducing fungicide use by weather-based disease forecasts for control of *Venturia inaequalis* in apples. NZ J Crop Hortic Sci 22:113–120

Bower CC, Penrose LJ, Dodds K (1993) A practical approach to pesticide reduction on apple crops using supervised pest and disease control – preliminary results and problems. Plant Prot Quart 8:57–62

Cook RTA (1974) Pustules on wood as sources of inoculum in apple scab and their response to chemical treatments. Ann Appl Biol 77:1–9

Ferron P, Deguine JP (2005) Crop protection, biological control, habitat management and integrated farming. A review. Agronomy Sustainable Dev 25:17–24

Hill SA (1975) The importance of wood scab caused by *Venturia inaequalis* (Cke) Wint. as source of infection for apple leaves in the spring. J Phytopathol 82:216–223

Holb IJ (2000) Disease progress of apple scab caused by *Venturia inaequalis* in environmentally friendly growing systems. Intern J Hortic Sci 6(4):56–62

Holb IJ (2005a) Effect of pruning on apple scab in organic apple production. Plant Dis 89:611–618

Holb IJ (2005b) Effect of pruning on disease incidence of apple scab and powdery mildew in integrated and organic apple production. Intern J Hortic Sci 11(1):57–61

Holb IJ (2006a) Effect of six sanitation treatments on leaf decomposition, ascospore production of *Venturia inaequalis* and scab incidence in integrated and organic apple orchards. Eur J Plant Pathol 115:293–307

Holb IJ (2006b) Scab control strategies in environmentally-benign apple production systems (Review). Phytopathologica Polonica 39:31–39
Holb IJ (2007a) Classification of apple cultivar reactions to scab in integrated and organic apple production systems. Can J Plant Pathol 29:251–260
Holb IJ (2007b) Effect of four non-chemical sanitation treatments on leaf infection by *Venturia inaequalis* in organic apple orchards. Eur J Hortic Sci 71:60–65
Holb IJ (2008) Timing of first and last sprays against apple scab combining with leaf removal and pruning in organic apple production. Crop Prot 27:814–822
Holb IJ, Heijne B (2001) Evaluating primary scab control in organic apple production. Gartenbauwissenschaft 66:254–261
Holb IJ, Heijne B, Jeger MJ (2003a) Summer epidemics of apple scab: the relationship between measurements and their implications for the development of predictive models and threshold levels under different disease control regimes. J Phytopathol 151:335–343
Holb IJ, de Jong PF, Heijne B (2003b) Efficacy and phytotoxicity of lime sulphur in organic apple production. Ann Appl Biol 142:225–233
Holb IJ, Heijne B, Jeger MJ (2004a) Overwintering of conidia of *Venturia inaequalis* and the contribution to early epidemics of apple scab. Plant Dis 88:751–757
Holb IJ, Heijne B, Withagen JCM, Jeger MJ (2004b) Dispersal of *Venturia inaequalis* ascospores and disease gradients from a defined inoculum source. J Phytopathol 152:639–646
Holb IJ, Heijne B, Jeger MJ (2005a) The widespread occurrence of overwintered conidial inoculum of *Venturia inaequalis* on shoots and buds in organic and integrated apple orchards across the Netherlands. Eur J Plant Pathol 111:157–168
Holb IJ, Heijne B, Withagen JCM, Gáll JM, Jeger MJ (2005b) Analysis of summer epidemic progress of apple scab in different apple production systems in the Netherlands and Hungary. Phytopathology 95:1001–1020
Holb IJ, Heijne B, Jeger MJ (2006) Effects of a combined sanitation treatment on earthworm populations, leaf litter density and infection by *Venturia inaequalis* in integrated apple orchards. Agric Ecosyst Environ 114:287–295
Keitt GW (1936) Some problems and principles of orchard disease control with special reference to sanitation and related measures. J Econ Entomol 29:43–52
Keitt GW, Jones LK (1926) Studies of the epidemiology and control of apple scab. Univ Wisconsin Agric Exp Station Res Bullet 73:1–104
Köller W, Parker DM, Reynolds KL (1991) Baseline sensitivities of *Venturia inaequalis* to sterol demethylation inhibitors. Plant Dis 75:726–728
Kranz J (1968) Eine Analyse von annuellen Epidemien pilzlicher Parasiten. III. Über Korrelationen zwischen quantitativen Merkmalen von Befallskurven und Ähnlichkeiten von Epidemien. J Phytopathol 61:205–217
Lancon J, Wery J, Rapidel B, Angokaye M, Gérardeaux E, Gaborel C, Ballo D, Fadegnon B (2007) An improved methodology for integrated crop management systems. Agronomy Sustainable Dev 27:101–110
MacHardy WE (1996) Apple scab, biology, epidemiology and management. The American Phytopathology Society, St. Paul, USA
MacHardy WE, Gadoury DM, Rosenberger DA (1993) Delaying the onset of fungicide programs for control of apple scab in orchards of low potential ascospore dose of *Venturia inaequalis*. Plant Dis 77:372–375
MacHardy WE, Gadoury DM, Gessler C (2001) Parasitic and biological fitness of *Venturia inaequalis*: relationship to disease management strategies. Plant Dis 85:1036–1051
Montag J, Schreiber L, Schönherr J (2005) An in vitro study on the postinfection activities of hydrated lime and lime sulphur against apple scab (*Venturia inaequalis*). J Phytopathol 153:485–491
Penrose LJ (1995) Fungicide reduction in apple production potentials or pipe dreams? Agric Ecosyst Environ 53:231–242
Reganold JP, Glover JD, Andrews PK, Hinman HR (2001) Sustainability of three apple production systems. Nature, London 410:926–930

Sansavini S (1990) Integrated fruit growing in Europe. HortScience 25:842–846
Sansavini S (1997) Integrated fruit production in Europe: research and strategies for a sustainable industry. Sci Hortic 68:25–36
Sansavini S, Wollesen J (1992) The organic farming movement in Europe. Hortic Technol 2:276–281
Sutton DK, MacHardy WE, Lord WG (2000) Effect of shredding or treating apple leaf litter with urea on ascospore dose of *Venturia inaequalis* and disease buildup. Plant Dis 84:1319–1326
Van der Scheer HATh (1992) Management of scab and powdery mildew on apple with emphasis on threshold values for control of both diseases. Acta Phytopathologica et Entomologica Hungarica 27:621–630
Vincent C, Rancourt B, Carisse O (2004) Apple leaf shredding as a non-chemical tool to manage apple scab and spotted tentiform leafminer. Agric Ecosyst Environ 104:595–604
Vogt G (2000) Origins, development and future challenges of organic farming. Proceedings of 13th international congress of International Federation of Organic Movements (IFOAM), August 24–30, Basel, Switzerland, pp 708–711
Weibel F (2002) Organic fruit production in Europe. Compact Fruit Tree 35(3):77–82
Weibel F, Häseli A (2003) Organic apple production – with emphasis on European experiences. In: Ferree DC, Warrington IJ (eds) Apples: botany, production and uses. CABI, Wallingford, UK, pp 551–583

Part III
Natural Compounds

Chapter 14
Exploitation of Natural Compounds in Eco-Friendly Management of Plant Pests

N.K. Dubey, Ashok Kumar, Priyanka Singh, and Ravindra Shukla

Abstract The intensive use of synthetic pesticides and their environmental and toxicological risks have generated increased global interest to develop alternative sources of chemicals to be used in safe management of plant pests. Recently, in different parts of the world, attention has been paid towards exploitation of higher plant products as novel chemotherapeutics for plant protection because they are mostly non phytotoxic and easily biodegradable. Currently, different plant products have been formulated for large scale application as botanical pesticides in eco-friendly management of plant pests and are being used as alternatives to synthetic pesticides in crop protection. These products have low mammalian toxicity and are cost effective. Such products of higher plant origin may be exploited as eco-chemical and biorational approach in integrated plant protection programmes. The current status and future prospects of botanical pesticides in eco-friendly management of different plant pests are reviewed and discussed.

Keywords Biorational • Botanical pesticides • Eco-chemical • Eco-friendly • Pests

14.1 Introduction

The continuous growth of the world's population requires substantial resources for the production of food. One of the greatest challenges is the production of enough food for the growing population. Agriculture is the driving force for broad-based economic growth in developing countries where food demand is particularly critical because of the slow rate of net food production in relation

N.K. Dubey (✉), A. Kumar, P. Singh, and R. Shukla
Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi, 221005, India
e-mail: nkdubey2@rediffmail.com

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,*
Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_14,

to population growth. Compared to temperate zones tropical and sub-tropical regions have a greater potential for food production and can grow multiple crops annually. On the other hand, based on congenial climatic conditions and the particular environment, agriculture in tropical and subtropical countries suffers from severe losses due to pests (Roy, 2003). Heavy rains and flash floods are very common in some tropical and subtropical countries which enhance the moisture content of grain thus being more vulnerable to different pests and pathogens (Wakdikar, 2004). Despite the use of all means of plant protection, approximately one-third of the global food production is estimated to be destroyed annually by pests and pathogens contamination (Varma and Dubey, 1999; Arthur and Thorne, 2003). Even today losses can even lead to famine in some countries which are densely populated. In addition to fungal contamination, considerable insect damage to stored food grains has been reported in countries where modern storage technologies have not been introduced (Shaaya et al., 1997). Production of mycotoxins by several fungi has added a new dimension to the gravity of post harvest problems of food commodities. About 4.5 billion persons living in developing countries are chronically exposed to mycotoxins. Aflatoxin contamination is represented in six of the ten most important health risks identified by WHO for developing countries (Williams et al., 2004). Hence, quality and safety of stored food commodities have to be guaranteed by avoiding fungal and insect infestations as well as mycotoxin production by storage fungi.

14.2 Synthetic Pesticides and Control of Plant Pests

In order to minimize crop losses caused by pests and diseases, pesticides are being used widely. In the early 1940s and following decades, the discovery of insecticides such as DDT, BHC (Benzene hexachloride), chlorinated cyclodienes, organophosphates and carbamates made a major step forward in the field of plant protection. The use of synthetic pesticides has undoubtedly resulted in increased crop production and achievement of green revolution by different countries. On the other hand, the intensive application of such synthetic pesticides has raised environmental and toxicological concerns, especially questions on hormonal imbalance (Omura and Hirata, 1995; Pandey, 2003), poisoning of applicators and wildlife, groundwater contamination and residue in food. Methyl bromide, a widely used fumigant for insect pest control in stored products has been reported to cause ozone depletion (WMO, 1991; Lee et al., 2001a) and phosphine induces the development of resistance to certain pests (Subramanyam and Hagstrum, 1995). Due to the development of resistance in a number of pathogenic fungi, failures of disease control have been reported for the benzimidazole fungicides such as benomyl, thiabendazole, carbendazim and thiophanate methyl (Wilson et al., 1997). The number of insects resistant to certain pesticides has increased

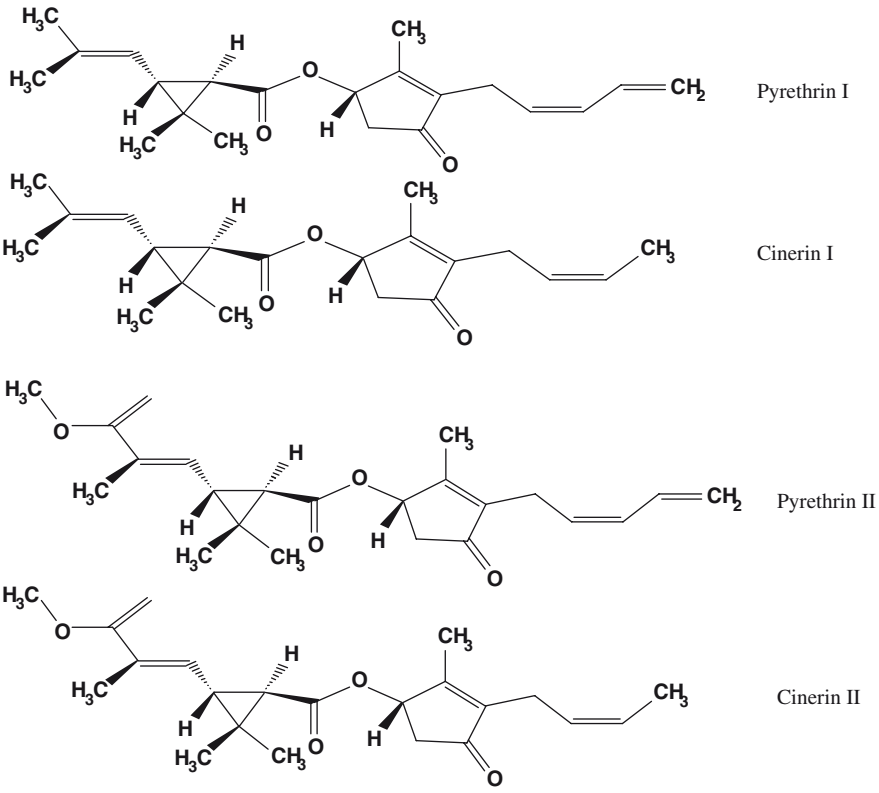
significantly over the last years. Hence, there is an urgent need to develop alternative sources of chemicals to be used in the management of plant pests without causing unacceptable side effects to the environment and mammals after their application (Sahaf et al., 2007).

14.3 Botanical Pesticides in Agricultural Pest Management

Recently, attention has been paid towards the exploitation of higher plant products as novel chemotherapeutics in plant protection. Because most of them are not phytotoxic, easily biodegradable and sometimes stimulatory to the host metabolism, plant products possess a high potential for pest management (Mishra and Dubey, 1994). Higher plants contain a wide spectrum of secondary metabolites such as phenolics, flavonoids, quinones, tannins, essential oils, alkaloids, saponins and sterols. Such plant-derived chemicals may be exploited for their different biological properties (Tripathi et al., 2004). Many of these are thought to defend the plants producing them against herbivores and pathogens (Isman and Akhtar, 2007). Therefore, higher plants can be exploited for the discovery of new insecticides or novel structures that could serve as lead compounds in pesticide development. Such structures produced by plants are thought to have novel modes-of-action as natural insecticides (Regnault-Roger et al., 2005). Used widely until the 1940s, natural pesticides were partly displaced by synthetic pesticides that at the time seemed easier to handle and longer lasting. In the presence of sunlight botanical pesticides may break down into harmless compounds within hours or days. They are expected to be decomposed as easily as the plants they derive from by a variety of microbes common in most soils. Because of greater consumer awareness and concerns regarding synthetic chemicals, food preservation against insects with natural additives is becoming more popular (Kumar et al., 2007b). Such plant products have also been formulated for large scale application in crop protection.

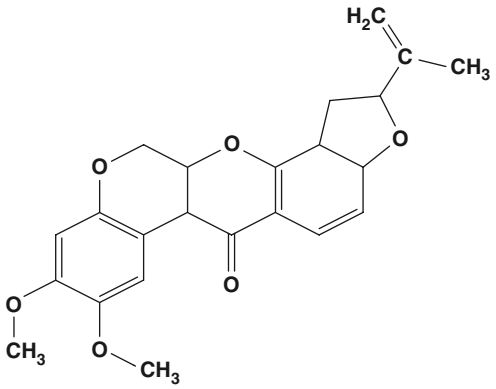
14.3.1 Botanical Pesticides in Current Use

Pyrethrum Pyrethrum is one of the oldest and safest insecticides which was extracted from the flowers of *Chrysanthemum* grown in Kenya and Ecuador. The ground, dried flowers were used in the early nineteenth century to control body lice during the Napoleonic Wars. Pyrethrum is a mixture of four compounds: pyrethrins I and II and cinerins I and II (chemical structures see below; Ware, 2002). As pure compounds pyrethrins are moderately toxic to mammals (oral acute LD_{50} values for rats range from 350 to 500 mg kg^{-1}), but technical grade pyrethrum is considerably less toxic (≈1,500 mg kg^{-1}) (Casida and Quistad, 1995).



Mode of Action Like DDT, Pyrethrum is an axonic poison affecting the electrical impulse transmission along the axons which are the elongated extensions of the neuron cell body. It affects both the peripheral and central nervous system of insects. Pyrethrum initially stimulates nerve cells to produce repetitive discharges, leading ultimately to paralysis of insects. These effects are produced in the insect nerve cord which contains ganglia and synapses, as well as in giant nerve fibre axons. Pyrethrum has a greater insecticidal effect at low temperature. The compounds are susceptible to enzymatic degradation by heat and light. Unless they are formulated in mixture with synergists, most of the paralyzed insects recover once again. In order to retain their activity pyrethrins are mixed (1:4) with natural synergists such as piperonyl butoxide derived from sassafras or *n*-octyle bicycloheptane dicarboximide (Ware, 2002).

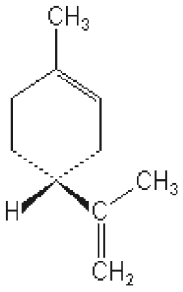
Rotenone Rotenone or rotenoids are produced in the roots of *Derris* and *Lonchocarpus* grown in South America. They were used for the last century as stomach and contact insecticides to control leaf-eating caterpillars. Initially rotenone was used in South America to paralyze fish causing them to come towards surface and be easily captured (Ware, 2002). Pure rotenone is comparable to synthetic insecticides in terms of acute toxicity to mammals (oral LD_{50} for rats is 132 mg kg^{-1}) (Isman, 2006).



Rotenone

Mode of Action Rotenone is a respiration inhibitor affecting the electron flow between NAD^+ (a coenzyme involved in oxidations and reductions in metabolic pathways) and coenzyme Q (a respiratory enzyme responsible for carrying electrons) (Ware, 2002).

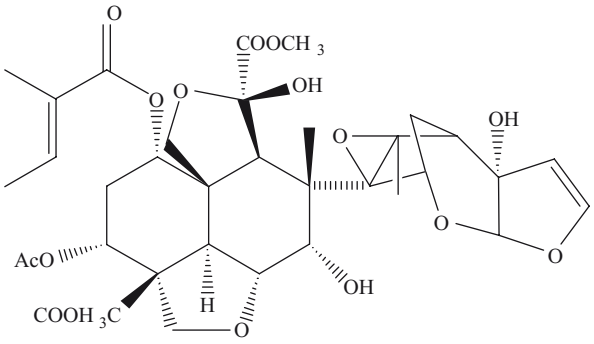
Limonene Limonene or d-Limonene, extracted from citrus peel, is the latest addition to the botanical pesticides. Limonene belongs to a group of compounds often called florals or scented plant chemicals. It is effective against external pests of pets including fleas, lice, mites and ticks, and is nontoxic to mammals. Limonene constitutes about 98% of the orange peel oil by weight (Ware, 2002). Two other recently introduced floral products are eugenol (oil of clove) and cinnamaldehyde (derived from Ceylon and Chinese cinnamon oils). Both compounds are generally regarded as safe to mammals (GARS) by the United States Food and Drug Administration (Ware and Whitacre, 2004).



Limonene

Mode of Action of Limonene Its mode of action is similar to that of pyrethrum. It affects the sensory nerves of the peripheral nervous system of insects.

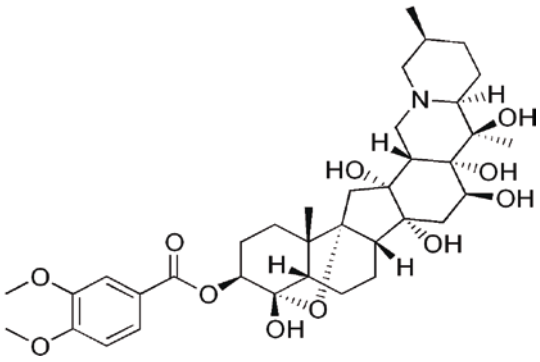
Neem From prehistoric times, neem has been used primarily against household and storage pests and to some extent against pests related to field crops in the Indian subcontinent. It was a common practice in rural India to grind and use dried leaves of the neem tree *Azadirachta indica*. Azadirachtin (molecular formula: $C_{35}H_{44}O_{16}$, chemical structure see below) is a highly oxidised triterpenoid, it is the most widely publicized bioactive molecule in neem. It is well known as an insect growth inhibitor which affects feeding and moulting in a wide variety of insects. Neem contains several other bioactive ingredients such as salanin, nimbin, nimbidin, meliantriol belonging to the tetranortriterpenoids (TNTT) which are used both as pesticides and pharmaceuticals. When present in pure form, azadirachtin degrades rapidly in the presence of moisture and light. However, azadirachtin is stable when formulated in neem oil medium together with the other natural products of neem. Hence, it is preferable to use neem oil enriched with azadirachtin as a stable feed stock for making pesticide formulations. Azadirachtin has shown some rather extraordinary insecticidal, fungicidal and bactericidal properties, including insect growth regulating qualities. Azadirachtin is considered non-toxic to mammals (oral acute LD_{50} for rats is >5,000 mg kg^{-1}), fish (Wan et al., 1996), and pollinators (Naumann and Isman, 1996).



Azadirachtin

Mode of Action Azadirachtin disrupts molting of insects by inhibiting biosynthesis or metabolism of ecdysone, the juvenile molting hormone.

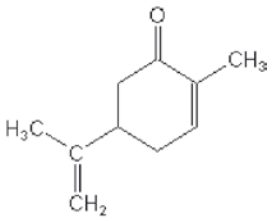
Sabadilla Sabadilla is derived from the ripe seeds of *Schoenocaulon officinale* (Liliaceae), also known as cevadilla, a tropical lily that grows in Central and South America (Soloway, 1976). The plant produces veratrine alkaloids that are insecticidal. The mode of action of sabadilla is similar to that of pyrethrins. Sabadilla was used historically for the control of insects on crops, animals and humans (Allen et al., 1944). The insecticidal alkaloids in sabadilla degrade rapidly in air and sunlight, resulting in only very short residual control after application.



Veratrine

Sabadilla is commonly used in organic fruit and vegetable production against squash bugs, harlequin bugs, caterpillars, leaf hoppers, and stink bugs. It is highly toxic to honeybees and should only be applied in the evening after bees have returned to their hives.

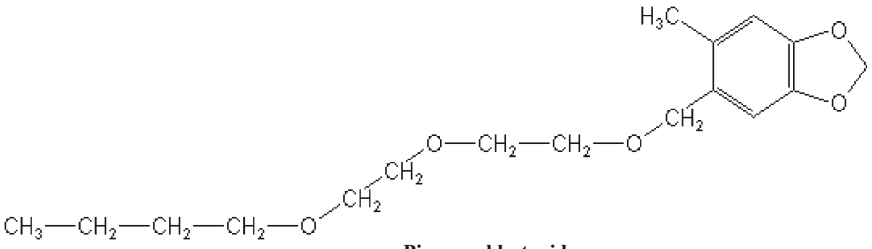
Carvone It is a monoterpene standardised from essential oil of *Carum carvi* used as botanical pesticide with the trade name TALENT in The Netherlands. Carvone inhibits sprouting of potato tubers during storage and protects them from bacterial rotting without exhibiting mammalian toxicity. Thus, it enhances the shelf life of stored fruits and vegetables and inhibits microbial deterioration without altering the taste and odour of the fruits after treatment (Varma and Dubey, 1999).



Carvone

14.3.2 Higher Plant Products as Synergists or Activators of Pesticides

Synergists are compounds which are not toxic or insecticidal when used alone, but synergize or enhance the activity of insecticides when used in mixture with them. Piperonyl butoxide is a synergist for a wide variety of insecticides. It is frequently used to increase the effectiveness of pyrethrum (Ware and Whitacre, 2004; Kakko et al., 2000). Piperonyl butoxide contains the methylenedioxyphenyl moiety; a molecule found in sesame oil, hence named sesamin. The oral LD_{50} for rats is 6,150 mg kg^{-1} and for mice 3,800 mg kg^{-1}.



Piperonyl butoxide

Mode of Action Piperonyl butoxide inhibits cytochrome P-450 dependent polysubstrate monooxygenases (PSMOs), which are produced in microsomes of the liver of mammals and in certain insect tissue. The PSMOs bind to the enzymes that degrade pyrethrum and rotenone. Piperonyl butoxide is not insecticidal, but binds to the oxidative enzymes preventing them from degrading the toxicants (Ware, 2002).

14.3.3 Essential Oils

Among the different plant products, essential oils are a very attractive method for crop protection. Production of essential oils by plants is believed to be predominantly a defence mechanism against pathogens and pests (Oxenham, 2003). Essential oils and their components are gaining increasing interest because of their relatively safe use and potential for multi-purpose functional use (Feng and Zheng, 2007). The development of resistant strains of fungi and insects against essential oils may not become a real issue as it is the case for many synthetic pesticides because several active components are often present in the final product and synergistic interactions may exist between the different components of the oils (Dubey et al., 2006). Most of the components of essential oils are specifically active against particular insect groups (Huang et al., 1997; Isman, 2000). Many essential oils are less persistent in their insecticidal activity (Shaaya et al., 1997; Ngamo et al., 2001), readily biodegradable (Baysal, 1997) and less detrimental to non-target organisms as compared to synthetic pesticides. Essential oils are mostly a mixture of many different volatile compounds, their composition often varies between species (Mishra and Dubey, 1994) and exert differential effects depending on both the mode of action and the target pest (Liu et al., 2006). They have been tested against a wide range of pests such as food spoiling organisms (Janssen et al., 1988; Ouattara et al., 1997), food poisoning organisms (Deans and Ritchie, 1987; Lis-Balchhin and Deans, 1997), and mycotoxigenic filamentous fungi (Knobloch et al., 1989) and pathogenic and dimorphic yeasts (Ghannoum, 1988). Essential oils of many edible and medicinal plants are also used as food preservatives and in different pharmaceutical preparations (Holley and Patel, 2005). They are in many cases endowed with antimicrobial, allelopathic, antioxidant and bioregulatory properties (Caccioni and Guizzardi, 1994; Holley and Patel, 2005). The volatility,

ephemeral nature and biodegradability of flavour compounds of angiosperm may be especially advantageous for treatment of food commodities because only low residues can be expected. Some essential oils acquired through the diet are actually beneficial to human health (Huang et al., 1994).

Dubey et al. (1983) demonstrated the efficacy of essential oils of *Ocimum canum* and *Citrus medica* as volatile fungitoxicants for the protection of some spices against post harvest fungal deterioration. Essential oils of *Cymbopogon citratus*, *Caesulia axillaris* and *Mentha arvensis* have shown *in vivo* fumigant activity for the control of storage fungi of some cereals (Mishra et al., 1992; Varma and Dubey, 2001). Essential oils are generally volatile substances composed of mono- and sesqui-terpenoids, aldehydes, esters, acids, ketones, alcohols, coumarins. Their composition varies within the same species as a result of genetic and environmental factors. Numerous studies have documented the antifungal (Suhr and Nielsen, 2003; Elgayyar et al., 2001) and antibacterial (Canillac and Mourey, 2001) effects of plant essential oils.

Essential oils of *Thymus serpyllum* (rich in thymol and carvacrol) and *Origanum majorana* (rich in terpinen-4-ol) were very effective when treated as fumigants against the bean weevil *Acanthoscelides obtectus* (Bruchidae) (Regnault-Roger et al., 1993). Shaaya et al. (1991) evaluated the fumigant toxicity of 28 essential oils and 10 of their major constituents against four different species of stored product coleopterans. Some of the oils showed insecticidal activity against a broad spectrum of pests. Sarac and Tunc (1995) investigated the fumigant activity of four essential oils against three species of stored product pests. A number of investigations (Ho et al., 1997; Huang et al., 1998) have demonstrated contact, fumigant and antifeedant effects of a range of essential oil constituents (cinnamaldehyde, a-pinene, anethole), as well as extracts of cloves (*Syzygium aromaticum*) and star anise (*Illicium verum)* against the red flour beetle (*Tribolium castaneum*) and the maize weevil (*Sitophilus zeamais*). Eugenol, the major constituent of oil of cloves and holy basil, *Ocimum suave*, was shown to be effective against these and two additional coleopterans, *S. granarius* and *Prostephanus truncatus* (Obeng-Ofori and Reichmuth, 1997). There is evidence that certain essential oils and their constituents are effective also against *Varroa jacobsoni*, an ectoparasite of the honey bee (Calderone et al., 1997).

Essential oils and their combinations were also useful as fumigants for the protection of stored wheat against storage fungi (Weaver and Subramanyam, 2000) and of rice against the rice weevil (Lee et al., 2001b). Major constituents from aromatic plants, mainly monoterpenes, are of special interest to industrial markets because of their insecticidal, anti-bacterial, anti-fungal and anti-inflammatory activities (Isman, 2000; Shakarami et al., 2003; Negahban and Moharramipour, 2005). Perhaps the most attractive aspect of using essential oils and/or their constituents for pest management is their favourable mammalian toxicity (Shaaya and Kostjukovsky, 1998). Some of the essential oils are exempted from the usual data requirements for registration of pesticides particularly in the USA. American companies have recently taken advantage of this situation and have introduced essential-oil-based pesticides to the market. Mycotech Corporation produces Cinnamite TM, as an aphidicide/miticide/fungicide for glasshouse and horticultural crops, and ValeroTM, as a miticide/fungicide for use

in grapes, berry crops, citrus and nuts. Both products are based on cinnamon oil, with cinnamaldehyde (30% EC formulation) as the active ingredient (Isman, 2000). Recently, essential (volatile) oils have received increased attention due to a growing interest in the need for alternative techniques to assure quality and safety of perishable food (Burt, 2004; Holley and Patel, 2005).

14.3.4 Efficacy of Botanical Pesticides as Semiochemicals Against Agricultural Pests

Chemicals that deliver behavioural messages in the pests rather than killing them are termed semiochemicals. They may be exploited to manage agricultural pests in different ways.

14.3.4.1 Insect Growth Regulators

Insect growth regulators (IGRs) are chemical compounds that alter growth and development of insects. The IGRs disrupt insect growth and development in three different ways: As juvenile hormones, as precocenes and as chitin synthesis inhibitors. Juvenile hormones (JHs) include ecdysone (the molting hormone), JH mimics, JH analogues (JHAs), and are known as their broader synonyms, juvenoids and juvegens. They disrupt insect maturation and emergence as adults. Precocenes interfere with the normal function of glands that produce juvenile hormones while chitin synthesis inhibitors (e.g. synthetic pesticides as benzoylureas, buprofezin and cyromazine) affect the ability of insects to produce new exoskeletons when molting. Instead of exhibiting a killing effect, IGRs interfere with the normal mechanisms of development and cause the insects to die before reaching the adult stage. One famous JH is juvabione which was found in the wood of balsam fir. Its effect was discovered by accident when paper towels made from this source were used to line insect-rearing containers resulting in a suppression of insect development (Varma and Dubey, 1999). Analogues of insect juvenile hormones have been found including juvocimenes in *Ocimum basilicum* and juvabione in *Abies balsamea* (Balandrin et al., 1985).

14.3.4.2 Antifeedants and Attractants

The concept of antifeedants was discovered already in the 1970s with the demonstration of the potent feeding deterrent effect of azadirachtin and neem seed extracts in a large number of pest species. Indeed, a considerable amount of literature documents the antifeedant potential of neem. Under agronomical conditions, it is the physiological action of azadirachtin that is responsible for field efficacy of neem insecticides (Immaraju, 1998). Many natural plant chemicals deter insects from feeding (antifeedant effect), although none of them have been developed commercially

so far. Azadirachtin and limonoids such as limonin and nomilin originating from many different plant species in Meliaceae and Rutaceae (e.g. from *Citrus* fruits) have long been used successfully for insect control, especially in India. Azadirachtin has also some systemic properties as it also protects newly grown leaves of crop plants from feeding damage (Varma and Dubey, 1999).

Certain essential oil constituents are effective attractants for some insects. Geraniol and eugenol are used as lures in traps for the Japanese beetle *Popillia japonica* and methyl eugenol has been used to trap Oriental fruit fly *Dacus dorsalis* (Vargas et al., 2000). Cinnamyl alcohol, 4-methoxy-cinnamaldehyde, cinnamaldehyde, geranylacetone and a-terpineol are attractants for adult corn rootworm beetles (*Diabrotica* spp.) (Hammack, 1996; Petroski and Hammack, 1998).

14.3.4.3 Chemosterilants

The compound β-asarone extracted from rhizomes of *Acorus calamus*, possesses antigonadial activity. The vapour of the oil exhibits complete inhibition of ovarian development of different insects (Varma and Dubey, 1999). Therefore, oils showing chemosterilant activity may be utilised as botanical fumigants especially in the management of storage pests without running a big risk of selecting for physiological (resistant) races of the pest species. *Quassia amara* (Surinam Wood), belonging to the family Simaroubaceae, is a tree species naturally distributed in several tropical countries. Traditionally, the bark and leaves are used in herbal remedies and as medicine because the major secondary metabolites of this tree, quassin and neo-quassin exhibit pharmacological properties such as anti-malarial, anti-fungal, anti-ulcerative, anti-edimogenic and anti-cancer activity. The male reproductive system, particularly spermatogenesis, sperm maturation and androgen biosynthesis, are highly sensitive to the metabolites of *Q. amara* which would be useful for insect pest control but may also affect male reproduction in non-target organisms. Therefore, their pharmacological effects on mammals should be worked out before recommendation to avoid any handling problems with such chemicals.

14.3.5 Higher Plant Products as Inhibitors of Aflatoxin Secretions

Aflatoxins are toxic, carcinogenic, mutagenic, immunosuppressive and teratogenic agents produced as secondary metabolites by *Aspergillus flavus* and *A. parasiticus* (Kumar et al., 2008). Among 18 different types of aflatoxins identified, major members are B_1, B_2, G_1, G_2, M_1 and M_2. Aflatoxin B_1 is produced most abundantly and is also most toxic followed by G_1, B_2 and G_2. Aflatoxins B_1, B_2, G_1 and G_2 are classified as Group I human carcinogens, whereas M_1 is classified as Group 2B probable human carcinogen (Ioannou et al., 1999). FAO and WHO have imposed regulatory guidelines with a maximum limit of 20 ppb of total aflatoxins in food or

feed substrates. In some European countries, a maximum level of 5 ppb aflatoxin is tolerated (Krishnamurthy and Shashikala, 2006). Knowing the hazards of aflatoxin exposure, the need for protection of food and feed against aflatoxin contamination is universally recognized and several approaches have been suggested. Amongst them, powders and extracts of many spices, herbs and higher plants have been reported to inhibit the production of aflatoxin (Paranagama et al., 2003).

Some of the natural products, such as cinnamon and clove oil (Bullerman et al., 1977), phenols (Singh, 1983), some spices (Hasan and Mahmoud, 1993) and many essential oils (Mahmoud, 1994) have been reported as effective inhibitors of fungal growth and aflatoxin production. The extracts of several wild and medicinal plants have also been tested against aflatoxin-producing fungi (Bilgrami et al., 1980). Essential oils extracted from *Cymbopogon citratus*, *Monodora myristica*, *Ocimum gratissimum*, *Thymus vulgaris* and *Zingiber officinale* were investigated for their inhibitory effect against food spoilage and mycotoxin producing fungi such as *Fusarium moniliforme*, *Aspergillus flavus* and *A. fumigatus*. Recently, the essential oils of *Cinnamomum camphora* (Singh et al., 2008), *Thymus vulgaris* (Kumar et al., 2008) and *Pelargonium graveolens* (Singh et al., 2008) have been reported to inhibit aflatoxin B_1 secretion by different toxigenic strains of *A. flavus*. These effects against food spoilage and mycotoxin producing fungi indicated the potential of each essential oil as a food preservative.

14.4 Current Status and Future Prospects

In the context of agricultural pest management, botanical pesticides are well suited for use in organic food production and may play a much greater role in future in the production and postharvest protection of food in developing countries (Isman, 2006). It has been claimed that natural plant products may successfully replace synthetic fungicides and provide an alternative method to protect cereals, pulses and other agricultural commodities from aflatoxin B_1 contamination by *A. flavus* (Krishnamurthy and Shashikala, 2006; Mabrouk and El-Shayeb, 1992). Different crude extracts and plant materials rich in polyphenolics are becoming increasingly important in food industries because of their antifungal, antiaflatoxigenic and antioxidant activity (Kumar et al., 2007a). Hence, such plant chemicals can improve shelf-life, quality and nutritional value of stored food commodities (Tripathi and Dubey, 2004).

Plants often contain more than one bioactive chemical and the biological activity may be due to synergistic effects between different active components. They may impart different modes of action during their pesticidal action (Varma and Dubey, 1999). This in turn reduces the chances for multiple genomic mutations in insects and the subsequent development of resistance. Recent results have demonstrated combined larvicidal and antifeeding effects (Park et al., 1997; Larocque et al., 1999), delay in development and adult emergence combined with egg mortality (Marimuth et al., 1997), deterrent effects on oviposition (Naumann and Isman,

1995), and arrestant and repellent action on insect pests strengthening the future prospectives of plant derived chemicals as novel eco-friendly measures for the management of agricultural pests (Landolt et al., 1999; Moretti et al., 1998). Plants and their secondary metabolites are important source for useful biopesticides. The recognition of the important role of these compounds has increased, particularly in terms of resistance to pests and diseases.

Plants used in traditional medicine are generally considered as non-toxic to mammals. Therefore, such plant products, especially essential oils can be recommended also as botanical pesticides (Singh and Upadhyay, 1993). Antimicrobial compounds of plant origin are effective against pests mostly through diverse modes of action and can express several properties such as growth retardation (Breuer and Schmidt, 1995; Pavela, 2007), feeding deterrent (Klepzig and Schlyter, 1999; Wheeler and Isman, 2001) oviposition deterrent and reduction in fertility (Zhao et al., 1998; Muthukrishnan and Pushpalatha, 2001). Hence, more emphasis is currently being given to plant based pesticides in integrated pest management programmes. Such products may be recommended as safe antimicrobials in place of some rather toxic organophosphate insecticides such as tetraethylpyrophosphate, parathion and fonofos (Coats, 1994). The opinion of modern society towards 'green consumarism' (Tuley de Silva, 1996; Smid and Gorris, 1999) desiring fewer synthetic ingredients in food may favour the recommendation of plant based antimicrobials and herbal products 'generally recognized as safe' (GRAS) food additives and alternatives in eco-friendly management of plant pests.

However, pesticides derived from plants cannot be easily produced due to insufficient quantities of the required plant material. For the availability of sufficient raw material, emphasis should be given to easily growing wild plants. The vast majority of botanical pesticides originate from tropical plants, because many of them are chemically well described and plant–insect interactions have been investigated in depth (MacKinnon et al., 1997). In addition, as the chemical profile of plant species can vary depending on geographic, genetic, climatic, annual or seasonal factors, pesticide manufacturers must take additional steps to ensure that product quality will remain unchanged. Many agrochemical companies have paid attention to natural products as sources for the development of new pesticides (Addor, 1995). In comparison to synthetic pesticides the market for botanical pesticides is very small (less than 2% of the total market of nearly $30 billion for chemical crop protection) (Khambay, 2002). Until the late 1980s, the market for biopesticides was stable with sales of around $20–25 million per year, but continues to grow since then, although it has reached much lower figures than anticipated 10 or 20 years ago. By 1997, the market for biopesticides was $85–90 million and the projected growth was estimated to be around 10–15% per year. While the world wide market for synthetic pesticides is more or less stable (Franck et al., 2009), the biopesticide market is growing steadily increasing from $672 million in 2005 to an estimate of over $1 billion in 2010, at an average growth rate of 9.9% (http://www.ien.com/article/biopesticides-market-to/8648). Public concern about the impact of synthetic pesticides on the environment and non target species may generate increased interest in the use of botanical pesticides

Natural plant chemicals will undoubtedly play a significant role in the future for pest control in both industrialised and developing countries. Because of the need for new, safe pesticides, chemical products of higher plant origin may gain more interest for an eco-chemical approach in integrated pest management programmes. More botanical pesticides are expected to come into development and reach global market in the near future.

References

Addor RV (1995) Insecticides [C]. In: Godfrey CRA (ed) Agrochemicals from natural products. Marcel Dekker, New York, pp 1–63

Allen TC, Dicke RJ, Harris HH (1944) Sabadilla, *Schoenocaulon* spp., with reference to its toxicity to houseflies. J Econ Entomol 37:400–408

Arthur FH, Thorne JE (2003) Efficacy of diatomaceous earth to control internal infestations of rice weevil and maize weevil (Coleoptera: Curculinoidae). J Econ Entomol 95:510–518

Balandrin MF, Klocke JA, Wurtele ES, Bollinger WH (1985) Natural plant chemicals: sources of industrial and medicinal materials. Science 228:1154–1160

Baysal O (1997) Determination of microorganisms decomposing essential oils of *Thymbra spicata* L. var. *spicata* and effect of these microorganisms on some soil borne pathogens. M.Sc. Thesis, Akdenniz University, Antalya (in Turkish)

Bilgrami KS, Misra RS, Sinha KK, Singh A (1980) Effect of some wild and medicinal plant extracts on aflatoxin production and growth of *Aspergillus flavus* in liquid culture. J Indian Bot Soc 59:123–126

Breuer M, Schmidt GH (1995) Influence of a short period treatment with *Melia azaderach* extract on food intake and growth of the larvae of *Spodoptera frugiperda* (J.E. Smith) (Lep. Noctuidae). J Plant Dis Prot 10:633–654

Bullerman LB, Lieu FY, Seier SA (1977) Inhibition of growth and aflatoxin production by cinnamon and clove oil, cinnamic aldehyde and eugenol. J Food Sci 42:1107–1116

Burt S (2004) Essential oils: their antibacterial properties and potential applications in foods – a review. Int J Food Microbiol 94:223–253

Caccioni DRL, Guizzardi M (1994) Inhibition of germination and growth of fruit and vegetable post-harvest pathogenic fungi by essential oil components. J Essent Oil Res 6:173–179

Calderone NW, Twilson W, Spivak M (1997) Plant extracts used for control of the parasitic mites *Varroa jacobsoni* (Acari: Varroidae) and *Acarapis wood* (Acari: Tarsonemidae) in colonies of *Apis mellifera* (Hymentoptera: Apidae). J Econ Entomol 90:1080–1086

Canillac N, Mourey A (2001) Antibacterial activity of the essential oil of *Picea excelsa* on *Listeria*, *Staphylococcus aureus* and coliform bacteria. Food Microbiol 18:261–268

Casida JE, Quistad GB (1995) Pyrethrum flowers: production, chemistry, toxicology and uses. Oxford University Press, Oxford, p 356

Coats JR (1994) Risks from natural verses synthetic intsecticides. Annu Rev Entomol 39:489–515

Deans SG, Ritchie G (1987) Antibacterial properties of plant essential oils. Int J Food Microbiol 5:165–180

Dubey NK, Bhargava KS, Dixit SN (1983) Protection of some stored food commodities from fungi by essential oils of *Ocimum canum* and *Citrus medica*. Int J Trop Plant Dis 1:177–179

Dubey SC, Suresh M, Singh B (2006) Evaluation of *Trichoderma* species against *Fusarium oxysporum* f. sp. *ciceris* for integrated management of chickpea. Biol Control 40:118–127

Elgayyar M, Draughon FA, Golden DA, Mount JR (2001) Antimicrobial activity of essential oils from plants against selected pathogenic and saprophytic microorganisms. J Food Prot 64:1019–1024

Feng W, Zheng X (2007) Essential oil to control *Alternaria alternata in vitro* and *in vivo*. Food Control 18:1126–1130

Franck ED, Charles LC, Stephen OD (2009) Natural products in crop protection. Bioorg Med Chem . doi:10.1016/j.bmc.2009.01.046

Ghannoum MA (1988) Studies on the anticandidal mode of action of *Allium sativum* (garlic). J Gen Microbiol 134:2917–2924

Hammack L (1996) Corn volatiles as attractants for northern and western corn rootworm beetles (Coleoptera: Chrysomelidae: *Diabrotica* spp.). J Chem Ecol 22:1237–1253

Hasan HA, Mahmoud ALE (1993) Inhibitory effect of spice oils on lipase and mycotoxin production. Zentralbl Mikrobiol 148:543–548

Ho SH, Ma Y, Huang Y (1997) Anethole, a potential insecticide from *Illicium verum* Hook F., against two stored product insects. Int Pest Control 39:50–51

Holley AH, Patel H (2005) Improvement in shelf life and safety of perishable food by plant essential oils and smoke antimicrobials. Int J Food Microbiol 22:273–292

Huang MT, Ferraro T, Ho CT (1994) Cancer chemoprevention by phytochemicals in fruits and vegetables. Am Chem Soc Symp Ser 546:2–15

Huang Y, Tan JMWL, Kini RM, Ho SH (1997) Toxic and antifeedant action of nutmeg oil against *Tribolium castaneum* (Herbst) and *Sitophilus zeamais* Motsch. J Stored Prod Res 33:289–298

Huang Y, Hee SK, Ho SH (1998) Antifeedant and growth inhibitory effects of α-pinene on the stored-product insects, *Tribolium castaneum* (Herbst) and *Sitophilus zeamais* Motsch. Int Pest Control 40:18–20

Immaraju JA (1998) The commercial use of azadirachtin and its integration into viable pest control programmes. Pestic Sci 54:285–89

Ioannou KL, Aletrali A, Christou E, Hadjioannou-ralli A, Koliou A, Akkelidou D (1999) Surveillance and control of aflatoxins B1, B2, G1, G2, and M1 in foodstuffs in the Republic of Cyprus: 1992–1996. J AOAC Int 82:883–892

Isman MB (2000) Plant essential oils for pest and disease management. Crop Prot 19:603–608

Isman MB (2006) Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. Annu Rev Entomol 51:45–66

Isman MB, Akhtar Y (2007) Plant natural products as a source for developing environmentally acceptable insecticides. In: Ishaaya I, Nauen R, Horowitz AR (eds) Insecticides design using advanced technologies. Springer, Berlin, Heidelberg, pp 235–248

Janssen MA, Scheffer JJC, Parhan-Van Atten AW, Svendsen AB (1988) Screening of some essential oils for their activities on dermatophytes. Pharm Weekbl 10:277–280

Kakko I, Toimela T, Tahti H (2000) Piperonyl butoxide potentiates the synaptosome ATPase inhibiting effect of pyrethrin. Chemosphere 40:301–305

Khambay BPS (2002) Botanicals. In: Pimental D (ed) Encyclopedia of pest management. Marcel Dekker, New York, pp 107–108

Klepzig KD, Schlyter F (1999) Laboratory evaluation of plant derived antifeedants against the pine weevil *Hylovius avietis* (Coleoptera: Curculionidae). J Econ Entomol 92:644–650

Knobloch K, Pauli P, Iberl B, Weigand H, Weiss N (1989) Antibacterial and antifungal properties of essential oil components. J Essent Oil Res 1:119–128

Krishnamurthy YL, Shashikala J (2006) Inhibition of Aflatoxin B_1 production of *Aspergillus flavus*, isolated from soyabean seeds by certain natural plant products. Lett Appl Microbiol 43:469–474

Kumar R, Mishra AK, Dubey NK, Tripathi YB (2007a) Evaluation of *Chenopodium ambrosioides* oil as a potential source of antifungal, antiaflatoxigenic and antioxidant activity. Int J Food Microbiol 115:159–164

Kumar R, Srivastava M, Dubey NK (2007b) Evaluation of *Cymbopogon martinii* oil extract for control of postharvest insect deterioration in cereals and legumes. J Food Prot 70:172–178

Kumar A, Shukla R, Singh P, Prasad CS, Dubey NK (2008) Assessment of *Thymus vulgaris* L. essential oil as a safe botanical preservative against post harvest fungal infestation of food commodities. Innovative Food Sci Emerg Technol 9:575–580

Landolt PJ, Hofstetter RW, Biddick LL (1999) Plant essential oils as arrestant and repellents for neonate larvae of the codling moth Lepidoptera: Tortricidae. Environ Entomol 28:954–960

Larocque N, Vincent C, Belanger A, Bourassa JP (1999) Effects of tansy essential oil from *Tanacetum vulgare* on biology of oblique banded leaf roller, *Choristoneura rosaceana*. J Chem Ecol 25:1319–1330

Lee B, Choi W, Lee S, Park B (2001a) Fumigant toxicity of essential oils and their constituent towards the rice weevil, *Sitophilus oryzae* (L.). Crop Prot 20:317–320

Lee SE, Lee BH, Choi WS, Park BS, Kim JG, Campbell BC (2001b) Fumigant toxicity of volatile natural products from Korean spices and medicinal plants towards the rice weevil, *Sitophilus oryzae* (L). Pest Manag Sci 57:548–553

Lis-Balchhin M, Deans SG (1997) Bioactivity of selected plant essential oils against *Listeria monocytogenes*. J Appl Microbiol 82:759–762

Liu CH, Mishra AK, Tan RX, Tang C, Yang H, Shen YF (2006) Repellent and insecticidal activities of essential oils from *Artemisia princeps* and *Cinnamomum camphora* and their effect on seed germination of wheat and broad bean. Bioresour Technol 97:1969–1973

Mabrouk SS, El-Shayeb NMA (1992) Inhibition of aflatoxin production in *Aspergillus flavus* by natural coumerins and chromones. World J Microbiol Biotechnol 8:60–62

MacKinnon S, Durst T, Arnason JT, Angerhofer C, Pezzuto JM, Sachez-Vidas PE, Poveda LJ, Gbeassor FM (1997) Antimalarial activity of tropical Meliaceae extracts and gedunin derivatives. J Nat Prod 60:336–341

Mahmoud ALE (1994) Antifungal action and antiaflatoxigenic properties of some essential oil constituents. Lett Appl Microbiol 19:110–113

Marimuth S, Gurusubramanian G, Krishna SS (1997) Effect of exposure of eggs to vapours from essential oils on egg mortality, development and adult emergence in *Earias vittella* (F.) (Lepidoptera: Noctuidae). Biol Agric Hortic 14:303–307

Mishra AK, Dubey NK (1994) Evaluation of some essential oils for their toxicity against fungi causing deterioration of stored food commodities. Appl Environ Microbiol 60:1101–1105

Mishra AK, Kishore N, Dubey NK, Chansouria JPN (1992) An evaluation of the toxicity of the oils of *Cymbopogon citratus* and *Citrus medica* in rats. Phytother Res 6:279–281

Moretti MDL, Peana AT, Franceschini A, Carta C (1998) *In vivo* activity of *Salvia officinalis* oil against *Botrytis cinerea*. J Essent Oil Res 10:157–160

Muthukrishnan J, Pushpalatha E (2001) Effects of plant extract on fecundity and fertility of mosquitoes. J Appl Entomol 125:31–35

Naumann K, Isman MB (1995) Evaluation of neem *Azadirachta indica* seed extracts and oils as oviposition deterrents to noctuid moths. Entomology Experimentalis et Applicata 76:115–120

Naumann K, Isman MB (1996) Toxicity of neem (*Azadirachta indica* A. uss) seed extracts to larval honeybees and estimation of dangers from field applications. Am Bee J 136:518–520

Negahban M, Moharramipour S (2005) Fumigant toxicity of essential oil from *Artemisia scoparia* Waldst and Kit against *Sitophilus oryzae* (Coleoptera: Curculionidae). In: Secretariat of the symposium, faculty of agriculture, university of Shahed (eds) Proceedings of the second symposium of medicinal plants, Shadnaghsh Printing House, Tehran, Iran, p 124, 26–27 January 2005

Ngamo TSL, Ngassoum MB, Jirovetz L, Ousman A, Nukenine EC, Moukala O (2001) Protection of stored maize against *Sitophilus zeamais* (Motsch.) by use of essential oils of spices from Cameroon. Medlinden Faculteit Landbouww Universiteit Gent 66:473–478

Obeng-Ofori D, Reichmuth CH (1997) Bioactivity of eugenol, a major component of essential oil of *Ocimum suave* (Wild.) against four species of stored-product Coleoptera. Int J Pest Manag 43:89–94

Omura M, Hirata MM (1995) Pesticide causes testicle damage reduces sperm count. Bull Environ Contam Toxicol 55:1–7

Ouattara B, Simard RE, Holley RA, Piette GJP, Begin A (1997) Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms. Int J Food Microbiol 37:55–162

Oxenham SK (2003) Classification of an *Ocimum basilicum* germplasm collection and examination of the antifungal effect of the essential oil of the basil. Ph.D. Thesis, Glasgow, University of Glasgow, UK

Pandey R (2003) Pesticides and sterility. Everyman's Sci XXXVIII:84–86
Paranagama PA, Abeysekera KHT, Abeywickrama K, Nugaliyadde L (2003) Fungicidal and anti-aflatoxigenic effects of the essential oil of *Cymbopogon citrata* (DC.) Stapf. (lemongrass) against *Aspergillus flavus* Link. isolated from stored rice. Lett Appl Microbiol 37:86–90
Park SJ, Lee SG, Shin SC, Lee BJ, Ahn YJ (1997) Larvicidal and antifeeding activities of oriental medicinal plant extracts against four species of forest insect pests. Appl Entomol Zool 32:601–608
Pavela R (2007) Possibilities of botanical insecticide exploitation in plant protection. Pest Technol 1:47–52
Petroski RJ, Hammack L (1998) Structure activity relationships of phenyl alkyl alcohols, phenyl alkyl amines, and cinnamyl alcohol derivatives as attractants for adult corn rootworm (Coleoptera: Chrysomelidae: *Diabrotica* spp.). Environ Entomol 27:688–694
Regnault-Roger C, Hamraoui A, Holeman M, Theron E, Pinel R (1993) Insecticidal effect of essential oils from mediterranean plants upon *Acanthoscelides obtecus* Say (Coleoptera: Bruchidae), a pest of kidney bean (*Phaseolus vulgaris* L.). J Chem Ecol 19:1233–1244
Regnault-Roger C, Philogene BGR, Vincent D (eds) (2005) Biopesticides of plant origin. Lavoisier, Paris, p 313
Roy AK (2003) Mycological problems of crude herbal drugs-overview and challenges. Indian Phytopathology 56:1–13
Sahaf BZ, Moharramipour S, Meshkatalsadat MH (2007) Chemical constituents and fumigant toxicity of essential oil from *Carum copticum* against two stored product beetles. Insect Sci 14:213–218
Sarac A, Tunc I (1995) Toxicity of essential oil vapours to stored-product insects. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz 102:69–74
Shaaya E, Kostjukovsky M (1998) Efficacy of phyto-oils as contact insecticides and fumigants for the control of stored product insects. In: Ishaaya I, Degheele D (eds) Insecticides with novel modes of action: mechanism and application. Springer, Berlin, pp 171–188
Shaaya E, Ravid U, Paster N, Juven B, Zisman U, Pissarev V (1991) Fumigant toxicity of essential oils against four major stored-product insects. J Chem Ecol 17:499–504
Shaaya E, Kostjukovski M, Eilberg J, Sukprakarn C (1997) Plant oils as fumigants and contact insecticides for the control of stored product insects. J Stored Prod Res 33:7–15
Shakarami J, Kamali K, Moharramipour S, Meshkatalsadat M (2003) Fumigant toxicity and repellency of essential oil of *Artemisia aucheri* on four species of stored pest. Appl Entomol Phytopathol 71:61–75
Singh P (1983) Control of aflatoxin through natural plant extracts. In: Bilgranii KS, Prasad T, Sinha KK (eds) The proceedings of symposium on mycotoxin in food and feed. Allied Press, Bhagalpur, pp 307–315
Singh G, Upadhyay RK (1993) Essential oils : A potent source of natural pesticides. J Sci Ind Res 52:676–683
Singh P, Srivastava B, Kumar A, Dubey NK (2008) Fungal contamination of raw materials of some herbal drugs and recommendation of *Cinnamomum camphora* oil as herbal fungitoxicant. Microb Ecol 56:555–560
Smid EJ, Gorris LGM (1999) Natural antimicrobials for food preservation. In: Rehman MS (ed) Handbook of food preservation. Marcel Dekker, New York, pp 285–308
Soloway SB (1976) Naturally occurring insecticides. Environ Health Perspect 14:109–117
Subramanyam B, Hagstrum DW (1995) Resistance measurement and management. In: Subramanyam B, Hagstrum DW (eds) Integrated management of insects in stored products. Marcel Dekker, New York, pp 331–397
Suhr KI, Nielsen PV (2003) Antifungal activity of essential oils evaluated by two different application techniques against rye bread spoilage fungi. J Appl Microbiol 94:665–674
Tripathi P, Dubey NK (2004) Exploitation of natural products as an alternative strategy to control post harvest fungal rotting of fruits and vegetables. Postharvest Biol Technol 32:235–245

Tripathi P, Dubey NK, Banerji R, Chansouria JPN (2004) Evaluation of some essential oils as botanical fungitoxicants in management of post-harvest rotting of Citrus fruits. World J Microbiol Biotechnol 20:317–321

Tuley de Silva K (1996) A manual of the essential oil industry. United nation industrial development organisation, Vienna

Vargas RI, Stark JD, Kido MH, Ketter HM, Whitehand LC (2000) Methyl eugenol and cue-lure traps for suppression of male oriental fruit flies and melon flies (Diptera: Tephritidae) in Hawaii: effects of lure mixtures and weathering. J Econ Entomol 93:81–87

Varma J, Dubey NK (1999) Prospectives of botanical and microbial products as pesticides of tomorrow. Curr Sci 76:172–179

Varma J, Dubey NK (2001) Efficacy of essential oils of *Caesulia axillaries* and *Mentha arvensis* against some storage pests causing biodeterioration of food commodities. Int J Food Microbiol 68:207–210

Wakdikar S (2004) Global health care challenge: Indian experiences and new prescriptions. Electron J Biotechnol 7:214–220

Wan MT, Watts RG, Isman MB, Strub R (1996) An evaluation of the acute toxicity to juvenile Pacific northwest salmon of azadirachtin, neem extract and neem-based products. Bull Environ Contam Toxicol 56:432–439

Ware GW (2002) An introduction to insecticides, 3rd edn. Department of Entomology, University of Arizona, Tuscon

Ware GW, Whitacre DM (2004) An Introduction to Insecticides, 4th edn. Meister Pro Information Resources Publisher, Ohio

Weaver DK, Subramanyam B (2000) Botanicals. In: Subramanyam B, Hagstrum DW (eds) Alternatives topesticides in stored product IPM. Kluwer, Dordrecht, pp 303–320

Wheeler DA, Isman MB (2001) Antifeedant and toxic activity of *Trichilia americana* extract against the larvae of *Spodoptera litura*. Entomology Experimentalis et Applicata 98:9–16

Williams HJ, Phillips TD, Jolly EP, Stiles KJ, Jolly MC, Aggarwal D (2004) Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences and interventions. Am J Clin Nutr 80:1106–1122

Wilson CL, Solar JM, El Ghaouth A, Wisniewski ME (1997) Rapid evaluation of plant extracts and essential oils for antifungal activity against *Botrytis cinerea*. Plant Dis 81:204–210

WMO (1991) Scientific assessment of Ozone depletion. World metrological organization report no. 25. World Metrological Organization of the United Nations, Geneva

Zhao B, Grant GG, Langevin D, McDonald L (1998) Deterring and inhibiting effects of quinolizidine alkaloids on spruce budworm (Lepidoptetra: Tortricidae) oviposition. Environ Entomol 27:984–992

Chapter 15
Regulation and Functional Analysis of Bioprotective Metabolite Genes from the Grass Symbiont *Epichloe festucae*

Barry Scott, Ruth E. Wrenn, Kimberley J. May, Daigo Takemoto, Carolyn A. Young, Aiko Tanaka, Damien J. Fleetwood, and Richard D. Johnson

Abstract Epichloë endophytes (*Neotyphodium*/*Epichloë* species) are biotrophic fungi that systemically colonise the intercellular spaces of leaves of grasses to form mutualistic symbiotic associations. The production of secondary metabolites by these fungi confers bioprotective benefits to the grass. However, in pastoral ecosystems some of these metabolites are toxic to grazing mammals. We have cloned and functionally analysed genes for the synthesis of three classes of the bioprotective molecules, peramine, lolitrem B and ergovaline. A single gene, *perA*, encoding a non-ribosomal peptide synthetase is required for peramine biosynthesis. Complex gene clusters with 10 and 11 genes, respectively, are required for lolitrem B (*ltm* genes) and ergovaline (*eas* genes) biosynthesis. The biochemical function of these genes is being elucidated by a systematic deletion analysis combined with chemical analysis of intermediates that accumulate *in planta*. These experiments allow us to propose biosynthetic schemes for the synthesis of the metabolites. Symbiota of these 'knock-out' mutants are being used to examine biological function of the metabolites. Spatial and temporal patterns of gene expression are being examined *in planta* using promoter fusions with the GUS reporter gene. An overview of recent progress to date combined with some original data are presented.

Keywords Lolitrem B • Ergovaline • Peramine • *Neotyphodium lolii* • *Epichloë festucae*

B. Scott (✉), R.E. Wrenn, K.J. May, D. Takemoto, and A. Tanaka
Institute of Molecular BioSciences and Bio-Protection Research Centre, Massey University, Private Bag, 11222, Palmerston North, New Zealand
e-mail: d.b.scott@massey.ac.nz

C.A. Young
The Samuel Roberts Noble Foundation, 2510, Sam Noble Parkway, Ardmore, OK 73401, USA

D.J. Fleetwood and R.D. Johnson
AgResearch, Grasslands Research Centre, Private Bag, 11008, Palmerston North, New Zealand

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,* Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_15,

15.1 Introduction

Neotyphodium lolii and *Epichloë festucae* are obligate seed-borne biotrophic fungi (Clavicipitaceae, Ascomycota) that systemically colonize the intercellular spaces of vegetative and reproductive aerial tissues of *Lolium* and *Festuca* grass species (Philipson and Christey, 1986; Leuchtmann et al., 1994; Christensen et al., 2002, 2008). Molecular phylogenetic studies indicate that *N. lolii* is a haploid asexual derivative of *Epichloë festucae*, a natural symbiont of *Festuca spp*. *E. festucae* is also capable of forming stable synthetic associations with perennial ryegrass (*L. perenne*), the natural host for *N. lolii* (Leuchtmann et al., 1994; Christensen et al., 1997; Tanaka et al., 2006). Numerous studies have established that *N. lolii*, *E. festucae* and related epichloë endophytes are mutualists that confer bioprotective benefits to their host plants, particularly under conditions of biotic and abiotic stress (Clay, 1990; Schardl and Clay, 1997; Easton, 1999). The ability of these endophytes to synthesize bioprotective metabolites has been proposed to constitute a major ecological benefit for the symbiotum (Schardl, 1996; Lane et al., 2000).

Three main classes of biologically active metabolites have been identified in *N. lolii* infected perennial ryegrass: peramine, indole diterpenes, principally lolitrem B, and ergot alkaloids, principally ergovaline (Rowan, 1993; Lane et al., 2000) (Fig. 15.1). Peramine has been shown to be a potent feeding deterrent of adult Argentine stem weevil (ASW; *Listronotus bonariensis*) (Rowan and Gaynor, 1986; Rowan et al., 1990), an economically important pest of perennial ryegrass in New Zealand. Lolitrem B has biological activity against ASW larvae

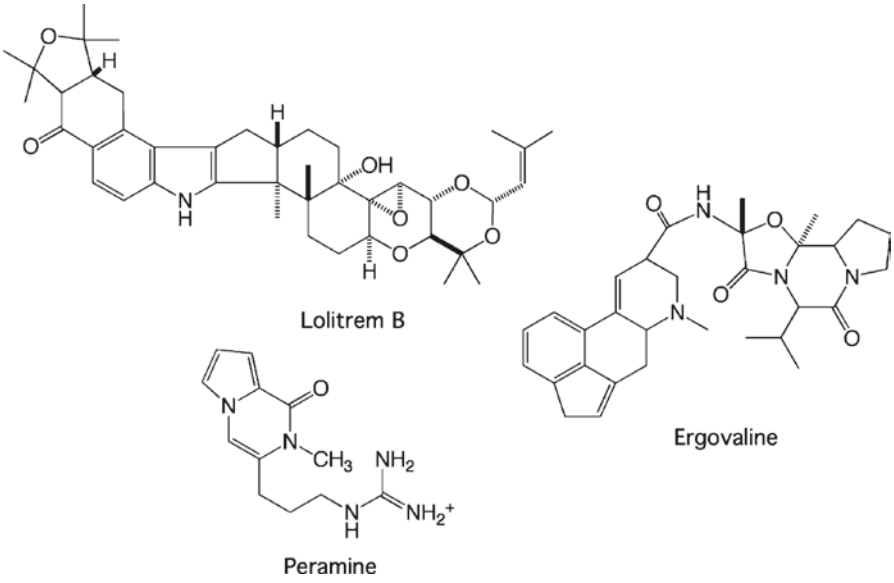


Fig. 15.1 Structure of lolitrem B, peramine and ergovaline

(Prestidge and Gallagher, 1988), but is better known as the causative agent of the neuromuscular disorder known as 'ryegrass staggers', associated with sheep grazing ryegrass dominant pastures during and after periods of water stress (Keogh, 1973; Fletcher and Easton, 1997). The importance of ergot alkaloids towards ecological fitness of perennial ryegrass has not been well defined, but ergovaline is implicated in protection against some insects (Ball et al., 1997).

Genes for the synthesis of peramine (Tanaka et al., 2005), indole diterpenes (Young et al., 2005, 2006) and ergot alkaloids (Panaccione et al., 2001; Wang et al., 2004; Fleetwood et al., 2007) have now been cloned and the function of the protein products inferred from a combination of genetic analysis in *E. festucae* and by comparison of known functions of orthologues in other filamentous fungi. The adoption of *E. festucae* strain Fl1 as a model experimental system to study epichloë endophyte-grass symbiotic interactions has been key to these recent rapid advances (Scott et al., 2007).

This paper provides an overview of recent advances in our understanding of the function and regulation of the genes required for the biosynthesis of peramine, indole diterpenes and ergot alkaloids in the *N. lolii*/*E. festucae* group of epichloë endophytes.

15.2 Peramine

15.2.1 Molecular Cloning and Genetic Analysis of perA, a Peramine Synthetase

Peramine, the only known natural occurring pyrrolopyrazine, is a potent insect feeding deterrent which is uniquely synthesized by clavicipitaceous fungal endophytes of the epichloë group in symbiotic association with their grass hosts (Rowan and Gaynor, 1986; Rowan et al., 1990; Rowan, 1993; Lane et al., 2000; Clay and Schardl, 2002). Taxonomically, peramine is the most widely distributed of the epichloë bioprotective metabolites identified to date and has been detected in symbiota containing *E. typhina*, *E. festucae*, *E. amarillans*, *E. bromicola*, *E. elymi*, as well as *N. lolii*, *N. coenophialum* and many other asexual epichloë species (Lane et al., 2000; Clay and Schardl, 2002). The lipophilic ring system and the hydrophilic guanidinium group of peramine (Fig. 15.1) are novel structural features not reported in any other insect feeding deterrent. Peramine is a potent feeding deterrent against both larvae and adults of ASW, a major pest of perennial ryegrass (Rowan et al., 1990). Protection from insect herbivory may provide strong selective pressure for maintenance of the fungal biosynthetic gene (Schardl, 1996).

The structure of peramine led Rowan to propose that this metabolite is derived from proline and arginine via a diketopiperazine intermediate (Rowan et al., 1986; Rowan, 1993). This led to the hypothesis that peramine synthetase would be a

two-module non-ribosomal peptide synthetase (NRPS), containing a methylation domain. This was recently verified by cloning a NRPS, designated *perA* (peramine synthetase), from an *E. festucae* cosmid library, using as probe, an RT-PCR generated product to the adenylation domain of an NRPS gene preferentially expressed *in planta* (Tanaka et al., 2005). The inferred PerA amino acid sequence was found to have a domain structure consistent with the expected functions of an enzyme required for the synthesis of peramine. Perennial ryegrass symbiota containing an *E. festucae perA* deletion mutant lacked detectable levels of peramine but otherwise had a wild-type symbiotic interaction phenotype. Unlike wild-type symbiota, those containing the *perA* mutant were susceptible to ASW feeding damage, confirming that peramine is the metabolite responsible for ASW feeding deterrence.

A comparison of the *perA* genome region from *E. festucae* with the corresponding region from *Fusarium graminearum* and other members of the Sordariomycetes, revealed a remarkable conservation of gene structure, order and orientation, with the exception of *perA*, which is absent from the genomes of these other filamentous fungi (Tanaka et al., 2005). The presence of duplicate 12-bp direct repeats flanking *perA* suggests a transposon-like recombination event leading to gene acquisition at this *E. festucae* locus. These results led Tanaka et al. (2005) to propose that PerA alone is responsible for peramine biosynthesis. The condensation domain of PerA is proposed to catalyse formation of a peptide bond between 1-pyrroline-5-carboxylate (an immediate precursor of proline) and arginine. The methylation domain of PerA is proposed to catalyse the *N*-methylation of the α-amine group of arginine. The reductase domain is proposed to reduce the thioester and cyclise the dipeptide to form an iminium ion that is concomitantly released. Deprotonation of this intermediate and oxidation of the pyrroline ring would give rise to peramine. Testing the validity of this proposed biosynthetic scheme will require feeding studies with isotopically labelled substrates as has been used for dissecting the loline biosynthesis pathway (Blankenship et al., 2005; Faulkner et al., 2006). This approach may only be feasible if epichloë endophytes can be identified that are naturally derepressed for peramine biosynthesis or if culture conditions under which they synthesize peramine can be identified.

15.2.2 Taxonomic Distribution of perA

To determine the distribution of *perA* among the *Epichloë*/*Neotyphodium* group, genomic DNA from 38 different isolates, representing the 10 known sexual species of *Epichloë* as well as a diverse range of asexual *Neotyphodium* species, was amplified by PCR using three different sets of primers designed to adenylation domain 1 (A1), adenylation domain 2 (A2) and the methylation and thiolation domains (M-T) of *E. festucae* Fl1 PerA (Fig. 15.2). Products of 1.1- and 0.6-kb

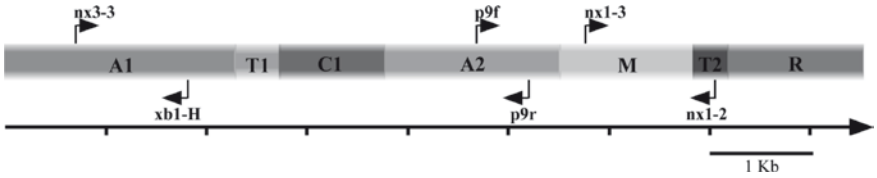


Fig. 15.2 Domain structure of PerA showing primers (*arrows*) used to amplify various regions of this gene. The two modules of PerA (peramine synthetase) contain domains for adenylation (A1 and A2), thiolation (T1 and T2), condensation (C1), methylation (M) and reduction (R). Primer names are given above and below arrows

were generated for the A1 and A2 domains, respectively for 36 of the 38 isolates (Table 15.1). No product was obtained for the A1 domain of *E. glyceriae* strain E2772 and the A2 domain of *E. sylvatica* strain E503. Both strains also lacked a product to the M-T domain. A product of 1.3-kb was amplified for the M-T domain of 28 of the isolates. Given all 38 templates could be amplified with primers designed to the positive control *tubB* (β-tubulin), any lack of *perA*-domain product was unlikely to be due to the quality of DNA. Alternatively, either the sequence was absent or there was a primer mismatch due to polymorphisms present in the template DNA. To distinguish between these possibilities, the entire *perA* or *perA*-gene remnant would have to be amplified and sequenced. Even where all three gene-products are present, the gene may still be non-functional due to point mutations or frame shifts.

As discussed above, peramine has been detected in symbiota containing *E. typhina*, *E. festucae*, *E. amarillans*, *E. bromicola*, and *E. elymi* (Clay and Schardl, 2002). Representative strains of these species contained all three PCR products, a result consistent with their known capability to produce peramine. However, one strain of *E. amarillans* (E57), one strain of *E. bromicola* (E501) and three strains of *E. typhina* (E8, E2463, E348) lacked a M-T domain product. Given *E. typhina* strain E8 is a known peramine producer (Tanaka et al., 2005), the lack of amplification of the M-T region of *perA* in this strain must be due to primer mismatch. Whether symbiota of the other strains produce peramine remains to be determined. To date, peramine has not been detected in symbiota of *E. sylvatica*, a result consistent with the absence of an M-T product in both strains analysed. Symbiota of *E. glyceriae* have yet to be tested for peramine, but the absence of an A1 product in the one strain tested suggests that *perA* may be non-functional in this species.

While symbiota of only a few of the asexual strains have been analysed for peramine, PCR products for all three regions of the *perA* gene could be amplified from templates of all strains, suggesting that they all contain an intact *perA*, but whether it is functional or not, in most cases remains to be determined. With the exception of *N. lolii*, all other asexual species examined are known to be interspecific hybrids of varied ancestry. All are derived from at least one sexual species known to produce peramine.

Table 15.1 PCR screen of perA domains in epichloë endophytes

Species	Strain[a]	Domain[b]			Peramine producer[c]
		A1	A2	M-T2	
E. festucae	Fr1	+	+	+	(+)
E. festucae	Frc5	+	+	+	(+)
E. festucae	Frr1	+	+	+	(+)
E. festucae	Fg1	+	+	+	(+)
E. festucae	Fl1	+	+	+	+
E. festucae	Frc7	+	+	+	(+)
E. festucae	E189	+	+	+	(+)
E. amarillas	E52	+	+	+	(+)
E. amarillas	E57	+	+	−	(+)
E. baconii	E248	+	+	−	(−)
E. baconii	E1031	+	+	+	(−)
E. bromicola	E799	+	+	+	(+)
E. bromicola	E501	+	+	−	(+)
E. elymi	E56	+	+	+	(+)
E. glyceriae	E2772	−	+	+	nt
E. brachyelytri	E1040	+	+	+	nt
E. clarkii	E422	+	+	−	(−)
E. typhina	E505	+	+	−	(+)
E. typhina	E1022	+	+	+	(+)
E. typhina	E425	+	+	+	(+)
E. typhina	E348	+	+	−	(+)
E. typhina	E2463	+	+	−	(+)
E. typhina	E8	+	+	−	(+)
E. sylvatica	E354	+	+	−	(−)
E. sylvatica	E503	+	−	−	(−)
N. lolii	Lp19	+	+	+	+
N. lolii	Lp14	+	+	+	−
N. lolii	AR1	+	+	+	(+)
N. melicola	E822	+	+	+	nt
N. tembladarae	E1169	+	+	+	(+)
N. australiense	E938	+	+	+	nt
N. typhinum	Poa	+	+	+	nt
N. siegelii	E915	+	+	+	(−)
N. huerfanum	E4055	+	+	+	nt
N. coenophialum	Tf28	+	+	+	(+)
N. sp. *LpTG-2*	Lp1	+	+	+	+
N. sp. *"sleepygrass"*	E4096	+	+	+	nt
N. sp.	Hd1	+	+	+	nt

[a]More detailed information on the grass host, closest non-hybrid ancestor, ATCC number where available and reference is available from Young et al. (2009).

[b]Primers used to amplify domain regions of *perA* were; nx3-3 (ATGTCCGATTCTAGGTGCAC)/xb1-H (CGTAGAAGCTTCAGGACTGA) for A1, p9f (GCAAACGCCGTCTCTGCTCA)/p9r (GGATCCCCTTAACAACCACT) for A2 and nx1–3 (GTCGGGCATGATGCTCTTCA)/nx1–2 (CTGACTCGACTCGATACTCA) for M-T2.

[c]Known peramine producer (Tanaka et al., 2005). +: Known non-producer of peramine (Tanaka et al., 2005). −: Known peramine producer for host of this species, but strain not known (Clay and Schardl, 2002). (+): Known non-producer of peramine for host of this species, but strain not known (Clay and Schardl, 2002). (−): To date not tested for peramine production (Clay and Schardl, 2002) nt.

15.3 Indole Diterpenes

15.3.1 Molecular Cloning and Genetic Analysis of Lolitrem (ltm) Biosynthetic Genes

Lolitrems are an important subgroup of a structurally diverse group of indole diterpene metabolites found in leaves and seeds of perennial ryegrass and tall fescue (*Lolium arundinanceum*) symbiota containing *E. festucae*, *N. lolii* and interspecific asexual hybrids with *E. festucae* ancestry. The most abundant indole-diterpene found in the *N. lolii*-perennial ryegrass association is lolitrem B. The structural similarity between paxilline, an abundant metabolite of *Penicillium paxilli*, and lolitrem B, suggests that the more complex indole-diterpenes found in epichloë endophytes are derived from either paxilline or proximate precursors such as paspaline (Parker and Scott, 2004; Saikia et al., 2008). The molecular cloning of genes for paxilline (*pax*) biosynthesis provided a molecular strategy to isolate genes for lolitrem B (*ltm*) biosynthesis (Young et al., 2001).

A *P. paxilli* geranylgeranyl diphosphate synthase gene, *paxG*, was used as a probe to find the corresponding gene, *ltmG*, in *N. lolii* (Young et al., 2005). Two additional *pax* homologues, *ltmC* and *ltmK* were linked to *ltmG* (Fig. 15.3). Chromosome walking from this original locus, using sequence information derived from ESTs isolated from *N. lolii* SSH libraries, identified additional *ltm* genes (Young et al., 2006). Sequence analysis revealed a complex lolitrem biosynthetic locus (*LTM*) comprised of at least ten genes, organized in three clusters, in both *N. lolii* and *E. festucae* (Young et al., 2005, 2006) (Fig. 15.3). The first cluster comprises three genes, *ltmG*, *ltmM* and *ltmK*, two of which have been shown to be functional orthologues of *paxG* (encoding a geranylgeranyl diphosphate synthase) and *paxM* (FAD dependent monooxygenase), genes that encode enzymes required for early steps in paxilline biosynthesis. Immediately adjacent to the *N. lolii ltmK* is a 17-kb retrotransposon relic sequence followed by a polyketide synthase pseudogene and an additional AT-rich sequence. While the retrotransposon relic is absent from *E. festucae* Fl1, the presence of the other AT-rich sequences suggests that *ltmK* defines one boundary of the *LTM* locus (Fig. 15.3).

The second *ltm* gene cluster comprises five genes, *ltmP*, *ltmQ*, *ltmF*, *ltmC* and *ltmB*, four of which appear to be homologues of *paxP* (P450 monooxygenase), *paxQ* (P450 monooxygenase), *paxC* (prenyl transferase) and *paxB* (unknown function) (Young et al., 2001; McMillan et al., 2003; Saikia et al., 2007). Cluster two is separated from cluster one by a block of AT-rich retrotransposon relic sequences of approximately 35- and 32-kb in strains Lp19 and Fl1, respectively. The third *ltm* gene cluster comprises just two genes, *ltmE* and *ltmJ*, that appear to be unique to the epichloë endophytes and therefore, lolitrem biosynthesis. A 16-kb AT-rich sequence separates cluster two from cluster three (Fig. 15.3). The other side of cluster three is comprised of a further AT-rich sequence. Whether *ltmE* defines the other boundary of the *LTM* locus remains to be determined, as no additional linked sequence has been cloned for analysis.

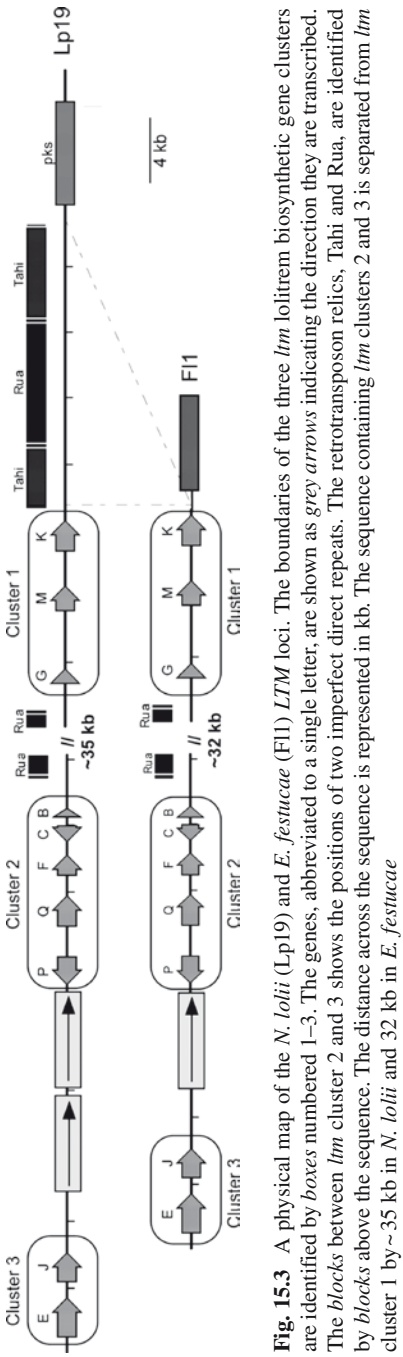


Fig. 15.3 A physical map of the *N. lolii* (Lp19) and *E. festucae* (Fl1) *LTM* loci. The boundaries of the three *ltm* lolitrem biosynthetic gene clusters are identified by *boxes* numbered 1–3. The genes, abbreviated to a single letter, are shown as *grey arrows* indicating the direction they are transcribed. The *blocks* between *ltm* cluster 2 and 3 shows the positions of two imperfect direct repeats. The retrotransposon relics, Tahi and Rua, are identified by *blocks* above the sequence. The distance across the sequence is represented in kb. The sequence containing *ltm* clusters 2 and 3 is separated from *ltm* cluster 1 by ~ 35 kb in *N. lolii* and 32 kb in *E. festucae*

By analogy to the proposed functions of PaxG, PaxM, PaxB and PaxC, in *P. paxilli*, and the ability of *ltmM* and *ltmC* to complement *paxM* and *paxC* mutants, respectively, LtmG, LtmM, LtmB and LtmC are likely to be required for paspaline biosynthesis in *N. lolii* and *E. festucae* (Young et al., 2005, 2006). LtmG is proposed to catalyse the synthesis of geranylgeranyl diphosphate (GGPP), the first step in lolitrem biosynthesis. GGPP then condenses with indole-3-glycerol phosphate to form 3-geranylgeranylindole, a linear intermediate that has been shown, by radioisotope-labelling, to be incorporated into paxilline (Fueki et al., 2004). LtmM is proposed to catalyse the epoxidation of the two terminal alkenes of the geranylgeranyl moiety, which is then cyclised by LtmC to paspaline. A comparison of the structure of lolitrem B with paxilline would suggest that LtmP and LtmQ, homologues of PaxP and PaxQ, catalyse analogous biosynthetic steps in *N. lolii* and *E. festucae*. LtmP is proposed to catalyse the demethylation of C-12 of paspaline and subsequent hydroxylation of C-10; LtmQ is proposed to hydroxylate the C-13 position of the paspaline ring (McMillan et al., 2003; Young et al., 2006).

Formation of the A- and B-rings of lolitrem B requires prenylation of the indole ring of paspaline. A candidate enzyme for one or both of these prenylations is LtmE, given the domain structure of this protein appears to be a fusion of two prenyl transferases (Young et al., 2006). To complete the oxidation and closure of ring-A of lolitrem B, two additional catalytic steps are required. LtmJ, a P450 monooxygenase, is a candidate enzyme for these steps (D. Takemoto, B.A. Tapper and B. Scott, unpublished results, pers. comm.). At least two additional catalytic steps are required to form the epoxide between C-11 and C-12 of paspaline, and to oxidize and prenylate C-10 to allow formation of ring-I of lolitrem B. Candidate enzymes for the latter reactions are LtmF and LtmK (D. Takemoto et al., unpublished results, pers. comm.). The isolation of naturally occurring mutations in *E. festucae ltmF* strains that accumulate indole diterpenes that lack a fused ring I, supports a role for LtmF in prenylating C-10 (Young et al., 2009). The chemical diversity of indole-diterpenes identified in *N. lolii*-infected perennial ryegrass seed indicates that the A and B rings can form independently of the I ring (Munday-Finch et al., 1998; Gatenby et al., 1999). This observation suggests that lolitrem biosynthesis is modular, proceeding by way of a metabolic grid rather than a linear pathway. Chemical analysis of the metabolites that accumulate in *E. festucae*-perennial ryegrass symbiota containing deletions of each of the Fl1 *ltm* genes will identify the major and minor biosynthetic pathways that comprise this metabolic network.

15.3.2 Regulation of ltm Gene Expression

Given lolitrem B is readily detectable in plant-infected material but not detectable in culture-grown mycelium, it was not surprising to find that the pattern of *ltm* gene expression corresponded to the indole diterpene metabolic state (Young et al., 2006). RT-PCR analysis showed that *ltm* transcript levels for all 10 genes are very

high *in planta* but barely detectable in mycelium. Attempts to de-repress expression of these genes in culture have to date been unsuccessful (Young et al., 2005; May et al., 2008). To further analyse the patterns of *ltm* gene expression throughout the life cycle of perennial ryegrass, the promoter of *ltmM* was fused to the *gusA* reporter gene and ectopically integrated into the genome of strain Fl1 (May et al., 2008). In mature vegetative tillers infected with these transformants, *gusA* was expressed in all infected aerial tissues, as well as in epiphyllous hyphae. At pre-anthesis, *gusA* expression was observed in all floral organs except the immature gynoecium. In post-anthesis florets, GUS activity was observed almost exclusively in the fertilised gynoecium. GUS activity was also detected in germinating seeds and seedlings. These results demonstrate that *E. festucae ltmM* is actively expressed throughout the life cycle of perennial ryegrass.

15.3.3 Taxonomic Distribution of ltm Genes

A PCR screen of genomic DNA from 44 different isolates, representing the 10 known sexual species of *Epichloë* as well as a diverse range of asexual *Neotyphodium* species showed that all ten genes were present in only three isolates, including one *N. lolii* isolate and two *E. festucae* isolates (Young et al., 2009). Consistent with this gene composition, symbiota of the three isolates were shown to synthesize lolitrems. The majority of the sexual species lack *ltm* genes; however, the presence of remnant sequences in some isolates would suggest that indole diterpene biosynthetic capability was once more widespread, but is now confined to *E. festucae* and asexual derivatives of this species. A discontinuous pattern of distribution has also been observed for a set of 12 different NRPS genes, recently characterised from epichloë endophytes (Johnson et al., 2007). Many of the asexual hybrid endophytes contained cluster one and cluster two genes but were missing cluster 3. The inferred function and proposed position of the gene products from these two clusters in the metabolic biosynthetic network suggested that these isolates were capable of synthesizing simple indole diterpenes including paspaline, 13-desoxypaxilline and terpendoles. LC–MS analysis of extracts from symbiota containing these isolates confirmed these predictions. All asexual hybrid endophytes shown to contain a *ltm* gene sequence are derivatives of hybridisation events involving *E. festucae* as one of the ancestral parents. The analysis of *ltm* gene composition by PCR provides a method for pre-screening endophytes for their potential indole diterpene biosynthetic capability. While absence of a gene precludes synthesis of a particular indole diterpene product(s), presence of the gene does not necessarily mean the gene is active, as illustrated by the fact that two isolates of *E. festucae* were found to have point mutations in *ltmF* that led to the introduction of a premature stop codon and expression of a non-functional gene product. Such naturally occurring mutations give rise to symbiota with diverse indole diterpene metabolic profiles and potential for novel bioprotective activities.

15.4 Ergot Alkaloids

15.4.1 Molecular Cloning and Genetic Analysis of Ergot Alkaloid (eas) Biosynthetic Genes

The ergot alkaloids are among the most well known group of fungal secondary metabolites, principally because of their association with ergot alkaloid poisoning and the long research history. Much of the chemistry of ergot alkaloid biosynthesis has been elucidated from the ergot producing fungus, *Claviceps purpurea*. The first committed step is the formation of dimethylallyl tryptophan (DMAT) from the primary metabolites, tryptophan and dimethylallyl diphosphate, catalysed by DMAT synthase. The gene *dmaW*, encoding DMAT synthase, has been cloned and characterised from *Claviceps fusiformis*, *C. purpurea*, *Aspergillus fumigatus* and *Neotyphodium* sp. Lp1 (Schardl et al., 2006). DMAT is converted by a series of oxidative steps to various clavine intermediates including chanoclavine, agroclavine and elymoclavine, with the latter converted to D-lysergic acid by a cytochrome P450 monooxygenase encoded by *cloA*. D-lysergic acid can then be converted into ergopeptines by NRPS-catalysed linkage of activated lysergic acid to a tripeptide. In *C. purpurea*, lysergic acid is activated and tethered to the single-module LpsB (LPS2). The lysergyl group then forms a peptide bond with a specific amino acid tethered to the first of three modules found in LpsA (LPS2). Two further amino acids are sequentially linked, and the tripeptide cyclized and released from LpsA as lysergyl peptide lactam. The final ergopeptine product is formed by one further heterocyclization step. The most abundant ergopeptine synthesized by epichloë endophytes is ergovaline, which contains a tripeptide of alanine, valine and proline. The genes encoding LpsA and LpsB have been cloned from both *C. purpurea* and *N. lolii* (Panaccione et al., 2001; Correia et al., 2003; Haarmann et al., 2005; Fleetwood et al., 2007).

Genes for ergot alkaloid biosynthesis in *C. purpurea* and *A. fumigatus* are organised in discrete clusters comprising 13 and 14 genes, respectively (Coyle and Panaccione, 2005; Haarmann et al., 2005). The *eas* genes in *N. lolii* are also clustered, but like the *LTM* locus are interspersed with a complex array of Type 1 and Type II transposon relic sequences (Fleetwood et al., 2007). The first cluster comprises *easH*, *easA*, *easG*, *easF*, *easE* and *lpsB*, flanked on either side by AT-rich retrotransposon relic sequences (Fig. 15.4). Two degenerate Type II Toru elements with features of miniature inverted repeat transposable elements (MITE) are located in the intergenic region between *easG* and *easA*. A second MITE-like element was identified between *easE* and *lpsB*. Subsequent to this study, two additional *eas* clusters were identified in the genome sequence of *E. festucae* strain 2368 (Fleetwood, 2007). Cluster two comprising *dmaW*, *cloA*, *easC* and *easD* is flanked on both sides by retrotransposon relic sequences and interspersed with Toru-like MITEs. Cluster three contains just *lpsA* and is again flanked by AT-rich retrotransposon relic sequences. Southern blot analysis of *Not*I digests of *N. lolii* and *E. festucae* DNA established that all three clusters are physically linked within a region of no more than 340 and 114-kb, respectively (Fleetwood et al., 2007).

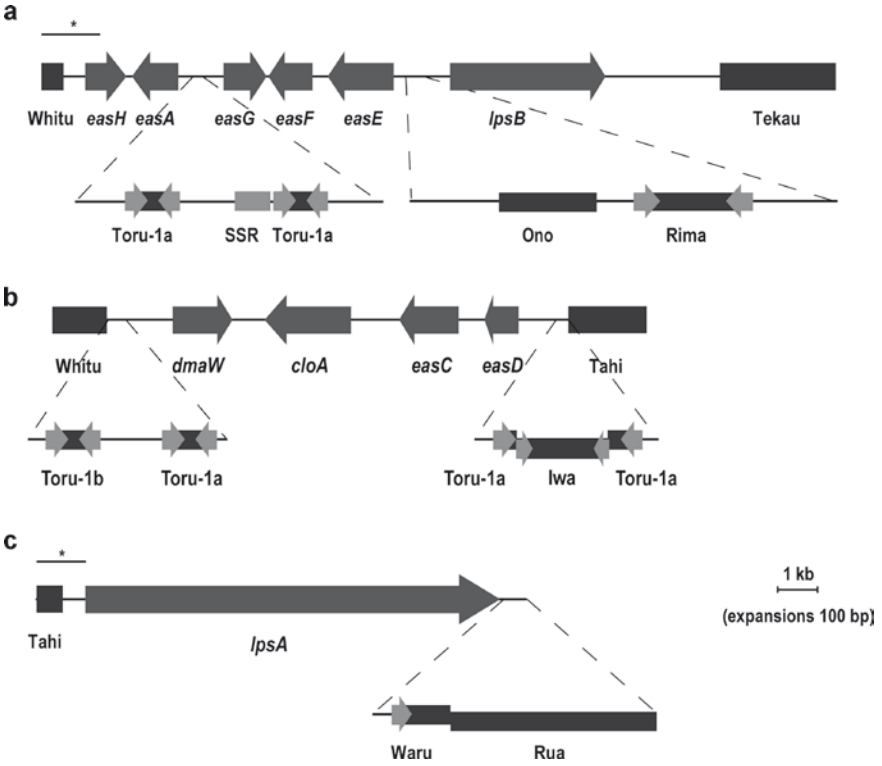


Fig. 15.4 A physical map of the *N. lolii*/*E. festucae EAS* locus. The three *eas* (ergot alkaloid biosynthesis) clusters (a, b & c) were assembled from either *N. lolii* Lp19 or *E. festucae* E2368, whichever had the more complete sequence for each cluster, supplemented with sequence from the other species where noted. Genes of unknown function are designated *easA*, etc., whereas genes of known function have a specific three letter name, including *lpsA* (lysergyl peptide synthase A) and *lpsB* (lysergyl peptide synthase B), *dmaW* (DMAT synthase) and *cloA* (cytochrome P450 monooxygenase). The *expanded regions* show the structure and organization of transposon relics identified at this locus including the miniature inverted repeat elements Toru (Maori for 3), Rimu (5) and Iwa (9), the type II transposon relics, Wha (4) and Waru (8), and the retrotransposons Tahi (1), Rua (2), Ono (6), Whitu (7) and Tekau (10). (**a**) *N. lolii* cluster one.* Overlined sequence from *E. festucae* 2368. (**b**) *E. festucae* E2368 cluster two. (**c**) *E. festucae* E2368 cluster three.* Overlined sequence from *N. lolii* Lp19

15.4.2 Bioprotection from Ergot Alkaloids

Deletion of *lpsB* confirmed that this gene is required for ergovaline biosynthesis in *E. festucae* (Fleetwood et al., 2007). Symbiota containing the Δ*lpsB* mutant lacked ergovaline and lysergic acid amides, but instead accumulated lysergic acid and other clavine intermediates. While ergovaline has been implicated in pasture-deterrence of African Black Beetle (Ball et al., 1997), studies with perennial ryegrass infected with the Δ*lpsB* mutant showed that ergovaline was not necessary

for black beetle feeding deterrence in these symbiota (Fleetwood, 2007). Whether clavine intermediates or a different alkaloid altogether are responsible for black beetle deterrence in these mutant symbiota remains to be determined.

15.5 Future Prospects

The molecular cloning of genes encoding products for the biosynthesis of the three bioprotective metabolites peramine, lolitrem B and ergovaline will allow the biochemistry of the pathways to be elucidated, the mechanisms for plant-regulated gene expression to be explored, and the gene composition of any epichloë endophyte to be determined.

References

Ball OJ-P, Miles CO et al (1997) Ergopeptine alkaloids and *Neotyphodium lolii*-mediated resistance in perennial ryegrass against adult *Heteronychus arator* (Coleoptera: Scarabaeidae). J Econ Entomol 90:1382–1391

Blankenship JD, Houseknecht JB et al (2005) Biosynthetic precursors of fungal pyrrolizidines, the loline alkaloids. Chembiochem 6(6):1016–1022

Christensen MJ, Ball OJ-P et al (1997) Fungal and host genotype effects on compatibility and vascular colonization by *Epichloë festucae*. Mycol Res 101(4):493–501

Christensen MJ, Bennett RJ et al (2002) Growth of *Epichloë/Neotyphodium* and p-endophytes in leaves of *Lolium* and *Festuca* grasses. Mycol Res 106:93–106

Christensen MJ, Bennett RJ et al (2008) Epichloë endophytes grow by intercalary hyphal extension in elongating grass leaves. Fungal Genet Biol 45:84–93

Clay K (1990) Fungal endophytes of grasses. Annu Rev Ecol Syst 21:275–297

Clay K, Schardl C (2002) Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. Amer Naturalist 160(Suppl):S99–S127

Correia T, Grammel N et al (2003) Molecular cloning and analysis of the ergopeptine assembly system in the ergot fungus *Claviceps purpurea*. Chem Biol 10(12):1281–1292

Coyle CM, Panaccione DG (2005) An ergot alkaloid biosynthesis gene and clustered hypothetical genes from Aspergillus fumigatus. Appl Environ Microbiol 71(6):3112–3118

Easton HS (1999) Endophyte in New Zealand ryegrass pastures: an overview. In: Woodfield DR, Matthew C (eds) Ryegrass endophyte: an essential New Zealand symbiosis Grasslands Research and Practice Series No 7. New Zealand Grasslands Association, Palmerston North, pp 1–9

Faulkner JR, Hussaini SR et al (2006) On the sequence of bond formation in loline alkaloid biosynthesis. Chembiochem 7(7):1078–1088

Fleetwood DJ (2007) Molecular characterisation of the EAS gene cluster for ergot alkaloid biosynthesis in epichloe endophytes of grasses. Ph.D. Massey University, Palmerston North, New Zealand

Fleetwood DJ, Scott B et al (2007) A complex ergovaline gene cluster in epichloe endophytes of grasses. Appl Environ Microbiol 73(8):2571–2579

Fletcher LR, Easton HS (1997) The evaluation and use of endophytes for pasture improvement. In: Bacon CW, Hill NS (eds) Neotyphodium/grass interactions. Plenum, New York, pp 209–227

Fueki S, Tokiwano T et al (2004) Biosynthesis of indole diterpenes, emindole, and paxilline: involvement of a common intermediate. Org Lett 6(16):2697–2700

Gatenby WA, Munday-Finch SC et al (1999) Terpendole M, a novel indole-diterpenoid isolated from *Lolium perenne* infected with the endophytic fungus *Neotyphodium lolii*. J Agric Food Chem 47(3):1092–1097

Haarmann T, Machado C et al (2005) The ergot alkaloid gene cluster in Claviceps purpurea: extension of the cluster sequence and intra species evolution. Phytochemistry 66(11):1312–1320

Johnson R, Voisey C et al (2007) Distribution of NRPS gene families within the Neotyphodium/Epichloe complex. Fungal Genet Biol 44(11):1180–1190

Keogh RG (1973) Induction and prevention of ryegrass staggers in grazing sheep. N Z J Exp Agric 1:55–57

Lane GA, Christensen MJ et al (2000) Coevolution of fungal endophytes with grasses: the significance of secondary metabolites. In: Bacon CW, White JF Jr (eds) Microbial endophytes (books in soils plants and the environment). Marcel Dekker, New York, pp 341–388

Leuchtmann A, Schardl CL et al (1994) Sexual compatibility and taxonomy of a new species of *Epichloë* symbiotic with fine fescue grasses. Mycologia 86:802–812

May KJ, Bryant MK et al (2008) Patterns of expression of a lolitrem biosynthetic gene in the Epichloe festucae-perennial ryegrass symbiosis. Mol Plant Microbe Interact 21(2):188–197

McMillan LK, Carr RL et al (2003) Molecular analysis of two cytochrome P450 monooxygenase genes required for paxilline biosynthesis in *Penicillium paxilli*, and effects of paxilline intermediates on mammalian maxi-K ion channels. Mol Gen Genomics 270:9–23

Munday-Finch SC, Wilkins AL et al (1998) Isolation of lolicine A, lolicine B, lolitriol, and lolitrem N from *Lolium perenne* infected with *Neotyphodium lolii* and evidence for the natural occurrence of 31-epilolitrem N and 31-epilolitrem F. J Agric Food Chem 46(2):590–598

Panaccione DG, Johnson RD et al (2001) Elimination of ergovaline from a grass-*Neotyphodium* endophyte symbiosis by genetic modification of the endophyte. Proc Natl Acad Sci USA 98:12820–12825

Parker EJ, Scott DB (2004) Indole-diterpene biosynthesis in ascomycetous fungi. In: An Z (ed) Handbook of industrial mycology. Marcel Dekker, New York, pp 405–426

Philipson MN, Christey MC (1986) The relationship of host and endophyte during flowering, seed formation, and germination of *Lolium perenne*. N Z J Bot 24:125–134

Prestidge RA, Gallagher RT (1988) Endophyte fungus confers resistance to ryegrass: argentine stem weevil larval studies. Ecol Entomol 13:429–435

Rowan DD (1993) Lolitrems, peramine and paxilline: mycotoxins of the ryegrass/endophyte interaction. Agricultue, ecosystems and environment. Elsevier Science, Amsterdam, pp 103–122

Rowan DD, Gaynor DL (1986) Isolation of feeding deterrents against Argentine stem weevil from ryegrass infected with the endophyte *Acremonium loliae*. J Chem Ecol 12(3):647–658

Rowan DD, Hunt MB et al (1986) Peramine, a novel insect feeding deterrent from ryegrass infected with the endophyte Acremonium loliae. J Chem Soc, Chem Commun:935–936

Rowan DD, Dymock JJ et al (1990) Effect of fungal metabolite peramine and analogs on feeding and development of Argentine stem weevil (*Listronotus bonariensis*). J Chem Ecol 16(5):1683–1695

Saikia S, Parker EJ et al (2007) Defining paxilline biosynthesis in *Penicillium paxilli*: functional characterization of two cytochrome P450 monooxygenases. J Biol Chem 282(23):16829–16837

Saikia S, Nicholson MJ et al (2008) The genetic basis for indole-diterpene chemical diversity in filamentous fungi. Mycol Res 112(Pt 2):184–199

Schardl CL (1996) *Epichloë* species: fungal symbionts of grasses. Annu Rev Phytopathol 34:109–130

Schardl CL, Clay K (1997) Evolution of mutalistic endophytes from plant pathogens. In: Carroll GC, Tudzynski P (eds) The Mycota V plant relationships, Part 3. Springer, Berlin, Heidelberg, pp 221–238

Schardl CL, Panaccione DG et al (2006) Ergot alkaloids-biology and molecular biology. In: Cordell GA (ed) Alkaloids: chemistry and biology. Academic, San Diego, CA, pp 425–443

Scott B, Takemoto D et al (2007) Functional analysis of the *Epichloë festucae*-perennial ryegrass symbiosis. In: Popay A, Thom ER (eds) 6th international symposium on fungal endophytes of

grasses grasslands research and practice series no 13. New Zealand Grassland Association, pp 433–441

Tanaka A, Tapper BA et al (2005) A symbiosis expressed non-ribosomal peptide synthetase from a mutualistic fungal endophyte of perennial ryegrass confers protection to the symbiotum from insect herbivory. Mol Microbiol 57(4):1036–1050

Tanaka A, Christensen MJ et al (2006) Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic association. Plant Cell 18:1052–1066

Wang J, Machado C et al (2004) The determinant step in ergot alkaloid biosynthesis by an endophyte of perennial ryegrass. Fungal Genet Biol 41:189–198

Young C, McMillan L et al (2001) Molecular cloning and genetic analysis of an indole-diterpene gene cluster from *Penicillium paxilli*. Mol Microbiol 39:754–764

Young CA, Bryant MK et al (2005) Molecular cloning and genetic analysis of a symbiosis-expressed gene cluster for lolitrem biosynthesis from a mutualistic endophyte of perennial ryegrass. Mol Gen Genomics 274:13–29

Young CA, Felitti S et al (2006) A complex gene cluster for indole-diterpene biosynthesis in the grass endophyte *Neotyphodium lolii*. Fungal Genet Biol 43:679–693

Young CA, Tapper B et al (2009) Indole-diterpene biosynthetic capability of epichloe endophytes as predicted by ltm gene analysis. Appl Environ Microbiol. doi:10.1128/AEM.00953-08 75:2200–2211

Part IV
Soilborne Plant Diseases and their Control

Chapter 16
IPM for Soilborne Disease Management for Vegetable and Strawberry Crops in SE USA

Frank J. Louws

Abstract Major shifts in agricultural practices are complex with highly inter-dependent biological, environmental, social, economic, business, and other (agri)culture factors. Seeking alternatives to methyl bromide (MeBr) to manage soilborne pathogens provided a model system that could be simulated where other large scale and dramatic changes need to be made. Parallel priorities were set in place. The first priority was to assemble an inter-disciplinary and inter-state response and vision team of key private and public sector stakeholders. The second priority was to determine the risk-aversion and biological basis for fumigation. The third priority was to implement a plan that did not simply focus on chemical alternatives, but sought to advance the science of plant pathology and conduct discovery research about the biology, ecology and management of the primary plant pathogens and cropping systems. Therefore, three strategic levels of research and extension were identified: (1) Tactic substitution – addressing short term needs of growers who sought non-ozone depleting fumigant alternatives; (2) Tactic Diversification – focused on medium term alternatives that included non-fumigant and IPM based tactics; (3) Tactic Development – focused on long-term goals to explore microbial ecology and farming systems-based approaches to replace MeBr-dependent production systems. The fourth priority was to effectively extend research based information to primary clientele. Combined efforts resulted in technically and economical feasibility assessments of alternatives, exploration of viable diversification and development of plant disease management tactics, and a region-wide advanced understanding of the biology and ecology of key plant pathogens.

Keywords Soilborne diseases • Fumigation • Integrated pest management • Effective extension

F.J. Louws (✉)
Department of Plant Pathology, North Carolina State University, 27695, Raleigh, NC, USA
e-mail: frank_louws@ncsu.edu

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,*
Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_16,

16.1 Background

Methyl bromide-dependent plasticulture crop production has been an integral part of small-acreage farm operations throughout the Southeastern United States (SEUS). The plasticulture system is a "tool-box" to obtain high productivity in strawberry (O'Dell and Williams, 2000; Poling et al., 2005) and vegetable production systems (Sanders et al., 1996). Methyl bromide (MeBr) has been the primary soil fumigant as a tactic within the plasticulture system to manage a wide spectrum of pests including weeds, nematodes, insects and soilborne plant pathogens (Martin, 2003). However, it is an ozone depleting substance and therefore is being phased out for most agricultural uses in accordance with the Montreal Protocol (Ristaino and Thomas, 1997). Since 2005, agricultural uses in the SEUS have been possible based on existing stocks and Critical Use Nominations (CUN). Through the CUN process, grower stakeholders and the extent of acreage in the Southeast (excluding FL) and surrounding States was documented. There are 2235 ha of strawberries, 6376 ha of tomatoes, 3055 ha peppers and 5941 ha of cucurbit crops. Fifty to eighty-five percent of this acreage, depending on the crop, has relied on MeBr. A 2002 CUN analysis estimated these crops account for over $215 million dollars annually in total sales to the growers in the region. Based on a USDA Economic Research Service study (Carpenter et al., 2000) and regional calculations, losses to strawberry, tomato, and other vegetable growers were estimated to be $4, $6, and $4 million dollars per year, respectively, if suitable replacements to MeBr were not developed and implemented.

Clearly, growers in the SEUS face a major challenge. Our clientele's challenge tends to be very different than the clientele in other major MeBr-dependent production systems. We typically do not work with large farming enterprises that have the capability to allocate personnel time and financial resources to specifically seek out viable alternatives for their enterprises, to buy and/or modify new equipment, or to allocate large areas to test alternatives (with a perceived high risk). Rather, we work with many growers who tend to have limited acreages that are essential to farm viability. This is well documented for strawberry enterprises (Safley et al., 2004) where the average strawberry enterprise is about 2 ha. As articulated in the 2008 CUN, that includes our clientele within the eastern US industry, "Farms in this region are typically small family farms requiring transition adjustment to newer technologies. Significant uncertainties exist when a change in management strategy is considered. Extension information is slower because of the diversity and size of the farms. Transition to alternatives will occur, but these farmers require the most effective and timely treatment to make what frequently is a marginal profit" (http://www.epa.gov/ozone/mbr/cun2009/cun2009_StrawberryFruit.pdf). Thus, our region faces multiple definable problems: there are technical and economic issues associated with alternatives and there are adoption issues and barriers that need to be overcome.

16.2 Multi-state and Interdisciplinary Response

Major shifts in agricultural practices are complex with highly inter-dependent biological, environmental, social, economic, business, and other (agri)culture factors. Therefore, our first priority was to assemble an inter-disciplinary and inter-state response that sought to include key stakeholders including individual growers and grower associations, fumigant and alternative product manufactures, suppliers and applicators, county extension personnel, private consultants, state and federal expert employees and Land-Grant University specialists (weed science, plant pathology, horticultural science, agricultural engineering, agricultural economics), among others. The goal was to enhance the over-all infrastructure of knowledge, experience and sources of solutions during the transition away from MeBr and to foster broad regional impact. Although not articulated in formal documentation, a fair summary of the short-term goal was to develop and implement crop production systems and inputs that alleviated the predicted economic losses associated with the current phase-out of MeBr and the long-term goal was, and is, to foster a strawberry and vegetable industry that is competitive, sustainable, and conducive to SEUS farm viability.

By necessity, this chapter focuses on components of the region-wide program that are related to plant pathology or managed through the plant pathology initiatives at North Carolina State University (NCSU). Other disciplines within NCSU and other states including SC, GA, VA and FL, in particular, have effective and complimentary programs looking at alternatives in vegetables, providing good inter-disciplinary and inter-state linkages.

16.3 Implementation of a Plan of Action

The “plasticulture” system relies on low- or high-density polyethylene (LDPE; HDPE) mulches to cover raised beds of fumigated soil with buried drip irrigation lines within the bed. Raised beds ensure optimum drainage, reduce contact with non-fumigated soil and facilitate harvest operations. Black LDPE/HDPE warms soils and suppresses weeds, among other benefits. Drip irrigation enables precise application of water, nutrients and other chemicals or additives for optimum crop productivity. The combination of mulch, precision irrigation and management of soilborne pests enhances early and total yield and improves produce quality. MeBr was adopted as a component of the plasticulture system throughout much of the SEUS and has been used routinely, often without due consideration about target pests and production issues. Therefore, the second priority was to determine the risk-aversion and biological basis for fumigation.

16.3.1 Determine the Risk-Aversion and Biological Basis for Fumigation

Although not scientifically documented through structured surveys in the SEUS, grower implementation of alternative pest management tactics appears to be limited due to risk aversion. Growers relied on MeBr due to its wide spectrum of activity and reliable application for efficacious outcomes despite a wide range of management, biological, technology and environmental variables. Other contributing factors may be the perceived and real changes. Changes in cultural systems require changes in scheduling of activities, changes in combination of pest management materials, changes in scouting, changes in target pest delineation, and in some cases, paradigm shifts. All of these changes add uncertainty to the production systems. These changes in 'comfort' level of producers add additional uncertainty and risk to the production system for these high value crops. Despite the level of high activity in research and extension in our region, especially by our team, surprisingly, many growers have not made strategic plans for transition. This relates to our clientele, more risk adverse and less able to allocate human and financial resources to implement alternatives or modify equipment, etc. However, there is more recent and increased interest in transition. In part, this is because the availability of MeBr supplies are dwindling rapidly and the smaller acreage grower cannot compete for the MeBr stock (preferentially allocated to large customers), let alone afford the ever increasing price. To date, few growers have transitioned to other products but have preferred to use reduced rates of MeBr under virtually impermeable films (VIF). The phase out of MeBr has forced the industry to better understand the biological basis for fumigation.

The primary biological consideration has been the pest complexes to be managed. Determining the primary pest complex in each crop has been documented (Table 16.1). For example in strawberries, detailed analysis of the plant pathogens associated with root rot was conducted at multiple sites and States over several years. Sixty different taxa amongst 1300 fungi and stramenopiles isolated from strawberry roots documented *Pythium irregulare* and *Rhizoctonia fragariae* AG-A, AG-G predominated. Important and less prevalent pathogens included *Pythium* HS and Group "F", *Phytophthora cactorum* and a newly named species *Phytophthora bisheria* Abad, Abad & Louws sp. nov (Abad et al., 2008).

16.3.2 Parallel Development and Implementation of Tactics to Replace MeBr

The third priority was to implement a plan that did not simply focus on chemical alternatives, but sought to advance the science of plant pathology and conduct discovery research about the biology, ecology and management of the primary plant pathogens and cropping systems. Three strategic levels of research and extension

Table 16.1 Primary pest problems associated with strawberry and vegetable crops in different ecological zones in NC

Crop	Production region	Relative importance of weeds[a]	Major pathogens[b]	Minor pathogens[c]
Strawberry	All regions	A	*Rhizoctonia fragariae* *Pythium* species *Phytophthora cactorum* Nematodes	*Phytophthora fragariae* *Sclerotium rolfsii* *Fusarium* sp.
Tomatoes	Coastal and Piedmont	B	*Sclerotium rolfsii* *Ralstonia solanacearum* Nematodes	*Fusarium oxysporum* f.sp. *lycopercisi* (race 3) *Phytophthora capsici* *Pythium* sp.
Tomatoes	Mountains	B	*Verticillium dahlia* (race 2) *Fusarium oxysporum* f.sp. *lycopercisi* (race 3)	*Phytophthora capsici* *Pythium* sp. *Ralstonia solanacearum*
Peppers	Coastal and Piedmont	A	Nematodes *Phytophthora capsici* *Sclerotium rolfsii*	*Pythium* sp. *Rhizoctonia solani*
Peppers	Mountains	A	*Phytophthora capsici*	*Pythium* sp. *Rhizoctonia solani*
Water-melons	Coastal (primary production region)	A	Nematodes *Fusarium oxysporum* f.sp. *melonis*	*Pythium* sp.
Squash	Coastal and Piedmont	A	Nematodes *Phytophthora capsici*	*Pythium* sp.
Squash	Mountains	A	*Phytophthora capsici*	*Pythium* sp.

[a] Reflects experience of weed pressure to limit crop productivity and is a score of alternative weed management options available to growers.
[b] Represents the primary target pest of methyl bromide fumigation.
[c] Represents pathogens that occur occasionally, on a limited number of farms, or during early plant growth when environmental conditions favor disease progress.

were identified: (1) Tactic substitution – focused on addressing short term needs of growers who sought non-ozone depleting fumigant alternatives; (2) Tactic Diversification – focused on medium term alternatives that included non-fumigant and IPM based tactics; (3) Tactic Development – focused on long-term goals to explore microbial ecology and farming systems-based approaches to replace MeBr-dependent production systems.

Research and extension efforts comprised three phases: Phase I trials included research on research stations and evaluated alternative farming systems, new products, and novel methods of application; Phase II trials focused on implementing leading alternatives in cooperation with growers and on limited land areas, and Phase III trials tended to be grower driven and focused on tactic substitution on large acreages in the SEUS region. From 2000 to 2007, 60 Phase I, 27 Phase II and 10 Phase III (NCSU-led) trials were implemented. In all experiments, plant pathology, weed science, horticulture science and economic data were documented.

16.3.2.1 Tactic Substitution

The primary alternative fumigants evaluated singly or in combination included Telone-C35 (1,3-dichloropropene 61.1% + chloropicrin 34.7%), chloropicrin (Chlor-o-pic 99% and TriClor EC), InLine (1,3-dichloropropene 60.8% + chloropicrin 33.3%), metam sodium (42% Sodium N-methyldithiocarbamate), and Midas (iodomethane 50% + chloropicrin 50%). The MeBr formulation was 67% methyl bromide and 33% chloropicrin (Terr-O-Gas) or a 50:50 formulation. Products were applied using standard shank injections or injection through the drip irrigation system, depending on the product and experiment. A detailed economic analysis of strawberry production systems based on 15 Phase I trials demonstrated chloropicrin, Telone-C35, and metam sodium generated higher profits than MeBr (Sydorovych et al., 2006). Implementing the fumigant alternatives was also technically feasible in most cases. Limitations included the length of the plant-back time and poor efficacy against specific weed complexes. A similar economic analysis of tomato production systems based on nine Phase I trials generated similar results (Sydorovych et al., 2008). Research since 2006 also demonstrated iodomethane at low use rates combined with VIF appears to be a viable alternative.

On-farm-research (OFR) trials (Phase II and III trials) were implemented on multiple farms and under diverse conditions. In most cases, the OFR was conducted using 2–4 treatments per farm and treatments were arranged in a randomized complete block design. Moreover, growers managed yield data records for the majority of experiments and our team collected additional detailed data (e.g. root rot severity, disease incidence, plant growth data, etc.). A summary of strawberry trials is detailed in Table 16.2. In all cases, alternatives proved viable on each farm and the strength of OFR was realized – novel ideas to address specific problems were generated by growers or collaboratively.

Table 16.2 On-farm research or demonstrations to collect on-farm data and enable strawberry growers to transition

Year and location	Telone-C35[a]	Metam sodium	Pic	Other or combinations	Control	Design
2004–2005						
Str-N GA[c]				T-C35 + ms	MeBr	RCBD, 4 reps
Str-SE VA	×		×		MeBr, non	RCBD, 3 reps[b]
Str-E NC	×		×		MeBr	RCBD, 3 reps
2005–2006						
Str-E. NC	×		×		MeBr	RCBD, 3 reps[b]
2006–2007[d]						
Str-E NC	×			Pic + ms(drip)	MeBr	RCBD, 4 reps
Str-P NC1	×		×		MeBr	RCBD, 4 reps
Str-P NC2				ms + TC35; ms + pic	MeBr, non	RCBD, 3 reps
Str-WP NC	×	×	×	ms + Tc35; ms + pic	MeBr, non	RCBD, 5 reps
	1 rep	1 rep	1 rep			
2007–2008						
Str-P SC	×			Midas	MeBr	RCDB, 3 reps
				2 Film types		
Str-E NC				Pic + 1,3-D (60:40)	MeBr	RCDB, 4 reps
				InLine		
Str-P NC	×	×		Pic + 1,3-D (60:40)	MeBr, non	RCDB, 3 reps
	+Goal	+Goal				

[a] Fumigant broadcast rates are Telone-C35 (T-C35) 262–327 L/ha (28–35 gal/A); metam sodium (ms) 327–655 L/ha (35–70 gal/A); chloropicrin (Pic) 112–168 kg/ha (100–150 lb/A), MeBr (67:33 or 50:50) 224–448 kg/ha (200–400 lb/A), Midas 168 kg/ha (150 lb/A), PicClor 60 (chloropicrin 60% + 1,3-dichloropropene 40%; Pic+1,3-D) 211 kg/ha (188 lb/A). Several trials included virtually impermeable film and often included reduced rates (50–75%) of MB or alternative fumigants.

[b] The MB and/or the non-fumigated (non)treatment was not replicated.

[c] N – north, SE – southeast, E – east, W – west, P – piedmont (central); Georgia (GA), Virginia (VA), South Carolina (SC), North Carolina (NC).

[d] 2006–2008 trials are part of the Area Wide Program (Phase III).

16.3.2.2 Tactic Diversification

Diversification strategies included evaluation of host resistance (Driver and Louws, 2004), seed treatment technologies (Driver and Louws, 2006) and crop rotation combined with no fumigation, among others. A major emphasis was placed in exploring grafting as a potential tool to manage key soilborne pathogens (Rivard and Louws, 2006, 2008). Initial work focused on collaborations with certified organic growers who faced serious crop losses due to southern bacterial wilt (*Ralstonia solanacearum*), Fusarium wilt (*Fusarium oxysporum* f.sp. *lycopersici*) and root knot nematodes (*Meloidogyne incognita*). After experience was gained with a highly receptive clientele, work was expanded to explore the utility of grafting within non-organic production systems. This "diversity tactic" will require considerable more research and extension efforts. However, growers that have severe problems, such as bacterial wilt infested fields, can realize a complete crop loss in infested areas using conventional practices, or 100% plant stand using selective rootstocks (Rivard and Louws, 2008) – a dramatic economic impact for those fields.

16.3.2.3 Tactic Development

Tactic development is rooted in a core belief that biologically-based solutions or radically different farming systems can be developed and economically implemented. We are exploring three main areas where this may occur. First, we are involved in large scale farming systems research that relies on diverse and complementary enterprises to sustain farm viability (Mueller et al., 2001). This work includes assessment of the impact of farming systems on the microbial ecology of soils (Zhang et al., 2005). Microbial ecology is primarily a descriptive science but the vision is that it can become prescriptive i.e. that microbial communities can be reliably managed for specific functions such as plant disease suppression and plant health benefits or greater crop profitability. A second area of exploration is the use of high tunnels to complement grafting for enhanced tomato production per unit area and to enhance fruit quality and enterprise stability and profitability. Finally, a third area of tactic development has been to evaluate the utility of compost, cover crops, crop rotations and beneficial microbes within alternative management systems as an alternative to MeBr dependent systems. Such systems are complex and require more management and more knowledge of biological processes as compared to tactic substitution solutions. A three year study demonstrated a compost-based system generated marketable yields comparable to MeBr, even in the absence of crop rotations (Grabowski, 2001). Subsequent work documented the dynamics of beneficial and indigenous *Trichoderma* species within a compost-based system, but the yield benefits were not realized in the second series of experiments (Leandro et al., 2007a,b) – highlighting the site and management specific intensity of such alternative systems. Never-the-less, there is optimism that viable alternative management systems that do not over-rely on soil fumigants can be developed and integrated into highly productive agricultural systems.

16.4 Extension

The fourth priority was to effectively extend research based information to primary clientele on a continuous basis. Multiple methods were adopted to communicate research-based outcomes. Phase I trials on research stations were complemented with grower field days, with up to 250 people in attendance, particularly at the tomato field days in Western NC. OFR was a critically important component of extension efforts. OFR generated grower driven and novel knowledge, addressed site specific issues, and more importantly engaged industry leaders who in turn were featured at local field days or state- and region-wide conferences. Grower testimony seems to bear more weight than public-sector efforts. Several OFR Phase II and III experiments were linked to county organized field days. Local county agents and field faculty were full partners in these experiments. They in turn helped organize in-field meetings drawing growers from surrounding counties. Field meetings were timed optimally to observe fumigant effects but not disrupt the busy season of work growers encountered. Agent training was also a priority in extension efforts. A coalition of organizers enabled the pooling of resources and expertise to attract 25–30 county agents or field faculty representing AL, FL, GA, SC, TN, VA and NC. The workshops consisted of multiple teaching modules and included: an overview of the pests and issues that drive the need for effective pest management programs; research and extension-based information on current and novel chemical and non-chemical alternatives; hands-on training about equipment and calibration issues; current status and issues associated with the phase-out of MeBr; and a discussion on current "best" and IPM-based alternatives. The workshops featured 14 speakers that could offer a regional expertise in major priority topics. In addition, traditional outlets using web-based dissemination, industry and public-sector newsletters, fruit and vegetable conferences and one-to-one contacts complemented extension efforts.

16.5 Summary

Research efforts associated with the phase out of MeBr generated new knowledge about the biology, ecology, and management of soilborne pathogens associated with strawberry and vegetable crops, on the biology and dynamics of weed species, and horticultural issues associated with optimum crop production. Biological-based and chemical alternatives to MeBr were evaluated in multiple replicated and observational strawberry, double-cropped, and vegetable trials on research stations and through on-farm-research. This fundamental knowledge was simultaneously translated to stakeholders to advance implementation of MeBr alternative products or farming systems. Issues unique to each alternative need to be resolved through additional research but our research provides a solid scientific basis for strawberry and vegetable growers who desire to implement alternatives in site and problem specific ways.

A completed budget analysis of strawberry and tomato production provides growers a tool to make sound economic decisions about pre-plant pest control options. Team efforts in research and extension helped to address the short-term needs of growers and mitigate the long-term impacts on production. Thus, we have clear and definable solutions that will be useful to many growers – and these can be adopted. They are effective and some are sustainable. Numerous issues remain relating to plant back for early crops or under adverse weather conditions, inadequate tools to manage some major soilborne pests, and regulatory challenges related to personal protective equipment and worker exposure, need for buffers, and presence of soil types that limit use of certain products. Never-the-less, many growers could transition or, at a minimum, explore the viability of transitional strategies, and we desire to enable the process.

References

Abad ZG, Abad JA, Coffey MD, Oudemans PV, Man in 't Veld WA, de Gruyter H, Cunnington J, Louws FJ (2008) *Phytophthora bisheria* sp. nov., a new species identified in isolates from the Rosaceae raspberry, rose and strawberry in three continents. Mycologia100:99–110

Carpenter J, Gianessi L, Lynch L (2000) The economic impact of the scheduled U.S. phaseout of methyl bromide. National Center for Food and Agricultural Policy. http://www.ncfap.org/documents/mb.pdf

Driver JG, Louws FJ (2004) IPM and fumigation: host resistance to manage *Phytophthora* in peppers. Proceedings of the international research conference on methyl bromide alternatives and emissions reduction. 88/1-88/2. http://mbao.org/2004/Proceedings04/088%20DriverJ%20Louws%20Pepper%202004.pdf

Driver JG, Louws FJ (2006) Effects of seed treatments to manage nematodes as an alternative to methyl bromide on cantaloupe. Proceedings of the international research conference on methyl bromide alternatives and emissions reduction. 51/1-51/2.

Grabowski MA (2001) Biologically- and chemically-based alternatives to methyl bromide fumigation in strawberry production. NCSU MS thesis, p.113. http://mbao.org/2006/06Proceedings/051DriverandLouws2006.pdf

Leandro LFS, Ferguson LM, Louws FJ, Fernandez GE (2007a) Strawberry growth and productivity in fumigated compared to compost-amended production systems. HortScience 42:227–231

Leandro LFS, Guzman T, Ferguson LM, Fernandez GE, Louws FJ (2007b) Population dynamics of *Trichoderma* in fumigated and compost-amended soil and on strawberry roots. Appl Soil Ecol 35:237–246

Martin FN (2003) Development of alternative strategies for management of soilborne pathogens currently controlled with methyl bromide. Ann Rev Phytopathol 41:325–350

Mueller JP, Barbercheck ME, Bell M, Brownie C, Creamer NG, Hu S, King L, Linker HM, Louws FJ, Marra M, Raczkowski CW, Susko D, Wagger MG (2001) Development and implementation of long-term agricultural systems studies: challenges and opportunities. HortTechnology 12:362–368

O'Dell C, Williams J (2000) Hill system plastic mulched strawberry production guide of the colder areas. Virginia Tech Cooperative Extension, Publication 438-018, 32 pp

Poling EB, Krewer G, Smith JP (2005) Southeast Regional Strawberry Plasticulture Production Guide. http://www.smallfruits.org/SmallFruitsRegGuide/Guides/2005culturalguidepart1bs1.pdf

Ristaino JB, Thomas W (1997) Agriculture, methyl bromide, and the ozone hole: can we fill the gaps? Plant Dis 81:964–977

Rivard CL, Louws FJ (2006) Grafting for disease resistance in heirloom tomatoes. NC cooperative Extension Service Bulletin AG-675

Rivard CL, Louws FJ (2008) Grafting to manage soilborne diseases in heirloom tomato production. HortScience 43:2104–2111

Safley CD, Poling EB, Sydorovych O, Louws FJ, Fernandez GE, Ferguson LM (2004) Estimated costs of producing, harvesting, and marketing strawberries in North Carolina. Agricultural and resource economics report number 28, North Carolina State University, Raleigh, NC, p 26

Sanders DC, Cook WP, Granberry D (1996) Plasticulture for commercial vegetables. North Carolina Cooperative Extension Service, Bulletin AG-489, p 22

Sydorovych O, Safley CD, Ferguson LM, Poling EB, Fernandez GE, Brannen PM, Monks DM, Louws FJ (2006) Economic evaluation of the methyl bromide alternatives for the production of strawberries in the Southeastern US. HortTechnology 16:118–128

Sydorovych O, Safley CD, Welker RM, Ferguson LM, Monks DM, Jennings K, Driver J, Louws FJ (2008) Economic evaluation of methyl bromide alternatives for the production of tomatoes in North Carolina. HortTechnology 18:705–713

Zhang W, Wu J, Tu C, Diab HG, Louws FJ, Mueller JP, Creamer NG, Bell M, Wagger MG, Hu S (2005) Responses of soil microbial community structure and diversity to agricultural deintensification. Pedosphere 15(4):440–447

Chapter 17
Integrated Control of Soilborne Pathogens of Wheat

Timothy C. Paulitz, Patricia A. Okubara, and Kurtis L. Schroeder

Abstract There are no resistant varieties or chemical controls for the major soilborne pathogens of wheat in the Pacific Northwest of the United States. Root and crown diseases of wheat include Rhizoctonia root rot and bare patch (caused by *R. solani* and *R. oryzae*), Fusarium crown rot (caused by *F. pseudograminearum* and *F. culmorum*), Pythium root rot (caused by numerous *Pythium* spp.) and take-all (caused by *Gaeumannomyces graminis* var. *tritici*). Growers rely almost completely on cultural control measures. Our research program has evaluated many of these techniques, some of which are effective against *Rhizoctonia*, including greenbridge (weed and crop volunteer) management, fallow (both chemical and mechanical), seed opener disturbance, precision seed row placement, crop rotation, residue and nitrogen management, and chemical seed treatment. Until recently, there was no way of accurately detecting and quantifying pathogens in soil. We have developed real-time PCR assays to quantify nine species of *Pythium* and seven groups of *Rhizoctonia*, based on ITS sequences of the rDNA. In the last 3 years, soils were extensively sampled in eastern Washington, including grower fields and breeder variety-testing sites. By developing a pathogen profile for each testing site, breeders can focus on locations with high pathogen densities to select for tolerance. This survey has shown that pathogen species composition is affected by cropping system and rotation. With accurate detection and quantification of soilborne pathogens, growers can determine risk before planting and make management decisions to mitigate the effects of soilborne fungal pathogens.

Keywords Soilborne fungal pathogens of wheat • quantitative PCR • greenbridge management • take-all • Fusarium crown rot • Rhizoctonia bare patch and root rot • Pythium root rot

T.C. Paulitz (✉), P.A. Okubara and K.L. Schroeder
United States Department of Agriculture, Agricultural Research Service,
Root Disease and Biological Control Research Unit, Pullman, WA, 99164-6430, USA
e-mail: paulitz@wsu.edu

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,*
Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_17,

17.1 Introduction

Wheat is the third most important staple cereal crop in the world after rice and corn. In 2003, 204 million ha of wheat were grown worldwide and 549 million metric tons were produced (Food and Agriculture Organization, United Nation, 2008). The United States is the fourth largest producer in the world (56 million metric tons, $13.6 billion in 2007) and a major wheat exporter, accounting for about one third of the world wheat trade. The Pacific Northwest (PNW) of the United States, consisting of the states of Washington, Oregon, and Idaho, is one of the largest wheat producing regions, growing 7 million metric tons for a value of $1.92 billion in 2007 (National Agricultural Statistics Service, 2008). Most of the wheat production in the PNW is winter wheat (79%), primarily soft white wheat exported to the Asian and Middle Eastern markets for noodle and flat bread (Washington State Grain Alliance, 2008). As in most industrialized countries, wheat in the PNW is produced on large farms averaging 1,000–2,000 ha in size, highly mechanized with expensive specialized equipment for tillage, cultivation, planting, and harvesting.

Most of PNW wheat is produced east of the Cascade Mountains without irrigation, in zones ranging from 150 to 650 mm precipitation per year. This area has a mediterranean climate typified by warm, dry summers and most of the precipitation occurs in late fall, winter and early spring as rain or snow. Because most of the precipitation falls outside of the growing season, the plants must rely on stored soil moisture. Due to the of the lack of summer rainfall, foliar diseases are not a major problem as they are in the Midwest of the United States and Europe, where Septoria foliar pathogens and Fusarium head blight pathogens require numerous applications of foliar fungicides. Stripe (yellow) rust, however, can cause significant yield losses in the PNW in epidemic years. As a result of this unique climate, soilborne pathogens of wheat are the major biotic yield constraint in the PNW, after weeds.

Cropping systems and rotations in the PNW vary by precipitation zones. In the higher precipitation areas, winter wheat, which is the most profitable crop, is grown in a 3-year rotation with a spring cereal (wheat or barley) in the second year and a spring pulse crop (pea, lentil or chickpea) in the third year. Spring canola is sometimes used in rotation in place of the pulse crop. Because of the higher precipitation, a crop can be grown every year. In contrast, in the low and intermediate precipitation areas, there is only enough stored water in the soil profile to grow a crop every other year, and the land is fallowed during the second year.

In the eastern part of the dryland region of Washington State, the soils are rich, wind-deposited loess silt loams with a hilly topography (hills 50–150 m in height, slopes up to 40% with 2–3 m depth of top soil). However, soil erosion from water runoff can be a severe problem, especially when the soil is frozen in the winter. In the lower rainfall areas, soil erosion from wind can cause environmental problems, especially particles of <10 μm in diameter which can be a health hazard (Papendick, 2004). Much of this soil erosion problem is eliminated by use of no-till or direct-seeding, where the crop residue is left undisturbed prior to planting, and the crop is sown directly into the existing crop stubble. However, less than

20% of the growers in the PNW practice no-till or direct-seeding, in part because of the problem of some soilborne pathogens which can increase with the lack of tillage (Paulitz, 2006).

This synopsis of management of soilborne pathogens of wheat will primarily focus on practices, methods, and techniques, both existing and experimental, used by growers in the PNW. However, these concepts can also apply to wheat cropping systems in other parts of the world. Unlike higher-value crops, there are no economical fungicides or chemicals effective against soilborne pathogens that rot the root systems of wheat. Likewise, there is no genetic resistance effective in adapted cultivars against these necrotrophic generalist pathogens. Thus, growers must rely on integration of a range of cultural practices and methods to reduce the impacts of these soilborne pathogens. Such cropping systems require complex management decisions that change from season to season and from field to field, as the grower seeks to balance the economic, crop health, and environmental constraints.

17.2 The Pathogens

The following four root and crown diseases are the most important in the PNW, and will be the focus of this chapter. Because of the limitation of space, we will not cover nematode diseases. Lesion nematodes (*Pratylenchus neglectus* and *P. thornei*) and cereal cyst nematode (*Heterodera avenae*) have been shown to have impacts on wheat in the PNW (Smiley et al., 2005a, b, c). In addition, there are a number of minor diseases that have been recently managed with genetic resistance: strawbreaker foot rot or eyespot, caused by *Tapesia yallundae* and *T. acuformis* (= *Pseudocercosporella herpotrichoides* [anamorph]); Cephalosporium stripe, caused by *Cephalosporium gramineum*; and snow molds caused by *Typhula*, *Pythium* and *Microdochium* spp.

17.2.1 Fusarium Crown Rot

This disease, which also goes by the name of dryland foot rot, is caused by a complex of pathogens in the PNW, including *Fusarium pseudograminearum*, *F. culmorum* and *Bipolaris sorokiniana*. This disease is also is important in Australia and the Middle East. The pathogens survive as macroconidia or chlamydospores in soil or as mycelium in infected residues. Roots and crowns become infected early in the growing season, but the pathogen can remain latent until the plant undergoes water stress. Under these conditions, the pathogen will colonize the lower stem, cutting off the supply of nutrients and water to the grain, resulting in whiteheads and shriveled grain. One of the major cultural practices contributing to increased disease is overfertilization with N, which results in lush vegetative growth early in the season,

but as plants deplete water from the soil profile, they undergo drought stress which predisposes them to infection by the pathogen (Papendick and Cook, 1974). Fusarium crown rot can cause up to 35% yield losses, with an average of 9% loss (Smiley et al., 2005d). This disease has become more important in recent years, with the increased cultivation of hard red varieties which require higher levels of N fertilization to attain sufficient protein levels.

17.2.2 Take-All

This disease, caused by the ascomycete *Gaeumannomyces graminis* var. *tritici* (*Ggt*), is the most important soilborne disease of wheat worldwide, especially in areas with high rainfall or irrigation, since high soil moisture is required for infection (Asher and Shipton, 1981). It can infect wheat and barley as well as other grasses. *Ggt* infects the roots and produces dark runner hyphae that can move along the surface of the root and into the crown, blocking the flow of water and nutrients. Whiteheads frequently occur in a patchy distribution in the field, and the disease can significantly reduce yields. Under high moisture conditions, the pathogen causes a blackening of the roots and lower crown. Surveys in the PNW found low levels of disease in most dryland fields, but the disease can be very destructive in irrigated crop circles grown with continuous wheat (T.C. Paulitz, 2008).

17.2.3 Rhizoctonia Bare Patch and Root Rot

This disease was first observed in the PNW in the mid 1980s (Weller et al., 1986). The characteristic symptom is bare patches in the field, ranging several meters across, where the plants are severely stunted, and show signs of nutrient deficiency, especially P deficiency. However, it also has a chronic phase, without patches, where the root rot causes stunted plants or uneven stands on wheat or barley in higher rainfall areas (Paulitz et al., 2002). The bare patch is associated with *Rhizoctonia solani* AG-8, but the etiology of this disease is much more complex, with other species and anastomosis groups present and detected (discussed in next section). *R. solani* AG-8 causes distinct symptoms on the root, including browning and killing of the root tips (spear-tipping) and rot of the cortex, leaving a constriction with just the stele intact. Of the four major root pathogens, *Rhizoctonia* is the most associated with no-till or reduction in tillage (Rovira, 1986; Pumphrey et al., 1987). Recent work in the PNW has shown that *R. solani* becomes a major problem during the third and fourth year of the transition from conventional tillage to no-till (Schroeder and Paulitz, 2006). Tillage may break up hyphal networks in the soil, or may stimulate a flush of microbial growth that inhibits the pathogen.

17.2.4 Pythium Root Rot

Of all the four diseases, Pythium root rot has the least distinct symptoms, either above or below ground. Pathogenic *Pythium* species are ubiquitous in most soils and can attack the embryo of cereals within 24 h after planting, but rarely causes seedling death or pre-emergence damping-off of wheat, unlike with large-seeded dicots. Instead, *Pythium* attacks the root tips, feeder roots, root hairs, and juvenile tissue, rotting the root system while soil conditions are moist. It was not until the development of metalaxyl, a compound with specific activity on oomycetes, that the true impact of the disease was appreciated. In a series of trials, Cook et al. (1987) showed that *Pythium* caused from 3% to 36% in yield reduction, and metalaxyl could increase yields by 2.2 to 4.4 tons/ha. As with *Rhizoctonia*, an increasing number of species have been found on wheat in the PNW, which will be discussed below. *Pythium* spp. survive primarily as oospores in dead roots. The disease is favored by cool, poorly drained wet conditions, which are favorable for the production of zoospores, motile swimming spores that are chemotactically attracted to roots and can initiate infection.

17.3 Molecular Detection and Quantification of Soilborne Pathogens

In order to manage soilborne pathogens, growers must first know *what* pathogens are present, *where* they are prevalent, and finally *how much* inoculum is in the soil in order to define risk. In addition, by knowing the biogeography of the pathogens and their association with different cropping systems and rotations, growers can take this into account to make their decisions.

In 2000, we initiated a research program to understand the pathogen diversity of *Rhizoctonia* and *Pythium* in the PNW. It was thought that *Rhizoctonia solani* AG-8 was the main pathogen responsible for Rhizoctonia root rot and bare patch, but surveys in 2000–2001 indicated that *R. oryzae* (perfect stage = *Waitea circinata*) was much more widespread and predominant (Paulitz et al., 2002). This pathogen had been previously detected in the PNW (Ogoshi et al., 1990), but was thought to be less virulent. Paulitz et al. (2002) showed that many isolates were highly virulent, and could also attack peas (Paulitz, 2002). We have also shown the widespread distribution of *R. solani* AG 2-1, primarily a pathogen on brassica crops (Paulitz et al., 2006), binucleate *Rhizoctonia* spp. (AG-I-like *Ceratobasidium* spp.) and *R. solani* AG-10 (Okubara et al., 2008). The latter two groups do not appear to cause much damage to cereals, but can cause stunting of broadleaf rotation crops.

A similar complexity of *Pythium* species was found in eastern Washington. Previously, Chamswarng and Cook (1985) found 10 different species, including *Pythium ultimum* var. *ultimum*, *P. ultimum* var. *sporangiferum*, *P. irregulare*, *P. torulosum* and *P. heterothallicum*. In 2000–2001, 80 sites were surveyed in eastern Washington, and a collection of over 500 isolates were identified using classical

morphology and sequencing of the ITS region of the rDNA of a subset of the collection (Paulitz and Adams, 2003). Fourteen species were found, including a new species described as *Pythium abappressorium* (Paulitz et al., 2003a). Other newly found species were *P. paroecandrum*, two types of *P. irregulare* (Groups I and IV *sensu* Matsumoto, Group IV originally identified as *P. debaryanum* in this chapter), *P. rostratifingens* (originally described as *P. rostratum*), *P. attrantheridium* (originally described as *P. intermedium*), and *P. sylvaticum*. These species were tested for virulence in greenhouse assays, and all species caused significant root loss on wheat. The most virulent were *Pythium ultimum*, *P. irregulare* Group I, and *P. irregulare* Group IV (Higginbotham et al., 2004a).

A real-time PCR assay was developed for quantification of DNA from soils for nine species of *Pythium* (Schroeder et al., 2006). Primers were designed from the ITS regions of the rDNA. Standard curves were constructed by spiking soil with known levels of inoculum grown on autoclaved soil amended with oatmeal. Using a capillary PCR system with SYBR green and DNA extracted using commercial kits (MoBio, Carlsbad, CA, USA), we can detect as little as 500 fg DNA/g of soil, or between 1–5 propagules/g. A similar system was developed for detection and quantification of *Rhizoctonia* groups, including *R. solani* AG-8, 2-1, and 10; *Ceratobasidium* spp. (AG-I-like), and three genotypes of *Rhizoctonia oryzae* (Okubara et al., 2008). Using these methods, we have conducted extensive sampling from variety testing sites and grower's fields over the last 3 years, and have made several interesting observations about the distribution of these pathogens and correlations with rotations and cropping systems. *R. solani* AG-8 is found in high levels only in areas of the state where the bare-patch symptoms are predominant, in lower precipitation (winter wheat–summer fallow) areas with sandy soil, especially at no-till sites. Interestingly, this pathogen can be detected in very low level in higher precipitation areas with continuous cropping, but is often too low to quantify. Why the pathogen occurs at low population densities and does not cause bare patch in these areas is not understood – do edaphic (soil) or microbiological conditions limit its distribution? A similar geographical gradient is seen with *Pythium* spp., in which high populations are found in the continuous cropping zones with higher precipitation and much lower populations in the low precipitation – summer fallow regions. Of all the *Pythium* spp., *P. irregulare* Group IV appears to have the widest distribution, whereas the most virulent species, *P. ultimum* is rare. Both *P. ultimum* and *P. irregulare* Group I appear to be associated with rotation with legumes, especially lentils. *R. solani* AG-2-1, although a Brassica pathogen, seems to be associated with legume rotations. The ability to quantify individual species will provide useful information for wheat breeders. We are developing a pathogen profile for the variety testing sites across the state and identifying sites with high pathogen pressure, so breeders can screen and select germplasm for tolerance or resistance to these soilborne pathogens. This technology also will be useful for growers, to determine the risk of pathogen damage before planting and make management decisions based on what is present in the soil. We still need to accumulate agronomic and field data to develop these economic thresholds, and this technology is currently being transferred to a commercial testing laboratory.

17.4 Integrated Control Methods

17.4.1 Genetic Resistance or Tolerance

At the present time, there is no identified genetic tolerance or resistance to Rhizoctonia root rot. Smith et al. (2002a, b) screened spring wheat, spring barley, and synthetic wheat hexaploids in inoculated greenhouse and field tests, but did not detect any resistance or tolerance. However, *Dasypyrum villosum*, a wild grass of the Mediterranean that can be hybridized with wheat, showed resistance. A similar screening of spring wheat cultivars with *Pythium ultimum* and *P. irregulare* Group IV (identified as *P. debarynum* in this chapter) showed that cultivars differed in susceptibility, but displayed no true resistance or tolerance (Higginbotham et al., 2004b). A similar lack of resistance to take-all has been noted, despite extensive testing (Tineline et al., 1989).

However, resistance to Fusarium crown rot has been detected and moved to adapted wheat cultivars in Australia (Wallwork et al., 2004; Wildermuth and Morgan, 2004). In addition, QTLs have been identified from mapping populations made from crosses with highly resistant Australian lines (Collard et al., 2005). Presently, efforts are underway to incorporate this resistance in to cultivars adapted to PNW conditions and to identify QTLs that can be used in marker-assisted selection.

17.4.2 Chemical Control

Because of the low economic value of wheat and lack of chemicals effective in soil, there are no soil-applied fungicides registered for wheat in the United States. However, most growers in the PNW use seed treatments, both protectant and systemic, to protect against smuts and bunts. Tebuconazole, triticonazol, difenoconazole, or demethylation inhibitors (DMIs) are often used in combination with metalaxyl or mefanoxam, protectants against *Pythium*. Thiram and fludioxonil, a phenylpyrrol, are also used. These chemicals are not translocated to the roots to protect against root rot and often do not increase yield (Smiley et al., 1990), although sometimes improve seedling health (Paulitz and Scott, 2006) and give slight yield increases (Cook et al., 2002b).

Two recently registered seed treatment chemicals have been shown to control take-all-silthiofam, which inhibits ATP transport from mitochondria, and fluquinconazole, a triazole fungicide with broad spectrum activity. Both chemicals are fungistatic and neither is registered in North America.

17.4.3 Crop Rotation

Crop rotation is the most effective method of controlling take-all. A 1- or 2-year break with a non-host crop such as a broadleaf legume, oats or corn is enough to

reduce the disease below economic levels. Barley is ineffective as a rotation crop as it is also a host to the take-all pathogen, but is less susceptible. However, rotation is not as effective for the other three pathogens. *Rhizoctonia solani* AG-8 has a wide host range, and can attack peas, lentils and canola in greenhouse trials. In the field, rotation with canola, mustard or safflower did not reduce bare patches (Cook et al., 2002a). Likewise, *R. oryzae* attacks both cereals and broadleaf crops. *Pythium* species are generalized necrotrophs with wide host ranges, and rotation may not be effective. However, recent results from real-time PCR quantification of *Pythium* populations in soils indicate that pathogen species prefer specific hosts (K.L. Schroeder et al., 2008). For example, *P. irregulare* Group I appears to be highly correlated with lentils in rotation. Further work may show that crop rotation shifts species composition, and may be useful for disease management. Crop rotation does not appear to reduce Fusarium crown rot, because the pathogen can survive in the soil for long periods as chlamydospores (in the case of *F. culmorum*) or as mycelium in crop residue (in the case of *F. pseudograminearum*) (Inglis and Cook, 1986). DNA concentrations of *F. culmorum* and *F. pseudograminarum* in soil were not affected by rotation with canola, peas or barley (Davis et al., 2008).

17.4.4 Tillage

Tillage is effective in reducing Rhizoctonia bare-patch, which is more severe under no-till conditions (Rovira, 1986; Pumphrey et al., 1987). *Rhizoctonia solani* causes severe yield losses in the third year after tillage is stopped (Schroeder and Paulitz, 2006). However, tillage reduces soil quality and structure, requires increased energy inputs, and causes soil loss and erosion (Pimentel et al., 1995). One strategy that may be useful for no-till is to provide more disturbance in the seed row (Roget et al., 1996), thus creating a zone where *Rhizoctonia* is temporarily suppressed. We have tested two types of no-till openers in fields with severe patches: low disturbance single disk openers compared to higher disturbance hoe or shank type openers. The high disturbance openers gave better seedling performance early in the season, but did not resulted in yield increases (T.C. Paulitz, 2006).

The effectiveness of tillage in controlling the other three diseases is not clear. Since *Fusarium* can survive in the above-ground residue and crowns, one could hypothesize that turning under the crop debris would reduce disease. Indeed, Bailey et al. (2001) found more disease in no-till systems, based on a multivariate study of 7 trial-years. However, no-till may conserve water, and the reduction in water stress may reduce disease. In fact, in the PNW, the disease is very common in conventional tillage. A similar discrepancy has been seen in research with take-all: some studies showed less disease with no-till (Bailey et al., 2001), whereas others showed more disease with no-till (Roget et al., 1996). Tillage probably has no direct effect on *Pythium*, because the oospores are long-lived and can survive outside of crop residue. But the lack of tillage leaves increased crop residue on the surface. In the spring, the increased residue may keep the soils wet and cooler for a longer period

of time, providing a larger window and more conducive conditions for *Pythium* infection. But tillage can create tillage pans and poor drainage which is expected to increase *Pythium* damage. No-till improves soil infiltration and drainage, which may make soil conditions less conductive for *Pythium.*

17.4.5 Greenbridge Management

The strategy of controlling greenbridge carryover of inoculum to a new crop by timely use of herbicides is widely recognized and used by growers, and applies to most soilborne pathogen, but especially *Rhizoctonia* (Smiley et al., 1992). Most soilborne pathogens with wide host ranges will also colonize grassy weeds and volunteer crops. When weeds and volunteers are killed immediately before planting, using a non-specific herbicide such as glyphosate, the pathogen can extensively colonize the dying roots. This is hypothesized to occur because glyphosate inhibits the shikimic acid pathway, a crucial intermediate in the biosynthesis of aromatic defense compounds. The resulting 'glyphosate synergy' (Lévesque and Rahe, 1992), allows the pathogen to produce high levels of inoculum before the crop is planted. It is recommended that growers wait at least 3 weeks after spraying, to allow time for the inoculum to decline and be reduced by microbial action. Recent research by our group has shown an asymptotic response: 3 to 4 weeks is the optimum time to spray out the crop, but longer intervals do not increase plant growth. The greenbridge control strategy also has been demonstrated with take-all (Hornby et al., 1998) and *Pythium* (Lévesque and Rahe, 1990).

17.4.6 Row Spacing and Precision Placement

Increasing the row spacing by using paired rows increases the time that the canopy is open to allow the soil to warm and dry, making conditions less favorable for take-all and *Pythium*. By using paired rows, the total population density of plants is not decreased. Cook et al. (2000) found this strategy could reduce take-all incidence. Another strategy in no-till systems is precision placement of seed rows to avoid the previous year's relic row, which contains the inoculum in intact undecomposed roots and crowns (Bockus and Schoyer, 1998). This can be achieved using precision GPS-located steering on tractors or by seeding at an angle from the previous year. As the distance from the inoculum source was increased, infection of seedlings by *Ggt* decreased (Kabbage and Bockus, 2002) and mathematical models suggested that planting between the old seed rows could reduce take-all losses by 50% (Garrett et al., 2004). However, a similar strategy applied to field trials in eastern Washington did not result in any reduction of *Rhizoctonia,* probably because this pathogen can survive in intact roots that extend in between the rows, rather than in infected crowns, as is the case for take-all (Davis et al., 2008).

17.4.7 Fallow

Theoretically, the inoculum of a soilborne pathogen may be reduced during a period without a host, due to depletion of energy in the resting structures and the suppressive action of microflora and microfauna. The concept of a short chemical fallow period for reducing *Rhizoctonia* is well accepted; this is part of the greenbridge management strategy. For example, MacNish and Fang (1987) showed that fallow periods of 26 days did not reduce *Rhizoctonia* on wheat, but periods of 3 to 6 weeks reduced *Rhizoctonia* severity in direct-seeded wheat (Roget et al., 1987). The survival of *Rhizoctonia* in longer-term fallow conditions under no-till was studied in a series of experiments using a toothpick baiting method (Paulitz and Schroeder, 2005) to quantify the activity of *Rhizoctonia* in the soil. In the higher rainfall zones of eastern Washington, *Rhizoctonia oryzae* was not reduced by 3 years of chemical fallow, and *R. solani* AG 2-1 was only reduced in the third season of fallow. In another experiment, both chemical fallow and a low-disturbance mechanical fallow reduced the activity of *R. solani* AG-8 in a field with active bare patches. Reduced activity was observed both during the fallow period and the following year when the field was planted (T.C. Paulitz, 2007). However, *R. oryzae* was not influenced by fallow. This indicated that *R. oryzae* can survive as microsclerotia in the absence of the host, but inoculum can decline over time without the host. However, because both *R. solani* AG 2-1 and AG-8 survive in roots, which can remain intact in no-till systems and provide protection for the pathogens, the decline in inoculum is very slow and may not fall below economic thresholds in all situations.

Because the take-all pathogen has limited saprophytic ability and must survive on roots, fallow is an effective management technique for this disease. For example, both fallow and sorghum as a summer crop were more effective than soybean in reducing take-all on wheat (Rothrock and Langdale, 1989). Wheat after fallow had significantly less take-all disease in long-term trials in the southeastern United States (Cunfer et al., 2006). On the other hand, fallow does not appear to reduce Fusarium crown rot in dryland PNW, since the pathogens can survive as chlamydospores, macroconidia, or mycelium in crop residue in dry soils through the summer fallow period. Information on fallow effects on *Pythium* is lacking. However, using molecular quantification of DNA from extensive surveys taken during the last few years in eastern Washington, most species of *Pythium* drop to below detectable levels in fallow soil, except for *P. abappressorium* and *P. irregulare* Group IV (K.L. Schroeder and T.C. Paulitz, 2006).

17.4.8 Residue Management

In direct seed or no-till systems, the crop residue is left on the soil surface. This has benefits for building up soil organic matter, improving soil structure, and reducing soil erosion. Soil infiltration is also improved, because the intact roots create channels in the soil. High populations of earthworms also create burrows and stable aggregates that improve water movement. However, excessive residue may create problems for

seeding the following year, due to the absence of cultivation or seed bed preparation in no-till. Excessive straw can clog seed openers or tuck into the seed row, preventing adequate soil coverage of the seed. Some growers use burning, mowing, or harrowing to break up the straw or chaff and incorporate straw spreaders on the back of combines. Excessive residue can exacerbate pathogens that survive as inoculum in crop residue, resulting in more diseases. For example, higher levels of stubble can increase Fusarium crown rot (Summerell et al., 1989; Smiley et al., 1996).

Burning and mechanical removal of straw did not reduce inoculum levels of *R. solani* AG-8 in a no-till cropping system study (Paulitz et al., 2009). *R. solani* AG-8 survives primarily in the intact root systems, rather than in the crowns, so removal of stubble may have little effect. This is contrary to other reports (Pumphrey et al., 1987; Weller et al., 1986), in which Rhizoctonia root rot was more severe with high residues. Another effect of excessive residue is that it keeps the soil cooler and wetter in the spring by acting as a mulch and reflecting solar radiation. This may exacerbate Rhizoctonia root rot, which is favored by cooler soil conditions (Ogoshi et al., 1990; Mazzola et al., 1996). The two most pathogenic *Pythium* spp. on wheat in the PNW, *P. ultimum* and *P. irregulare*, are active at soil temperatures of 10°C and 5°C, respectively (Ingram and Cook, 1990). Therefore, Pythium diseases are also favored by cold, wet, high residue seed beds (Cook et al., 1990).

17.4.9 Altering Planting Date

Since Rhizoctonia and Pythium diseases are favored by wet, cool conditions in the spring, less damage may result from later planting when the soils warm up. However, in practice, spring cereals have a narrow planting window in the PNW, because later planting severely reduces yields. But, with fall-planted cereals, there is a greater window, because of a longer growing season (9 months compared to 4 months), although yield is reduced with later planting. Later planting of winter wheat reduced Fusarium crown rot compared to early plantings, because later plantings did not outstrip the water supply (Cook, 1980). Early sowing dates for winter wheat in the fall can increase take-all, whereas cooler soil temperatures later in the fall are less favorable for infection by *G. graminis* var. *tritici* (Cook and Veseth, 1991).

17.4.10 Seed Quality

Growers use certified seed to prevent introducing seed borne pathogens such as smuts and bunts. None of the four soilborne pathogens considered here are seed-borne. However, seed quality has been shown to affect Pythium diseases. Older seed takes longer to germinate and emerge, increasing the time that the embryo

and emerging seedling are susceptible to the pathogen (Hering et al., 1987). Deterioration of the seed coat in older seed also results in the release of more exudates that attract *Pythium*.

17.4.11 Plant Nutrition

Plant nutrition can play a critical role in plant health when plants are attacked by soilborne pathogens. In the case pathogens that rot the roots of seedlings, application of starter fertilizer in the seed row can compensate for the root pruning and stripping of root hairs, which are crucial for the uptake of relatively immobile nutrients such as phosphorus. One of the symptoms of both *Pythium* and *Rhizoctonia* is nutrient deficiency, such as P deficiency (purpling of leaves) in the case of *Rhizoctonia*. Aqueous solutions of fertilizers are often applied right below the seed or to the side of the seed, to provide quick access to the plant. Nutrients do not directly affect plant resistance, but enhance seedling vigor to compensate for pathogen damage (Patterson et al., 1998). Under Zn deficient conditions, application of Zn decreased disease severity and patch area caused by *R. solani* AG-8 (MacNish and Neate, 1996).

However, additional Zn applications in patches in an experiment in the PNW did not provide a benefit (Cook et al., 2002a).With take-all, the type of N source can affect disease severity, as take-all is more severe at neutral to alkaline conditions (Asher and Shipton, 1981). Ammoniacal forms of N decrease soil pH and decrease take-all, while NO_3 forms can increase the disease (Brennan, 1993). Take-all can be more severe under Mn deficiency, which can be alleviated by application of manganese sulfate to the soil. *Ggt* can oxidize Mn, an ability correlated with virulence (Wilhelm et al., 1988; Thompson et al., 1996). In the case of *Fusarium*, as discussed earlier, excessive N leads to higher levels of disease.

17.4.12 Suppressive Soils

Suppressive soils are those that contain a virulent pathogen, a susceptible host, and an environment favorable for disease, but the disease occurs at low incidence or severity, or is absent. The paradigm for suppressive soils is take-all decline, documented by R. J. Cook in the 1970s and 1980s in the PNW (Weller et al., 2002). After several years of monoculture of wheat, the disease levels increase, but with continuing monoculture, the disease declines. This phenomenon has been shown to be due to antagonistic microflora, specifically bacteria of the genus *Pseudomonas*, which produce antifungal compounds such as phenazine-1-carboxylic acid or 2,4-diacetylphloroglucinol (Weller et al., 2002). These bacteria are present in the take-all lesions on the host roots, and reach high population in the presence of the disease and wheat monoculture (Cook et al., 1995; Raaijmakers et al., 1999; Cook, 2003).

There also is evidence of natural suppression to Rhizoctonia disease. In Australia, Rhizoctonia root rot declined to almost nil after 7 to 9 years of continuous wheat cropping (MacNish, 1988). Similarly, Roget (1995) observed that Rhizoctonia patch development decreased after 5 years of continuous no-till wheat and reached negligible levels after 10 years. Several lines of evidence indicate that suppressiveness may be developing at sites in the PNW, particularly at a site with a 10-year history of bare patches. Firstly, many bare patches mapped using GPS over 8 years have disappeared over time. Secondly, cores taken from the center of patches and planted with cycles of monoculture barley over a 9-month period in the greenhouse yielded stunted barley at the start of the experiment, but did not produce stunting at the end of the experiment in over half of the cores (Paulitz et al., 2003b). Thirdly, wheat following barley had significantly fewer patches and greater yield than did continuous wheat, but this occurred only after 5 years of continuous no-till (Schillinger and Paulitz, 2006). This is unexpected because barley is highly susceptible to *Rhizoctonia*, arguing against a typical crop rotation response from a resistant host. Finally, recently we have isolated unique groups of phenazine-producing pseudomonads in high populations from the roots of wheat in patches; these bacteria may be involved in this suppression (D. Mavrodi, 2008).

17.4.13 Biological Control

Over the past 30 years, a wide range of fungi and bacteria have been tested to control soilborne pathogens on many crops. However, many would not be economical even if they were effective, because of the large amount of biocontrol inoculum that would have to be applied to the soil. One strategy of biological control that would be economical with wheat cropping systems is seed treatments, especially with rhizosphere-competent bacteria that effectively colonize the roots at high population densities. A number of experiments and studies with *Pseudomonas* and *Bacillus* have shown the effectiveness of seed treatment for reducing take-all, Pythium and Rhizoctonia root rot under PNW conditions (Weller and Cook, 1986; Kim et al., 1997; Raaijmakers and Weller, 2001). *Trichoderma* has also been shown to be effective (Duffy et al., 1996). Despite numerous attempts at commercialization and registration, only two biocontrol agents are registered on wheat as seed treatments for the PNW – Kodiak (*Bacillus subtillus* GB03) marketed by Bayer CropScience and T-22 Planter Box (*Trichoderma harzianum*) marketed by BioWorks, Inc.

17.5 Conclusions

Wheat yield is limited by numerous soilborne pathogens. These pathogens and diseases can be difficult to diagnose, because of non-specific above-ground symptoms and the difficulty of quantifying pathogens in the soil. Molecular DNA-based

quantitative PCR techniques can be used to quantify individual species in the soil, and offer new tools for growers to predict the risk and use appropriate management techniques. Because of the lack of economic and effective soil-applied fungicides and genetic resistance/tolerance in adapted cultivars, wheat growers must manage soilborne pathogens by numerous cultural techniques. This requires an intimate knowledge of a complex cropping system, including plant nutrition, soils, weeds, insects, engineering, economics, and marketing. Any decision about management of root diseases must be integrated with these other factors. In the future, the development of stable genetic resistance may further reduce the chronic yield losses caused by these pathogens.

References

Asher MJC, Shipton PJ (1981) Biology and control of take-all. Academic, New York

Bailey KL, Gossen BD, Lafond GP, Watson PR, Derksen DA (2001) Effect of tillage and crop rotation on root and foliar diseases of wheat and pea in Saskatchewan from 1991–1998: univariate and multivariate analyses. Can J Plant Sci 81:789–803

Bockus WW, Schoyer JP (1998) The impact of reduced tillage on soilborne plant pathogens. Annu Rev Phytopathol 36:485–500

Brennan RF (1993) Effect of ammonium chloride, ammonium sulphate, and sodium nitrate on take-all and grain yield of wheat grown on soils in south-western Australia. J Plant Nutr 16:349–358

Chamswarng C, Cook RJ (1985) Identification and comparative pathogenicity of *Pythium* species from wheat roots and wheat-field soils in the Pacific Northwest. Phytopathology 75:821–827

Collard BCY, Grams RA, Bovill CD, Percy CD, Jolley R, Lehmensiek A, Wildermuth G, Sutherland MW (2005) Development of molecular markers for crown rot resistance in wheat: mapping of QTLs for seedling resistance in a '2–49' x 'Janz' population. Plant Breed 124:532–537

Cook RJ (1980) *Fusarium roseum* foot rot of wheat and its control in the Pacific Northwest. Plant Dis 64:1061–1066

Cook RJ (2003) Take-all of wheat. Physiol Mol Plant Pathol 62:73–86

Cook RJ, Veseth RJ (1991) Wheat health management. APS, St. Paul, MN

Cook RJ, Sitton JW, Haglund WA (1987) Influence of soil treatments on growth and yield of wheat and implications for control of Pythium root rot. Phytopathology 77:1172–1198

Cook RJ, Chamswarng C, Tang W-H (1990) Influence of wheat chaff and tillage on *Pythium* populations and *Pythium* damage to wheat. Soil Biol Biochem 22:939–947

Cook RJ, Thomashow LS, Weller DM, Fujimoto D, Mazzola M, Bangera G, Kim DS (1995) Molecular mechanisms of defense by rhizobacteria against root disease. Proc Nat Acad Sci USA 92:4197–4201

Cook RJ, Ownley BH, Zhang H, Vakoch D (2000) Influence of paired-row spacing and fertilizer placement on yield and root diseases of direct-seeded wheat. Crop Sci 40:1079–1087

Cook RJ, Schillinger WF, Christensen NW (2002a) Rhizoctonia root rot and take-all of wheat in diverse direct-seed spring cropping systems. Can J Plant Pathol 24:349–358

Cook RJ, Weller DM, El-Banna AY, Vakoch D, Zhang H (2002b) Yield responses of direct-seed wheat to fungicide and rhizobacteria treatments. Plant Dis 87:780–784

Cunfer BM, Buntin GD, Phillips DV (2006) Effect of crop rotation on take-all of wheat in double-cropping systems. Plant Dis 90:1161–1166

Davis RA, Huggins D, Cook RJ, Paulitz TC (2008) Can placement of seed away from relic stubble limit Rhizoctonia root rot in direct-seeded wheat? Soil Tillage Res 101:37–43

Davis RA, Huggins D, Cook RJ, Paulitz TC (2009) Nitrogen and crop rotation effects on Fusarium crown root rot in no-till spring wheat. Can J Plant Path submitted

Duffy BK, Simon A, Weller DM (1996) Combination of *Trichoderma koningii* with fluorescent pseudomonads for control of take-all on wheat. Phytopathology 86:188–194

Food and Agriculture Organization, United Nation (2008) FAOSTATS. http://faostat.fao.org/site/339/default.aspx

Garrett KA, Kabbage M, Bockus WW (2004) Managing for fine-scale differences in inoculum load: seeding patterns to minimize wheat yield loss to take-all. Precision Agric 5:291–301

Hering TF, Cook RJ, Tang W-H (1987) Infection of wheat embryos by *Pythium* species during seed germination and the influence of seed age and soil matric potential. Phytopathology 77:1104–1108

Higginbotham RW, Paulitz TC, Kidwell KK (2004a) Virulence of *Pythium* species isolated from wheat fields in eastern Washington. Plant Dis 88:1021–1026

Higginbotham RW, Paulitz TC, Kidwell KK (2004b) Virulence of *Pythium* species isolated from wheat fields in eastern Washington. Plant Dis 88:1021–1026

Hornby D, Bateman GL, Gutteridge RJ, Lucas P, Osbourn AE, Ward E, Yarham DJ (1998) Take-all and cereal production systems. In: Take-all disease of cereals. CAB International, Cambridge, UK, pp 103–164

Inglis DA, Cook RJ (1986) Persistence of chlamydospores of *Fusarium culmorum* in wheat field soils of eastern Washington. Phytopathology 76:1205–1208

Ingram DM, Cook RJ (1990) Pathogenicity of four *Pythium* species to wheat, barley, peas, and lentils. Plant Pathol 39:110–117

Kabbage M, Bockus WW (2002) Effect of placement of inoculum of *Gaeumannomyces graminis* var. *tritici* on severity of take-all in winter wheat. Plant Dis 86:298–303

Kim D-S, Cook RJ, Weller DM (1997) *Bacillus* sp. L324-92 for biological control of three root diseases of wheat grown with reduced tillage. Phytopathology 87:551–558

Lévesque CA, Rahe JE (1990) Site, host specificity, and pathogenicity of *Pythium* spp. colonizers of glyphosate treated seedlings. Can J Plant Pathol 12:336

Lévesque CA, Rahe JE (1992) Herbicide interactions with fungal root pathogens, with special reference to glyphosate. Annu Rev Phytopathol 30:579–602

MacNish GC (1988) Changes in take-all (*Gaeumannomyces graminis* var. *tritici*), rhizoctonia root rot (*Rhizoctonia solani*) and soil pH in continuous wheat with annual applications of nitrogenous fertilizer in Western Australia. Aust J Exp Agric 28:333–341

MacNish GC, Fang CS (1987) Effect of short chemical fallow on rhizoctonia bare patch and root rot of wheat at Esperance, Western Australia. Aust J Exp Agric 27:671–677

MacNish GC, Neate SM (1996) Rhizoctonia bare patch of cereals – an Australian perspective. Plant Dis 80:965–971

Mazzola M, Wong OT, Cook RJ (1996) Virulence of *Rhizoctonia oryzae* and *R. solani* AG-8 on wheat and detection of *R. oryzae* in plant tissue by PCR. Phytopathology 86:354–360

National Agricultural Statistics Service, USDA (2008) Crops and Plants. http://www.nass.usda.gov/ QuickStats/indexbysubject.jsp?Pass_group =Crops+%26+Plants

Ogoshi A, Cook RJ, Bassett EN (1990) *Rhizoctonia* species and anastomosis groups causing root rot of wheat and barley in the Pacific Northwest. Phytopathology 80:784–788

Okubara PA, Schroeder KL, Paulitz TC (2008) Identification and quantification of *Rhizoctonia solani* and *R. oryzae* using real-time polymerase chain reaction. Phytopathology 98:837–847

Papendick RI (2004) Farming with the Wind II: wind erosion and air quality control on the Columbia Plateau and Columbia Basin. University Publishing, Washington State University, Pullman, WA

Papendick RI, Cook RJ (1974) Plant water stress and development of *Fusarium* (*roseum*) foot rot in wheat subjected to different cultural practices. Phytopathology 64:358–363

Patterson L-M, Smiley RW, Alderman SM (1998) Effect of seed treatment fungicides and starter fertilizer on root diseases and yield of spring wheat. Fungic Nematicide Tests 53:425

Paulitz TC (2002) First report of *Rhizoctonia oryzae* on pea. Plant Dis 86:442

Paulitz TC (2006) Low input no-till cereal production in the Pacific Northwest of the U.S.: the challenges of root diseases. Eur J Plant Pathol 115:271–281

Paulitz TC, Adams K (2003) Composition and distribution of *Pythium* communities from wheat fields in eastern Washington State. Phytopathology 93:867–873
Paulitz TC, Schroeder KL (2005) A new method for quantification of *Rhizoctonia solani* and *R. oryzae* from soil. Plant Dis 89:767–772
Paulitz TC, Scott R (2006) Effect of seed treatments for control of Rhizoctonia root rot in winter wheat, 2003–2004. Fungicide and Nematicide Reports, 61:ST014
Paulitz TC, Smith J, Kidwell K (2002) Virulence of *Rhizoctonia oryzae* on wheat and barley cultivars from the Pacific Northwest. Plant Dis 87:51–55
Paulitz TC, Adams K, Mazzola M (2003a) *Pythium abappressorium* – a new species from eastern Washington. Mycologia 95:80–86
Paulitz TC, Schillinger WF, Cook RJ (2003b) Greenhouse studies of Rhizoctonia bare patch disease in soil cores from direct-seeded fields. [CD-ROM]. American Society of Agronomy Annual Meeting, 2–6 Nov., Denver, CO. ASA, CSSA, and SSSA Abstracts
Paulitz TC, Okubara PA, Schillinger WF (2006) First report of damping-off of canola caused by *Rhizoctonia solani* AG 2-1 in Washington State. Plant Dis 90:829
Paulitz TC, Schroeder KL, Schillinger WF (2009) Root diseases of cereals in an irrigated cropping system: effect of tillage, residue management and crop rotation. Plant Dis submitted
Pimentel D, Harvey C, Resosudarmo P, Sinclair K, Kurz D, McNair M, Crist S, Shpritz L, Fitton L, Saufourri R, Blair R (1995) Environmental and economic costs of soil erosion and conservation benefits. Science 267:1117–1123
Pumphrey FV, Wilkins DE, Hane DC, Smiley RW (1987) Influence of tillage and nitrogen fertilizer on Rhizoctonia root rot (bare patch) of winter wheat. Plant Dis 71:125–127
Raaijmakers JM, Weller DM (2001) Exploiting genotypic diversity of 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp.: characterization of superior root-colonizing *P. fluorescens* strain Q8r1-96. Appl Environ Microbiol 67:2545–2554
Raaijmakers JM, Bonsall RF, Weller DM (1999) Effect of population density of *Pseudomonas fluorescens* on production of 2,4-diacetylphloroglucinol in the rhizosphere of wheat. Phytopathology 89:470–475
Roget DK (1995) Decline in root rot (*Rhizoctonia solani* AG-8) in wheat in a tillage and rotation experiment at Avon, South Australia. Aust J Exp Agric 35:1009–1013
Roget DK, Venn NR, Rovira AD (1987) Reduction of Rhizoctonia root rot of direct-drilled wheat by short-term chemical fallow. Aust J Exp Agric 27:425–430
Roget DK, Neate SM, Rovira AD (1996) Effect of sowing point design and tillage practice on the incidence of Rhizoctonia root rot, take-all and cereal cyst nematode in wheat and barley. Aust J Exp Agric 36:683–693
Rothrock CS, Langdale GW (1989) Influence of nonhost summer crops on take-all in double-cropped winter wheat. Plant Dis 73:130–132
Rovira AD (1986) Influence of crop rotation and tillage on Rhizoctonia bare patch of wheat. Phytopathology 76:669–673
Schillinger WF, Paulitz TC (2006) Reduction of Rhizoctonia bare patch in wheat with barley rotations. Plant Dis 90:320–306
Schroeder KL, Paulitz TC (2006) Root diseases of wheat and barley during the transition from conventional tillage to direct seeding. Plant Dis 90:1247–1253
Schroeder KL, Okubara PA, Tambong JT, Lévesque CA, Paulitz TC (2006) Identification and quantification of pathogenic *Pythium* spp. from soils in eastern Washington using real-time polymerase chain reaction. Phytopathology 96:637–647
Smiley RW, Wilkins DE, Klepper EL (1990) Impact of fungicidal seed treatments on Rhizoctonia root rot, take-all, eyespot, and growth of winter wheat. Plant Dis 74:782–787
Smiley RW, Ogg AG, Cook RJ (1992) Influence of glyphosate on Rhizoctonia root rot, growth, and yield of barley. Plant Dis 76:937–942
Smiley RW, Collins HP, Rasmussen PE (1996) Diseases of wheat in long-term agronomic experiments at Pendleton, Oregon. Plant Dis 80:813–820
Smiley RW, Whittaker RG, Gourlie JA, Easley SA, Ingham RE (2005a) Plant-parasitic nematodes associated with reduced wheat yield in Oregon: *Heterodera avenae*. J Nematol 37:297–307

Smiley RW, Whittaker RG, Gourlie JA, Easley SA (2005b) Suppression of wheat growth and yield by *Pratylenchus neglectus* in the Pacific Northwest. Plant Dis 89:958–968

Smiley RW, Whittaker RG, Gourlie JA, Easley SA (2005c) *Pratylenchus thornei* associated with reduced wheat yield in Oregon. J Nematol 37:45–54

Smiley RW, Gourlie JA, Easley SA, Patterson LM, Whittaker RG (2005d) Crop damage estimates for crown rot of wheat and barley in the Pacific Northwest. Plant Dis 89:595–604

Smith JD, Kidwell KK, Evans MA, Cook RJ, Smiley RW (2002a) Evaluation of spring cereal grains and wild *Triticum* relatives for resistance to *Rhizoctonia solani* AG 8. Crop Sci 43:701–709

Smith JD, Kidwell KK, Evans MA, Cook RJ, Smiley RW (2002b) Assessment of spring wheat genotypes for disease reaction to *Rhizoctonia solani* AG-8 in controlled environment and direct-seeded field evaluations. Crop Sci 43:694–700

Summerell BA, Burgess LW, Klein TA (1989) The impact of stubble management practices on the incidence of crown rot of wheat. Aust J Exp Agric 29:91–98

Thompson IA, Huber DM, Schulze DG (1996) Evidence of a multicopper oxidase in Mn oxidation by *Gaeumannomyces graminis* var. *tritici*. Phytopathology 96:130–136

Tineline RD, Bailey KL, Harding H (1989) Role of plant breeding in controlling soil-borne diseases. Can J Plant Pathol 11:158–165

Wallwork H, Butt M, Cheong JPE, Williams KJ (2004) Resistance to crown rot in wheat identified through an improved method for screening adult plants. Aust Plant Pathol 33:1–7

Washington State Grain Alliance (2008) Washington Wheat Facts. http://www.wawg.org/images/E0177801/2007WFWeb.pdf

Weller DM, Cook RJ (1986) Increased growth of wheat by seed treatments with fluorescent pseudomonads and implications of *Pythium* control. Can J Plant Pathol 8:328–334

Weller DM, Cook RJ, MacNish G, Bassett EN, Powelson RL, Petersen RR (1986) Rhizoctonia root rot of small grains favored by reduced tillage in the Pacific Northwest. Plant Dis 70:70–73

Weller DM, Raaijmakers JM, McSpadden Gardener BB, Thomashow LS (2002) Microbial populations responsible for specific soil suppressiveness to plant pathogens. Annu Rev Phytopathol 40:309–348

Wildermuth GB, Morgan JM (2004) Genotypic differences in partial resistance to crown rot caused by *Fusarium pseudograminearum* in relation to an osmoregulation gene in wheat. Aust Plant Pathol 33:121–123

Wilhelm NS, Graham RD, Rovira AD (1988) Application of different sources of manganese sulfate decreases take-all (*Gaeumannomyces graminis* var. *tritici*) of wheat grown in a manganese deficient soil. Aust J Agric Res 39:1–10

Chapter 18
Challenges in Controlling Verticillium Wilt by the Use of Nonchemical Methods

George Lazarovits and Krishnamurthy Subbarao

Abstract Verticillium wilt is one of the most serious soilborne diseases worldwide. Three non-fumigant control methods that appear to have great potential for reducing losses due to wilt and other soilborne pathogens are detailed here. High nitrogen organic amendments and products containing volatile fatty acids (VFAs) can significantly reduce disease severity and inoculum density but only under specific soil conditions. Identification of the modes of action for these products provides new avenues to improve their efficacy. Broccoli amendments also effectively reduce Verticillium wilt and have great potential for use on a large scale where economics allow. Grafting susceptible cultivars onto Verticillium resistant root stocks has become widely adopted in many countries. Eggplants and tomatoes provide a good model system for testing this technology. Promising results have been obtained under diverse disease pressure and soil and climatic conditions.

Keywords Soilborne diseases • Organic amendments • Modes of action • Grafting • Volatile fatty acids • Ammonia • Nitrous acid • Swine manure • Fish emulsion • Brassicas • Green manures • Glucosinolates • Root stocks

18.1 Introduction

Verticillium wilt, caused by the fungus *Verticillium dahliae* Kleb, remains one of the most important soilborne plant diseases worldwide. Since the fungus infects dozens of crop and ornamental plants, losses incurred to this pathogen likely

G. Lazarovits (✉)
Agriculture and Agrifood Canada, 1391, Sandford St, London, Ontario, Canada, N5V 4T3
e-mail: George.Lazarovits@AGR.GC.CA

K. Subbarao
Department of Plant Pathology, University of California, Davis
1636 E, Alisal St, Salinas, 93905, CA, USA
e-mail: kvsubbarao@ucdavis.edu

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,*
Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_18,

amount to billions of dollars worldwide (Pegg and Brady, 2002). Actual dollar values however, are difficult to assess and are likely underestimated as most often infections result in reduced plant growth rather than the outright death of plants. The best known and most important causal agents of Verticillium wilt are *Verticillium dahliae* and *V. albo-atrum. V. dahliae* produces only microsclerotia (MS) as resting structures and *V. albo-atrum* produces only dark resting mycelium (Pegg and Brady, 2002). A third species, *V. longisporum* that predominantly affects the members of Brassicaceae is now emerging as an important pathogen (Karapapa et al., 1997) although the validity of this new species has not been universally accepted (Barbara and Clewes, 2003; Collins et al., 2003). In this paper therefore all MS-producing, plant pathogenic species will be referred to as *V. dahliae*.

Disease management has focused on reducing the populations of MS in soil, which are hardy resting structures that are produced in the plant by the millions. MS can survive in soils and plant debris for decades and once introduced into soil are nearly impossible to eradicate. The most successful technology for eliminating MS from soil has been the use of broad spectrum soil fumigants. However, due to numerous negative health and environmental concerns associated with these pesticides and increasing urban sprawl, their use has been greatly curtailed. Disease resistance for *Verticillium* spp. in crop plants has been difficult to identify. For tomato plants, the *Ve* resistance gene was the primary method for disease control for decades but the evolution of biotypes that overcame resistance has now made this crop highly susceptible to wilt again. Efforts are now being placed into finding non-chemical control methods for control of Verticillium wilt and other soilborne diseases. In this article, we describe three approaches that have found limited success but are seen to have tremendous future potential.

18.2 Organic Amendments

18.2.1 Disease Reductions in the Field

Almost 60 years ago Wilhelm (1951) tested a spectrum of chemical pesticides and organic amendments for the control of Verticillium wilt of tomato, and found that only blood meal and fishmeal reduced the incidence of wilt to zero. Why these products controlled Verticillium wilt, however, was never questioned or explained. Subsequent studies also found that a number of similar amendments provided some level of disease control but the efficacy was variable and difficult to reproduce. Over the last decade, the Lazarovits laboratory examined how these products may be controlling plant diseases. Products tested included organic by-products derived from animal and plant processing industries, including blood meal, meat and bone meal, feather meal, fish emulsion (Abbasi et al., 2006), soymeal, lignosulfonates from the pulp and paper industry (Lazarovits et al., 2008), condensed distillers solubles from alcohol production (Abbasi et al., 2007),

and several types of manures. The products were selected for their potential use at the field scale. Summaries of the results of such work have been previously published (Lazarovits, 2001, 2004; Lazarovits et al., 2001, 2005; Bailey and Lazarovits, 2003). As most of the products were considered waste products by the industries that generated them, they were mostly inexpensive, reasonably consistent from batch to batch, deemed environmentally safe, and had fertility values that enhanced crop growth.

In field trials on sandy soils, such products as poultry manure and soymeal reduced the incidence of Verticillium wilt and potato scab, and populations of plant pathogenic nematodes to the extent seen by Wilhelm (1951) in his pot trials (Conn and Lazarovits, 1999). In many cases, the disease reduction persisted for two crop seasons. The efficacy of these products, however, was often site- and product-specific. Overall, the level of control was lower than that found with chemical treatments but the added fertility resulted in increased plant vigor and yield. Field testing of organic products proved to be more complicated than testing of chemicals as there were many unknowns associated with the use of the products including the rates required, the effect of soil type, the impact of climatic conditions such as moisture and temperature, the nature and source of the amendment, the methods and times of application, etc. To examine even a few of these factors in the field would have required hundreds of plots to be established. Thus, studies were moved into the laboratory and model systems developed to simplify evaluating these products' impact on pathogen survival and to learn how best to deploy them in the field.

18.2.2 Mechanisms of Action by Which High Nitrogen Containing Amendments Reduce Pathogen Populations

Conversion of Ammonium to Ammonia A microcosm assay was developed to assess the survival of MS buried in amended soils and the result used as an indicator of inoculum reduction (Lazarovits et al., 2005). This assay allowed the identification of several modes of action for the various amendment types. In soils amended with high nitrogen containing material, mortality of MS was observed within one week after incorporation and was caused by the production of ammonia (Tenuta and Lazarovits, 2002a,b, 2004). When microbes degrade proteins, NH_4^+ ions are released and as they accumulate in the soil, an increase in soil pH occurs. When soil pH increases above 8, NH_4^+ is converted into NH_3 with the equilibrium (pK_a) between $NH_4^+ \leftrightarrow NH_3$ occurring at pH 9.3. NH_4^+ is non-toxic even at high concentrations, but NH_3 is very toxic (Warren, 1962). This mechanism becomes progressively less effective with increasing levels of soil organic matter primarily because the high populations of microorganisms in such soils rapidly convert NH_4^+ into nitrate (NO_3^-) (Tenuta and Lazarovits, 2004). In soils with high organic matter content, such amendments often fail to be pathogen-suppressive (Tenuta and Lazarovits, 2002a,b, 2004).

Conversion of Nitrite to Nitrous Acid The second mechanism by which high nitrogen amendments reduce pathogen populations occurs 4–6 weeks after incorporation of the amendments (Tenuta and Lazarovits, 2002a). When NH_4^+ is converted to nitrite (NO_2^-), $4H^+$ ions are freed up and soil pH drops. When the pH drops below 5.5, NO_2^- is converted to HNO_2 (nitrous acid). The equilibrium (pK_a) between $NO_2^- \leftrightarrow HNO_2$ occurs at pH 3.3. Nitrous acid is about 300–500 times more toxic to MS than NH_3 (Tenuta and Lazarovits, 2002a) and is also toxic to many plant pathogens including *Streptomyces scabies*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum*, as well as to crop and weed seeds. The formation of HNO_2 is most influenced by soil buffering capacity (Tenuta and Lazarovits, 2004) as nitrification lowers soil pH only in poorly buffered soils. The toxicity of ammonia and nitrous acid to plant pathogens was predicted by Tsao and Oster (1981) from their work on controlling soilborne pathogens with poultry manure.

18.2.3 Mechanism of Action of Materials Containing Volatile Fatty Acids

Single applications of liquid swine manure (LSM) significantly reduced the severity and incidence of Verticillium wilt and potato scab but disease reductions were site specific even when the same manure was used (Conn and Lazarovits, 1999). Laboratory assays using manure and soil from a field where disease reductions were optimal revealed that MS were killed within 1–2 days after manure application. The mechanism for this was shown to be caused by the presence of high concentrations of volatile fatty acids (VFA) in the manure, with acetic acid representing 60% of the active ingredients, and propionic, butyric, isobutyric, valeric, isovaleric and caproic the remainder (Tenuta et al., 2002). When commercially purchased VFAs were adjusted to the identical concentration found in LSM they reproduced all the LSM killing activity. It is important to note that only the acidic forms of VFA molecules are toxic and therefore disease control can only occur in acidic soils (Conn and Lazarovits, 2000). This explains most of the soil-specific activity of LSM. Most VFAs are at equilibrium with their non-ionic counterpart at pH 4.7 (pK_a). However, LSM quality can also vary, and of the dozens of LSM tested from different farms, only about 50% had sufficient VFAs to kill MS (Conn et al., 2005, 2007). Chemical analysis of manure can be used to predict the disease control potential of different batches and this can be used to spike the manures with deficient VFAs to provide consistent results.

High concentrations of VFAs have also been identified in fish emulsion (Abbasi et al., 2009), molasses (G. Lazarovits, unpublished), and composts (Bailey and Lazarovits, 2003). In some cases, the disease suppressive and phytotoxic activity of composts may be related to VFA content but this has not been examined. Immature composts that were phytotoxic to crop plants were found to have high concentrations of VFAs (Bailey and Lazarovits, 2003). Anaerobic decomposition of wheat straw resulted in the phytotoxicity due to production of acetic acid (Lynch, 1977, 1978).

Additions of large quantities of organic matter were used to create anaerobic conditions, and possibly to VFA formation, to reduce inocula of *F. oxysporum*, *Rhizoctonia solani*, and *V. dahliae* (Blok et al., 2000) and populations of *Meloidogyne hapla* and *M. incognita* (Browning et al., 2006). As VFAs are components of edible products and have very low toxicity to mammals with no mutagenic potential, they can be used in organic farming systems. VFAs however, persist in soil for only short times as they are readily metabolized by bacteria and fungi. In our tests, VFAs rarely lasted in soil for more than a week and in fact, rapid degradation may explain why they do not work at some locations.

18.2.4 Formulation for Site Specific Activity

The increasing cost of synthetic fertilizers makes the use of organic amendments more attractive to growers and if disease control can also obtained their utilization will greatly escalate. Through a greater understanding of mechanisms involved in disease reduction, we can select materials for optimal benefits to plant health based on soil and other conditions that favor their chemical and biological activity. There are also numerous options available for modifying the composition of such amendments for increased disease control. By anaerobically digesting LSM, Xiao et al. (2007) enriched both VFAs and NH_4^+ levels in the manure after 4 weeks of incubation. The enriched manure was superior to untreated manure for reducing egg production of the soybean cyst nematode. VFA-containing mixtures can be easily generated from almost any organic byproduct of agriculture or forestry. By acidifying the materials prior to treatment or by applying them in the fall when soil pH levels are lowest, their disease reduction activity can be enhanced (Conn and Lazarovits, 2007). There is also potential for formulating these to extend efficacy. Combining fish emulsion with biological control agents increased its disease control efficacy (El-Tarabily et al., 2003). Several companies are looking to generate such custom amendments and to commercially market them in the near future.

Combining organic amendments with solarization often results in a synergistic affect which improves the efficacy of both treatments for control of soilborne pathogens, although not many studies have focused specifically on the control of Verticillium (Gamliel and Stapleton, 1993a,b; Oka et al., 2007). The combination of the two treatments allows for reductions in the quantity of organic matter applied, as well as the time needed for effective solarization for reduction of pathogen populations that would normally take 6–8 weeks to as low as 3 weeks. This can be partially attributed to the efficacy by which plastic traps and enhances the toxicity of the volatiles formed, especially when residues of brassicas and Compositae are used. The presence of organic matter also helps to stabilize or increase the soil microbial biomass, which may have a disease suppressive impact (Scopa and Dumontet, 2007). A component that likely has been overlooked is the interaction of heat and organic amendments in the formation of ammonia and nitrous acid. With increasing temperature the concentrations of these toxicants is likely to

greatly increase as the equilibrium constant for both (pKa) shifts toward the neutral soil pH range (Lazarovits et al., 2005). Increased levels of ammonium are known to form in solarized soils (Oka et al., 2007) and thus its toxic forms would also likely be present. The combined technologies thus would greatly increase the range of soils where either technology alone would not produce desired control levels.

Organic products offer a technology for growers who wish to incorporate it as part of a sustainable and holistic crop production system to keep populations of key pathogens to levels that are below crop loss thresholds. The only potato grower in Ontario that did not have high levels of Verticillium wilt applied five tons of poultry manure every fall to his soil. It is likely that this practice not only accelerated the degradation of the potato plant debris that releases microsclerotia from the plant tissues into the soil but also produced sufficient nitrous acid to kill most of these MS such that Verticillium inoculum levels were kept at below disease threshold levels. For many growers, the lack of availability of manure or manure of uniform quality or other by-products has prevented adoption of this disease-suppressive tool. There are also substantial costs for moving such products long distances and incorporating them into soil, and the technology for application is still not available. Populations of microorganisms always increase in the soil following applications of organic amendments but the role these microbes play in disease reduction has not yet been clearly defined. Some amendment may increase microorganism populations that lead to disease-suppressive conditions (Trankner, 1992; Mazzola, 2004; Mazzola et al., 2007). We need to refine this selective management of microbiological ecosystems for agriculture to the same extent as is now occurring in the probiotic movement found in human health protection. Here, disease is prevented by products that enrich the native microflora, thereby keeping pathogens from becoming established either directly, or indirectly. We already know that disease suppressive soils exist. The goal is to learn how create them at will.

18.2.5 Crop Rotation and Green Manures

The use of crop rotation and green manure crops for the control of *V. dahliae* has been used interchangeably although the two techniques differ both conceptually and in their effects on MS survival and populations, as wells as to their overall benefits and limitations. The practice of growing a sequence of taxonomically different, economically valuable plant species on the same piece of ground is known as crop rotation. Continuous cropping with susceptible hosts can result in the build-up in soil of populations of specific plant pathogens, resulting in a decline in crop yield and quality. Crop rotation is effective for limiting the increase of soilborne pathogen populations that have a limited host range, but is less effective when pathogen population densities are high. In contrast, incorporating into soil green biomass of a plant species that may not be an economically important crop or crop residue brought in from elsewhere, is referred to as green manuring. *Brassica* species are particularly well suited as green manures because of their large taproot

with a dense network of fine surface roots (Matthiessen and Kirkegaard, 2006). These species reduce wind erosion, improve water infiltration of the soil and soil structure, and are effective at mineralizing soil nitrogen and thus reducing leaching, etc. (Thorup-Kristensen et al., 2003).

Crop rotation, cover cropping and green manures are tactics that provide multiple benefits and are therefore of vital importance to agroecosystems (Haramoto and Gallandt, 2004) and their benefits in general soilborne disease suppression well known. However, their success in the management of *V. dahliae* has been less clear primarily because of the unique survival characteristics of the MS (Isaac and MacGarvie, 1966; Hoes, 1971). *V. dahliae* is able to infect over 200 plant species, including high value annual and perennial crop species (Subbarao et al., 1995; Bhat and Subbarao, 1999a) and the list of new hosts infected is continually expanding, with cauliflower and lettuce two major crops in California being prime examples (Koike et al., 1994; Subbarao et al., 1995; Bhat and Subbarao, 1999a,b). This capability of *V. dahliae* to infect numerous crop plants, as well as natural flora and weeds which sometimes remain asymptomatic (Vallad et al., 2005a), and to contaminate seeds of various plants make *V. dahliae* a chronic disease problem (Bhat and Subbarao, 1999a; Qin et al., 2006; Vallad et al., 2005a, b). Reducing MS populations prior to planting to below the crop-dependent critical threshold for crop loss to occur is essential for disease management. However, the successful implementation of a crop rotation strategy has been confounded by long survival times of MS, the bewildering array of hosts the fungus infects, and that it can also be a seed resident (Vallad et al., 2005a, b). Even when rotations included resistant or immune crops, *V. dahliae* can colonize roots and maintain soil population levels with no apparent impact on the crop; even on non-hosts such as graminaceous crops (Krikun and Bernier, 1990). The use of *Brassica* spp. as green manure crops for reducing plant diseases was thoroughly reviewed by Matthiessen and Kirkegaard (2006) and this section focuses on crop rotations for the control of *Verticillium* species.

18.2.6 Attributes of Successful Rotation Crops

The basic criteria for developing successful rotation crops to minimize the impact of *V. dahliae* include: (i) the crop should result in a reduction of MS in soil and a concomitant reduction of wilt in the susceptible crop, (ii) be compatible with current production practices, and (iii) result in grower acceptance of the crop for rotation. Identifying crops that fit these criteria is difficult for the reasons given previously. During the evaluation of Verticillium wilt on cauliflower Koike et al. (1994) observed that Verticillium wilt did not affect a related host, broccoli. *V. dahliae* isolates from cauliflower were either weakly-pathogenic or non-pathogenic on broccoli and no MS developed on inoculated plants (Subbarao et al., 1995). The broccoli plants suffered no yield losses even when planted into highly infested fields; nor could the pathogen be isolated from mature plants from these fields (Koike et al., 1994). Broccoli and cauliflower however, are closely related taxonomically as both are

Brassica olearacea var. *botrytis* L., but broccoli and cauliflower are separated into the subvarieties *cymosa* and *cauliflora,* respectively. Most other cultivated *Brassica* species are susceptible to *V. dahliae* but a few exhibit varying levels of resistance (Ciccarese et al., 1987; Subbarao et al., 1995).

A detailed analysis of the host range of *V. dahliae* isolates from artichoke, cabbage, cotton, pepper, potato, strawberry, tomato, watermelon and two virulent isolates from cauliflower when tested on all of the above crops, lettuce, and other crucifer crops showed that there was no host specificity associated with *V. dahliae* (Subbarao et al., 1995). Isolates from cauliflower were only weakly pathogenic on broccoli and Brussels sprouts and were non-pathogenic on lettuce. To clarify if this was cultivar-specificity or a more general reaction of broccoli, a number of commercial cultivars of broccoli were evaluated against *V. dahliae* isolates from 15 different hosts as also against *V. albo-atrum* from alfalfa. All cultivars evaluated were resistant to *V. albo-atrum* and *V. dahliae* from all hosts except from crucifer hosts against which they were weakly susceptible (Bhat and Subbarao, 2002). Strains re-isolated from internally discolored broccoli plants were unable to cause symptoms on broccoli but caused severe wilt on cauliflower. The immunity of broccoli against *V. dahliae* isolates from non crucifer hosts and resistance against crucifer isolates and *V. alboatrum,* coupled with its importance as a commercial vegetable, makes broccoli an attractive rotation crop for the management of Verticillium wilt.

While broccoli as a rotational crop possessed all attributes listed above, it was necessary to determine if fresh broccoli residue is as effective as dry broccoli and the temperature at which the benefits of broccoli are maximized; this information was also useful in determining the timing of broccoli crop planting and residue incorporation. Fresh broccoli residue suppressed *V. dahliae* MS more than dry broccoli residue over the entire temperature range tested (10°C to 35°C) (Subbarao and Hubbard, 1996). Furthermore, the greatest reductions in MS occurred at soil temperatures above 20°C with both fresh and dry broccoli residue (with fresh broccoli significantly more effective than dry broccoli) and most of this reduction occurred within 15 days after incorporation. In multiple greenhouse experiments cauliflower plants grown in fresh broccoli treatments were consistently taller, had greater root and shoot biomass, and had the least number of infected plants than in other treatments. These studies demonstrated that for maximal reductions of soilborne *V. dahliae* MS and subsequent lower wilt incidence in cauliflower, the broccoli residue incorporation should occur when the soil temperatures are at least 20°C.

Following these laboratory studies, rotations of susceptible cauliflower with broccoli as well as incorporating post-harvest residue was evaluated in an experimental field as well as a grower's field. The experiment in a grower's field involved the comparison of broccoli in a highly infested field with treatments of methyl bromide plus chloropicrin, Vapam, broccoli residue with tarp, broccoli residue without tarp, control with tarp, and control without tarp, and was arranged in a randomized block design with four replications. Approximately 11 Kg chopped broccoli per m^2 was uniformly spread and incorporated into the corresponding plots by disking. For treatments with tarping, clear plastic was spread over the plots and sealed at the edges. Tarps were removed after two weeks. The numbers of *V. dahliae* propagules in broccoli-treated

plots was lower than in control plots and were comparable to those in fumigated plots. Similarly, plant height, marketable heads, and head weight were significantly higher in broccoli treatments than in control plots. Tarping alone did not reduce propagule numbers. These results suggested that broccoli residue has the potential for Verticillium wilt control. The ideal means of exploiting this, however, was by rotating cauliflower with broccoli (Subbarao et al., 1999).

In a follow-up experiment in an experimental field, actual broccoli–cauliflower rotations were tested for their efficacy to control Verticillium wilt on cauliflower (Xiao et al., 1998). Treatments tested were a factorial combination of three main plots (broccoli crop grown, harvested, and residue incorporated in *V. dahliae*-infested plots, no broccoli crop or residue in infested plots, and fumigated control plots), two sub-plots (furrow and subsurface-drip irrigation), and three sub-sub-plots (deficit, moderate, and excessive irrigation regimes) arranged in a split-split-plot design with three replications. Number of propagules in all broccoli plots declined significantly after residue incorporation and continued to decline throughout the cauliflower season. The overall reduction in propagule numbers after two broccoli crops was 94%, in contrast to the fivefold increase in infested plots without broccoli after two cauliflower crops. Disease incidence and severity were both reduced approximately 50% in broccoli treatments compared with no broccoli treatments. Differences between furrow and subsurface drip irrigation were not significant, but incidence and severity were significantly lower in the deficit irrigation regime compared to the other two regimes. MS of *V. dahliae* on infected cauliflower roots 8 weeks after cauliflower harvest was significantly lower in treatments with broccoli compared to treatments without broccoli. Rotating broccoli would be successful regardless of the irrigation methods or regimes followed on susceptible crops for Verticillium wilt control. These two studies proved the efficacy of broccoli rotations to control Verticillium wilt.

18.2.7 Mode of Action

As early as the late 1930s, the toxicity of mustard oils and their breakdown products were demonstrated on *Colletotrichum circinans*, *Botrytis alii*, *Aspergillus niger*, *A. alliaceus*, and *Gibberella saubinetii* (Subbarao and Hubbard, 1996) in laboratory studies. Subsequent studies identified that this broad spectrum toxicity to plant pathogens and pests resulted from the release of toxic products from crucifer residues (Ramirez-Villapadua and Munnecke, 1988; Gamliel and Stapleton, 1993b). The efficacy of disease suppressive effects was related to the degree of dryness of the crucifer residue at the time of soil amendments (Ramirez-Villapadua and Munnecke, 1988) and to the glucosinolate content in a crop (Mayton et al., 1996). However, the use of this technology has not been widely adapted by growers for disease management primarily owing to the difficulty of obtaining dry crucifer residue and the potential cost of application. Subbarao and Hubbard (1996) demonstrated that fresh broccoli residue is more effective than dry broccoli powder at

equivalent amounts at all temperatures tested. They also established the temperature at which maximal pathogen suppression occurred. A comprehensive review by Matthiessen and Kirkegaard (2006) focused on the bioactive compounds in Brassicaceae members as the mechanism responsible for the pathogen/pest suppression observed with these plants. Alternative mechanisms are likely because pathogen suppression has been observed with isothiocyanates from plants with relatively low antimicrobial activity (Mancini et al., 1997). Furthermore, the suppression of soilborne pathogens and pests by Brassicaceae residues continued long after isothiocyantes had been volatilized or degraded (Lewis and Papavizas, 1971) or was observed independent of the glucosinolates content (Mazzola et al., 2001). The suppression observed with Brassicaceae and other plant products was also attributed to the depletion of oxygen in soil through anaerobiosis (Blok et al., 2000), accumulation of acetic and butyric acid (Momma, 2008) and hydrogen cyanide (Bjarnholt et al., 2008) in amended soils, oxidation of N in soil amendments to nitric oxide by soil bacteria (Cohen et al., 2005) which is known to stimulate plant defense pathways (Durner et al., 1998).

With broccoli specifically, attempts to isolate *V. dahliae* from inoculated plants or plants collected from fields heavily infested with MS were unsuccessful (Koike et al., 1994). Inoculated broccoli plants seldom showed symptoms consistently across isolates from 14 hosts (Subbarao et al., 1995), all commercial broccoli cultivars responded similarly to isolates of *V. dahliae* (Bhat and Subbarao, 2002). Despite the apparent lack of foliar symptoms and few root symptoms, broccoli root cortex was still colonized *V. dahliae* to the same degree as cauliflower (Shetty et al., 2000). Under high inoculum density however, colonization rates of cauliflower roots were 1.5-fold higher than in broccoli. Empirical data evaluating the mechanisms of broccoli-induced *V. dahliae* suppression are only now becoming available. Although bacterial populations, especially actinomycetes, increased by as much as three orders of magnitude following the incorporation of broccoli residue, identifying an actual cause and effect relationship has proven difficult (K.V. Subbarao, unpublished data). Instead, the ontogenic changes in the type and levels of glucosinolates, structural components such as lignin and phenolic compounds explain why broccoli is resistant to *V. dahliae* relative to cauliflower. Colonization patterns of *V. dahliae* in cauliflower and broccoli were compared using immunohistochemical staining (Shetty et al., 2000) and a green-fluorescent-protein (GFP)-tagged *V. dahliae* isolate from cauliflower (Njoroge et al., 2008a). Minimal differences in the colonization of cortex were observed between broccoli and cauliflower (Shetty et al., 2000) but the vascular tissue in broccoli was uncompromised in contrast to the extensive colonization in cauliflower (Njoroge et al., 2008b). The type of glucosinolates and the range of their catabolic products have been associated with the suppressive effects of crucifer crops in general (Matthiessen and Kirkegaard, 2006) but it is clear from the high susceptibility of many crucifer crops to *V. dahliae*, including strains from crucifer crops, that not all glucosinolates and their catabolic products are involved in pathogen suppression. However, *V. dahliae* suppression by broccoli remains effective long after the volatilization of the isothiocyanates and is independent of glucosinolate content suggesting that the suppression is due more to biological than

chemical reasons. The reduction in *V. dahliae* soil population was perhaps caused by the combined effects of broccoli acting as a trap crop to force the germination of MS, and the activation of resident microflora with an ability to degrade lignin-rich broccoli residue in addition to the melanized MS of *V. dahliae* (Shetty and Subbarao, 1999). Microorganisms with melanolytic activity may be selectively enhanced by the incorporation of broccoli residue in soil (Butler and Day, 1998; Shetty and Subbarao, 1999). Interestingly, broccoli rotations were also suppressive to *S. minor*, another pathogen producing melanized sclerotia (Hao et al., 2003).

A related area that was thoroughly researched is the employment of cyanogenic green manure crops for pathogen suppression. Davis et al. (1996) determined that incorporating sudangrass and corn residues increased potato yields in fields infested by *V. dahliae*. The release and accumulation of hydrogen cyanide in amended soils is believed to be responsible for the pathogen suppression in these crops (Matthiessen and Kirkegaard, 2006).

18.2.8 Successes and Frustrations

Rotations with broccoli have proven successful in not only experimental plots but also in grower fields in repeated large scale studies in the management of Verticillium wilt and diseases caused by *Sclerotinia* spp. Based on these successful studies, there has also been an encouraging adaptation of this procedure by vegetable and strawberry growers in both conventional and organic production systems. Nevertheless, the wider adaptation of this technique has been less than total due to the low economic returns from broccoli crops that have also depressed the overall broccoli production, and the very high land costs in coastal California (>$100,000 per ha) with rentals costing as high as $30,000 per year. Ultimately, the economics of crop production trumps all other factors and there is little researchers could do to alter this reality.

18.2.9 Grafted Plants

Until 2005, when methyl bromide (MB) was banned as a soil fumigant, Italy ranked first in Europe and second in the world in its use of MB for horticultural crop production. Preplant soil fumigation was practiced in Southern Italy for the protection of solanaceous plants where Verticillium wilt was a serious problem. Grafting commercial cultivars susceptible to Verticillium onto resistant rootstocks was developed as a replacement for fumigation. However, the practice of growing grafted vegetables started in Japan and Korea in the late 1920s (Lee, 1994). Grafting vegetables onto resistant rootstocks represents a technically and economically feasible alternative particularly in Japan and in Korea where 54% and 81%, respectively, of vegetables grown are grafted (Rivero et al., 2003). In the

Mediterranean region, grafting represented an opportunity to maintain productivity of crops such as watermelon, cantaloupe, tomato, pepper, and eggplant (Bletsos et al., 2003; Diánez et al., 2007). It was rapidly adopted and for instance in Greece, 90–95% of watermelon, 40–50% of cantaloupe, 5–8% of tomato and 5–8% and 2–4% of cucumber and eggplant are now grafted (Traka-Mavrona et al., 2000); in Spain 98% of watermelon and 10% of tomato, in Morocco and Netherlands more than 25% and 50% of protected tomatoes and in Cyprus 80% of watermelon (Diánez et al., 2007). Over 5 million eggplants and 5.8 million tomato plants were produced from grafted seedlings in Italy in 2005 (Minuto et al., 2007).

Grafting vegetables onto resistant rootstocks offers numerous advantages including: resistance to soil pathogens, specifically *Verticillium* and *Fusarium* (Lockwood et al., 1970; Lee, 1994; Bletsos et al., 2003), improved yield in infested soils (Bletsos et al., 2003), greater tolerance against low and high temperatures and salt stress (Rivero et al., 2003) and higher plant vigour that can support longer crop cycles under adverse climatic conditions. Bletsos et al. (2003) found that grafted eggplants had not only increased fruit yield of up to 79% over non-grafted plants in Verticillium-infested soil (Bletsos et al., 2003) but they also produced fruit a week earlier (Khahm, 2005). Fruits from grafted eggplants contain fewer seeds than from non-grafted plants (Khahm, 2005) and this is regarded as another qualitative benefit to the consumer.

Several rootstocks are available for grafting of both tomato and eggplant, the most common being tomato hybrids (Energy, Kyndia) and interspecific hybrids of *L. esculentum* and *L. hirsutum* (He Man, Beaufort, Maxifort, Trifort). For grafted eggplant *Solanum torvum* was introduced and now represents more than the 70% of the total market of eggplant rootstocks in the south Italy (Minuto et al., 2007). Other *Solanum* species could be adopted for grafting eggplant including *S. sisymbriifolium*, but *S. torvum* guarantees the highest resistance against Verticillium wilt (Bletsos et al., 2003) and also carries traits of resistance to the most serious disease of eggplant namely bacterial wilt (*Ralstonia solanacearum*) and nematodes (Gousset et al., 2005).

18.2.10 Limitations of Adoption of Grafted Plants

Among the major constraints and limitations of grafted rootstocks is that resistance may break down under high pathogen population pressure, that new races of the pathogen may evolve, and under some environmental stresses such as high temperature and salinity, the plants may prematurely collapse. Furthermore, pathogens generally considered minor can become major pathogens on the rootstocks in the absence of soil fumigation. As an example, novel root rots caused by *Colletotrichum coccodes* were repeatedly observed on rootstocks currently used for grafting tomatoes and eggplant (Garibaldi and Minuto, 2003). Although *C. coccodes* was previously reported to infect *L. hirsutum* rootstocks, it was never observed on *L. lycopersicum* × *L. hirsutum* hybrids, the most widely used rootstocks.

Grafted hybrids of *L. lycopersicum* × *L. hirsutum* ("Beaufort", "He Man") and of *L. lycopersicum* ("Energy") were infected by *Phytophthora nicotiane* and *Rhizoctonia solani* accompanied by some plant stunting (Minuto et al., 2007). Finally, eggplants (cv Black Bell and Mirabell) grafted onto rootstock of *S. torvum* that confer a high degree of nematode tolerance exhibited low levels of Verticllium wilt in several greenhouses in Sicily (Garibaldi et al., 2005) that in subsequent crops increased to about 10 times in the same greenhouses. *S. torvum* exhibited partial tolerance to *V. dahliae* in artificially inoculated conditions (20–27% of infected plants) compared with non grafted Black Bell eggplants (87–100% of infected plants).

The relatively low tolerance of *S. torvum* to *V. dahliae* was known previously known (Ginoux and Laterrot, 1991; Gousset et al., 2005). Ginoux and Laterrot (1991) confirmed the resistance of *S. torvum* against *V. dahliae* particularly under mild climate conditions and in sandy soils and when 70–80-days-old grafted plants were transplanted. In trials carried out with 15 day old *Solanum spp* seedlings belonging to 14 different species vertical resistance to *V. dahliae* was not found but there was only tolerance to wilt symptoms (Nothamann and Ben-Yephet, 1979). Experiments conducted in highly infested fields confirmed that *S. torvum* conferred only partial wilt resistance (30–50% infection plants compared with non grafted eggplant 80–100% infection), while *L. lycopersicum* × *L. hirsutum* and *L. lycopersicum* hybrid rootstocks always showed low infection (7–10% infected plants) (Minuto et al., 2007).

18.2.11 Physiological Disorders Caused by Grafting Adoption

In Northern Italy since 1997, sudden collapse of grafted plants in protected and open field tomatoes (cv Cuore di Bue and cv Marmande-Raf) grafted on "He-Man" and on "Energy" rootstocks were observed (Garibaldi and Minuto, 2003). The collapse before or after fruit setting during spring and summers was in the 15–70% range. Sudden collapses were also observed on cv Iride, Naomi, Cuore di Bue, and Marmande-Raf grafted on "He-Man", "Energy" and sometimes on "Beaufort", regardless of the season or phenological stage of plants in Southern Italy (Garibaldi and Minuto, 2003). This collapse appears to be a direct consequence of the incompatibility between scion and rootstock or the climatic conditions during fruit setting and ripening.

Similar collapses were observed on eggplant grafted on tomato rootstocks (Ginoux and Laterrot, 1991) demonstrating the importance of rootstock selection. *S. torvum* performs best as an eggplant rootstock during warm seasons, but may reduce plant vigor during other seasons. Tomato rootstocks should be more vigorous and possess cold tolerance, graft incompatibility may reduce cold tolerance (Minuto et al., 2007). With tomato rootstocks grafted onto eggplant one often finds that the diameter of the rootstocks is double that of the scion but this is not the main reason for graft incompatibility. This is inferred from the fact that plants with

tomato rootstocks transplanted in late spring to early summer do not show signs of damage although the size differences between rootstock and scion are present.

Catara et al. (2001) found a widespread dieback of eggplant (Mission Bell), grafted onto the interspecific hybrid (Beaufort) and on tomato hybrid (Energy) during winter cultivation. Bacteria isolated from symptomatic tissues were identified as *Pectobacterium carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *atrosepticum* and confirmed to be pathogenic. Ginoux and Laterrot (1991) recognized these same symptoms as a graft incompatibility enhanced by low temperature and by heavy leaf guttation and water soaked leaf areas and lesions. Since the wide scale adoption of *S. torvum* for eggplant grafting, this type of plant dieback is no longer considered important.

18.2.12 Potential for Future

Since the use of grafting is likely to increase in the future (Edelstein, 2004), especially following the ban of MB for soil disinfestation, the adoption of grafting robots and healing chambers (Kurata, 1994) has assumed major importance. The cost of grafted plants is still the major constraint to their wide adoption together with the risks of unexpected plant dieback caused by biotic and/or abiotic factors. Grafting robots, healing chambers, and quick predictive methods for graft compatibility–incompatibility will improve and increase the adoption of grafting as a tool to reduce many soilborne diseases.

References

Abbasi PA, Lazarovits G, Jabaji-Hare S (2009) Detection of high concentrations of organic acids in fish emulsion and their role in pathogen or disease suppression. Phytopathology 99:274–281

Abbasi PA, Conn KL, Lazarovits G (2006) Effect of fish emulsion used as a pre-plant soil amendment on Verticillium wilt, common scab, and tuber yield of potato. Can J Plant Pathol 28:509–518

Abbasi PA, Conn KL, Lazarovits G (2007) Managing soilborne diseases of vegetable crops with a pre-plant soil or substrate amendment of a corn distillation product. Biocontrol Sci Tech 17:331–344

Bailey KL, Lazarovits G (2003) Suppressing soilborne diseases with residue management and organic amendments. Soil Tillage Res 72:169–180

Barbara DJ, Clewes E (2003) Plant pathogenic *Verticillium* species: how many of them are there? Mol Plant Pathol 4:297–304

Bhat RG, Subbarao KV (1999a) Green manure. In: The encyclopedia of plant pathology, vol 1, Wiley, New York, pp 519–520

Bhat RG, Subbarao KV (1999b) Host range specificity in *Verticillium dahliae*. Phytopathology 89:1218–1225

Bhat RG, Subbarao KV (2002) Reaction of broccoli to *Verticillium dahliae* isolates from various hosts. Plant Dis 85:141–146

Bjarnholt N, Laegdsmand M, Hansen HCB, Jacobsen OH, Moller BL (2008) Leaching of cyanogenic glucosides and cyanide from white clover green manure. Chemosphere 72:897–904

Bletsos F, Thanassoulopoulos C, Roupakias D (2003) Effect of grafting on growth, yield, and Verticillium wilt of eggplant. HortScience 38(2):183–186

Blok WJ, Lamers JG, Termorshuizen AJ, Bollen GJ (2000) Control of soilborne plant pathogens by incorporating fresh organic amendments followed by tarping. Phytopathology 90:253–259

Browning M, Wallace DB, Dawson C, Alm SR, Amador JA (2006) Potential of butyric acid for control of soil-borne fungal pathogens and nematodes affecting strawberries. Soil Biol Biochem 38:401–404

Butler MJ, Day AW (1998) Destruction of fungal melanins by ligninases of *Phanerochaete chrysosporium* and other white rot fungi. International. J Plant Sci 159:989–995

Catara V, Bella P, Polizzi G, Paratore A (2001) First report of bacterial stem rot caused by *Pectobacterium carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *atrosepticum* on grafted eggplant in Italy. Plant Dis 85:921

Ciccarese F, Frisullo S, Cirulli M (1987) Severe outbreaks of Verticillium wilt on *Cichorium intybus* and *Brassica rapa* andpathogenic variations among isolates of *Verticillium dahliae*. Plant Dis 71:1144–1145

Cohen MF, Yamasaki H, Mazzola M (2005) *Brassica napus* seed meal soil amendment modifies microbial community structure, nitric oxide production and incidence of *Rhizoctonia* root rot. Soil Biol Biochem 37:1215–1227

Collins A, Okoli CAN, Morton A, Parry D, Edwards SG, Barbara DJ (2003) Isolates of *Verticillium dahliae* pathogenic to crucifers are of at least three distinct molecular types. Phytopathology 93:364–376

Conn KL, Lazarovits G (1999) Impact of animal manures on Verticillium wilt, potato scab, and soil microbial populations. Can J Plant Pathol 21:81–92

Conn KL, Lazarovits G (2000) Soil factors influencing the efficacy of liquid swine manure added to soil to kill *Verticillium dahliae*. Can J Plant Pathol 22:400–406

Conn KL, Lazarovits G (2007) Reduction of potato scab with acidified liquid swine manure soil amendment. Can J Plant Pathol 29:440

Conn KL, Tenuta M, Lazarovits G (2005) Liquid swine manure can kill *Verticillium dahliae* microsclerotia in soil by volatile fatty acid, nitrous acid, and ammonia toxicity. Phytopathology 95:28–35

Conn KL, Topp E, Lazarovits G (2007) Factors influencing the concentration of volatile fatty acids, ammonia, and other nutrients in stored liquid pig manure. J Environ Qual 36:440–447

Davis JR, Huisman OC, Westermann DT, Hafez SL, Everson DO, Sorensen LH, Schneider AT (1996) Effects of green manures on Verticillium wilt of potato. Phytopathology 86:44–453

Diánez F, Díaz M, Santos M, Huitrón V, Ricárdez M, Camacho F (2007) The use of grafting in Spain. Proceedings of technical meeting on non-chemical alternatives for soil-borne pest control, Budapest, pp 87–97

Durner J, Wendehenne D, Klessig DF (1998) Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. Proc Natl Acad Sci 95:10328–10333

Edelstein M (2004) Grafting vegetable-crop plants: pros and cons. Acta Horticulturae 659:235–238

El-Tarabily KA, Nassar AH, Hardy GE St. J, Sivasithamparam K (2003) Fish emulsion as a food base for rhizobacteria promoting growth of radish (*Raphanus sativus* L. var. *sativus*) in a sandy soil. Plant Soil 252:397–411

Gamliel A, Stapleton JJ (1993a) Effect of chicken compost or ammonium phosphate and solarization on pathogen control, rhizosphere microorganisms, and lettuce growth. Plant Dis 77:886–891

Gamliel A, Stapleton JJ (1993b) Characterization of antifungal volatile compounds evolved from solarized soil amended with cabbage residues. Phytopathology 83:899–905

Garibaldi A, Minuto A (2003) The application of grafting against soilborne pests and diseases of tomato in Italy: present situation and perspectives. Proceedings of international congress "Greenhouse tomato – Integrated crop production – Organic production", Avignon, France, pp 55–59

Garibaldi A, Minuto A, Gullino ML (2005) Verticillium wilt incited by *Verticillium dahliae* in eggplant grafted on *Solanum torvum* in Italy. Plant Dis 89:777

Ginoux G, Laterrot H (1991) Greffage de l'aubergine: réflexion sur le choix du porte-greffe P.H.M. Revue Horticole 321:49–54

Gousset C, Collonnier C, Mulya K, Mariska I, Rotino GL, Besse P, Servaes A, Sihachakr D (2005) *Solanum torvum*, as a useful source of resistance against bacterial and fungal diseases for improvement of eggplant (*S. melongena* L.). Plant Sci 168:319–327

Hao JJ, Subbarao KV, Koike ST (2003) Effects of broccoli rotation on lettuce drop caused by *Sclerotinia minor* and on the sclerotial population in soil. Plant Dis 87:159–166

Haramoto ER, Gallandt ER (2004) Brassica cover cropping for weed management: A review. Renew Agric Food Sys 19:187–198

Hoes JA (1971) Development of chlamydospores in *Verticillium nigriscens* and *V. nubilum*. Can J Bot 49:1863–1866

Isaac I, MacGarvie QD (1966) Dormancy and germination of resting structures of *Verticillium* spp. Trans Br Mycol Soc 49:669–678

Karapapa VK, Bainbridge BW, Heale JB (1997) Morphological and molecular characterisation of *Verticillium longisporum* comb. nov., pathogenic to oil seed rape. Mycol Res 101:1281–1297

Khahm EM (2005) Effect of grafting on growth, performance and yield of aubergine (*Solanum melongena* L) in the field and greenhouse. J Food Agric Environ 3:92–94

Koike ST, Subbarao KV, Gordon TR, Davis RM, Hubbard JC (1994) Verticillium wilt of cauliflower in California. Plant Dis 78:1116–1121

Krikun J, Bernier CC (1990) Morphology of microsclerotia of *Verticillium dahliae* in roots of gramineous plants. Can J Plant Pathol 12:439–441

Kurata K (1994) Cultivation of grafted vegetables II. Development of grafting robots in Japan. HortScience 29:240–244

Lazarovits G (2001) Management of soilborne plant pathogens with organic soil amendments: a disease control strategy salvaged from the past. Can J Plant Pathol 23:1–7

Lazarovits G (2004) Managing soilborne plant diseases through selective soil disinfestation by a knowledge based application of soil amendments. Phytoparasitica 32:427–431

Lazarovits G, Tenuta M, Conn KL (2001) Organic amendments as a disease control strategy for soilborne diseases of high-value agricultural crops. Australas Plant Pathol 30:111–117

Lazarovits G, Conn KL, Abbasi PA, Tenuta M (2005) Understanding the mode of action of organic soil amendments provides the way for improved management of soilborne plant pathogens. Acta Horticulturae 698:215–225

Lazarovits G, Conn KL, Abbasi PA, Soltani N, Kelly W, McMillan E, Peters RD, Drake KA (2008) Reduction of potato tuber diseases with organic soil amendments in two Prince Edward Island fields. Can J Plant Pathol 30:37–45

Lee JM (1994) Cultivation of grafted vegetables. Current status, grafting methods and benefits. HortScience 29:235–239

Lewis JA, Papavizas GC (1971) Effect of sulfur-containing volatile compounds and vapors from cabbage decomposition on *Aphanomyces euteiches*. Phytopathology 61:208–214

Lockwood JL, Yoder OL, Bente MK (1970) Grafting eggplants on resistant rootstocks as a possible approach for control of Verticillium wilt. Plant Dis 54:846–848

Lynch VM (1977) Phytotoxicity of acetic acid produced in the anaerobic decomposition of wheat straw. J Appl Bacteriol 42:81–87

Lynch VM (1978) Production and phytotoxicity of acetic acid produced in anaerobic soils containing plant residues. Soil Biol Biochem 10:131–135

Mancini LM, Lazzeri L, Palmieri SJ (1997) In vitro fungitoxic activity of some glucosinolates and their enzyme-derived products toward plant pathogenic fungi. J Agric Food Chem 45:2768–2773

Matthiessen J, Kirkegaard J (2006) Biofumigation and enhanced biodegradation: opportunity and challenge in soilborne pest and disease management. Crit Rev Plant Sci 25:235–265

Mayton HS, Olivier C, Vaughn SF, Loria R (1996) Correlation of fungicidal activity of *Brassica* species with allyl isothiocyanate production in macerated leaf tissue. Phytopathology 86:267–271

Mazzola M (2004) Assessment and management of soil microbial community structure for disease suppression. Annu Rev Phytopathol 42:35–59

Mazzola M, Granatstein DM, Elfving DC, Mullinix K (2001) Suppression of specific apple root pathogens by *Brassica napus* seed meal amendment regardless of glucosinolate content. Phytopathology 91:673–679

Mazzola M, Brown J, Izzo AD, Cohne MF (2007) Mechanism of action and efficacy of seed meal-induce pathogen suppression differ in *Brassica* species and time-dependent manner. Phytopathology 97:454–460

Minuto A, Serges T, Nicotra G, Garibaldi A (2007) Applicazione dell'innesto erbaceo per le solanacee allevate in coltura protetta: problematiche e prospettive. Informatore fitopatologico – La difesa delle piante 57:30–36

Momma N (2008) Biological soil disinfestation (BSD) of soilborne pathogens and its possible mechanisms. Jpn Agric Res Quar 42:7–12

Njoroge S, Park S, Kang S, Subbarao KV (2008a) Comparative analysis of infection of broccoli and cauliflower by a GFP-tagged *Verticillium dahliae* isolate. (Abstr.). Phytopathology 98:S114

Njoroge SMC, Riley MB, Keinath AP (2008b) Effect of incorporation of *Brassica* spp. residues on population densities of soilborne microorganisms and on damping-off and Fusarium wilt of watermelon. Plant Dis 92:287–294

Nothamann J, Ben-Yephet Y (1979) Screening of eggplant and other *Solanum* species for resistance to *Verticillium dahliae*. Plant Dis Reporter 63:70–73

Oka Y, Shapira N, Fine P (2007) Control of root-knot nematodes in organic farming systems by organic amendments and soil solarization. Crop Prot 26:1556–1565

Pegg GF, Brady BL (2002) Verticillium wilts. CABI, New York, p 552

Qin Q-M, Vallad GE, Wu BM, Subbarao KV (2006) Phylogenetic analyses of phytopathogenic isolates of *Verticillium*. Phytopathology 96:582–592

Ramirez-Villapudua J, Munnecke DE (1988) Effect of solar heating and soil amendments of cruciferous residues on *Fusarium oxysporum* f. sp. *conglutinans* and other organisms. Phytopathology 78:289–295

Rivero RM, Ruiz JM, Romero L (2003) Role of grafting in horticultural plants under stress conditions. J Food Agric Environ 1:70–74

Scopa A, Dumontet S (2007) Soil solarization: effects on soil microbiological parameters. J Plant Nutr 30:537–547

Shetty KG, Subbarao KV (1999) Melanolytic activity of microorganisms and antagonism to *Verticillium dahliae*. Phytopathology 89 (Suppl.):S72

Shetty KG, Subbarao KV, Huisman OC, Hubbard JC (2000) Mechanism of broccoli-mediated Verticillium wilt reduction in cauliflower. Phytopathology 90:305–310

Subbarao KV, Hubbard JC (1996) Interactive effects of broccoli residue and temperature on *Verticillium dahliae* microsclerotia in soil and on wilt in cauliflower. Phytopathology 86:1303–1309

Subbarao KV, Chassot A, Gordon TR, Hubbard JC, Bonello P, Mullin R, Okamoto D, Davis RM, Koike ST (1995) Host range of *Verticillium dahliae* from cauliflower and genetic relationships and cross pathogenicities of isolates from different crops. Phytopathology 85:1105–1112

Subbarao KV, Hubbard JC, Koike ST (1999) Evaluation of broccoli residue incorporation into field soil for Verticillium wilt control in cauliflower. Plant Dis 83:124–129

Tenuta M, Lazarovits G (2002a) Ammonia and nitrous acid from nitrogenous amendments kill microsclerotia of *Verticillium dahliae*. Phytopathology 92:255–264

Tenuta M, Lazarovits G (2002b) Identification of specific soil properties that affect the accumulation and toxicity of ammonia to *Verticillium dahliae*. Can J Plant Pathol 24:219–229

Tenuta M, Lazarovits G (2004) Soil properties associated with the variable effectiveness of meat and bone meal to kill microsclerotia of *Verticillium dahliae*. Appl Soil Ecol 25:219–236

Tenuta M, Conn KL, Lazarovits G (2002) Volatile fatty acids in liquid swine manure can kill microsclerotia of *Verticillium dahliae*. Phytopathology 92:548–452

Thorup-Kristensen K, Magid J, Jensen LS (2003) Catch crops and green manures as biological tools in nitrogen management in temperate zones. Adv Agron 51:227–302

Traka-Mavrona E, Koutsika-Sotiriou M, Pritsa T (2000) Response of squash (*Cucurbita* spp.) as rootstock for melon (*Cucumis melo* L.). Sci Hortic 83:353–362

Trankner A (1992) Use of agricultural and municipal organic wastes to develop suppressiveness to plant pathogens. In: Tjamos ES, Papavizas GC, Cook RJ (eds) Biological control of plant diseases. Plenum, New York, pp 35–42

Tsao PH, Oster JJ (1981) Relation of ammonia and nitrous acid to suppression of *Phytophthora* in soils amended with nitrogenous organic substances. Phytopathology 71:53–59

Vallad GE, Bhat RG, Koike ST, Subbarao KV, Ryder EJ (2005a) Weedborne reservoirs and seedborne transmission of *Verticillium dahliae* in lettuce. Plant Dis 89:317–324

Vallad GE, Qin QM, Subbarao KV (2005b) Verticillium wilt of cool season vegetable crops: their distribution, impact and management. In: Recent research developments in plant pathology. Research Signpost, Trivandrum, India, pp 189–210

Warren KS (1962) Ammonia toxicity and pH. Nature 195:47–49

Wilhelm S (1951) Effect of various soil amendments on the inoculum potential of the Verticillium wilt fungus. Phytopathology 41:684–690

Xiao CL, Subbarao KV, Schulbach KF, Koike ST (1998) Effects of crop rotation and irrigation on *Verticillium dahliae* microsclerotia in soil and wilt in cauliflower. Phytopathology 88:1046–1055

Xiao J, Zhu J, Chen S, Ruan W, Miller C (2007) A novel use of anaerobically digested liquid swine manure to potentially control soybean cyst nematode. J Environ Sci Health, Part B 42:749–757

Chapter 19
Soil Solarization – 30 Years On: What Lessons Have Been Learned?

Jaacov Katan and Abraham Gamliel

Abstract Soil solarization (SH) in its present form was first introduced in 1976 in Israel. Shortly thereafter, it was investigated in the USA. Since then, it has been studied in over 60 countries, both developed and developing, in hot climate regions, but to some extent also in more humid and cooler regions. It is used by farmers in many countries. As with any new method, introduction of SH involved several stages covering both fundamental and applied aspects: (1) Exploring and documenting SH effectiveness (with respect to spectrum of pest control) in various regions and cropping systems. (2) Studying mechanisms of pathogen control (both physical and especially biological) and of crop-growth improvement. Models referring to physical and biological processes were developed. (3) Integrating SH with nonchemical and chemical (at reduced dosages) means. (4) Implementing SH. (5) Improving SH and adapting it to various uses. (6) Developing extension and training tools. SH is climate-dependent and has advantages and limitations. It is not connected with commercial companies, making its dissemination more difficult. Multidisciplinary studies, governmental support, knowledge transfer and international cooperation are essential for introducing nonchemical methods of control.

Keywords Soil disinfestation • Soilborne pathogens • Soil solarization • Solar heating

19.1 Introduction

Soilborne pathogens (SBP) cause heavy losses to all major crops, leading to reductions in both yield and quality. In severe cases, the farmer is forced to abandon the infested soil or to shift to less profitable crops. Thus, the impact of SBP is both economic and social.

J. Katan (✉)
Facuty of Agricultural, Food and Environmental Quality Sciences,
Rehovot 76100, Israel
e-mail: katan@agri.huji.ac.il

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,*
Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_19,

Moreover, the management of SBP often involves the use of pesticides, which have environmental consequences. The severe impact of SBP is connected with their long-term effect, since most of these pathogens can survive in the soil for years, or even decades (Cook and Baker, 1983).

There are many approaches and tools for the management of SBP which, alone or in combination, are aimed at preventing the introduction or establishment of the pathogen in soil, suppressing it, or eradicating – or at least reducing – the size of the existing population. Soil disinfestation is one of these approaches, which was developed in the early days of plant pathology, in connection with the *Phylloxera* outbreak at the end of the nineteenth century. The main goal of soil disinfestation is to eradicate, usually before planting, harmful soilborne biotic agents with minimal effect on the soil biota or on its chemical or physical properties, for the improvement of plant and soil health. This has to be achieved by using an effective, feasible, economic and environmentally acceptable technology. By the end of the nineteenth century, two basic approaches for soil disinfestation had already been developed: a physical one, involving mainly heating the soil with steam, and a chemical one, involving the use of fumigants. The latter approach became, and remains, the dominant one. Soil solarization (SH) is a third approach, developed much later (Katan et al., 1976; Katan and DeVay, 1991). It is based on both physical and biological principles and is achieved through mild heating of the soil in daily cycles for a period of several weeks. SH and biofumigation are nonchemical tools for soil disinfestation.

The subject of SH has been thoroughly discussed in many reviews, articles and books (Katan, 1981; Stapleton and DeVay, 1986; Katan and DeVay, 1991; Katan, 1996; McGovern and McSorely 1997; Stapleton et al., 1998; Stapleton, 2000; and others). Therefore, we shall only briefly review some major developments in this field. We will then describe some of the major questions that have been raised or addressed and discuss the limitations of SH, and the lessons that have been or should have been learned after three decades of research and development of a method which has been successfully adopted by farmers in many countries. It should be noted that many of the issues that are discussed in this article are also relevant to other methods of nonchemical pest management.

19.2 Exploring Soil Solarization

Many attempts have been made to harness solar energy for pest control in the last decades, and even centuries. For example, mulching (covering) the soil with cellophane in order to heat it and control nematodes was reported decades ago (Hagan, 1933). However, SH in its present form, as a soil-disinfestation tool based on mulching the soil, before planting, with a transparent material (usually polyethylene) during a climatically appropriate period, was first described in 1976 in an article reporting the results from laboratory and field studies, suggesting the principles of this approach (termed “solar heating of soil” at the time) (Katan et al., 1976).

These reported studies on disease control concentrated on one disease, Verticillium wilt (of eggplant and tomato), and were carried out in a single region, the hot Jordan and Bet Shean valleys in Israel. The achieved disease control was significant and yield increase was in the range of 200%. However at that time, it was not known whether these results were unique to the specific agricultural, climatic and edaphic conditions of valleys in which the studies were carried out, or if they would have wider implications for other pathogens, pests and regions. The 1976 publication was in essence a call to the scientific community to assess and evaluate this new approach under various conditions, the world over. The response was very encouraging: nearly 180 studies were published in the first decade (1976–1986) of SH research, referring to many pests (fungal pathogens, nematodes and weeds) and many crops, covering both fundamental and applied investigations carried out in 22 countries (Katan et al., 1987). In many cases, pathogen, disease and weed control, as well as yield increase, were satisfactory. Thus, SH was indeed found to have wide implications and its adoption as a practical management tool began. In the following years, SH research and development continued to expand, with studies being reported from over 60 countries (Gamliel and Katan, 2009). The researcher JE DeVay and his associates from UC Davis pioneered SH in the USA (Pullman and DeVay, 1977) and made major contributions to the field. The phase-out of methyl bromide (MBr) and other pesticides and increasing public concern over pesticide usage triggered further interest in SH and in other environmentally acceptable methods of pest control.

19.3 Some Major Developments in SH Study

We cannot review here all of the developments in SH research that have taken place since its inception. Instead, we refer to and highlight some key points of selected topics which are relevant to our discussion and which also yielded useful lessons for the future.

19.3.1 Mechanisms of Pathogen and Disease Control

This is one of the most investigated topics in solarization research. After all, revealing the mode of action of any management method provides tools for its improvement. Solarization involves soil heating and, therefore, thermal killing of pathogens is expected. Indeed, the elevated temperatures, especially under hot conditions, are responsible for a major or partial reduction in pathogen populations and consequently, for disease reduction, as usually occurs with fumigation or any disinfestation method. This is the physical effect. However, it was realized early on that the rate of pathogen control was frequently higher than expected from merely thermal killing, indicating the involvement of additional mechanisms. Indeed, it was shown

in many studies, performed by many researchers the world over, that pathogen biocontrol takes place in the soil during, and even after termination of the solarization process. This would explain the unexpectedly effective control achieved by SH under marginal conditions, such as in lower soil layers (where temperatures are low) or in regions which are climatically marginal for SH, e.g. Idaho (Davis and Sorensen, 1986) or Canada (Lazarovits et al., 1991). We have to expect that when a control agent is applied, not only will the pathogen be affected, but so may all biotic and abiotic components in the system, and this can have some surprising consequences. This is especially true when a control agent is applied to the soil, where so many processes are occurring, and is just one more example of a case in which studies on pathogen responses to killing agents, carried out under controlled conditions, e.g. developing dosage response curves for thermal killing, can be of very limited value, if not totally misleading. Such studies alone would not have predicted that SH could become an effective tool for disease management. Monitoring the temporal decline of a pathogen population in soil (at various depths) has a better chance of reflecting the effectiveness of SH. However, it should be remembered that although inoculum density of the pathogen is crucial in determining disease level, this is not the sole factor involved, since the capacity of inoculum to cause disease (inoculum potential) as well as host response might be also affected. Nevertheless, data on pathogen population dynamics during and after solarization can be useful, as shown in many studies, in that they constitute a tool for studying the mechanisms of disease control.

The biotic components which can be affected by SH (and this has been demonstrated) are the pathogen, the soil microbial activity and the host response. There are also many abiotic soil components that can be affected (see below). It should be stressed that during the solarization process, the soil is kept wet in order to increase the sensitivity of the propagules to heat and to improve heat conduction, but this situation also favors biological activity. The final result of these interactions can be an increase, a decrease or no change at all in disease incidence. Thus, it is possible that a certain control agent, although harmful to the pathogen, will still increase disease incidence if its effects on the existing antagonists in the soil, or on plant resistance, are more detrimental than its effect on the pathogen. The opposite situation can also occur. Although most studies on SH which were carried out under appropriate climatic conditions report various levels of disease reduction (up to 100% in some cases), increases in disease incidence by solarization have also been recorded, e.g. when heat-tolerant weeds or pathogens (e.g. *Macrophomina*) are involved (Gamliel and Katan, 2009). Disease outbreaks in previously fumigated or steamed soils are well known (Cook and Baker, 1983). In most cases, it appears that the mild soil heating generated by SH is not detrimental to soil biological activity. However, we cannot exclude the possibility of a biological vacuum being created by SH in certain cases. Detecting such cases and determining their causes is another tool to improve SH and to avoid unexpected and undesirable effects.

Several mechanisms are involved in the nonthermal control that is induced or enhanced by SH (DeVay and Katan, 1991). They need not all exist in every system,

but many of them have been reported for various pests and cropping systems, indicating the broad implications of this phenomenon. We shall refer briefly to three biocontrol mechanisms and to induced resistance.

a. *The Weakening Effect* When assessing the effect of a control agent on a pathogen, we usually measure the level of mortality that it causes. In most cases, less attention is paid to the fate of the surviving, but injured and apparently weakened propagules. It has been shown that treating a pathogen with a sublethal dosage of a stress agent (e.g. heat or fumigant) renders it more vulnerable to the action of antagonists existing in the soil, and therefore, the pathogen population may decline further with time, even after the stress agent has dissipated. Therefore, assessing population level of the pathogen shortly after treatment does not necessarily reflect the whole impact of a stress agent on a pathogen: weakening leads to induced and enhanced biocontrol. Garrett (1956) emphasized the potential of sublethal dosages in enhancing biocontrol, as demonstrated with *Armillaria* which became extensively colonized by *Trichoderma* after exposure to sublethal dosages of CS_2. Apparently, the weakening phenomenon is common after soil disinfestation since a gradient of reduced sublethal dosages exists around every treated soil site. Since SH involves mild, namely sublethal, heating, especially at deep soil layers, the weakening effect would appear to be very important. Indeed, this mechanism has been described by many researchers in connection with SH (e.g., Lifshitz et al., 1983; Freeman and Katan, 1988; Tjamos and Fravel, 1995; Arora et al., 1996; and others). Assaraf et al. (2002) studied heated conidia of *Fusarium*: although apparently not killed when examined shortly after heating, their population was nevertheless reduced later, suggesting delayed mortality.
b. *Shift in Microbial Populations* There are reports showing that in solarized soil, the populations of beneficial organisms, e.g., fluorescent pseudomonads, *Bacillus*, *Pasteuria*, *Talaromyces*, *Trichoderma* and others, are frequently increased (e.g. Stapleton and De Vay, 1984; Walker and Wachtel, 1988; Gamliel and Katan, 1991; Tjamos et al., 2000; Stevens et al., 2003). This might be because saprophytic thermotolerant organisms survive, at least partially, under the mild heating of solarization, or it may be for other unknown reasons. Therefore, SH appears to create a soil environment that favors saprobes. It should be noted that in a typical agricultural soil, the phytopathogens comprise only a very small proportion of the total flora and therefore, chances are good that certain antagonist species will survive the mild heating involved in SH. This important issue needs to be further studied.
c. *Induced Soil Suppressiveness* Solarized soils are frequently less vulnerable to reinfestation (Kassaby, 1985; Greenberger et al., 1987; Freeman et al., 1990; Gamliel and Katan, 1993). This would explain the frequently observed long-term effect of solarization (Katan et al., 1983; Tjamos and Paplomatas, 1988), or the effectiveness of SH even when strip solarization, in which the solarized soil is surrounded by infested soil, is applied (Katan et al., 1980), or under furrow irrigation (Abdel-Rahim et al., 1988). Moreover, it was found that in out of ten solarized soils, eight became suppressive and one became conducive, while one out of two MBr-fumigated soils became conducive (Greenberger et al., 1987).

d. *Induced Host Resistance* SH aims to control pathogens that live in the soil, by heating that soil. Nevertheless, surprising results have been obtained with respect to reductions in *foliar* diseases by this method. This phenomenon was attributed to among others, the control of primary inoculum in the soil. Since physiological, including hormonal (Gruenzwieg et al., 1993, 2000, changes were recorded in the foliar parts of plants whose roots were the only parts exposed to the solarized soil, induced resistance, triggered by solarized soil, should also be considered, as has been previously demonstrated (Stevens et al., 1992; Levy et al., 2005). Incidence of gray mold caused by *Botrytis cinerea* or powdery mildew (caused by *Sphaerotheca fuliginea*) in foliage of cucumbers decreased when the plants were grown in solarized soil. This phenomenon was first reported by Hassasn and Younis (1984). Since SH also increases populations of beneficial microorganisms, which are also known to induce resistance in plants, this indirect effect should also be considered.

The above four mechanisms, and additional ones which are not discussed here, clearly show that SH achieves more than simply thermally killing pests by heating the soil.

19.3.2 Increased Growth Response of Plants in Solarized Soils

The phenomenon of plant-growth enhancement in disinfested soils, frequently with a subsequent increase in yield even in the absence of known pests, was discovered at the end of the nineteenth century, and has since been repeatedly reported with all disinfestation methods, including solarization (Chen and Katan, 1980; Stapleton et al., 1985; Chen et al., 1991). Such a phenomenon is unexpected in noninfested soil and therefore, mechanisms beyond pest control appear to be involved. Indeed, significant changes in the biotic and abiotic components of the soil occur upon disinfestation, whether the soil is infested with pathogens or not (Chen et al., 1991). These changes were found to be correlated with IGR. Different mechanisms that are not related to control of major pathogen have been suggested to explain IGR in disinfested soils, such as increased micro- and macro-elements in the soil solution (Chen and Katan, 1980; Stapleton et al., 1985; Patrıcio et al., 2006), reduced soil salinity (Abdel-Rahim et al., 1988), elimination of minor pathogens or parasites, destruction of phytotoxic substances in the soil, release of growth-regulator-like substances, and stimulation of mycorrhizae or other beneficial microorganisms (Stapleton et al., 1985; Chen et al., 1991; Gamliel and Katan, 1991). When plants were grown in pathogen-free solarized soil, they had significantly higher levels of chlorophyll and protein contents than controls (Gruenzwieg et al., 1993). In addition, the degradation of these compounds and the decrease in net photochemical yield with age were delayed in plants growing in solarized soil, as compared to the control. In that case, delayed leaf senescence appeared to be a plant response contributing to IGR. In another study, the level of soluble organic substances, e.g. humic substances, was higher in the solarized soil (Chen et al., 2000). These soluble substances increased the growth of corn plants as well as of populations of

fluorescent pseudomonads, indicating an additional mechanism for improved plant growth. An association between plant-growth improvement in the solarized soil and microbial activities, such as the stimulation of beneficial rhizobacteria and suppression of minor pathogens, has been reported (Gamliel and Katan, 1991; Stevens et al., 2003; Tjamos et al., 2004).

The increases in plant growth in IGR studies range from a few to several hundred percent, depending on the soil, the plant, and the parameter used. For example, a 29% increase in onion yield was found due to IGR (Satour et al., 1989). However, the effect on yield components is not always analyzed. This may have very important economic implications which should be taken into account when considering the use of solarization. The major difficulty is that we cannot predict whether and to what extent a soil will respond with IGR. Developing predictive methods for this purpose and developing measures to further increase IGR would improve the economic benefits of solarization (or of any other disinfestation method for that matter). The definition of IGR depends largely on that of a healthy plant. It is now widely accepted that plant health involves more than simply disease control. As Browning (1983) states: "Plant health is far more than the opposite of plant disease as used in plant pathology." In the twenty-first century, such fundamental questions still need to be addressed.

19.3.3 Simulation and Modeling

A main difficulty with SH is its dependence on climate. Therefore, one needs to be able to predict the effectiveness of solarization in various climatic regions and seasons. This can be achieved experimentally, by following soil temperatures and pathogen mortality in the solarized soil, and by modeling.

A variety of models for predicting temperatures of solarized soils under various climatic conditions, arid conditions in particular, have been developed and validated (Mahrer, 1991). Simplified models (Cenis, 1989), and a model that is also suitable for more humid areas (Wu et al., 1996), have also been developed. However, information on soil temperatures in the solarized soil, although necessary and very helpful, is not sufficient for predicting the effectiveness of SH for pathogen control, since it can only consider thermal inactivation of the pathogen.

Attempts were made to correlate soil heating with pathogen or disease control, utilizing data collected under constant temperatures. Traditionally, thermal inactivation is considered a first-order reaction, characterized by a logarithmic change in the organism's population with time. A logarithmic relationship was indeed found between time and temperature (at constant temperatures) for the thermal inactivation of four soilborne plant pathogens (Pullman et al., 1981). Studies on thermal inactivation under fluctuating temperatures, such as those naturally prevailing in the field, are much more complicated to perform, because the partial effects of varying temperatures on pathogens is difficult to weigh, and they mandate numerical integration to account for their complexity (Shlevin et al., 2003). Another approach to

simulating and modeling pathogen control by solarization is to plot the level of mortality versus accumulated hours above a certain temperature, i.e. the degree-hours (Chellemi et al., 1994), or by giving different values for various temperatures (Shlevin et al., 2005).

19.3.4 Integrated Pest Management

IPM is now a widely accepted concept in crop protection. This approach addresses pest control, environmental, economic, legal and public issues simultaneously in an attempt to achieve effective, economical, environmentally and publicly acceptable pest control via diverse methods, used alone or in combination, which are adapted to the specific cropping system (Gupta, 1996; Katan, 1996; Kendrick 1988; Davis et al., 2008; Gamliel and Katan, 2009). An appropriate IPM program can achieve better control with minimal use of pesticides and environmental hazards, a broader spectrum of control and even a long-term effect. Combining and alternating methods of control are at the heart of IPM. Solarization has been successfully combined with various chemical (especially at reduced dosages) and nonchemical methods of control in order to improve SH control levels and reliability. McGovern and McSorely (1997) emphasized that combining solarization with other pest-management practices may be necessary to ensure acceptable reduction of difficult-to-control pests, especially under suboptimal conditions. Combining SH with biocontrol agents or organic amendments is especially attractive and its effectiveness was demonstrated in various studies (Elad et al., 1980; Chet et al., 1982; Ristaino et al., 1991; Sivan and Chet, 1993; Lodha, 1995; Tjamos et al., 2000; Minuto et al., 2006).

Combining methods of control is more than merely mixing two methods. The combination has to be optimal. For example, sequence of application needs to be considered (Eshel et al., 2000). Certain elements of integrated management are powerful tools for improving solarization. Therefore, combining solarization with other methods of control enables us to address the limitations of the former, (see also below). Knowledge transfer and the development of appropriate tools for extension are crucial in disseminating the message of IPM. These tools have been developed for SH.

19.4 Improving Solarization

The basic principle of SH is to manipulate solar energy into a force for pest control. Transparent plastic mulch traps the solar irradiation and allows the heat to be transferred to deep soil layers. This is the simplest method of applying soil disinfestation. However, solarization is limited to the hot seasons, in which solar irradiation is maximal. Moreover, SH is not energy-efficient, leading to loss of a significant

fraction of the solar energy which could further heat the soil. The loss of energy results from the film's reflectance and lack of insulation capacity. Since SH's introduction, these attributes have been addressed, resulting in continual improvement and technological innovation in order to adapt SH to a wide variety of conditions and cropping systems. Advanced solarization seeks to achieve additional goals, such as improving the level of control – particularly of heat-tolerant pests, and especially expanding its use to climatically marginal regions. Some of the other sought-after improvements are cost reduction, and increased reliability and reproducibility of the method.

Improvement of SH can be achieved by either increasing transmittance of the irradiation through the plastic mulch or improving the insulation capacity of the heated soil. Over the years, much effort has been invested in moving from transparent polyethylene plastic sheets to other polymers and other formulations in order to maximize heat absorbance under the tarp. The following describes some of the current advances in film formulation and technology and other approaches.

19.4.1 Technological Improvements

SH can be improved by minimizing the volume of the soil to be disinfested. For example, by solarizing shallow layers of growth substrates, the temperatures can be increased to very high levels, thus leading to the control of even the thermotolerant pathogen *Monosporascus cannonballus* (Pivonia et al., 2002). Gullino et al. (1998) found that improved method of solarization of raised benches was very effective in controlling soilborne pathogens of sweet basil.

19.4.2 Double Tarps

Double-layer films form an insulating air space under and between the plastic layers, thereby reducing heat loss from the soil to the atmosphere, especially at night. Raymundo and Alcazar (1986) achieved an increase of 12.5°C at a depth of 10 cm using a double-layer film compared to a single-layer one (60 versus 47°C, respectively). A similar increase in soil temperature with double-layer films was achieved by Ben-Yephet et al. (1987), who observed a 98% reduction in the viability of *F. oxysporum* f. sp. *vasinfectum* after 30 days under the double mulch compared with a 58% reduction under a single mulch, at a depth of 30 cm. This approach was also followed successfully in Australia with nursery potting mix (Duff and Connelly, 1993). SH in a closed greenhouse represents another use of the double-layered film which was also found to improve disease control (Garibaldi and Gullino, 1991; Streck et al., 1996).

The use of a sprayable black polymer as the bottom layer is another approach to increasing solar heating with a double layer. The use of double mulch which

consists of a transparent film over a black polymer coating is based on the same principle as solar collectors for water heating in sunny countries. Stevens et al. (1999) reported a 5°C increase in soil temperature when applying solarization in strips in a cloudy climate. Similarly, Arbel et al. (2003) achieved an increase in soil temperature by mulching transparent polyethylene sheet over a layer of sprayable black mulch. They achieved effective control of Fusarium crown and root rot of tomatoes and vine collapse of melons (caused by *M. cannonballus)* with double-mulch solarization, while solarization with regular films was not effective. Nevertheless, the cost of double-mulching and the technology that needs to be developed for simultaneous tarping limit the implementation of this approach. Alternatively, it might be successfully applied in strip solarization and small farm plots.

19.4.3 Improved Films

Many studies have attempted to produce plastic films based on infrared (IR) blocking material. Studies conducted under rainy and cloudy conditions in Alabama and Florida reported minor, if any, differences in soil heating using IR films (Chase et al., 1999; Stevens et al., 1999). A different plastic formulation was tested using the addition of anti-drip (AD) components (Arbel et al., 2003). This formulation prevents condensation of water droplets on the film surface, leading to a 30% increase in irradiation transmittance over regular film. Soil temperatures under AD film were 2°C to 7°C higher than under regular film. Solarization with AD film in field experiments resulted in effective control of sudden wilt of melons, while solarization with common transparent film had no effect on disease levels (A. Gamliel, 2003). Another film formulation designed to increase heating capacity is virtually impermeable films (Chellemi et al., 1997; Tjamos et al., 2000).

19.4.4 Sprayable Films

Sprayable polymers offer a feasible and cost-effective alternative to plastic tarps for SH. The plastic-based polymers are sprayed on the soil surface in the desired quantity and form a membrane film, which can maintain its integrity in soil and elevate soil temperatures, but is still porous, allowing overhead irrigation. Overall, soil temperatures under sprayable mulch are lower than those obtained under plastic film. The thickness of the sprayed coat is critical to obtaining effective heating (Skutelsky et al., 2000). Initial research with these polymers was conducted by Stapleton and Gamliel (1993), who achieved effective soil heating and a reduction in the viability of *Pythium* propagules. The application, however, was not cost-effective, since a high amount of polymer was required to achieve a continuous and uniform coating. In Israel, a sprayable polymer product, “Ecotex”, was developed together with the technology to apply it economically on soils for various purposes (Skutelsky et al., 2000). Soil coating using this technology with a black polymer formulation resulted in a membrane film

that could raise soil temperatures to close to solarization levels. Soil heating with sprayable mulch is faster than with plastic film, but the soil also cools down to lower temperatures at night. SH using sprayable mulches was effective in controlling Verticillium wilt and potato scab in potato (Gamliel et al., 2001), at a level matching that achieved by solarization using plastic films. However, there is room for further improvement in the use of sprayable polymers.

19.5 Special Uses or Modification of Solar Heating

A prominent indication of a successful technology is the adoption of its principles beyond the original objectives. Indeed, the use of solar irradiation as a heating source has expanded since 1976 beyond the purpose of SH for soil disinfestation. The adoption of this method by many researchers has led to various uses, some of them quite unusual. Moreover, the experience and knowledge which have been accumulated regarding the mechanisms of SH (e.g. heat, humidity) have served as useful tools for extending the use of solar energy beyond its original scope. An excellent example is the extension of SH to existing, perennial crops such as pistachio (Ashworth and Gaona, 1982) and olive (Tjamos et al., 1991): the moderate heating pattern of solarization enables control of the pest while not killing the existing, deep-rooted crop. Below are more examples of other uses of solarization. SH is the only soil-disinfestation method that can be used in organic farming (CDFA, 2004) and indeed, it is used by these farmers. Also, it is especially useful in home gardens.

19.5.1 Solarization Inside the Greenhouse

Solarization was initially developed and studied for outdoor use (Katan et al., 1976). However, the strong trend toward indoor production has led to developments in the solarization of soils and substrates. Stapleton (2000) developed a method to eradicate phytoparasitic pests in closed horticultural applications (Stapleton, 2000; Stapleton et al., 2002). This is accomplished by covering the greenhouse structure with a double-layered tent, thereby creating increased heating efficacy. The soil or substrate are either mulched or left bare. The temperature inside the tent rises to above 70°C, a level of heat which, even if applied for only a short time, can completely eradicate both phytoparasitic and free-living nematodes.

19.5.2 Structural Solarization

Unlike soil treatment, solarization of structures aims to control the inoculum which is left within the greenhouse or any other structure and its components (the structure itself, irrigation lines, wires, etc.) which might be contaminated with inoculum

(Gamliel et al., 1996). This is achieved by closing the greenhouse during the summer time, thereby elevating the temperatures (dry heating) to 60°C, and even close to 70°C. Structural solarization is carried out under dry conditions and is considered a form of solar sanitation (Shlevin et al., 2003).

19.5.3 Solar Disinfection of Infested Objects

The concept of trapping solar irradiation to create lethal temperatures has been adopted for various purposes. Besri and Diop (1985) demonstrated disease reduction in greenhouse tomatoes by solarization of the wooden tomato stakes, thereby eliminating the inoculum of *Didymella lycopersici*. This approach was further elaborated by solarizing soils in small (40-cm high) mulched piles to eliminate root knot nematodes in olive nurseries in Spain (Nico et al., 2003).

19.5.4 Solar Collectors

Ghini (1993) demonstrated the use of solar collectors for soil disinfestation and the treatment of potting medium. The solar collector is comprised of soil inside metal tubes which are placed in a box and covered with transparent plastic. Her study showed that the solar collector is an efficient method for treating potting medium infested with soilborne pests. This equipment is used by many growers, nurseries and research institutions in Brazil as an alternative method of control. The easy construction and operation of the solar collector offers a low-cost, efficient and safe system for the production of healthy seedlings. This application of solarization could potentially come into common use for the disinfestation of soil seedbeds, containerized planting media, and cold frames in small and simple structures.

19.6 Solarization and the Methyl Bromide Crisis

The MBr crisis shocked the crop-protection community and has had a significant impact, especially in soil disinfestation. Thus, farmers have been left in desperate need of innovative and environmentally acceptable alternative approaches. Indeed, SH alone or in combination with other fumigants or other methods has replaced MBr in some hot regions. Furthermore, this crisis has served as a driving force for further adoption of SH as an alternative solution. In many countries, solarization is widely used and has proven particularly successful when combined with reduced doses of metham sodium (MBTOC, 2007). Extensive studies conducted over the past 30 years regarding combining solarization with fumigants have provided a useful background for the adoption of this approach. Indeed, it appears that

solarization in combination with reduced rates of fumigants has provided a rapid solution to the MBr crisis in certain situations.

Solarization, unlike MBr, provides long-term beneficial effects, which make this approach more cost-effective. Such benefits are important for both conventional cropping agriculture and organic farming. In recent years, several studies have examined the effects of long-term, large-scale use of SH and organic amendments on pest control, yields and soil fertility and compared them to chemical treatments (Chellemi et al., 1997; Roe et al., 2004; Benlioglu et al., 2005; Ozores-Hampton et al., 2005). Solarization has been shown to be cost-effective, compatible with other pest-management tactics and a valid alternative to pre-planting fumigation with MBr.

The MBr phase-out showed that dependence on a single method or chemical can lead to an agricultural crisis. Thus, integrated approaches are now the preferred solution for pest control, and solarization can be a major component in such integrated programs.

19.7 Limitations of Soil Solarization

As with any control method, SH has limitations and difficulties, many of them connected with its climate dependency. Thus, SH can be used only in certain regions and during certain periods. Climatic variations can lead to uncertainty. During the solarization process, land is without crop for 3 to 6 weeks. Thermotolerant pathogens are not controlled by SH. As with most nonchemical methods, SH is usually not backed by commercial companies (Tjamos et al., 2000). Plastics disposal also needs to be considered, with sprayable and degradable plastics offering potential solutions. Some of these difficulties can be partially or fully alleviated by adopting IPM approaches. For example, improved technologies for solarization or combining SH with biocontrol agents enable to reduce length of solarization (Tjamos et al., 2000). As with any newly introduced method of control, monitoring treated fields for potential failures and their causes is a very effective tool for the improvement of the method and avoiding harmful effects.

19.8 Lessons Which Can and Should Be Learned

Developing nonchemical methods of pest management is difficult but achievable, and much needed, especially in an era of heightened environmental concern.

Developing nonchemical methods of control should be a continuous task, not one that only comes into play in emergency situations. Indeed, SH was developed long before the methyl bromide crisis and was ready for use in certain regions by the 1980s.

It is not possible, nor is it desirable, to develop a method which controls all pathogens. This can be achieved by using an appropriate combination of control methods. SH is not a universal tool for control, but rather one more tool to be

incorporated into IPM programs. In certain situations it totally replaces chemicals, in other it enables to reduce chemical use (Gamliel et al., 1993).

Reliable diagnostic facilities are crucial for the optimal use of SH, or any soil-disinfestation method for that matter.

SH is especially effective when combined with other methods, including pesticides at *reduced* dosages.

Since nonchemical methods are usually not backed by commercial companies, the farmers consider their use a risk. Therefore, these methods have to be partially subsidized for a certain period in order to enhance their adoption.

Decision-making tools, e.g. assessing pathogen population before planting, can further improve SH use.

Education and extension tools are crucial for the proper introduction of any new control method.

Treated fields should be monitored for undesirable side effects as early as possible, in order to avoid or control them.

A network for the exchange of information, cooperation and international conferences have been key factors in the acceptance and adoption of SH. International conferences dealing with SH such in those which took place in Sicily, Jordan and Syria as well as SH sessions in scientific meetings contributed to the SH research.

Economic analyses are helpful in adjusting SH to the appropriate agricultural situations (Yaron et al., 1991).

All of the aforementioned are also relevant to all nonchemical methods of control, and some of them also to chemical methods.

19.9 Concluding Remarks

After three decades of research and development, it is time to examine the potential of a new method of control, its achievements, failures and acceptance. However, three decades cover only a small chapter in modern agriculture. A new method of control not only brings hope for the future, but may also bear the seeds of hazard. Our aim in developing any method is its adoption and application by farmers: this is the ultimate indicator of success. SH is used on thousands of acres, especially in the Mediterranean and Latin American regions, but also on a smaller scale in many countries and also by organic farmers and in home gardens. It has become a full-fledged citizen of the crop-protection community. However, its potential has not yet been fully realized, for the reasons discussed above, among others. Further research and development will expand its use and its applicability. A major (but not sole) reason for its success is the fact that leading scientists in many countries, along with their graduate students, have studied it, placing a major emphasis on revealing its mode of action and the mechanisms involved. These scientists have also created a useful network of information exchange.

The solarization approach was originally developed for soil disinfestation to control SBP. However, many additional uses were developed in the years that

followed, as described above. There may be additional uses for the solarization approach which have yet to be discovered.

It should be realized that any soil treatment, especially the drastic application of disinfestation, creates many changes in the biotic and abiotic components of the soil. Only a deep understanding of these changes will enable full exploitation its control potential and the avoidance of undesirable and unexpected phenomena. We now have better and more sophisticated tools at our disposal to address these challenges. Today, the biggest challenge in crop-protection sciences is to effectively control pests, while avoiding environmental hazards and the deterioration of natural resources. SH is an additional tool for achieving this task, when used in the appropriate situations. Although the positive effects of SH outweigh the negative ones (e.g., plant-growth retardation due to harmful effects on beneficial microorganisms, such as *Rhizobium* or mycorrhizae), the emphasis in research should be placed on the negative effects and on developing means to detect and avoid them. Pathogen populations usually build up in soils of intensive crops where monoculture is common. These organisms should be regarded as biopollutants of agricultural soils. Therefore, effective management of these harmful organisms, e.g., by SH, should be considered a remediation of agricultural soils.

After more than 100 years of soil disinfestation, the arsenal of chemical disinfestants is still very limited, and the arsenal of nonchemical agents even more so. Therefore, the integration of pest-management methods, rather than relying on one powerful control agent, is not only desirable but also the only feasible solution for coping with our need for methods of controlling SBP in an atmosphere of environmental awareness. SH began with an idea conceived by farmers and extension workers which was first tested in the Jordan and Bet Shean valleys, near the ancient towns of Bet Shean and Jericho and the Jordan River, the arena for so many Biblical events. SH is only one more way-station on our long journey toward better and safer sustainable agriculture; additional milestones await (Katan and DeVay, 1991).

References

Abdel-Rahim MF, Satour MM, Mickail KY, El Eraki SA, Grinstein A, Chen Y, Katan J (1988) Effectiveness of soil solarization in furrow-irrigated Egyptian soils. Plant Dis 72:143–146

Arbel A, Siti M, Barak M, Katan J, Gamliel A (2003) Innovative plastic films enhance solarization efficacy and pest control. Proceedings of the 10th annual international research conference on Methyl bromide alternatives and emission reduction, San Diego, CA, pp 91.1–91.3

Arora DK, Randey AK, Srivastava AK (1996) Effects of heat stress on loss of F. graminearum and pathogenicity from chlamydospores of *Fusarium oxysporum* f. sp. *ciceri.* Soil Biol Biochem 28:399–407

Ashworth LJ, Gaona SA (1982) Evaluation of clear polyethylene mulch for controlling Verticillium wilt in established pistachio nut groves. Phytopathology 72:43–246

Assaraf MP, Ginzburg C, Katan J (2002) Weakening and delayed mortality of *Fusarium oxysporum* by heat treatment: flow cytometry and growth studies. Phytopathology 92:956–963

Benlioglu S, Boz O, Yildiz A, Kaskavalci G, Benlioglu K (2005) Alternative soil solarisation treatments for the control of soilborne diseases and weeds of strawberry in the western Anatolia of Turkey. J Phytopathol 153:423–430

Ben-Yephet Y, Stapleton JJ, Wakeman RJ, DeVay JE (1987) Comparative effects of soil solarization with single and double layers of polyethylene on survival of *Fusarium oxysporum* f. sp. *vasinfectum*. Phytoparasitica 15:181–185

Besri M, Diop M (1985) Control of *Didymella lycopersici* in tomato by storing the supports in plastic tunnels: new application of the solar heating or solarization. Rev Hortic 58:99–102

Browning A (1983) Goal for plant health in the age of plants: a national plant heath system. In: Kammedahl T, Williams PH (eds) Challenging problems in plant health. APS, St. Paul, MN, pp 45–57

CDFA (2004) Approved treatment and handling procedures to ensure against nematode pest infestation of nursery stock. Nursery inspection procedures manual, NIPM item 7. In: California Department of Food and Agriculture (CDFA), Plant health and pest prevention services, pest exclusion branch, sacramento web page: http://www.cdfa.ca.gov/phpps/pe/nipm.htm

Cenis JL (1989) Temperature evaluation in solarized soils by Fourier analysis. Phytopathology 79:506–510

Chase CA, Sinclair TR, Chellemi DO, Gilreath JP, Locascio ST, Olson SM (1999) Heat-retentive films for increasing soil temperature in a humid, cloudy climate. HortScience 34:1085–1089

Chellemi DO, Olson SM, Mitchell DJ (1994) Effects of soil solarization and fumigation on survival of soilborne pathogens of tomato in northern Florida. Plant Dis 78:1167–1172

Chellemi DO, Olson SM, Mitchell DJ, Secker I, McSorly R (1997) Adaptation of soil solarization to the integrated management of soilborne pests of tomato under humid conditions. Phytopathology 87:250–258

Chen Y, Katan J (1980) Effects of solar heating of soils by transparent polyethylene mulching on their chemical properties. Soil Sci 130:271–277

Chen Y, Gamliel A, Stapleton JJ, Aviad T (1991) Chemical, physical, and microbial changes related to plant growth in disinfested soils. In: Katan J, DeVay JE (eds) Soil solarization. CRC, Boca Raton, FL, pp 103–129

Chen Y, Katan J, Gamliel A, Aviad T, Schnitzer M (2000) Involvement of soluble organic matter in increased plant growth in solarized soils. Biol Fertil Soils 31:28–34

Chet I, Elad Y, Kalfon A, Hadar Y, Katan J (1982) Integrated control of soilborne pathogens in iris. Phytoparasitica 10:2229–2366

Cook RJ, Baker KF (1983) The nature and practice of biological control of plant pathogens. APS, St. Paul, MN

Davis JR, Sorensen LH (1986) Influence of soil solarization of moderate temperatures on potato genotypes with differing resistance to *Verticillium dahliae*. Phytopathology 76:1021–1026

Davis JR, Katan J, Gamliel A (2009) The potential of using soil solarization as a means of controlling diseases caused by soilborne pathogens and weeds in potatoes. In: Petiss RD (ed) Current concepts in potato disease management, Research Signpost, Kerala, India (in press)

DeVay JE, Katan J (1991) Mechanisms of pathogen control in solarized soil. In: Katan J, DeVay JE (eds) Soil solarization. CRC, Boca Raton, FL, pp 87–101

Duff JD, Connelly MI (1993) Effect of solarization using single and double layers of clear plastic mulch in Pythium Phythophthora and Sclerotium species in a potting mix. Aus Plant Pathol 22:28–35

Elad Y, Katan J, Chet I (1980) Physical, biological and chemical control integrated for soilborne diseases in potatoes. Phytopathology 70:418–422

Eshel D, Gamliel A, Grinstein A, Di Primo P, Katan J (2000) Combined soil treatments and sequence of application in improving the control of soilborne pathogens. Phytopathology 90:751–757

Freeman S, Katan J (1988) Weakening effect on propagules of *Fusarium* by sublethal heating. Phytopathology 78:1656–1661

Freeman S, Sztenjnberg A, Shabi E, Katan J (1990) Long-term effect of soil solarization for the control of *Rosellinia necatrix* in apple. Crop Prot 9:312–316

Gamliel A, Katan J (1991) Involvement of fluorescent pseudomonads and other microorganisms increased growth response of plants in solarized soils. Phytopathology 81:494–502

Gamliel A, Katan J (1993) Suppression of major and minor pathogens by fluorescent pseudomonads in solarized and nonsolarized soils. Phytopathology 83:68–75
Gamliel A, Katan J (2009) Control of plant diseases through solarization. In: Walter D (ed) Disease control in crops: environmentally-friendly approaches. Blackwell, Oxford 196–220
Gamliel A, Hadar E, Katan J (1993) Improvement of growth and yield of *Gypsophila paniculata* by solarizaiton or fumigation of soil or container medium in continuous cropping systems. Plant Dis 77:933–938
Gamliel A, Katan T, Younis H, Katan J (1996) Fusarium wilt and crown rot of basil: involvement of soilborne and airborne inoculum. Phytopathology 86:56–62
Gamliel A, Skutelski Y, Peretz-Alon Y, Becker E (2001) Soil solarization using sprayable plastic polymers to control soilborne pathogens in field crops. Proceeding of the 8th annual international research conference on methyl bromide alternatives and emission reduction, San Diego, CA, pp 10.1–10.3
Garibaldi A, Gullino ML (1991) Soil solarization in southern European countries with emphasis in soilborne disease control of protected crops. In: Katan J, DeVay JE (eds) Soil solarization. CRC, Boca Raton, FL, pp 227–235
Garrett SD (1956) Biology of root-infecting fungi. Cambridge University Press, Cambridge
Ghini R (1993) A solar collector for soil disinfestation. Neth J Plant Pathol 99:45–50
Greenberger A, Yogev A, Katan J (1987) Induced suppressiveness in solarized soils. Phytopathology 77:1663–1667
Gruenzwieg JM, Rabinowitch HD, Katan J (1993) Physiological and developmental aspects of increased plant growth in solarized soils. Ann Appl Biol 122:579–591
Grunzweig JM, Rabinowitch HD, Katan J, Wonder M, Ben Tal Y (2000) Involvement of endogenous gibberellins in the regulation of increased tomato shoots growth in solarized soil. Plant Growth Regul 30:233–239
Gullino ML, Minuto A, Garibladi A (1998) Improved methods of bench solarization for the control of soilborne diseases in basil. Crop Prot 17:497–501
Gupta UK (1996) Integrated management of soil-borne diseases. In: Utkhede RS, Gupta VK (eds) Management of soil-borne diseases. Kalyani, New Delhi, pp 40–64
Hagan HR (1933) Hawaiian pineapple field soil temperatures in relation to the nematode *Heterodera radicicola* (Greef). Soil Sci 36:83–95
Hassasn MS, Younis MA (1984) Cucumber cultivation with soil solarization and plastic mulching. Arab J Plant Prot 2:65–69 (Arabic with English summary)
Kassaby FY (1985) Solar heating soil for control of damping off diseases. Soil Biol Biochem 17:429–434
Katan J (1981) Solar heating (solarization) of soil for control of soilborne pests. Annu Rev Phytopathol 19:211–236
Katan J (1996) Soil solarization: integrated control aspects. In: Hall R (ed) Strategies for managing soilborne plant pathogens. APS, St Paul, MN, pp 250–278
Katan J, DeVay JE (eds) (1991) Soil solarization. CRC, Boca Raton, FL
Katan J, Greenberger A, Alon H, Grinstein A (1976) Solar heating by polyethylene mulching for the control of diseases caused by soilborne pathogens. Phytopathology 66:683–689
Katan J, Rotem I, Finkel Y, Daniel J (1980) Solar heating of the soil for the control of pink root and other soilborne diseases in onions. Phytoparasitica 8:39–50
Katan J, Fishler G, Grinstein A (1983) Short- and long-term effects of soil solarization and crop sequence on Fusarium wilt and yield of cotton in Israel. Phytopathology 73:1215–1219
Katan J, Grinstein A, Greenbergr A, Yarden O, DeVay JE (1987) The first decade of soil solarization (solar heating): a chronological bibliography. Phytoparasitica 15:229–255
Kendrick JB (1988) A view point of integrated pest management. Plant Dis 72:647–648
Lazarovits G, Hawke MA, Tomlin AD, Olthof ThHA, Squire S (1991) Soil solarization to control *Verticillium dahliae* and *Pratylenchus penetrans* on potatoes in central Ontario. Can J Plant Pathol 13:106–111

Levy N, Elad Y, Katan J (2005) Induced resistance in plants treated with solarized soil or *Trichoderma*. Phytoparasitica 33:288 (Abstract)

Lifshitz R, Tabachnik M, Katan J, Chet I (1983) The effect of subleathal heating on sclrerotia of *Sclerotium rolfsii*. Can J Microbiol 29:1607–1610

Lodha S (1995) Soil solarization, summer irrigation, and amendments for the control of *Fusarium oxysporum* f. sp. *cumini* and *Macrophomina phaseolina* in arid soils. Crop Prot 14:215–219

Mahrer Y (1991) Physical principles of solar heating of soils by plastic mulching in field and in glasshouses are simulation models. In: Katan J, DeVay JE (eds) Soil solarization. CRC, Boca Raton, FL, pp 75–86

MBTOC (2007) 2006 report of the methyl bromide technical options committee (MBTOC). UNON, Publishing section services, Nairobi

McGovern RJ, McSoreley R (1997) Physical methods of soil sterilization for disease management including soil solarization. In: Rechcigel NA, Rechcigel, JE (eds) Environmentally safe approaches to disease control. CRC Lewis, Boca Raton, FL, pp 283–313

Minuto A, Spadaro D, Garibaldi A, Gullino ML (2006) Control of soilborne pathogens of tomato using a commercial formulation of *Streptomyces griseoviridis* and solarization. Crop Prot 25:468–475

Nico AI, Jimenez-Diaz RM, Castelo L (2003) Solarization of soils in piles for the control of *Meloidogyne incognita* in olive nurseries in southern Spain. Plant Pathol 52:770–778

Ozores-Hampton M, Stansly PA, McSorley R, Obreza TA (2005) Effects of long-term organic amendments and soil solarization on pepper and watermelon growth, yield, and soil fertility. HortScience 40:80–84

Patrıcio FRA, Sinigagli C, Barros BC, Freitas CC, Tessarioli Neto G, Cantarella H, Ghini R (2006) Solarization and fungicides for the control of drop, bottom rot and weeds in lettuce. Crop Prot 25:31–38

Pivonia S, Cohen R, Levita R, Katan J (2002) Improved solarization of containerized medium for the control of *Monosporascus* collapse in melon. Crop Prot 21:907–912

Pullman GS, DeVay JE (1977) Control of *Verticillium dahliae* by plstic tarping. Proc Am Phytopath Soc 4:210 (Abstr.)

Pullman GS, DeVay JE, Garber RH (1981) Soil solarization and thermal death: a logarithmic relationship between time and temperature for four soilborne plant pathogens. Phytopathology 71:959–964

Raymundo SA, Alcazar J (1986) Increasing efficiency of soil solarization in controlling root knot nematode by using two layers of plastic mulch. Nematology 18:626 (Abstract)

Ristaino JB, Perry KB, Lumsden RD (1991) Effect of solarization and *Gliocladium virens* on scleroia of *Sclerotium rolfsii*, soil microbiota, and the incidence of southern blight of tomato. Phytopathology 81:1117–1124

Roe N, Ozores-Hampton M, Stansly PA (2004) Solarization effects on weed populations in warm climates. Acta Horticulturae 638:197–200

Satour MM, Abdel-Rahim MF, Grinstein A, Rabinowitch HD, Katan J (1989) Soil solarization in onion fields in Egypt and Israel: short and long term effects. Acta Horticulturae 255:151–159

Shlevin E, Saguy S, Mahrer M, Katan J (2003) Modeling the survival of two soilborne pathogens under dry structural solarization. Phytopathology 93:1247–1257

Shlevin E, Mahrer I, Saguy S, Katan J, Siti M, Arbel A, Gamliel A (2005) Modeling thermal inactivation of soilborne pathogens under thermal structural (dry) and soil (wet) solarization. Acta Horticulturae 698:167–173

Sivan A, Chet I (1993) Integrated control of Fusarium crown and root rot of tomato with *Trichoderma harizianum* in combination with methyl bromide or soil solarization. Crop Prot 12:380–386

Skutelsky Y, Gamliel A, Kritzman G, Peretz-Alon I, Becker E, Katan J (2000) Soil solarization using sprayable plastic polymers to control soilborne pathogens in field crops. Phytoparasitica 28:269–270

Stapleton JJ (2000) Soil solarization in various agricultural production systems. Crop Prot 19:837–841

Stapleton JJ, De Vay JE (1984) Thermal components of soil solarization as related to changes in soil and root microflora and increased plant growth response. Phytopathology 74:255–259
Stapleton JJ, DeVay JE (1986) Soil solarization: a nonchemical approach for the management of plant pathogens. Crop Prot 5:190–198
Stapleton JJ, Gamliel A (1993) Feasibility of soil fumigation by seasling soil amended with fertilizers and crop residues containing biotoxic volatiles. Plant Prot Quart 3:10–13
Stapleton JJ, Quick J, De Vay JE (1985) Soil solarization: effects on soil properties, crop fertilization and plant growth. Soil Biol Biochem 17:369–373
Stapleton JJ, DeVay JE, Elmore CL (1998) Soil solarization and integrated management of soilborne pests. Proceedings of the 2nd international conference on soil solarization. FAO No. 147, Rome, 657 pages
Stapleton JJ, Prather TS, Mallek SB, Ruiz TS, Elmore CL (2002) High temperature solarization for production of weed-free container soils and potting mixes. HortTechnology 12:697–700
Stevens C, Kahn VA, Collins D, Rodriguez-Kabana R, Ploper LD, Adeyeye O, Brown J, Backman P (1992) Use of soil solarization to reduce the severity of early blight, southern blight and root knot in tomatoes. Phytopatholgy 82:500 (Abstract)
Stevens C, Khan VA, Wilson MA, Brown JE, Collins DJ (1999) Use of thermo film-IR single layer and double layer soil solarization to improve solar heating in a cloudy climate. Plasticulture 118:20–34
Stevens C, Khan VA, Rodriguez-Kabana R, Proper LD, Backman PA, Colins DJ, Brown JE, Wilson MA, Iqebe ECK (2003) Integration of soil solarization with chemical, biological and cultural control for the management of soilborne diseases of vegetables. Plant Soil 253:493–506
Streck NA, Schneider FM, Buriol GA (1996) Soil heating by solarization inside plastic greenhouse in Santa Maria, Rio Grande, DoSul, Brazil. Agric Forest Meteor 82:73–82
Tjamos EC, Fravel DR (1995) Detrimental effects of sub-lethal heating and *Talaromyces flavus* on microsclerotia of *Verticillium dahliae*. Phytopathology 85:388–392
Tjamos EC, Paplomatas EJ (1988) Long-term effect of soil solarization in controlling Verticillium wilt of globe artichokes in Greece. Plant Pathol 37:507–515
Tjamos EC, Biris DA, Paplomatas EJ (1991) Recovery of olive trees with Verticillium wilt after individual application of soil solarization in established olive orchards. Plant Dis 75:557–562
Tjamos EC, Antoniou PP, Tjamos SE (2000) Implementation of soil solarization in Greece: conclusions and suggestions. Crop Prot 19:843–846
Tjamos EC, Tsigiannis DI, Tjamos SE, Antoniou PP, Panayotis K (2004) Selection and screening of endorhizosphere bacteria from solarized soils as biocontrol agents against *Verticillium dahliae* of solaneceous hosts. Eur J Plant Pathol 110:35–44
Walker GE, Wachtel MF (1988) The influence of soil solarization and non-fumigant hematicides on infection of *Meloidozyne javanica* by *Pasteuria penetrans*. Nematologica 34:477–483
Wu Y, Perry KB, Ristaino J (1996) Estimating temperature of mulched and bare soil from meteorological data. Agric For Meteorol 81:299–323
Yaron D, Regev A, Spector R (1991) Economic evaluation of soil solarization as disinfestation. In: Katan J, DeVay JE (eds) Soil solarization. CRC, Boca Raton, FL, pp 171–190

Chapter 20
Back to the Future: Total System Management (Organic, Sustainable)

Dan O. Chellemi

Abstract Many soil disinfestation programs are implemented prior to crop cultivation due to the paucity of therapeutic interventions for controlling soilborne pests. In the 1950s a proliferation of chemical control options ushered in an era of soilborne pest control based upon a single or limited group of chemicals to control target pest organisms. Unfortunately, many chemicals also affected a broad and complex range of nontarget organisms comprising multiple trophic levels. This has necessitated their perpetual use to ensure pest control in agroecosystems where natural pest regulating mechanisms have been compromised. Presently, regulatory issues impact the availability of many chemical pesticides and urbanization of agricultural production regions restrict their use. Future trends further impacting growers include carbon sequestering and trading, increasing demand for biofuels and conservation of natural resources. An alternative, systems-based approach comprised of multiple economic, environmental and social goals is suggested for future crop production. In this total system management approach, creating and promoting conditions suppressive to soilborne pests and the damage they cause is incorporated into the design of the crop production system. For example, the establishment of long-term crop rotational sequences that enhance soil quality, mitigate damaging pest outbreaks, improve the quantity and quality of yields, increase soil carbon sequestration and provide sources of renewable energy. Examples of various approaches to soil disinfestation including a total system management approach are discussed.

Keywords Organic agriculture • Pest management • Soil disinfestation • Soil fumigation • Sustainable agriculture

D.O. Chellemi (✉)
United States Department of Agriculture, Agricultural Research Service, United States Horticultural Research Laboratory, 2001, South Rock Road, Fort Pierce, FL, 34945, USA
e-mail: Dan.Chellemi@ars.usda.gov

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,* Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_20,

20.1 Introduction

Soil disinfestation is defined as any formal process of eliminating soilborne pests or the damage they cause prior to planting susceptible crops (Louvet, 1979; Shanks et al., 2004). Intended to minimize economic risk associated with soilborne pests, soil disinfestation may be achieved through physical (e.g. heat), chemical (e.g. pesticides), biological (e.g. 2,4-diacetylphloroglucinol producing fluorescent *Pseudomonas* spp.), and evasive (e.g. soilless cropping systems) methods. Soil disinfestation practices may combine several different methods of eliminating soilborne pests or the damage they cause. For example, soil solarization combines physical, chemical and biological methods to control soilborne pests (Stapleton, 1998). The common thread uniting soil disinfestation methods and the practices used to implement them is that activities take place prior to planting the susceptible crop.

Many different practices have been developed to accommodate the implementation of soil disinfestation methods. Over the past 50 years, soil fumigation with methyl bromide or mixtures of methyl bromide and chloropicrin has emerged as the most popular soil disinfestation technique world-wide due to its ease of application, low cost, broad spectrum of control and rapid dissipation from soils. With its implication as a major contributor to stratospheric ozone depletion and subsequent phase-out by the signatory countries of the Montreal protocol, considerable attention and resources have been devoted to identify alternatives and several comprehensive reviews of those efforts have been published (Ajwa et al., 2003; Martin, 2003; Schneider et al., 2003). Most studies have focused on 'drop-in' replacements for methyl bromide, i.e. substitutes requiring minimal modifications to existing crop production practices. While direct input substitution may provide the quickest path to replacing methyl bromide it may not be the most desirable. Arriving at more sustainable solutions to soil disinfestation will require the refocusing of attention to the approaches used to manage soilborne pests. In the context of this paper, approach refers to the particular manner in which activities are directed towards eliminating soilborne pests or the damage they cause to agricultural crops prior to planting the susceptible plant hosts. Five basic approaches to developing successful soil disinfestation programs are discussed below with an emphasis placed on a total systems management approach.

20.2 Migratory Approach

A migratory approach to managing soilborne pests has long been practiced in the form of slash-burn or slash-mulch agriculture. This evasive method of soil disinfestation has its origins several millenniums ago and is still practiced today, particularly in the tropics (Peters and Neuenschwander, 1988; Thurston, 1997).

The advantage of slash-mulch over slash-burn is that decomposing, unburned plant debris can exert effects on soil fertility and microbiology (hence disease suppression) over longer durations than the ash produced from burning. In Florida, a migratory approach was advocated up to the 1950s as a means of eliminating risks form soilborne pests in fresh market tomato production (Hayslip et al., 1952). The diminishing availability of new (virgin) land and environmental concerns regarding the destruction of native forests and animal habitats limit the long-term utility of this approach. Another drawback limiting the long-term benefits of this approach is reinfestation of soil by pests after several seasons of crop production. Despite its drawbacks, a migratory approach still has relevancy in modern agriculture. For example, an alternative low-input production system for fresh market tomato employing minimum tillage practices into established bahiagrass (*Paspalum notatum*) pasture was designed, tested and shown to be technically feasible on a large scale (Chellemi et al., 1999). Rotation with bahiagrass pasture was selected because of its regionally availability (>1 million ha) and evidence that extended rotations can significantly reduce the impact of some major soilborne pests of tomato including southern blight (incited by *Athelia rolfsii*), Fusarium wilt (incited by *Fusarium oxysporum*) and root-knot (*Meloidogyne* spp.) nematodes (Dickson and Hewlett, 1989; Rodriguez-Kabana et al., 1991; Brennehaman et al., 1995).

20.3 Farm-Based Approach

Prior to mechanized agriculture and the exploitation of fossil fuel, a farm-based approach was necessary to achieve soil disinfestation in many regions. This approach relies on resources available at the farm level to minimize the impact of soilborne pests. In most cases, physical and biological methods of soil disinfestation are employed. Crop rotation, multi-plantings, and organic amendments were integrated into farm management plans, partly for their benefits to pest management and plant health but also because they could take advantage of locally available resources (Glynne, 1965; Thurston, 1992; Nene, 2003). It should also be noted that prior to the twentieth century, energy constraints were a critical concern during the selection of soil disinfestation techniques and these concerns have recently resurfaced as the price of crude oil continues to rise.

Despite its long history, a farm-based approach is still relevant in present day agriculture. Organic agriculture makes use of this approach by integrating organically based soil disinfestation practices into a farm management plant. Strict requirements regarding the use of inputs necessitate a long-term view of soil disinfestation and the utilization of resources available at the farm site. Sustainable pest management also relies on a farm based approach because farmers must rely upon soil disinfestation practices that make the most efficient use of non-renewable and on-farm resources in addition to biological cycles and controls.

20.4 Single Tactic Approach

A proliferation of chemical control options during the mid twentieth century ushered in an era of soilborne pest control based upon the use of broad-spectrum biocides. The goal of this single tactic approach is to eliminate soilborne pests via a single pesticide application (Chellemi, 2000). Consequently, research objectives focused upon the development and improvement of pesticides and their application, most notably soil fumigants. Under this approach reliable, consistent and economical pest control was achieved with soil fumigants and their effectiveness has contributed directly to the success of many high value crop production systems.

Reliance upon chemical fumigants for soil disinfestation has environmental, social and health consequences. Disruption of soil microbial community structure and the creation of biological vacuums can further exacerbate pest outbreaks (Bollen, 1974; Marois and Mitchell, 1981), leading to the perpetual use of fumigant to ensure pest control. Chemical fumigants are potential ground and surface water contaminants (Federal Register, 2001) and can contribute to stratospheric ozone depletion (WMO, 2007). Agricultural industries dependent upon a single chemical or group of chemicals for soil disinfestation are vulnerable to sudden changes in regulatory policies or input costs. Finally, focusing academic and governmental research programs and the funding that supports them upon additional research to identify, develop and improve pesticides may come at the expense of opportunities and motivation for long-term, high risk ecosystems-based research.

20.5 Integrated Pest Management

Integrated pest management (IPM) involves the coordinated use of multiple tactics to maintain damage from specific pests below an economic threshold and to conserve beneficial organisms. Using concepts introduced by Stern et al. (1959), IPM strives to manage pests using the ecological principals of natural pest mortality factors, predator-prey relationships, genetic resistance and cultural practices. IPM evolved as a successful approach to manage arthropod pests in the 1970s but its adaptation to soil disinfestation programs has proven to be more difficult. In addition to arthropods, soilborne pests include plants (weeds), fungi, bacteria and nematodes. Thus, a broad, multidisciplinary effort is required to develop comprehensive IPM programs. Soilborne plant pathogens are cryptic in nature, limiting the economic and technical feasibility of sampling programs for their detection. Pest populations are regulated by complex interactions involving soil edaphic factors, biological and microbial communities at several trophic levels, and plant hosts. Thus, a further understanding of ecological theories including diversity/stability relationships is required to predict damaging outbreaks. Because soil disinfestation techniques are implemented before planting, economic injury and action thresholds must be made well in advance of the current season's crop.

This is further complicated by a paucity of systemic therapeutic interventions and the technical difficulty of delivering them to active infection courts.

20.6 Total System Management

Central to the total system management approach to soil disinfestation is the premise that indigenous biological communities in agricultural soils limit outbreaks of soilborne pests through naturally occurring, self-regulating ecological feedback mechanisms. As discussed by Levins (1986) and Lewis et al. (1997) a fundamental difference to this approach is that the role of therapeutic interventions, whether biological, chemical, or physical, is deemphasized and they are used only as an occasional supplement rather than the primary means of controlling pests. Suppression of plant disease and parasitic nematodes are regulated by multi-trophic interactions occurring at or near the soil/root interphase including antibiosis, parasitization, competition for infection sites, interference with saprophytic colonization and stimulation of resistance elicitors in the plant hosts. Biological balances among biological communities are maintained within functional fluctuating bounds through a series of feedback loops.

While attractive in theory, consistent, reliable achievement of desired biological balance requires a continuation of scientific efforts to linking soil microbial community structure to ecosystem function and identifying crop management practices that promote the establishment and resilience/stability of desirable soil microbial communities. In recent years, culture-independent, molecular methods have revealed an extraordinary diversity of soil microorganisms (Anderson and Cairney, 2004; Kirk et al., 2004) and have been used to look at the implications of crop and land management practices on soil microbial communities and disease suppression (Buckley and Schmidt, 2001; Saison et al., 2006; Borneman and Becker, 2007; Wu et al., 2007, 2008). There is mounting evidence that substrate-mediated shifts in microbial community structure and activities are critical to establishing and maintaining desirable biological balances. Plant disease incidence and damage from plant parasitic nematodes are generally lower in soils where microbial communities are stimulated by applications of organically-based soil substrates (Drinkwater et al., 1995; van Bruggen, 1995) and where cultural practices have been implemented to improve fertility and stimulate the diversity of soil biota (Abawi and Widmer, 2000; Kratochvil et al., 2004). Examples of general plant disease suppression via substrate mediated stimulation of native microbial communities (Cotxarrera et al., 2002; van Os and van Ginkel, 2001) and specific plant disease suppression through substrate-mediated stimulation of native populations of 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. (McSpadden Gardener, 2007; Rotenberg et al., 2007) support the concept of developing persistent disease suppressive soils through enhanced activities of resident soil communities. Additional benefits can be achieved via other crop management practices including planting sequences, rotational crops, soil tillage and water management (Mazzola, 2004; McSpadden Gardener, 2007).

Integrating multiple economic, environmental, and social goals into the design of agricultural production systems is a key feature of total system management and will require broad multidisciplinary cooperation to be successfully accomplished. Soil disinfestation goals must not only be compatible with other economic, environmental and social goals, they must support achievement of those goals. For example, in the US corn belt, only 18% of farmers reported using cover crops despite knowledge of their beneficial effects on pest suppression and soil fertility (Singer, 2008). Reasons for the low adoption rate include the cost and time required to plant and manage them. To encourage their use, selection criteria for cover crops should be expanded to include economic goals (e.g. generating immediate cash revenue) and environmental goals (e.g. carbon sequestration and renewable energy). For example, in the southeastern US, high seed oil producing plants are being integrated into methyl bromide dependent vegetable production systems as a beneficial cover crop that also meets environmental and social goals as a source of biofuels that does not impact food supply and economic goals by generating immediate revenue (D.O. Chellemi, 2007, 2008). Another example is the integration of nitrogen fixing cover crops into methyl bromide dependent vegetable production systems to offset the escalating costs and environmental consequences of petroleum-based synthetic sources of N (Teasdale and Abdul-Bakai, 1998).

20.7 Summary

Soil disinfestation is defined as any formal process of eliminating soilborne pests or the damage they cause to agricultural crops prior to planting the susceptible plant hosts. Five different approaches for developing and implementing soil disinfestation programs are discussed: migratory, farm-based, single-tactic, integrated pest management and total system management. The migratory, farm-based, and single tactic approaches have been used successfully over the years, each having their benefits and draw-backs. A total systems management approach with multiple economic, environmental and social production goals is suggested for future crop production systems. In the total system management approach, mitigation of soilborne pest outbreaks is incorporated into the design of the crop production system. For example, selection criteria for cover and rotation crops that suppress soilborne pests and improve soil quality will be expanded to include sources of renewable energy, increased soil carbon sequestration and other economic incentives.

References

Abawi GS, Widmer TL (2000) Impact of soil health management practices on soilborne pathogens, nematodes and root diseases of vegetable crops. App Soil Ecol 15:37–47

Ajwa HA, Klose S, Nelson SD, Miuto A, Gullino ML, Laberti F, Lopez-Aranda JM (2003) Alternatives to methyl bromide in strawberry production in the United States of America and the Mediterranean region. Phytopathol Mediterr 42:220–224

Anderson IC, Cairney JW (2004) Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. Environ Microbiol 6:769–779
Bollen GJ (1974) Fungal recolonization of heat-treated glasshouse soils. Agro-Ecosystems 1:139–155
Borneman J, Becker JO (2007) Identifying microorganisms involved in specific pathogen suppression in soil. Annu Rev Phytopathol 45:153–172
Brennehaman TB, Sumner DR, Baird RE, Burton GW (1995) Suppression of foliar and soilborne peanut diseases in bahiagrass rotations. Phytopathology 85:948–952
Buckley DH, Schmidt TM (2001) The structure of microbial communities in soil and the lasting impact of cultivation. Microb Eocl 42:11–21
Chellemi DO (2000) Adaptation of approaches to pest control in low-input agriculture. Crop Prot 19:855–858
Chellemi DO, Rhoads FM, Olson SM, Rich JR, Murray D, Murray G, Sylvia DM (1999) An alternative low-input production system for fresh market tomatoes. Am J Altern Agric 14:59–68
Cotxarrera L, TRillas-Gay MI, Steinberg C, Alabouvette C (2002) Use of sewage sludge compost and *Trichoderma asperellum* isolates to suppress Fusarium wilt of tomato. Soil Biol Biochem 34:467–476
Dickson DW, Hewlett TE (1989) Effects of bahiagrass and nematicides on Meloidogyne arenaria on peanut. J Nematol (Supp.) 21:671–676
Drinkwater LE, Letourneau DK, Workneh R, van Bruggen AHC, Shennan C (1995) Fundamental differences between conventional and organic tomato agroecosystems in California. Ecol Appl 5(4):1098–1112
Federal Register (2001) 1,3-Dichloropropene (Telone), notice of final determination for termination of the telone special review. Fed Regist, November 21, 2001. 66(225):58468–58472
Glynne MD (1965) Crop sequence in relation to soil-borne pathogens. In: Baker KF, Snyder WC (eds) Ecology of soil-borne plant pathogens. University of California Press, Berkeley, pp 423–433
Hayslip NC, Allen RJ, Darby JF (1952) A vegetable-pasture rotation study at the Indian River Field Laboratory. Proc Fla State Hort Soc, 65:148–153
Kirk JL, Beaudette LA, Hart M, Moutoglis P, Klironomos JN, Lee H, Trevors JT (2004) Methods of studying soil microbial diversity. J Microbiol Meth 58:169–188
Kratochvil RJ, Sardanelli S, Everts K, Gallagher E (2004) Evaluation of crop rotation and other cultural practices for management of root-knot and lesion nematodes. Agron J 96:1419–1428
Levins R (1986) Perspectives in integrated pest management: from and industrial to an ecological model of pest management. In: Kogan M (ed) Ecological theory and integrated pest management. Wiley, New York, pp 1–18
Lewis WJ, van Lenteren JC, Phatak SC, Tumlinson JH III (1997) A total system approach to sustainable pest management. Proc Natl Acad Sci USA 94:12243–12248
Louvet J (1979) General aspects of soil disinfestation. In: Mulder D (ed) Soil disinfestation. Elsevier Scientific, New York, pp 1–8
Marois JJ, Mitchell DJ (1981) Effects of fungal communities on the pathogenic and saprophytic activities of *Fusarium oxysporum* f.sp. *radici-lycopersici*. Phytopathology 71:1251–1256
Martin FN (2003) Development of alternative strategies for management of soilborne pathogens currently controlled with methyl bromide. Annu Rev Phtyopathol 41:325–350
Mazzola M (2004) Assessment and management of soil microbial community structure for disease suppression. Annu Rev Phytopathol 42:35–59
McSpadden Gardener BB (2007) Diversity and ecology of biocontrol *Pseudomonas* spp. in Agricultural soils. Phytopathology 97:221–226
Nene YL (2003) Crop disease management practices in Ancient, Medieval, and Pre-Modern India. Asian Agri-Hist 7(3):185–201
Peters WJ, Neuenschwander LF (1988) Slash and burn: farming in the third world forest. Univiversity of Idaho Press, Moscow, ID, p 133
Rodriguez-Kabana R, Robertson DG, Weaver CF, Wells L (1991) Rotations of bahiagrass and castorbean with peanut for the management of *Meloidogyne arenaria*. J Nematology (Supp.) 23:658–661

Rotenberg D, Joshi R, Benitez MS, Chapin LG, Camp A, Zumpeta C, Osborne A, Dick WA, McSpadden Gardener BB (2007) Farm management effects on rhizosphere colonization by native populations of 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. and their contributions to crop health. Phytopathology 97:756–766

Saison C, Degrange V, Oliver R, Millard P, Commeaux C, Montange D, Le Roux X (2006) Alteration and resilience of the soil microbial community following compost amendment: effects of compost level and compost-borne microbial community. Environ Microbiol 8:247–257

Schneider SM, Rosskopf EN, Leesch JG, Chellemi DO, Bull CT, Mazzola M (2003) United States Department of Agriculture – Agricultural Research Service research on alternatives to methyl bromide: pre-plant and post-harvest. Pest Manag Sci 59:814–826

Shanks A, Mattner S, Brett R, Porter IJ, Tostovrsnik N, Dignam R (2004) Getting the most from methyl bromide alternatives: a guide to soil disinfestation strategies in the absence of methyl bromide. Department of Primary Industries, Victoria, AU, p 27

Singer JW (2008) How to boost cover crop plantings. Agricultural Research. US Dept Agric. Agric Res Sev 56(5):23

Stapleton JJ (1998) Modes of action of solarization and biofumigation. In: Stapleton JJ, DeVay JE, Elmore CL (eds) Soil solarization and integrated management of soilborne pests. FAO plant production and protection paper 147, Rome, pp 78–88

Stern VM, Smith RF, van den Bosch R, Hagen KS (1959) The integrated control concept. Hilgardia 29:89–101

Teasdale JR, Abdul-Bakai AA (1998) Comparison of mixtures vs. monocultures of cover crops for fresh-market tomato production with and without herbicide. HortScience 33:1163–1166

Thurston DH (1992) Sustainable practices for plant disease management in traditional farming systems. Westview, Boulder, CO, p 279

Thurston DH (1997) Slash-Mulch systems: sustainable agriculture in the tropics. Westview, Boulder, CO, p 224

Van Bruggen AHC (1995) Plant disease severity in high-input compared to reduced-input and organic farming systems. Plant Dis 79:976–984

Van Os GJ, van Ginkel JH (2001) Suppression of *Pythium* root rot in bulbous iris in relation to biomass and activity of the soil microflora. Soil Biol Biochem 33:1447–1454

WMO (2007) WMO (World Meteorological Organization) scientific assessment of ozone depletion: 2006, Global Ozone Research and Monitoring Project – Report No. 50, 572 pp., World Meteorological Organization, Geneva

Wu T, Chellemi DO, Graham JH, Martin KJ, Rosskopf EN (2007) Discriminating the effects of agricultural land management practices on soil fungal communities. Soil Biol Biochem 39:1139–1155

Wu T, Chellemi DO, Graham JH, Martin KJ, Rosskopf EN (2008) Comparison of soil bacterial communities under diverse agricultural land management and crop production practices. Microb Ecol 55:293–310

Chapter 21
Global Phaseout of Methyl Bromide Under the Montreal Protocol: Implications for Bioprotection, Biosecurity and the Ozone Layer

Ian Porter, Jonathan Banks, Scott Mattner, and Paul Fraser

Abstract The Montreal Protocol has been very effective in reducing the consumption of the major ozone depleting chemical, methyl bromide (MB), and represents an excellent model for future phaseout of other environmentally damaging products, such as those involved with climate change. Over a ten year period, 85% of MB (c. 45,000 tonnes) used for preplant soil fumigation has been phased out and a wide range of chemical and non chemical technologies adopted for disease and weed control in agriculture. Restrictions on the use of MB have stimulated new research and knowledge on: (1) soil health and relationships between soil microbial diversity and crop growth, and (2) new crop protection agents and production systems that moderate the need for harsh pesticides in agriculture. This has also led to increased use of substrate systems, grafting and plant resistance for disease control which, in most cases, avoid the need for soil disinfestation. Also other fumigants, such as 1,3-dichloropropene (1,3D)/chloropicrin (Pic), metham sodium, iodomethane (MI)/Pic, drip applied fumigants and barrier films have been adopted by growers as alternatives strategies for soil disinfestation. Implementation of a wide range of alternatives has led to a 50% fall in anthropogenic bromine in the troposphere and 30% reduction in effective chlorine load to date in the stratosphere. This has been hugely significant to the start of ozone layer recovery which should be observed within the next few years.

I. Porter (✉)
Department of Primary Industries, Knoxfield Centre,
PMB 15, Ferntree Gully Delivery Centre, Victoria, 3156, Australia
e-mail: ian.j.porter@dpi.vic.gov.au

J. Banks
Consultant, Piallago, Australian Capital Territory, 2609, Australia

S. Mattner
Department of Primary Industries, Knoxfield Centre, PMB 15, Ferntree Gully Delivery Centre, Victoria, 3156, Australia.

P. Frasert
Centre for Australian Climate and Weather Research, CSIRO Marine and Atmospheric Research, Aspendale, Victoria, 3195, Australia

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,* Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_21,

Internationally, pressure is mounting to restrict use of all fumigants worldwide (EC Reg 2037/US Cluster Analysis) and this will further stimulate new technologies for plant disease control. There are also moves to restrict MB, approx. 10,300 tonnes a year, used for quarantine and pre-shipment (QPS). The pressure on fumigant use globally is stimulating development of more sustainable crop protection, biosecurity and integrated pest management (IPM) strategies.

Keywords Biosecurity • Bioprotection • Fumigation • Methyl bromide • Ozone layer • Soil disinfestation

21.1 Introduction

The listing of methyl bromide (MB) for phase out in 1998 under the Montreal Protocol represented one of the biggest global challenges facing agriculture in the last century. MB is a very effective soil disinfestant which has provided excellent yields and high profits for industry, but it is a major ozone depleting substance and, with the other ozone depleting substances, led to the ozone hole developing during the 1970's. There has been fierce international debate about retention of MB for soil disinfestation, many countries embracing its phase out for environmental and social reasons and others keen to retain its use because of economic drivers for their industries.

It is estimated that over US $500 million has been invested to find alternatives to MB for soil disinfestation. During the process, there has been anger and denial from industry representatives, scepticism from politicians and governments, and remarkably huge debate by scientists about the phase out. Some have asserted that bromides have minimal effect on ozone degradation and that MB should be retained because no product can technically and economically provide the yield it affords (MBTOC, 2006). Others have chosen to ignore the connection of ozone degradation and increased UV radiation on increased skin cancer and cataracts in all populations over the last 20 years. Yet the majority of scientists have now accepted the models and global measurements that show major effects of anthropogenic bromides on stratospheric ozone (Butler, 1995) and have strived to find technical and economical solutions which allow mankind to sustainably coexist with the environment in the future.

This paper explores some of the major science issues that have influenced the phaseout of MB. It also shows why science has been so important to the success of the Montreal Protocol and why politicians could consider the use of this model to assist the Kyoto Protocol and climate change. It discusses the global trends in reduction of MB for all uses, the benefits this is having on reduction of bromine in the atmosphere, the relative efficacy of alternatives to MB, and the science behind the crop yield response induced by soil disinfestation.

21.2 Methyl Bromide Regulation under the Montreal Protocol Stimulates New Technologies

From the 1950s until 2005, MB was one of the most commonly used (by amount) agricultural pesticides throughout the world. In the early 1990s, c. 73,000 tonnes of MB was used globally with c. 58,000 tonnes for preplant soil fumigation (mainly to control pathogenic fungi, nematodes and weeds) and 4,000 tonnes for commodity treatments (mainly to control insect pests) not associated with quarantine and pre-shipment (QPS) applications (Fig. 21.1.). The remaining major uses of MB were 3,000 tonnes for feedstock to produce other chemicals and 8,000–10,000 tonnes for QPS. MB was listed as an ozone-depleting substance under the Montreal Protocol in 1992. By 1998, a graduated phaseout of MB for agricultural uses (soil, commodity and structural fumigation) was being implemented under Article 2H of the Montreal Protocol (UNEP, 2006), culminating in full withdrawal by 2005 in non developing (A5) countries and by 2015 in developing (non-A5) countries. However, there was provision for industries that could scientifically demonstrate they had no technically or economically feasible alternatives to continue using MB under a critical-use-exemption granted annually by the parties to the Montreal Protocol. The reduction in quantity used or available for use by major non A5 countries as of the end of 2007 is shown in Table 21.1. QPS use presently remains exempt from phaseout and represents the only major emissive use of a newly manufactured ozone degrading product that is not yet regulated under the Montreal Protocol.

MB has been an excellent fumigant because it is a highly volatile gas (Ruzo, 2006) which moves through soil easily in all directions from the point of application, therefore providing uniform treatment. Additionally, MB dissipates from soils

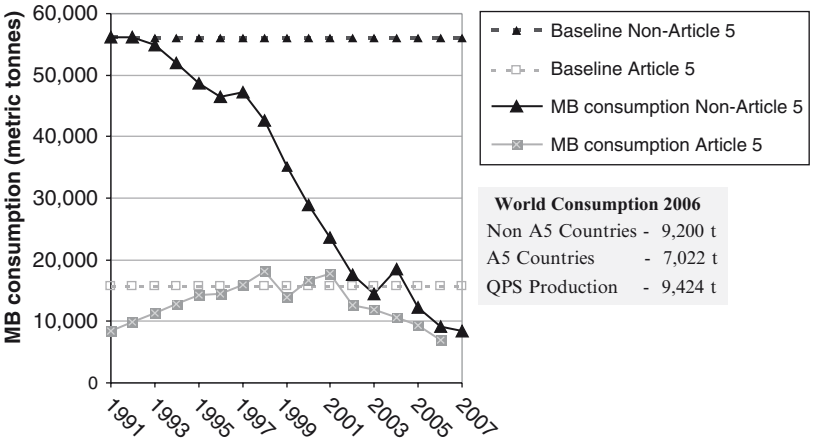


Fig. 21.1 Recent historical consumption of MB in Non-A 5 (Developed) and A5 (Developing) countries in relation to the baseline levels of MB capped by the Montreal Protocol in 1995. The most recent figures for total QPS production are also shown in 2006

Table 21.1 Summary of baseline amounts of MB for non QPS capped under the Montreal Protocol in 1995 and the proportional reductions in exempted consumption of MB in 2009

Party	Baseline Usage (including non QPS Commodity treatments)	Nominations for Preplant Soil Use in 2009	% Reduction
Australia	704	30	95.6
Belgium[a]	312	0	100
Canada	200	7	91.3
France[a]	4,195	0	100
Greece[a]	970	0	100
Israel	3,580	717	80.0
Italy[a]	6,974	0	100
Japan	6,107	503	91.7
Malta[a]	40	0	100
New Zealand	135	0	100
Poland[a]	200	0	100
Portugal[a]	65	0	100
Spain[a]	4,236	0	100
UK[a]	629	0	100
USA	25,529	4,473	82.5
TOTALS	53,876	5,730	89.4

[a] In 2008, the European Community ceased applying for critical uses for any of its member states for either soil or non-QPS commodity uses in 2009 or beyond.

rapidly allowing growers to plant their crops soon after treatment (Mattner et al., 2003). Fortunately, the search for alternatives has not only focussed on finding a 'silver bullet' fumigant replacement, but also on other non chemical options and 'softer' biorational strategies. The MB phaseout has ultimately been a stimulus for a wide range of technological advancements in bioprotection, particularly in development of methods that avoid the need for harsh soil disinfestation. This includes an increased adoption by growers of substrates, plant resistance, grafting, and solarisation combined with organic mulches and nutrient mechanisms for management of soil-borne diseases (Besri, 2004; MBTOC, 2006).

In addition, many new chemicals (eg. methyl iodide, dimethyl disulphide, cyanogen) and 'softer' biorational products (eg. mustard meals, neem, furfural) have been developed, as well as increased knowledge on the use of trap crops and biofumigation (MBTOC, 2006). New application methods have also been developed to improve the performance of established fumigants and their combinations (Donohoe et al., 2001). For example, emulsifiable formulations of 1,3-D and Pic have provided as effective method of application of these fumigants and the use of low-permeability barrier films has enabled reductions in effective dosage rates of fumigants by up to 50% (Ajwa et al., 2002; MBTOC, 2008). This latter technique can also lead to reductions in emissions of fumigants to the atmosphere and can improve user safety. These films may assist the industry meet requirements to reduce fumigant emissions under new volatile organic compound emission regulations (VOC) being considered in the USA (see CDPR, 2008).

In summary many of these new advancements stimulated by MB phase out are providing more sustainable technologies for modern day agriculture.

21.3 Implications of Methyl Bromide PhaseOut on Bioprotection

The following sections shows how science has played a major role in influencing the shift to MB alternatives by providing validation of the relative efficacy of a huge number of chemical and non-chemical options for soil disinfestation. Furthermore, an understanding of the increased growth response (IGR) of crops in disinfested soils and the changes that occur in soil microbial populations during disinfestation is useful to the development of new sustainable systems based on good management of the biological and nutrient parameters for good soil health. They also provide evidence why systems which avoid the need for methyl bromide can provide better yields, such as the use of hydroponics/substrate systems where the growth media and rhizosphere microflora are highly controlled.

21.3.1 Validation of Successful Alternatives to Methyl Bromide

In 2006, a 'world first' international metaanalysis study (Porter et al., 2006) was commissioned by the Parties to the Montreal Protocol to identify the relative efficacy of over 100 strategies for soil disinfestation against pathogens and weeds. This study was unique in agricultural science because it evaluated data from over 168 diverse studies worldwide across a wide range of crops and regions where MB was used as a soil disinfestant, using statistical methods well-established in other fields (e.g. medical epidemiology). A metaanalysis of a limited number of fumigant alternatives was reported by Shaw and Larson, 1999. Our study determined the statistical best estimate of the relative effectiveness of the major chemical alternatives to MB for strawberry and tomato production. In 2006, soil fumigation as a preplant treatment for production of strawberry fruit and tomatoes were the largest remaining uses of MB. The study confirmed that at least several alternatives provided similar yields to the standard MB/Pic mixtures and that most chemical alternatives perform similarly in different regions worldwide. Results from the analysis correlated well with the major alternatives being adopted by industries to replace MB soil fumigation. More importantly it flagged to the Parties of the Montreal Protocol and agricultural industries still using MB, that there were possible replacements for critical uses of MB.

The metaanalysis of published data from over 100 strawberry fruit trials showed that a large number of alternatives used alone or in various combinations had mean estimated yields which were within 5% of the estimated yield of the standard MB treatment (MB/Pic 67:33) (Fig. 21.2). These alternatives to MB included:

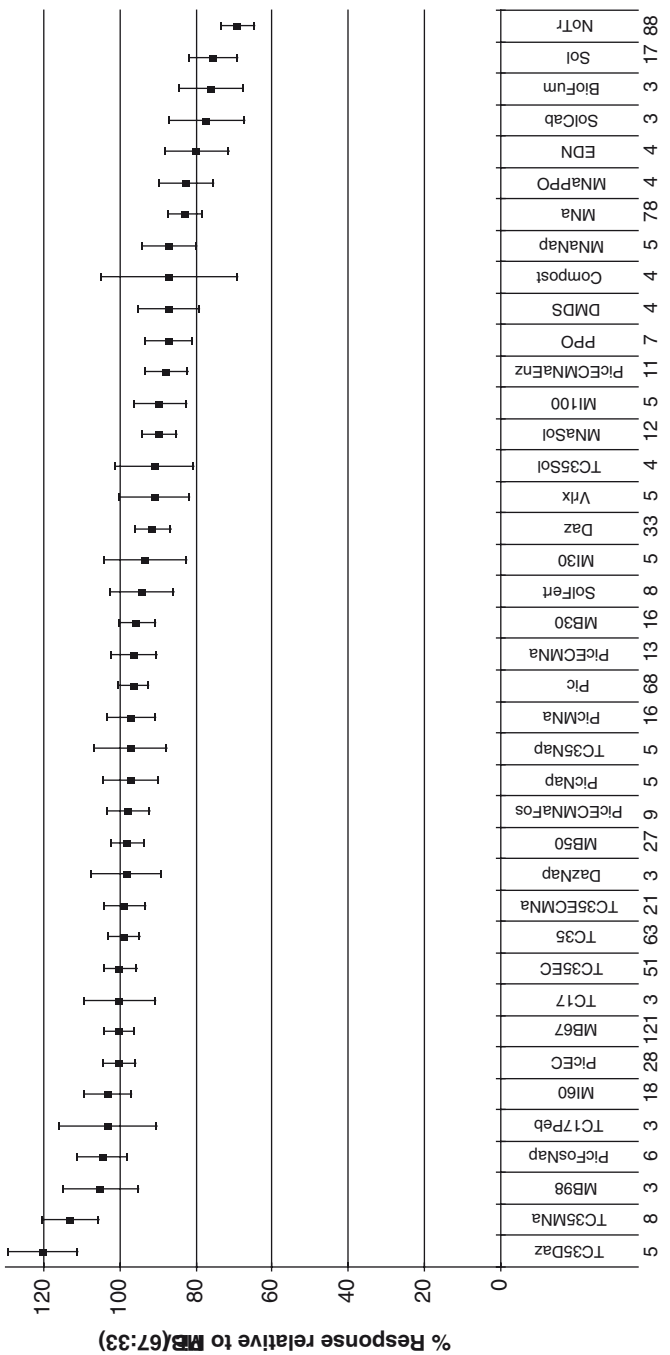


Fig. 21.2 Relative yield data from the full meta-analyses and LSI intervals for alternatives compared to methyl bromide/chloropicrin (67:33) from international research studies in strawberry fruit crops from 1997 to 2005 (A selected number of treatments is shown (modified from TEAP, 2006).) (Common abbreviations are MB – Methylbromide, Pic – Chloropicrin, MI – Methyl iodide, MNa – metham sodium, Fos – Fosthiazate, Nap – Napropamide, TC17 & TC35 – 1,3-D dichloropropene/chloropicrin, Daz – dazomet, SolFert– Solarisation and chicken manure, Vrlx – Vorlex, Enz – Enzone, PPO – propylene oxide, DMDS – dimethyl disulphide, EDN – ethane dintrile, Solcab -solarisation/cabbage residue, BioFum – biofumigation, NoTr – No Treatment, EC – Emulsifiable concentrate)

Pic emulsifiable concentrate (Pic EC), 1,3-D/Pic EC (TC35 EC), 1,3-D/Pic injected into soils (TC35), 1,3-D / Pic EC followed by metham sodium (TC35ECMNa) and MI/Pic (MI60). MI/Pic formulations recently received registration in the US as a soil fumigant and are undergoing reviews for registration in many other countries. In general, a similar range of MB alternatives was also effective for tomato production and much of this information has been useful for other horticultural crops relying on soil disinfestation for disease control.

The analysis also showed that several of the non chemical and more sustainable alternatives (eg compost), failed to provide the same crop yields (i.e. productivity) given by fumigant alternatives in short term studies (Fig. 21.2). However, some of these treatments may improve soil health in the long-term, and further research is required to understand the underlying mechanisms and how to make this effect more consistent. This aspect is seen as being particularly important to the development of future methods for disease suppression and bioprotection.

In addition to the metaanalysis, the Technical Economic Assessment Panel of the Montreal Protocol and its Methyl Bromide Technical Options Committee produce an assessment report every 4 years which review *inter alia* the benefits of a wide range of chemical and non chemical alternatives for soil disinfestation (MBTOC, 2006). There are also a large number of review articles that have been published on alternatives to MB for soil disinfestation and this article will not attempt to discuss these further (Duniway, 2002; Martin, 2003; Loumakis, 2004; Santos et al., 2006; Chellemi and Mirusso, 2006).

21.3.2 The Increased Growth Response After Soil Disinfestation

Disinfestation of soils, especially with fumigant chemicals, is often followed by an increased growth response (IGR) in crops, which results in yield gains of between 15 to 65% even in the absence of significant pathogen and weed pressure. This effect is also seen with other biocidal soil disinfestation treatments (steam and solarisation), although the level of response can vary widely (Katan, 1981; Stapleton and DeVay, 1984). Previously, the IGR has been shown to be attributed to a range of factors, including shifts in nutrients, changes in microbial populations (Gamliel and Katan, 1991) and control of non lethal or minor pathogens (Katan 1987). In addition, our research suggests that the shifts and interaction between the chemical and microbial populations in the few months after soil disinfestation are highly related with the IGR (Porter et al., 2005). In particular, increases in ammonium-N, reductions in total microbial populations, and different rates of recolonization of soil by different microbial groups after fumigation are important determinants of the IGR.

The following paragraphs report on research in Australia aimed at identifying some of the major factors responsible for the IGR and discusses the significance of these findings to future plant production practices and crop protection.

Studies have shown that fumigation (and to a lesser extent solarisation) of soils causes a mineralisation flush of ammonium-N and little change to nitrate-N (Porter

Table 21.2 Effect of soil fumigation on nitrate and ammonium in soil one to 7 weeks following fumigation treatment. Values assigned different letters (a,b) are significantly different at $P < 0.05$. (Untreated, covered with plastic only; MB:Pic = Methyl bromide: chloropicrin mixture 70:30 w/w; NS = Not significant)

Time after fumigation	One week	Two weeks	Five weeks	Seven weeks
Nitrate nitrogen (µg/ml)				
Untreated	2.10(NS)	4.35 (NS)	5.15(NS)	10.0(NS)
MB:Pic (70:30)	2.45(NS)	3.05 (NS)	4.20(NS)	7.3 (NS)
Ammonium nitrogen (µg/ml)				
Untreated	4.5a	4.5a	2.0a	1.0a
MB:Pic (70:30)	6.5a	14.5b	20.5b	22.0b

et al., 2005) (Table 21.2). Fumigation also affects soil pH and can increase electrical conductivity and many other nutrients depending on the soil type (Warcup, 1957; Rovira, 1976; Porter et al., 1999). Fumigation causes significant changes in specific bacterial populations (particularly pseudomonads and other gram negative bacteria) and this has a significant impact on nitrogen conversion in soil (e.g. ammonification, nitrification). In contrast to many beliefs, fumigation does not create a total biological vacuum in soils, but more so an environment where recolonization is rapid and selective to certain groups of microorganisms (Fig. 21.3). Studies have also shown that there is a positive relationship between the concentration of ammonium in soil after fumigation and the increase in total fresh weight of plants (Brett et al., 2001; Porter et al., 2005). The results suggest that the IGR observed with plants may be partly attributable to the altered nitrogen form and the fertilizer effect that this interaction gives to plant growth.

Our studies (Fig. 21.3) indicate that gram negative soil bacteria, particularly pseudomonads, are dramatically reduced by fumigation, but rapidly proliferate within the first few days after treatment (Brett et al., 2001; Porter et al., 2005). There are also significant imbalances in soil microbial populations for at least the first few months after fumigation, but these return to a more balanced state within 34 to 52 weeks. The *Pseudomonas* spp. and gram negative bacteria populations were consistently more affected by fumigation than total aerobic bacteria (mainly gram positive) and the actinomycetes. Their rapid recolonization however often coincides with the period when many host crops are growing rapidly in fumigated soils and have a high N demand. The pseudomonads include copiotrophic organisms that are able to rapidly recolonise soils with enhanced nutrient availability (i.e. fumigated soils). We hypothesise that these changes in soil biology following fumigation positively affect plant growth because many fluorescent pseudomonads in soils have been implicated as growth promoters and biological control agents (Xiao and Duniway, 1998; Hao et al., 2002). Also, many gram negative bacteria are capable of rapid nitrification of the high ammonia in fumigated soils, thereby making N more available for uptake by plants.

The advantage of the interaction between nitrogen form as a food source for microbial recolonization following fumigation is that this mechanism (i.e. nitrification) provides plants with a slow release of nitrates, which are most effective in promoting

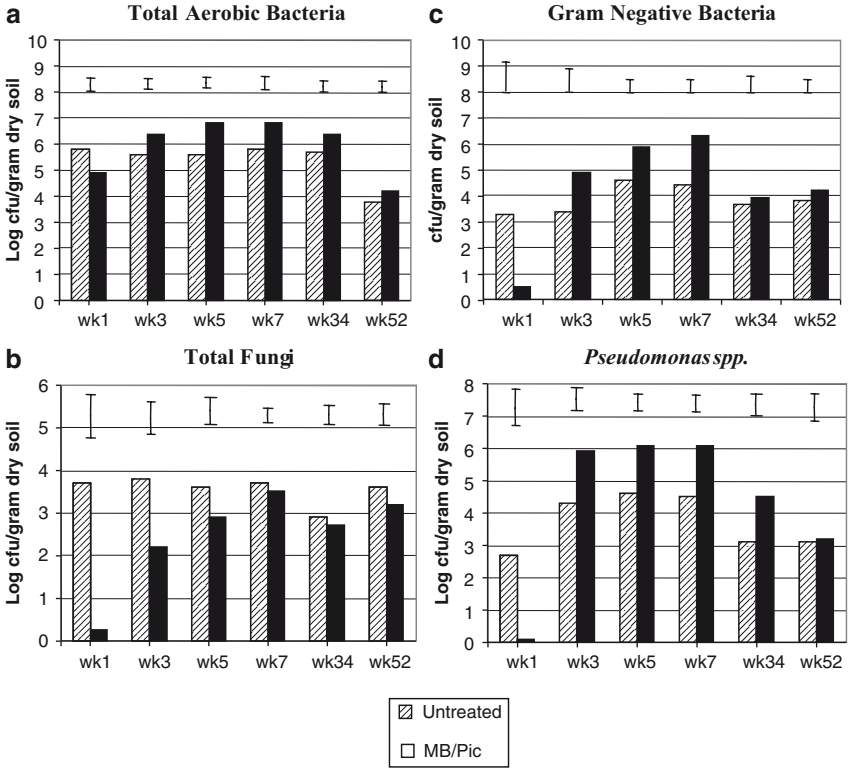


Fig. 21.3 Effect of fumigant treatments on population densities (colony forming units) of microorganisms in soil over 52 weeks (**a**) total aerobic bacteria, (**b**) total fungi (**c**) gram negative bacteria and (**d**) pseudomonads

plant growth. In contrast when nitrate is applied by synthetic fertilisers, much of it is leached. The results support that one of the major advantages of fumigation and other soil disinfestant strategies (solarisation and steam) in that they create a partial biological vacuum, which provides beneficial plant nutrients and microbial recolonization that favours plant growth and possibly rhizosphere health.

Understanding the interactions that occurs between microbial populations and plant nutrients is vital to further development of new strategies for bioprotection and crop growth. Current studies are aimed at understanding the effects of organic composts, biofumigants, fertilisers or crop rotations on nutrient flow and microbial shifts and to use this knowledge to develop systems that cause disease suppression, plant growth stimulation but maintain ecosystem resilience. Of major importance is the effect these treatments have on providing a food source in soils that can stimulate microbial populations that favour plant growth, such as those shown by the common soil disinfestants (chemical fumigants, solarisation and steam). In particular treatments which stimulate organic food sources for microbial activity and ammonium-N are likely to be most favourable.

21.4 Implications of Methyl bromide PhaseOut on Biosecurity

Whilst non quarantine pre plant and post harvest agricultural uses of MB are subject to phaseout under the Montreal Protocol, specific exemptions presently exist for use of MB for all QPS purposes (Article 2H, UNEP, 2006) and a wide range of biosecurity applications (Quarantine and Pre-shipment, QPS). This is despite its known damaging effect on the ozone layer. Most of these uses (Table 21.3) are to restrict movement of a wide range of insect pests, however there are a few fungal targets which require MB use specifically under phytosanitary certificates for export. For instance, treatment of export cottonseed from Australia to California, USA against a strain of Fusarium. Also, some wood and timber treatments with MB are against the pinewood nematode caused by *Bursaphelenchus xylophilus*.

In 2007, it was estimated that QPS use, which is relatively stable by amount, was about 10,250 tonnes (TEAP, 2008), representing about 35% of the total remaining uses for MB. Most of the recent reductions in the QPS use in some sectors (e.g. pre-shipment of grains) have been offset by increases in other sectors, notably on timber and wooden packaging materials.

There is a diversity of approved QPS treatments using MB worldwide, some with substantial consumption (Table 21.3), but many with total annual uses of only a few kilograms. This information is taken from the most recent available global survey of QPS applications.

The QPS use of MB for soil is predominantly for treatment of open field soils used for production of propagation stock (strawberry runners, trees and nursery stock) against fungal and nematode infection that can be transmitted on the stock. Many countries have phased out or are phasing out this use under the 'Critical Use' process of the Montreal protocol, whilst others maintain this use under QPS exemptions.

Quarantine databases, such as ICON (AQIS, 2008a), PHYTO (AQIS, 2008b) and the APHIS PPQ manual (APHIS, 2008), give QPS treatments for different applications, including many that rely on use of MB. In many cases, these databases also specify alternative treatments to MB, but in some cases, there are no recognised and approved alternative treatments. Examples of the latter include treatments

Table 21.3 Categories of use of methyl bromide for QPS purposes. Results of a survey given in MBTOC (2006)

QPS Use	Quantity (metric tonnes)	Table (%)
Soil (preplant)	1,527	29
Grain and cereals for consumption	1,262	24
Wood, including sawn timber	868	16
Fresh fruit and vegetables	722	14
Wooden packaging materials	335	6.4
Whole logs	209	4.0
Dried foodstuffs	160	3.0
Cotton and fibre	91	1.7
Totals	5,174[a]	98.1

[a] The survey only accounted for 5273 tonnes of about 10,600 metric tonnes produced in 2004, representing 54% of the estimated global consumption of methyl bromide.

Table 21.4 Alternative QPS treatments identified by the Parties to the Montreal Protocol when responding to Decisions XVI/10(4) and XI/13(4). Modified from MBTOC (2006)

QPS category of use	Principal alternative identified
Timber and wood packaging materials	Heat treatment
Export cereal grains	Phosphine fumigation
Perishables	Systems approach
Soils for production of certified propagation material	Fumigation with 1,3-D/chloropicrin mixture

against specific pests in some vegetable, fresh fruit and cut flower exports and treatments of export grain where logistic constraints do not permit use of phosphine fumigation as an alternative.

Methyl bromide, though requiring quite high concentration-time products for full effectiveness (e. g. Rhatigan et al., 1998), is one of the very few agents available for biosecurity treatments against fungal pathogens in plant material or soil. It is of concern that its use may be curtailed because of its ozone-depleting qualities. It may be that use of recapture technologies may allow continued use of this valuable material.

Most of the MB applied for QPS purposes is, at present allowed to leak or vent directly into the atmosphere during or after use. Commercial systems that recapture MB and thereby reduce emissions are available (Grullemans, 2008), however their use is not mandated under the Montreal Protocol. This means that even though the adoption of recapture units is increasing, their use is restricted by the lack of regulation on MB for QPS and the capital expenditure and logistics of use of commercial systems. It is anticipated that measures under the Protocol will soon be put in place to restrict MB use for biosecurity purposes. This is likely due to recognition of the continued dangers to the ozone layer of unconstrained MB emissions and the almost immediate benefits that can be obtained to ozone layer recovery by further regulation of MB. This may have an important outcome for biosecurity of fungal pathogens, as the effect of MB on many fungal pathogens is not known and there may be certain fungal species that are presently controlled under the present regulations that may not be under an alternative scenario.

In 2006 (MBTOC, 2006), the MBTOC committee concluded that on a global basis, there are technically effective and approved treatments available (Table 21.4) for more than half current QPS treatments by volume of MB consumed, but many individual QPS uses do not have proven, acceptable alternatives at this time. Frequently, actual application of technically effective and approved alternative treatments is constrained by local circumstances (MBTOC, 2006).

21.5 Implications of Methyl Bromide PhaseOut on the Ozone Layer

Man-made MB has been estimated to be responsible for up to 10% of past stratospheric ozone losses. The Montreal Protocol regulations combined with the scientific research that has allowed industries to transition to alternatives has led to a dramatic

decline in MB consumption over the last decade (Fig. 21.1) and this is having huge benefits in effecting a fall in stratospheric bromine levels. The MB phaseout has led to a 50% fall in anthropogenic bromine in the troposphere and a 30% fall in effective chlorine load to date in the stratosphere (Figs. 21.4 and 21.5). Owing to the short half life of MB (0.7 years) in the stratosphere, MB is one of the few regulated ODS gases that will have a rapid effect on ozone recovery. Also, recently the Scientific Assessment Panel (WMO, 2007) now rates the importance of MB, in its future contribution to ozone layer recovery higher than previously thought, because the role of bromine in stratospheric ozone loss is greater than previously estimated.

Prior to the onset of the widespread use of MB as a soil and structural fumigant in the 1960s, the background concentrations of MB in the atmosphere were 5–6 ppt (parts per 10^{12} molar). The historical trend (Fig. 21.6) shows that MB concentrations in the atmosphere were stable for hundreds of years and then grew rapidly through the 1970s to the late 1990s due to large anthropogenic (man-made) use of MB (up to 73,000 tonnes annually). In the mid 1990's their concentration reached 8–9 ppt (more than 50% above the 1950s background concentration).

In 2003, it was predicted that MB levels in the southern hemisphere would fall to about 7 ppt before levelling off (Fig. 21.6, A1 WMO, 2003). However, by 2007 the levels had continued to fall to 6.5 ppt and show signs of falling further. It is clear that the Montreal Protocol restrictions on the use of MB are having greater impact on atmospheric MB than thought possible 5 years ago. The latest WMO scenarios (Fig. 21.6, A1 WMO, 2007) suggest that further small reductions in atmospheric concentrations are possible over the next few years, but will occur only if the

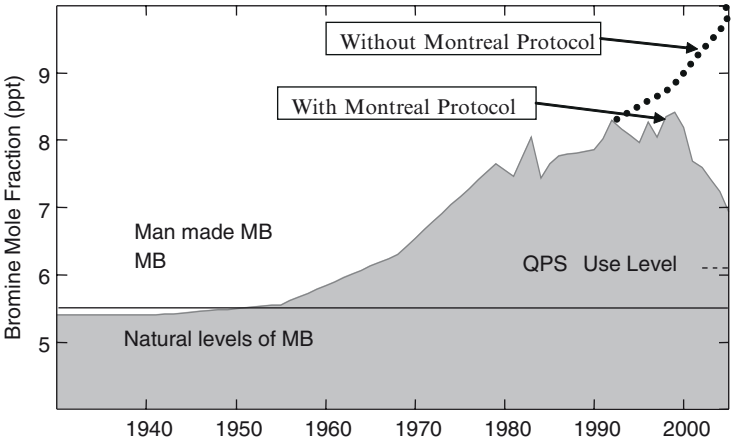


Fig. 21.4 The impact of the MB restrictions on non-Quarantine and Preshipment (QPS) use on reduction in bromine concentrations from MB in the troposphere since 1945. (The *solid line* indicates the bromide from natural sources (i.e. the historic baseline). The *dashed line* indicates the approximate level that the bromine concentration would presently fall if all non QPS MB was phased out). The possible scenario without the regulations of the Montreal Protocol is estimated from pastreds. The MB data are from Trudinger et al. (2004), Krummel et al. (2007) and CSIRO unpublished data

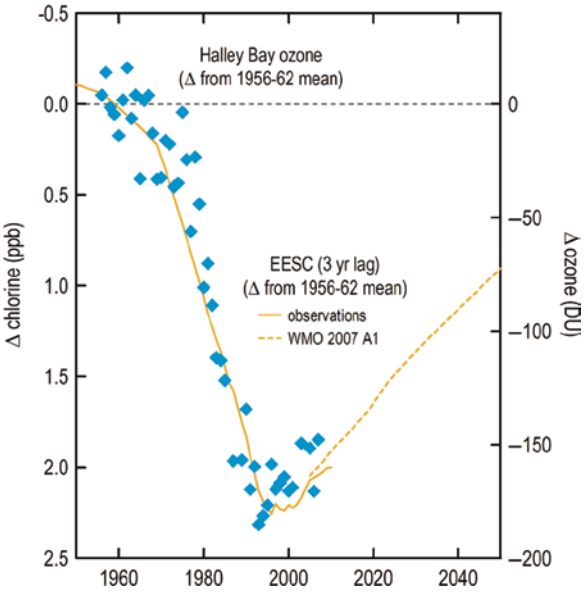


Fig. 21.5 Changes in observed Halley Bay, Antarctica, total ozone (Δ ozone, DU) – blue diamonds (BAS, 2008) – and actual – (solid yellow line) and predicted (dashed yellow line) changes in EESC based on atmosphere observations of all ODS's (Prinn *et al*., 2000; Krummel *et al*. 2007; CSIRO, unpublished data) and the WMO 2007 A1 scenario (Daniel and Velders 2007, Krummel and Fraser 2006).

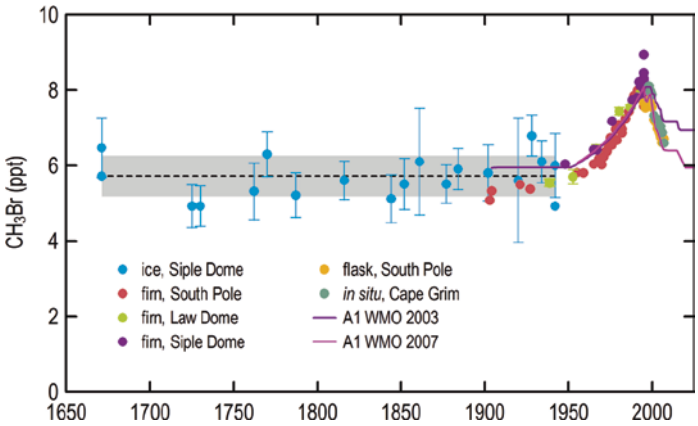


Fig. 21.6 Historic MB measurements (ppt = 10^{12} molar) in the southern hemisphere over the past 350 years (the dashed line represents the average unperturbed atmospheric MB level). Data are from Cape Grim, Tasmania, and various atmospheric and ice/firn sampling sites in Antarctica compared to modelled CH_3Br levels in 2003 (WMO, 2003) and 2007 (WMO, 2007), (Montzka and Fraser, 2003; Clerbaux and Cunnold, 2007; Daniel and Velders, 2007)

remaining uses in developing countries (A5 countries) and non A5 critical uses are phased out, and if emissions or use of MB for QPS is reduced significantly.

Figure 21.5 shows the atmospheric concentrations of all ozone depleting substances (ODSs) expressed as effective equivalent stratospheric chlorine (EESC). This metric allows a direct assessment of the impact of ODSs on stratospheric ozone depletion and in particular takes into account the fact that bromine radicals (from MB and halons) are 60 times more effective in ozone depletion than chlorine radicals from CFCs etc. The data show that EESC peaked in the late 1990s and is now in decline by about 0.8% per year.

Figure 21.5 also shows a strong correlation between observed ozone losses above Halley Bay in Antarctica (the Antarctic ozone hole) and the rise and recent fall of EESC, which is a direct measure of the bromine and chlorine levels derived from ODS gases. The data suggest that ozone recovery in Antarctica may have commenced and, from above, we now know that the biggest single driver of the start of the ozone recovery at present is the decline in MB in the atmosphere. As a consequence of the MB phase out and the reduction of other gases which have much longer half lives in the stratosphere, the complete ozone recovery over Antarctica (the closing of the ozone hole) is possible and expected to occur some time in the second half of this century. This is a great step for mankind.

21.6 Conclusions

In 1992, MB was added to the list of regulated ozone depleting substances and in 1995 its consumption was capped in developed countries (non A5 countries) for non-QPS uses. Since regulations have been enforced under the Protocol, 50,000 tonnes of MB has been phased out, (Fig. 21.1), but 23,000 tonnes is still used for a range of applications: critical uses in developed countries, post harvest and preplant soil uses in developing countries (A5 countries) and QPS uses. From 1998 to 2005, stepwise reductions in developed countries were implemented to achieve complete phaseout of non-QPS MB, except for those applications considered ‘critical’ uses. Critical uses are those where no technical and economical alternatives to MB exist. These have been subjected to annual review since 2003 and the amount of MB for such uses has been gradually declining from 17,000 tonnes to less than 5,000 tonnes in 2007. Phaseout of non-QPS MB for developing countries will occur in 2015, although considerable reduction in use is anticipated before this date (see Fig. 21.1 for recent history in levels of reduction).

Worldwide around 80% of MB was historically used for preplant fumigation, of which most (88%) has been replaced by 2007 by a range of alternatives without drastic effects to crop productivity and limited market disruption. To meet these reductions, growers worldwide have often moved to the next best fumigant alternative combination as they provide yields and control of diseases within 0–5% of those achieved with MB (Porter et al., 2006).

Some alternative fumigant systems have reliably given better yields/economics than MB use. Solarisation and steam have also been adopted as alternatives to MB for soil disinfestation, but to a lesser extent. Considerable adoption, however, has occurred of new hydroponics/substrate systems, grafting and use of plant resistance for disease control as these techniques avoid the need for soil disinfestation. Understandably the key alternatives adopted have been those which maximise crop productivity and minimise the effort required to control diseases and pests, and reduce the risk of crop loss. For this reason, many growers appear to have favoured fumigant alternatives over other IPM and biorational treatments, owing to the IGR, consistent yields and profit margins that soil fumigation provides.

Understanding the influence that alternative soil disinfestation techniques have on nutrients and microbial biomass is critical to the selection of treatments for future sustainable cropping systems. MB fumigation results in a change in the balance of soil nutrients and microbial biomass, which not only reduces plant pathogens but favours plant growth. Our studies suggest a key advantage of chemically fumigated systems and some physical systems of disinfestation (solarisation and, to a lesser extent, steam) is that treatment increases both ammonium concentration in soil and possibly other food sources (organic compounds released from dead organisms) resulting in rapid recolonization of soils by gram negative bacteria, including nitrifying bacteria. This leads to a slow release of nitrate-N from ammonium-N, which is readily absorbed by plants partly leading to the IGR of crops in fumigated soils. The challenge for researchers is to develop alternative soil disinfestation systems that also give enhanced crop growth, by either manipulation of organic matter and nutrient fertilizers, or by the use of soilless production systems where nutrient and microbiological populations can be highly controlled.

Worldwide, there has been a major trend in several industries previously underpinned by MB to shift to more controlled production systems. In particular, many high value commodities previously grown outdoors in MB fumigated soils have moved to hydroponics/substrate systems, viz. ornamental crops, tomatoes, capsicums, cucurbits and tobacco seedlings. The findings from this study indicate that this trend is set to continue, especially as regulations on fumigant pesticides make their use more difficult, and as farmers increase demand to use more sustainable treatments on farm.

The phaseout of MB as a plant protection chemical (soil fumigant) has led to significant reduction in the observed MB concentration of the stratosphere, while contributing to ‘technology forcing’. As a consequence of the MB phase out and the reduction of other gases which have much longer half lives in the atmosphere (MB – 0.7 yr, halons 20–60 yr, CFC: 50–100 yr), the complete ozone recovery over Antarctica (the closing of the ozone hole) is possible but expected to take at least until the second half of this century. This is a great step for mankind.

The development and adoption of new soil disinfestation alternatives and substitutes has been catalysed by the need to restrict methyl bromide use. There still remain a few outstanding technical challenges to complete reduction of emissions of this chemical to the atmosphere: some soil fumigation and other pest control uses of methyl bromide still remain, including particularly QPS uses. For instance, it is

not known how many important fungal diseases are presently prevented from worldwide spread by current QPS fumigation with MB. How these remaining issues are resolved is still in debate.

References

Ajwa HA, Trout T, Mueller J, Wilhelm S, Nelson SD, Soppe R, Shatley D (2002) Application of alternative fumigants through drip irrigation systems. Phytopathology 92:1349–1355

AQIS (2008a) ICON import conditions database. http://www.aqis.gov.au/icon32/asp/homecontent.asp. Accessed at 8 October 2008

AQIS (2008b) PHYTO export conditions database. http://www.aqis.gov.au/phyto/asp/ex_home.asp. Accessed at 8 October 2008

APHIS (2008) PPQ manual (in electronic form). http://www.aphis.usda.gov/import_export/plants/manuals/ports/treatment.shtml. Accessed at 8 October 2008

BAS (2008) British Antarctic Survey http://www.antarctica.ac.uk/met/jds/ozone/

Butler JH (1995) Methyl bromide under scrutiny. Nature 376:469–470

Besri M (2004) Progress in phasing out methyl bromide for tomato production in developing and developed countries. International Research Conference on Methyl Bromide Alternatives and Emissions Reductions, pp 24–1–3. Orlando, USA. October 31 – November 3, 2004

Brett RW, Mapson RJ and Porter IJ (2001) Microflora and nutrient changes after fumigation. Proceedings of the 2$_{nd}$ Australasian Soilborne Disease Symposium, pp 93–94. March 5–8, 2001. Lorne, Australia

CDPR (2008) http://www.cdpr.ca.gov/docs/emon/vocs/vocproj/ffm_discussion.pdf. Accessed at 8 October 2008

Chellemi DO, Mirusso J (2006) Optimizing soil disinfestation procedures for fresh market tomato and pepper production. Plant Disease 90:668–674

Clerbaux C, Cunnold D (2007) Long-Lived Compounds, Chapter 1 in *Scientific Assessment of Ozone Depletion 2006*, Global Ozone Research and Monitoring Project-Report No. 50, 1.1-1.63, WMO, Geneva, Switzerland, 2007

Daniel J, Velders G (2007) Ozone Depletion Potentials and Global Warming Potentials, Chapter 8 in *Scientific Assessment of Ozone Depletion 2006*, Global Ozone Research and Monitoring Project-Report No. 50, 8.1, 1.39, WMO, Geneva, Switzerland, 2007.

Donohoe H, Mattner S, Brett R, Bianco V, Shanks A, Gounder R, Porter I (2001) Status of methyl bromide phase-out in the Australian strawberry industry. International Research Conference on Methyl Bromide Alternatives and Emissions Reductions, pp 44–1–4. San Diego, USA November 5–9, 2001

Duniway JM (2002) Non-chemical alternatives used in the USA on horticultural crops. In: Batchelor TA, Bolivar JM (eds) Proceedings of the International Conference on Alternatives to Methyl Bromide: Sevilla, Spain, 5–8 March 2002. Office for Official Publications of the European Communities, Luxembourg, pp 258–60

Grullemans W (2008) Methyl bromide recapture technology. In: Proc. 8th International Conference on Controlled Atmosphere and Fumigation in Stored Products. Eds: Guo Daolin et al. 21–26 September 2008, Chengdu, China. Sichuan Publishing Group: Chengdu. pp 413–416

Hao JJ, Duniway JM, Dopkins DM, Xiao CL (2002) Effects of rhizobacteria on inhibition of soilborne pathogens and growth of strawberry. Phytopathology, S34

Katan J (1981) Solar heating (solarization) of soil for control of soilborne pests. Ann Rev of Phytopathol 19:211–216

Krummel P, Fraser P (2006) Ozone depletion over Australia and the Antarctic. Chapter 8 in *Ecology: An Australian Perspective*, 2nd Edition, P. Attiwill and B. Wilson (eds.), Oxford University Press, pp 123–140, 2006.

Krummel P, Fraser P, Steele P, Porter L, Derek N, Rickard C, Dunse B, Langenfelds RL, Miller B, Baly S, McEwan S (2007) The AGAGE *in situ* program for non-CO_2 greenhouse gases at Cape Grim, 2005–2006; methane, nitrous oxide carbon monoxide, hydrogen, CFCs, HCFCs, HFCs, PFCs, halons, chlorocarbons, hydrocarbons and sulphur hexafluoride, in: *Baseline Atmospheric Program Australia 2005–2006*, J. Cainey, N. Derek & P. Krummel (eds.), Australian Bureau of Meteorology and CSIRO Marine and Atmospheric Research, Melbourne, Australia, 65–77

Loumakis N (2004) Protected vegetable production in Mediterranean regions without the use of methyl bromide. In: TA Batchelor F Alfarroba, Proceedings of the fifth international conference on alternatives to methyl bromide; 27–30 September 2004; Lisbon, Portugal

Mattner SW, Porter IJ, Gounder RK, Shanks AL (2003) Phytotoxicity and plant-back – Critical issues in the Australian strawberry industry? Annual International Research Conference on Methyl Bromide Alternatives and Emissions Reductions, pp 41–1-4. San Diego, USA November 3–6, 2003

Martin FN (2003) Development of alternative strategies for management of soilborne pathogens currently controlled with methyl bromide. Ann Rev of Phytopathol 41:325–350

MBTOC (2006) Report of the Methyl Bromide Technical Options Committee (MBTOC). United Nations Environment Program (UNEP). 2006 Assessment. Nairobi, p 453

Montzka S, Fraser P (2003) Controlled Substances and other gas sources, Chapter 1 in: Scientific Assessment of Ozone Depletion, 2002, Global Ozone Research and Monitoring Project Report No. 47, Geneva, 1.1–1.83, WMO, Geneva, Switzerland, 2003

Porter IJ, Brett RW, Wiseman BW (1999) Alternatives to methyl bromide: chemical fumigants or integrated pest management systems? Austr Plant Pathol 28:65–71

Porter IJ, Brett RW, Mattner SW, Donohoe HE (2005) Implications of the increased growth response after fumigation on future crop protection and crop production strategies. Acta Horticulturae 698:229–238

Porter IJ, Trinder L, Partington D (2006) Validating the performance of alternatives to methyl bromide for preplant fumigation. Special Report of the Technical and Economic Assessment Panel, May 2006. United Nations Environment Program, Nairobi, p 91

Prinn R, Weiss R, Fraser P, Simmonds P, Cunnold D, Alyea F, O'Doherty S, Salameh P, Miller B, Huang J, Wang R, Hartley D, Harth C, Steele P, Sturrock G, Midgley P, and McCulloch A (2000) A history of chemically and radiatively important gases in air deducted from ALE/GAGE/AGAGE, *Journal of Geophysical Research - Atmospheres*, 105(D14):17751–17792

Rovira A (1976) Studies in soil fumigation – I. Effects of ammonium, nitrate and phosphate in soil on growth, nutrition and yield of wheat. *Soil Biology and Biochem* 8, 241–1247

Ruzo LO (2006) Physical, chemical and environmental properties of selected chemical alternatives for preplant use of methyl bromide as soil fumigant. Pest Manage Sci 62:99–193

Santos BM, Gilreath JP, Motis TN, Noling JW, Jones JP, Norton JA (2006) Comparing methyl bromide alternatives for soilborne disease, nematode and weed management in fresh market tomato. Crop Prot 25:690–695

Shaw DV, and Larson KD (1999) A meta-analysis of strawberry yield response to pre-plant soil fumigation with combinations of methyl bromide-chloropicrin and four alternative systems. HortScience 34:839–845

Stapleton JJ, DeVay JE (1984) Thermal components of soil solarization as related to changes in soil and root microflora and increased plant growth response. Phytopathology 74:255–259

Trudinger C, Etheridge D, Sturrock G, Fraser P, Krummel P, and McCulloch A (2004) Atmospheric histories of halocarbons from analysis of Antarctic fim air: methyl bromide, methyl chloride, chloroform, and dichloromethane, *Journal of Geophysical Research - Atmospheres*, 109(D22): 22310, doi: 10.1029/2004JD004932

Warcup JH (1957) Chemical and biological aspects of soil sterilisation. Soils Fertil 20:1–5

Xiao CL, Duniway JM (1998) Bacterial population responses to soil fumigation and their effects on strawberry growth. Phytopathology 88:S100

Chapter 22
Accelerated Degradation of Soil Fumigants: Occurrence and Agricultural Consequences

Abraham Gamliel and Shachaf Triky-Dotan

Abstract Accelerated degradation (AD) of pesticides occurs following repeated their application to the same soil, resulting in a rapid loss of the pesticide, and in ineffective control of soilborne pests. Accelerated degradation is well known with regular pesticides, much less so with soil fumigants. AD to fumigants can rapidly develop in certain soils even following single application. It frequently results from the enrichment of populations of degrading microorganisms in the soils. Cross degradation of structurally related chemicals was also reported with soil fumigants, resulting in AD of a fumigant in soils which were not treated with this fumigant before. AD persists in soil for a long period, as few years are needed for soil to recover once AD has been induced. Revealing the microbial mechanism of AD of soil fumigants and the factors which enhance or suppress these processes is very important as it can provide tools for managing it. Management of soil in which AD of fumigants has been developed, should include strategies to prevent the development of AD. These strategies should be exercised in every treated soil, as the potential for AD to develop can be realized. Crop rotation and combined methods of control are viable tools for avoiding the negative effect of accelerated degradation, as well as of for slowing down the buildup of population of soilborne plant pathogens. Management of soil in which AD is already present should involve approaches to suppress the populations of the degrading microorganisms, combined with strategies to ensure pest control and crop productivity.

Keywords Metam-sodium • Formalin • Dazomet • Soil disinfestation • Soilborne diseases

A. Gamliel (✉) and S. Triky-Dotan
Institute of Agricultural Engineering, ARO, The Volcani Center Bet Dagan, 50250, Israel
e-mail: agamliel@agri.gov.il

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,*
Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_22,

22.1 Introduction

Soilborne pests, i.e., nematodes, bacteria fungi, arthropods, and weeds play a major role in plant health and crop productivity. Their impact becomes more significant especially under intensive cropping practices. Therefore, ensuring economical crop production requires in many cases to disinfest the soil before the crop is planted. Soil disinfestation using chemicals (soil fumigation) consists of applying volatile, non selective biocides, which act at the vapor or liquid soil phase, and usually eliminate a wide spectrum of organisms. For many years fumigants have provided great benefits to agricultural production. The most common soil fumigant during the last decades is methyl bromide (MBr), a chemical that effectively controls a broad spectrum of soilborne pests and enables the production of acceptable commercial yield in a wide spectrum of agricultural systems (Klein, 1996). Being highly volatile, MBr dissipates from the soil after a short period, enabling to plant a new crop within a short time after the fumigation process. The inclusion of MBr in the list of ozone depleting agents, which was followed by its phase-out of in 2005, has stressed the need for other effective chemical and non-chemical alternatives to control soilborne pests. The list of the currently registered and available fumigants comprises only few fumigants with a narrower range of controlled pests (Anonymous, 2006). These include 1, 3-dichloropropene (1, 3-D, Telone), metam sodium (MS), Dazomet (Basamid), Chloropicrin (CP) and formaldehyde (formalin). The quest for new fumigants during the last 15 years has revealed only few potential molecules (Anonymous, 2002). However, after intensive efforts, only one, iodomethane, has been registered in 2007, only in the US. Furthermore, under the current environmental and public concern, the narrow road for registration a new fumigant seems today to be much longer and winding. Thus, only few fumigants are left, which do not always provide the anticipated control of soilborne pests, nor do they serve as satisfactory replacement for MBr (Anonymous, 2006).

In order to achieve effective control of soilborne pests, a soil fumigant should move rapidly from the application site and distribute uniformly within the cultivated soil layer. Since the currently available fumigants have high boiling point and low vapour pressure, it is obvious that mechanical incorporation and further delivery systems are essential for effective distribution of the fumigant in soil. Indeed, inconsistent and unsatisfactory control of soilborne pathogens following soil fumigation is reported more frequently since the phase-out of MBr. (Anonymous, 2002, 2006). These failures in pest control can often be a result of poor application technology and non-uniform distribution of the fumigant within the cultivated soil layer. The inferior performance of the fumigants leads also to a more frequent application in order to assure crop productivity. This approach, however, may result in accelerated degradation (AD) phenomenon, which causes a rapid loss of activity of the applied fumigant, and ineffective pest control.

Accelerated degradation usually occurs when a pesticide is applied repeatedly to the same soil, resulting in a shorter residual period of the pesticide in the soil. The rapid loss of the pesticide in soil reduces its effectiveness to the target organisms, leading to possible onset of a severe disease in the subsequent crop. Accelerated degradation of

pesticides is due in many cases to enhanced microbial degradation activity. This phenomenon was first reported with the herbicide 2,4-D (Audus, 1949), followed by the report of AD of carbofuran in 1970. Since then, AD of pesticides was extensively documented over the last few decades (Kaufman et al., 1984; Felsot, 1989). To date a long list of herbicide, fungicide, insecticides and nematicides are known to undergo AD (Kapouzas and Giannakou, 2002; Arbeli and Fuentes, 2007).

Accelerated degradation is well known with regular pesticides, much less so with soil fumigants. In 1989, Smelt et al. (1989a,b), have reported AD of methyl isothiocyanate (MITC) and 1, 3-D in soil after repeated application of metam-sodium and telone, respectively. Since 1989, more reports on AD of the fumigants MITC and 1, 3-D have been accumulated (Warton and Matthiessen, 2000; Di Primo et al., 2003) leading to more extensive research on this phenomenon and its implication on pathogen control and plant health. The following will elaborate more on the current knowledge on AD of soil fumigants, the causal factors, and the possible approaches to manage its development.

22.2 Degradation and Dissipation of Fumigants in Soil

Dissipation of pesticides and fumigants in soil is usually described as a first order kinetics (Nash, 1988), while deviation from this pattern to a significant sharp degradation rate can be regarded as AD. The evolvement of AD was originally defined in the literature as a "problem" or "history" soil (Kaufman et al., 1984), as opposed to "non problem" or "non history" soil in which AD was not observed. However, studies show that there are situations where there were no differences in degradation rates of a chemical between problem and non problem soil, i.e., rapid degradation occurred in a soil although the fumigant was applied for the first time in that soil (Kaufman et al., 1984; Triky-Dotan et al., 2007). Thus, few questions arise in order to study the role of AD evolvement in soil: (a) what is the "common" dissipation rate of a fumigant in a soil with no previous application of the parent compound (nonhistory soil)? (b) What is the role of repeated application on the development of AD? (c) What are physical, chemical and microbial mechanisms which are involved in the AD of fumigants in soil? (d) How does the dissipation rate affect pest control? (e) Can the dissipation pattern of a fumigant be predicted in a given soil, and related to the potential of successful soil fumigation, i.e., effective pest control? Below shall discuss relevant data on these issues and other which are in connection with AD phenomenon of soil fumigants.

22.2.1 Generation and Dissipation of the Active Ingredient in Soil

With certain fumigants, e.g. MBr and 1, 3-D and formalin, the parent compound which is applied into the soil and the active ingredient are the same. Therefore, the fumigant reaches maximal concentration of the active ingredient once it is applied

to the soil. However, application of other fumigants, e.g. MS and Dazomet, should involve chemical and microbial degradation of the parent compound to generate the active ingredient *methyl isothiocyanate* – MITC (Smelt and Leistra, 1974; Di Primo et al., 2003; Gamliel, 2005; Triky-Dotan et al., 2007). The overall balance of such a pesticide in soil is therefore a result of generation and dissipation processes, acting simultaneously especially at the initial period following application. These processes also dictate the generation curve and the maximal concentration which will be generated in a given soil. MS is chemically degraded in soil to generate MITC reaching the peak of its concentration after few hours (Gamliel et al., 2005). In contrast, application of dazomet, another MITC-generating compound, to soil involves its microbial degradation and a slower rate of MITC generation, reaching the peak after 24–48 h (Fritsch and Huber, 1995; Gamliel et al., 2005).

Dissipation of a pesticide from the soil is affected by the physical–chemical properties of the soil, such as pH, organic matter content, field water capacity, clay/sand/loam contents, and cation exchange capacity (Racke and Coats, 1990; Boesten et al., 1991). Being highly volatile, MBr moves along the air space in a porous medium such as soil or substrate and fills the soil spaces rapidly at the first stage after application. Only a small portion of the MBr interacts in the liquid phase, therefore its degradation in soil is at a rate of 6–7% per day (Klein, 1996). In fact, MBr escape into the atmosphere is much faster and plays the major dissipation factor, while degradation in soil is at much lower rates. For other alkyl halide fumigants such as, methyl iodide, or propargyl bromide, the main initial degradation is also chemical (Papiernik et al., 2000). This is not the case, however with the other fumigants, MS, 1, 3D, Formalin and mixtures which have low vapor pressure and Henry constant values (Ajwa et al., 2003).These move in the liquid phase interact with soil particles and are subjected to degradation. The behavior of these fumigants is strongly influenced by soil components by simultaneously occurring sink processes, such as chemical and physical sorption and degradation (Hartley and Graham-Bryce, 1980).

22.2.2 MITC

The half-life of MITC in soil is about 7 days (Gerstl et al., 1977; Draper and Wakeham, 1993; Di Primo et al., 2003: Triky-Dotan et al., 2007). However, a wide array of dissipation curves and half-life values are obtained with different soils as tested in studies conducted in the Netherlands and Israel (Verhagen et al., 1996; Triky-Dotan et al., 2007). Under the soil conditions in Australia, the loss of MITC from clay soils was also correlated with addition of lime, exhibiting rapid dissipation of the fumigant in high lime content (Warton and Matthiessen, 2005). However, in an extensive study in Israel, MITC dissipation was tested over a wide spectrum of soil, most of which are sandy and alkaline (pH = 6.9–8.2) with low organic matter contents (<1.9%; Triky-Dotan et al., 2007). In this study, there was no correlation between the rate of dissipation of MITC and these soil properties.

Furthermore, microbial analyses of the degrading organisms indicate that the occurrence of these organisms is the dominant factor in MITC dissipation (A. Gamliel, unpublished data). The study of Triky-Dotan et al. (2007) demonstrates that prediction of dissipation rate of MITC from a given soil is a difficult task. One way to evaluate the degradation of MITC in soil is by a preceding dissipation test in the laboratory (Triky-Dotan et al., 2007). Several studies indicated that dissipation of MITC from soils increases with increasing soil organic content, temperature, and pH (Ashley et al., 1963; Dungan et al., 2003a; Gan et al., 1999; Gerstl et al., 1977; Warton et al., 2001). On the other hand, a longer period for MITC dissipation was reported in water saturated soils or in soils that contained less than 20% clay (Ashley et al., 1963; Gerstl et al., 1977; Turner and Corden, 1963). Elevated temperature accelerates MITC degradation in the soil (Dungan et al., 2002; Gamliel, 2005). Dungan et al (2003a) found that degradation of MITC was three times higher at 40°C than at 20°C sandy loam soil.

22.2.3 1, 3-D

Dissipation of 1, 3-D from soil has no consistent differences between the degradation rates of the (*cis*)- and (*trans*)-isomers of 1, 3-D (Smelt and Leistra, 1974; van Dijk, 1980). Persistence of 1, 3-D in non-history agricultural soils is longer than MITC (Verhagen et al., 1996) . In the Netherlands (Smelt et al. 1996) found that degradation of 1, 3-D is delayed for a lag-time of 7–9 days, followed by a sharp increase in its degradation. During the lag-time, the decrease could be described as first-order kinetics, with half-lives of 1l–l3 days (van Dijk, 1980; Smelt et al., 1989a). The degradation of 1, 3-D after the lag-time is rapid and is similar to accelerated degradation pattern without any relations to previous 1, 3-D application (Smelt et al., 1996). This degradation pattern, however, was not evident in California; Zheng et al. (2003) found first order degradation kinetics of 1, 3-D without any lag time but with similar half time values. Degradation of 1, 3-D is increased with elevated soil temperatures, as a result of enhanced microbial activity (Gan et al., 1999; Dungan et al., 2001).

22.2.4 Chloropicrin (CP)

Chloropicrin was introduced as an insecticide in 1908 and later as a soil fumigant. The half life of CP in soil ranges from a short period of 0.2–4.5 days (Wilhelm et al., 1996; Gan et al., 2000). The major factor responsible for the CP dissipation under aerobic conditions is microbial degradation (Gan et al., 2000; Dungan and Yates, 2003). The major product of CP degradation in soil is nitromethane, while a small fraction of the parent compound is converted to CO_2 (Dungan and Yates, 2003). Under anaerobic soil conditions, CP degraded very rapidly (half-life of 1.3 h),

yielding only nitromethane as the major product. CP binds to the humic fraction in soils (humic and fulvic acids). A competitive degradation between chloropicrin and 1, 3-D has been reported (Zheng et al., 2003; Desaeger et al., 2004). This phenomenon is important to study since mixtures of 1, 3-D and CP are widely used as the major alternative to MBr for soil fumigation.

22.3 Development of Accelerated Degradation in Soil

Since 1950, MBr has been used repeatedly in fields, with no evidence for reduced efficacy due to accelerated degradation. Only Miller et al. (1997) have reported a level of increased rates of MBr dissipation following repeated application, without any support from field studies.

Controlled studies in the laboratory are well known and documented for many pesticides, and are usually employed to measure the potential of their AD in soils. These tests are usually done by incubating history soil with the tested soil and the tested fumigant and compare its degradation with that of non-history soil. However, such studies were not focused on soil fumigants until the late 1980s. At that time, studies regarding AD of soil fumigants have initiated following the phase-out of MBr in the Netherlands, resulting in intensive use of MS and 1, 3-D as alternative soil fumigants. Those studies have pointed out for the first time that soil fumigants MITC and 1, 3-D degradation are also vulnerable to AD as it was evident before with other pesticides. (Lebbink et al., 1989; Smelt et al. 1989a,b).

22.3.1 MITC

Studies conducted in the Netherlands, Israel and Australia clearly show that repeated application of MS in a controlled systems, leads to significant acceleration degradation and dissipation of MITC in soil (Smelt et al., 1989a,b; Warton and Matthiessen, 2000; Di Primo et al., 2003). This phenomenon was confirmed with a wide range of soils that differed in their physical and chemical properties, when tested. The AD of MITC was also accompanied by a significant reduction in pathogen control, indicating that repeated application of MS under field conditions may draw severe practical consequences (Di Primo et al., 2003). Verhagen et al. (1996) demonstrated the development of AD of after six repeated applications. However, AD to certain fumigants can develop even after one application. Smelt et al. (1989b) demonstrated that enhanced degradation of MITC occurred in soils that had not been previously treated, which may implies that degrading microbial activity exist in certain soils leading to rapid degradation of MITC even when it applied for the first time. Similar observation was reported by Di Primo et al. (2003), who also reported for the first time, accelerated degradation of MITC resulting from repeated application of dazomet, or after application of dazomet in soil which was

previously treated with MS. These findings indicate that AD to MITC can be induced by any chemical that generates MITC, e.g., soil fumigants, related pesticides, or other MITC precursors from plant debris.

22.3.2 1, 3-D

The potential of soil to rapidly degrade 1, 3-D was first studied in the Netherlands. Lebbink et al. (1989), reported that sustained annual application of 1, 3-D for 12 years resulted in 70% reduction in the control of cyst nematodes of potatoes in sandy soil. In another study, Smelt et al. (1989b) observed AD of 1, 3-D in either sandy soil or reclaimed peat soils without a correlation to previous treatments with these fumigants. Verhagen et al. (1996) have applied 1, 3-D intensively for 6 times over 12 months and also observed a rapid dissipation of the fumigant compared with the untreated soil. In a similar study which was conducted in Florida, a soil with history of 6 applications during a 12 year period was capable to rapidly degrade 1, 3-D, compared with the untreated soil (Ou et al., 1995). They found that only the *trans* isomer was degraded rapidly in the history soil, while there was no difference in the degradation of the *cis* isomer between the history and non history soil. These findings are in disagreement with those by Smelt et al. (1996), in which both isomers were rapidly degraded in history soil. Studies which were initiated in Israel did not show significant AD of 1, 3-D after three applications at two month intervals (A. Gamliel, unpublished data).

22.3.3 Chloropicrin (CP)

There are no reports of AD and reduced efficacy of CP in controlling soilborne pathogens following repeated application in the field. Gan et al. (2000) conducted a laboratory study in which they observed that sterilization of the soil prior to CP application increased its half-life by 3.2–13.5 time over the non-sterilized soils. The researchers suggested that the major factor responsible for the CP dissipation is microbial degradation. However, the literature lacks more details regarding the fate of CP in the environment and the effect of repeated application on its dissipation.

22.4 Accelerated Degradation Under Field Conditions

As noted above, controlled laboratory studies are a common approach to assess the potential of AD development. However, such studies do not necessarily predict the development of AD in the field and consequently the success of fumigation in disease control. Moreover, evidence for the development of AD in controlled laboratory

studies do not always result in poor disease control in crop production. A positive evidence of AD in the laboratory which is followed by contradicting results of satisfactory pest control in the field can be attributed to a complex of influencing factors which play important role in fumigant degradation. These factors include microbial communities, soil properties, and climatic conditions, and other agricultural practices, which are not present (or overlooked) in the laboratory studies. The list of studies which directly connect poor pest control in the field to AD is very limited, however recent information is reported. In studies conducted in Israel in potato fields, a significant relationship was obtained between the dissipation rate of MITC, the mortality of test pathogen *F. oxysporum* f. sp. *radicis lycopersici* (as a bioassay organism) and the incidence of Verticillium wilt disease in the field (Triky-Dotan et al., 2007). Furthermore, Gamliel et al. (2003) and Triky-Dotan et al. (2009) were able to connect AD of fumigants to the reduction of their efficacy in the field. In a peanut field, MS significantly reduced the incidence of Pythium pod rot and improved pod quality after a single application, but its effectiveness was greatly reduced after two applications. In a potato field, MS was significantly effective after a single application in controlling Verticillium wilt in potatoes (67% reduction), but its efficacy diminished after two and three consecutive applications (18% and 8%, respectively). In an additional experiment, fumigation with MS following single or double applications was more effective in reducing Verticillium wilt severity of potato as compared with triple application. In those studies it was possible to directly connect the reduction in disease control in the field with AD of MITC.

22.5 Mechanisms of Accelerated Degradation and the Conditions for Its Development

AD of a soil fumigant or any pesticide may result from enrichment of the population of microbial community in the soils, from increased enzymatic activity of the degraders, from the transfer of extra-chromosal elements from the degraders to the other components of the soil microbial community, or from a combination of these factors (Kaufman et al., 1985; Roeth, 1986; Katan and Aharonson, 1989). Studies have indicated that degradation of fumigant was faster in a history soil compared with the same sterilized soil indicating that biological degradation is the major force of MITC degradation (Taylor et al., 1996; Dungan et al., 2003a; Ibekwe et al., 2004). Furthermore, revealing the microbial mechanism of AD of soil fumigants and the conditions for its development is very important for better understanding this phenomenon, preventing it and for providing approaches and tools for managing of AD.

22.5.1 Microorganisms and Microbial Activity

The role of microorganisms and microbial activity in AD of soil fumigants has been clearly addressed. Triky-Dotan et al. (2009) were able to induce accelerated

degradation of MITC in six different agricultural soils, by inoculating natural (non-history) soil with 10% of MS history soil. Furthermore, they extracted the liquid fraction of the soil with AD which contained the degrading organisms. They used this fraction to inoculate a nonhistory soil and were able to induce again AD of MITC in the inoculated non-history soil. The microorganisms (mainly bacteria) which were found to degrade the various soil fumigants were listed in some reviews (Dungan et al., 2003b). These organisms were isolated from soil amended with the test fumigant, but not necessarily from soil in which AD has developed. The most common bacteria which were isolated from AD soil included the genera *Pseudomonas, Rhodococcus* and *Bacillus* were isolated from soil with AD to MITC and 1, 3-D (Lebbink et al., 1989; Ou et al., 2001). Verhagen et al. (1995) have suggested that a plasmid-located *dhlA*-like gene may be involved the degradation of 1, 3-D in soil. However the extent to which the gene is involved in the process of AD is yet to be clarified. Warton et al. (2001) reported that gram-positive heat-resistant bacterium *Rhodococcus* and *Bacillus* spp were isolated from soil exhibiting AD, and may be involved in the accelerated degradation of MITC. In contrast, Triky-Dotan et al. (2008) have extracted the liquid fraction of the soil which contained the degrading organisms from a history soil. They observed that AD activity has eliminated following heating of this extract to 60°C for 2 h. These findings are not necessarily contradicting, since they may indicate that a wide spectrum of organisms are capable of degrading the soil fumigant, and their activity is also a result of the appropriate soil conditions as is discussed further on. Indeed, Ibekwe et al. (2001) found a wider diversity of bacteria which were associated with the degradation of MITC and 1, 3-D following amendment of composted manure. They proposed that the addition of organic amendment to soil during fumigation practices has the potential to increase the diversity of different microbial species, thereby accelerating fumigant degradation.

22.5.1.1 Cross Accelerated Degradation

Application of a pesticide to the environment induces enzyme signal in microbial cultures. Adaptation of degrading organisms to a given fumigant will probably involve adaptation also to other breakdown products and to other homologous molecules. The ability of microorganisms to degrade other structurally related chemicals (cross degradation) is common in microbial activity (Kaufman et al., 1985; Felsot, 1989). The problem of accelerated biodegradation of a fumigant becomes more acute after observation the fumigants such as MITC and 1, 3-D are degraded rapidly in soil which were never treated with this fumigant before (Smelt et al., 1989a,b; Di Primo et al., 2003; Triky-Dotan et al., 2007). Di Primo et al. studied the dissipation rates of MITC, after application of MS, or Dazomet as the challenge fumigants, in soils previously treated with either MS or DAZ or 2-thiocyanatomethylthio, 1, 3-benzothiazole (TCMTB). TCMTB is a dithiocarbamate fungicide which is used for disinfection of peanut seeds before planting

in Israel; therefore the many soils are exposed to this compound, although at low rates. MITC (either from MS or Dazomet) was rapidly degraded in soils having previous treatments with the ‘‘cross-conditioning’’ homologous pesticides MS, Dazomet or TCMTB. These findings suggest that various agricultural practices of pesticides and other chemical application may trigger cross-AD to a soil fumigant later on.

Inducing AD by a homologous compound can originate also from a natural product. MITC is also a product from decomposition of crucifer plant debris in soil. Warton et al. (2003) have applied ITC compounds 2-propenyl isothiocyanate (PrITC), benzyl isothiocyanate (BeITC) and 2-phenylethyl isothiocyanate (2-PeITC) in a soil with AD to MITC, which was not previously exposed to the other ITCs. The rate of degradation of the same three ITCs was rapid indicating that the three ITCs are vulnerable to enhanced cross-biodegradation induced by previous application of MITC. The findings of Warton et al. (2003) have two significant implications on soilborne pest management. First, fumigation of with MITC fumigants in field previously cropped with cruciferous plant may result in a rapid degradation and poor pest control. The opposite is even a worse scenario; incorporation of ITC-producing cruciferous plants for the purpose of biofumigation may be ineffective since the soil was already induced to AD of MITC from previous fumigant application.

There is no indication for cross-AD between the major used fumigants. Verhagen et al. (1996) reported that application of 1, 3-D or MITC did not induce AD of soils to the other fumigant, nor does alternate application inhibit the development of AD, compared with application of each fumigant separately. In a similar study, Smelt et al. (1996) observed that addition of MITC to 1, 3-D-history soil prevented the accelerated degradation of 1, 3-D in the most frequently treated soil. This might be due to suppression of microbial activity by MITC, however, there is no further evidence for these findings.

22.5.1.2 Stability of AD

Little information is available as to the period required for soil to recover once AD occurred. Most of the reports deal with controlled environment studies. In general, it is well accepted that few years are need for soil to recover once AD has be induced. Soil with a history of treatment with Benomyl [methyl-l-(butyl-carbamoyl)-2-benzimidazole carbamate] preserved the capability for accelerated degradation of the fungicide for over 2 years without intermediate MBC application (Yarden et al., 1987; Di Primo et al., 2003), found that accelerated degradation of MITC was still evident 18–30 months after treatment. Verhagen et al. (1996) reported that three years were necessary before the accelerated degradation of MS was suppressed in a variety of soils in the Netherlands. However in the same study they found that AD of 1, 3-D was not restored to normal even after five years without additional application of the chemical. Similar results were observed by Smelt et al. (1996).

22.5.2 Soil Characteristic and Environmental Conditions

The effect of chemical and physical properties of the soil on the development of AD has been studied extensively with pesticides in general and with fumigants in certain cases. Many studies has indicated that AD to soil MITC and 1, 3-D can be developed in a wide spectrum of soil textures, including sand, clay and silt soils (Smelt et al., 1989a,b; Smelt et al., 1996; Verhagen et al., (1996); Di Primo et al., 2003). Read (1987) reported that acidic soils (pH < 5.6) cannot be induced for AD even after repeated application. Indeed, Smelt et al., (1996) have demonstrated that AD to MITC was rapidly developed in clay and silt soils with pH above 7, even when the soil was never fumigated before. Warton and Matthiessen (2005) have demonstrated that increasing soil pH with lime (calcium carbonate) resulted in AD following repeated application of MS. In contrast, increasing soil pH with magnesium carbonate in the same experimental system did not yield AD, indicating the interaction of Ca with the high pH are crucial for the development of AD of MITC. Soil pH probably affects AD either directly by affecting the chemical stability of the pesticide or indirectly by affecting the composition and activity of soil microflora (Suett et al., 1996a,b). Lower pH reduces bacterial numbers which are important degrading factor of pesticides in soil. Indeed, A. Gamliel and P. Di Primo (unpublished), have found that reducing the pH of MITC history soil from 8 to 5.5, resulted in a decreased rate of MITC dissipation and extending the half time of the fumigant from 6 to 72 h.

22.5.2.1 Soil Temperature and Water Content

Appropriate soil temperature and moisture are crucial factors for the rate of fumigant degradation in soil as they influence microbial activity. It was indicated in many studies that the degradation of MITC, 1, 3-D and other fumigants increased with increasing soil temperature, and the half time of this fumigants is shorten significantly (Turner and Corden, 1963; Gerstl et al., 1977; Gan et al., 1999; Dungan et al., 2001; Gamliel, 2005). Water content increases the availability of pesticides in the liquid phase, thereby enhancing their exposure to degradation (Hartley and Graham-Bryce, 1980; Ogram et al., 1985). The rapid degradation at increased temperature depends however upon soil moisture contents below saturation (Boesten et al., 1991). Water saturation reduces degradation rate as it is less favorable for microbial activity (Turner and Corden, 1963).

22.5.2.2 Organic Material

Organic content in soil was found to extend the rate of fumigant degradation following repeated application (Smelt et al., 1989a,b; Verhagen et al., 1996; Gan et al., 1999; Ibekwe et al., 2001; Dungan et al., 2003a,b; Ibekwe et al., 2004). Organic material can increase the degradation rate of a pesticide in soil. In contrast,

pesticides can be adsorbed to the organic material thus minimizing its availability to degradation in the liquid phase of the soil. Chapman et al. (1986) have reported that adsorption of these pesticides to soil particles was greater in organic amended soil. Additionally, insecticide degradation was slower when introduced to a soil with high organic material compared with sandy soil and lower organic content (Chapman and Harris, 1990). Similarly, Verhagen et al. (1996) have reported that degradation of MITC was lower in soils with pH ranging 4.5–5.2 and organic content 3.8–7.9% compared with soil with pH over 7 and organic content ranging 1.8–3.8%. However, it is difficult to determine from this study what is the role of the organic content compared with the pH. Today it is common to apply chemical and organic fertilizers in the shape of organic manure or various composts. These are colonized with microbial communities, some of which are foreign to the applied soil. Thus, enriching the soil with new microbial communities can involve the introduction of fumigant degrading microorganisms which can later establish in the soil. Indeed, Gan et al. (1998) have found that degradation of 1, 3-D was significantly enhanced in soil amended with composted manure compared with the respective unamended soil. They have attributed the AD to enhanced chemical and microbial degradation, since sterilization of the amended soil only partially reduced the enhanced degradation. A similar trend was observed by Dungan et al. (2003a,b) with the degradation of MITC 1, 3-D and propargyl bromide in soil following amendment with composted steer manure. The effect of organic content on the rate of fumigant degradation is usually a equation with multiple factors. Dungan et al. (2002) have found that compost amendment increased the degradation rate of MITC three times higher at 40°C than at 20°C. It should be noted, however that most of the findings were studied under controlled conditions with constant temperature, moisture and organic content. The influence of these factors on the expression of AD may be different and vary at different soil types, depth and different field conditions.

22.6 Management of Accelerated Degradation

A common reaction of farmers to AD is the tendency to treat soil more frequently and at higher dosages (Suett et al., 1996a,b). However, this reaction usually gives the opposite results, since by further enriching the populations of microorganisms which degrade the soil fumigants, AD is enhanced. Furthermore, there is an excessive use of pesticides. Thus, management of history soil in which AD of fumigants has been developed, should include an approach to suppress the microbial communities which are responsible for the AD. Additionally, strategies to prevent the development of AD should be exercised in every treated soil, since the potential for AD to develop can be realized in most soils. Appropriate crop rotations, and combined methods of control, are viable tools for avoiding the negative effect of accelerated degradation, and for suppressing population buildup of soilborne plant pathogens.

22.6.1 Soil Disinfestation for AD Management

Since AD of certain soil fumigants was usually attributed to the activity of bacterial populations, it is expected that chemical agents which suppress bacterial can reduce the accelerated activity. Indeed, Gamliel et al. (2003, 2005) found in certain cases that fumigation with formalin-MS mixture resulted in effective control of Verticillium wilt of potatoes and peanut pod rot in soil were accelerated degradation and loss of activity of MS was observed. Thus, the case of formalin-MS mixture is significant in soils where the phenomenon of accelerated degradation of MS occurs. In contrast, fumigation of soil with AD to MITC with either methyl bromide or methyl iodide did not restore the soil from AD to MITC (A. Gamliel, unpublished data). Smelt et al. (1996) have reported that addition of MITC prevented the AD of 1, 3-D in 1, 3-D history soils. A mixture of two or more fumigants is used in many cases in order to extend spectrum of controlled pests (Anonymous, 2006). Gamliel et al. (2005) observed that mixture of formalin and MS even at reduced rates, resulted in synergistic effect on the control of fungal pests in potatoes, peanuts, tomatoes and melons. Thus mixture of the appropriate fumigants can provide the desired pathogen control together with environmental benefits while minimizing the risk for AD development.

22.6.2 Combinations of Fumigants with Other Methods of AD Management

Soil solarization is a nonchemical method of control, which include tarping the soil for few weeks in order to heat it and actuate thermal killing and biological activity to suppress soilborne pathogens (Katan, 1996). Combination of fumigants with solarization further improves killing of fungal propagules and the control of several diseases (Frank et al., 1986; Gamliel and Katan, 2009). The appropriate combined application of pesticides and solarization should be at the heart of integrated disease management. Several studies have presented data of recovering AD soil and restoring pesticide performance following solarization (Katan and Aharonson, 1989; Aharonson and Katan, 1991). Gamliel et al. (2003) have found that disinfestation of a history soil by steam or solarization eliminated the AD phenomenon. Furthermore, solarization can be combined in order to prevent the development of AD in soils with repeated fumigation. Indeed in the study conducted by Triky-Dotan et al. (2009) AD of MITC did not develop in a soil which is annually solarized combined with MS.

22.7 Conclusions and Future Prospects

Dissipation of fumigants from soil is environmentally and agriculturally desirable since toxic residues may damage the subsequent crop and the environment. Nevertheless, accelerated degradation, leading to too rapid dissipation, has negative consequences

of excessive and useless input of chemicals along with insufficient pathogen control. The evidence regarding AD of soil fumigant is reported only during the last 20 years, while AD of other pesticides is known for over 65 years. Moreover, documentation of AD of soil fumigants under field conditions is very rare. Does this delay result from the predominance of MBr as soil fumigant, and lack of research efforts on the other fumigants, or is it the wide spectrum of organisms which are controlled by soil fumigants that suppressed the development of degrading organisms in soil? The answer for these questions is left open. However, the evidence regarding AD of soil fumigants during the last two decades clearly indicates that this phenomenon should be addressed.

Most of the studies regarding AD of soil fumigants were carried out under controlled conditions, indicating the potential of AD of the tested fumigants. These findings do not necessarily imply that AD will occur in any given agricultural field, nor will it indicate failure in pathogen control. Indeed, only few studies directly connect AD with the unsatisfactory control of soilborne pest in the field. Nevertheless, potential of AD of soil fumigants as indicated by many studies during the last 20 years, emphasizes the need to better understand this phenomenon, in order to prevent its development and protect the few soil fumigants which are still left in the market.

From the agricultural viewpoint, the organisms which are responsible for AD of fumigants can are similar in their behavior soilborne pathogens, since they cause a kind of soil "sickness" or "problem". Similar to soilborne plant pathogens, the populations of pesticide microbial degraders can increase in the appropriate substrate (namely, plant tissues or a pesticide) and under the appropriate conditions. The fumigant degraders also can be transferred to other soil, establish in it and induce AD similar to plant disease which is caused following pathogen infestation. Therefore, similar approaches should be developed in order to control AD or soilborne pathogens.

Effective control of soilborne pathogens by soil fumigants requires understanding all the aspects of the chemical aspects involved in order to tailor the optimal application. This includes a uniform distribution of the chemical to the desired soil profile, and maintaining the active ingredient in soil at the desired concentration and to a sufficient period of time. At the same time care should be taken regarding soil conditions especially soil temperature and moisture, in order to ensure optimal performance of the fumigant. On the other hand, accelerated degradation and too rapid dissipation of the chemical from the soil, before the pests were controlled, is a negative attribute which should be avoided. Combination of fumigants with methods which control different spectrum of target pests can extend the spectrum of control of these fumigants, in addition to a significant reduction of dosages and this can make such application more environmentally-acceptable. This will also reduce the chances for AD development.

AD of fumigants should be taken into consideration in order to protect the very few soil fumigants which are still available; it should be regarded also when developing new soil fumigants. Our concern regarding AD of soil fumigants should pave the way to the development of integrated pest control approach in order to maximize the effect of the available fumigant while keeping their activity.

Acknowledgements Contribution 709/08 series from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.

References

Aharonson N, Katan J (1991) Pesticide behavior in solarized and disinfected soils. In: Katan J, De Vay JE (eds) Soil solarization. CRC, Boca Raton, FL, pp 131–138

Ajwa HA, Klose S, Nelson SD, Minuto A, Gullino ML, Lamberti F, Lopez Aranda JM (2003) Alternatives to methyl bromide in strawberry production in the United States of America and the Mediterranean region. Phytopathologia Mediterranea 42:220–244

Anonymous (2002) Alternative to Methyl bromide for soil treatment. In: Report of the methyl bromide technical option committee (MBTOC). 2002 Assessment of the Montreal protocol on substances that deplete the ozone layer. UNEP Publishing Section Services, Nairobi, pp 41–77

Anonymous (2006) Alternative to Methyl bromide for soils uses. In: Report of the methyl bromide technical option committee (MBTOC). 2006 Assessment of the Montreal protocol on substances that deplete the ozone layer. UNEP Publishing Section Services, Nairobi, pp 100–188

Arbeli Z, Fuentes CL (2007) Accelerated biodegradation of pesticides: an overview of the phenomenon, its basis and possible solutions; and a discussion on the tropical dimension. Crop Prot 26:1733–1746

Ashley MG, Leigh BL, Lloyd LS (1963) The action of metham-sodium in soil II – Factors affecting the removal of methyl isothiocyanate residues. J Sci Food Agric 14:153–161

Audus LJ (1949) The biological detoxification of 2, 4-dichlorophenoxiacetic acid in soil. Plant Soil 2:31–36

Boesten JJ, Van Der Pas LJT, Smelt JH, Leistra M (1991) Transformation rate of methyl isothiocyanate and 1, 3-dichloropropene in water-saturated sandy subsoils. Net J Agric Sci 39:179–190

Chapman RA, Harris CR (1990) Enhanced degradation of insecticides in soil: factories influencing the development and effects of enhanced microbial activity. Am Chem Soc Symp 426:82–97

Chapman RA, Harris CR, Harris C (1986) Observations on the effect of soil type, treatment intensity, insecticide formulation, temperature and moisture on the adaptation and subsequent activity of biological agents associated with carbofurn degradation in soil. J Environ Sci Health 21:125–141

Desaeger JJ, Eger JE, Csinos AS, Gilreath JP, Olson SM, Webster TM (2004) Movement and biological activity of drip-applied 1, 3-dichloropropene and chloropicrin in raised mulched beds in the Southeastern USA. Pest Manag Sci 60:1220–1230

Di Primo P, Gamliel A, Austerweil M, Steiner B, Peretz I, Katan J (2003) Accelerated degradation of metam sodium and dazomet in soil: characterization and consequences for pathogen control. Crop Prot 22:635–646

Draper WM, Wakeham DE (1993) Rate constants for metam sodium cleavage and photodecomposition in water. J Agric Food Chem 41:1129–1133

Dungan RS, Gan J, Yates SR (2001) Effect of temperature, organic amendment rate, and moisture content on the degradation 1, 3-dichloropropene in soil. Pest Manag Sci 57:1107–1113

Dungan RS, Gan J, Yates SR (2003a) Accelerated degradation of methyl isothiocyanate in soil. Water Air Soil Pollut 142:299–310

Dungan RS, Ibekew MA, Yates SR (2003b) Effect of propargyl bromide and dichloropropene on microbial communities in an organically amended soil. FEMS Microbiol Ecol 43:75–87

Felsot AS (1989) Enhanced biodegradation of insecticides in soil: implications for agro systems. Annu Rev Entomol 34:454–476

Frank ZR, Ben-Yephet Y, Katan J (1986) Synergistic effect of metham and solarization in controlling delimited shell spots of peanut pods. Crop Prot 5:199–202

Fritsch HJ, Huber R (1995) Basamid granular – a halogen free soil disinfestant. Acta Horticulturae 382:76–85

Gamliel A (2005) Behaviour of fumigants in soil. Acta Horticulturae 698:41–51

Gamliel A, Katan J (2009) Control of plant diseases through solarization. In*:* Walters D (ed) Disease control in crops, biological and environmentally-friendly approaches. Wiley-Blackwell, Oxford 195–220

Gamliel A, Triky S, Austerweil M, Di Primo P, Peretz-Alon I, Heiman O, Beniches M, Steiner B, Katan J (2003) Accelerated degradation of metam sodium in the field and its management. Proceedings of the 10th annual international research conference on Methyl bromide alternatives and emission reduction, San Diego, CA. pp 10.1–10.3

Gamliel A, Triky S, Austerweil M, Di Primo P, Beniches M, Steiner B, Peretz-Alon I, Ucko O (2005) Combined soil fumigants: synergistic performance and improved yield. Acta Horticulturae 698:135–140

Gan J, Yates SR, Crowley D, Becker JO (1998) Acceleration of 1, 3-Dichloropropene degradation by organic amendments and potential application for emissions reduction. J Environ Qual 27:408–414

Gan J, Papiernik SK, Yates SR, Jury WA (1999) Temperature and moisture effects on fumigant degradation in soil. J Environ Qual 28:1436–1441

Gan J, Yates SR, Ernst FF, Jury WA (2000) Degradation and volatilization of the fumigant chloropicrin after soil treatments. J Environ Qual 29:1391–1397

Gerstl Z, Mingelgrin U, Yaron B (1977) Behaviour of Vapam and methylisothiocyanate in soils. Soil Sci Soc Am J 41:545–548

Hartley GS, Graham-Bryce IJ (1980) Behavior in soil and other porous granular material. In: Hartley GS, Graham-Bryce IJ (eds) Physical principles of pesticide behaviour, vol 1, Academic, London, pp 236–331

Ibekwe M, Papiernik SK, Yates SR, Crowley DE (2001) Microcosm enrichment of 1, 3-dichloropropene-degradaing soil microbial communities in a compost-amended soil. J Appl Microbiol 91:668–676

Ibekwe AM, Papiernik SK, Yang CH (2004) Enrichment and molecular characterization of chloropicrin and metam-sodium-degrading microbial communities. Appl Microbiol Biotechnol 66:325–332

Kapouzas DG, Giannakou IO (2002) Biodegradation and enhanced biodegradation: a reason for reduced biological efficacy of nematicides. Russ J Nematol 10:59–78

Katan J (1996) Soil solarization: integrated control aspects. In: Hall R (ed) Strategies for nanaging soilborne plant pathogens. APS, St Paul, MN, pp 250–278

Katan J, Aharonson N (1989) Accelerated degradation of pesticides. In: Gerstl Z, Chen Y, Mingelgrin U, Yaron B (eds) Toxic organic chemicals in porous media: ecological studies. Springer, Berlin, pp 193–207

Kaufman DD, Katan J, Edwards DF, Jordan EG (1984) Microbial adaptation and metabolism of pesticides. In: Hilton JL (ed) Agricultural chemicals in the future. BARC Symposia Number 8, Rowman & Allanheld, Totowa, NJ, pp 437–451

Klein L (1996) Methyl bromide as a soil fumigant. In: Bell CH, Price N, Chakrabarti B (eds) The methyl bromide issue. Wiley, New York, pp 191–235

Lebbink G, Proper B, Nipshagen A (1989) Accelerated degradation of 1, 3-dichloropropene. Acta Horticulturae 255:361–361

Miller LG, Connell TL, Guidetti JR, Oremland RS (1997) Bacterial oxidation of methyl bromide in fumigated agricultural soils. Appl Environ Microbiol 63:4346–4354

Nash RG (1988) Dissipation from soil. In: Grover E (ed) Environmental chemistry of herbicides. CRC, Boca Raton, FL, pp 131–142

Ogram AV, Jessup RE, Ou LT, Rao PSC (1985) Effects of sorption on biological degradation rates of 2,4-dichlorophenoxyaceitic acid in soils. Appl Environ Microbiol 49:582–587

Ou LT, Chung KY, Thomas JE, Obrenza TA, Dickson DW (1995) Degradation of 1, 3 dichloropropene (1, 3-D) in soils with different histories of field application of 1, 3-D. J Nematol 27:249–257

Ou LT, Tomas JE, Chung KY, Orgram CA (2001) Degradatio of 1, 3 dichloropreopen by soil bacterial consortium and *Rhodococcus* sp. *AS2C* isolated from the consortium. Biodegradation 12:39–47

Papiernik SK, Gan J, Yates SR (2000) Mechanism of degradation of methyl bromide and propargyl bromide in soil. J Environ Qual 29:1322–1328

Racke KD, Coats JR (1990) Enhanced biodegradation of pesticides in the environment. ACS symposium series, American Chemical Society, Washington DC, No 426

Read D.C. (1987) Greatly accelerated microbial degradation of aldicarb in the treated field soils, in flooded soil, and in water. J. Econ. Entomol. 80:156–161

Roeth FW (1986) Enhanced herbicide degradation in soil with repeated application. Weed Sci 2:45–65

Smelt JH, Leistra M (1974) Conversion of metam sodium to methyl isothiocyanate and basic data on the behaviour of methyl isothiocyanate in soil. Pest Sci 5:401–407

Smelt JH, Crum SJH, Teunissen W (1989a) Accelerated transformation of the fumigant methyl isothiocyanate in soil after repeated application of metam-sodium. J Environ Sci Health 24:437–455

Smelt JH, Teunissen W, Crum SJH, Leistra M (1989b) Accelerated 1, 3-dichloropropene transformation in loamy soils. Neth J Agric Sci 37:173–183

Smelt JH, van de Peppel Groen AE, van der Pas LJT, Dijksterhuis A (1996) Development and duration of accelerated degradation of nematicides in different soils. Soil Biol Biochem 28:1757–1765

Suett DL, Fournier JC, Papadopoulou-Mourkidou E, Pussemier L, Smelt J (1996a) Accelerated degradation: the European dimension. Soil Biol Biochem 28:1741–1748

Suett DL, Jukes AA, Parekh NR (1996b) Non-specific influence of pH on microbial adaptation and insecticide efficacy in previously-treated field. Soil Biol Biochem 28:1783–1790

Taylor GE, Schaller KB, Geddes JD, Gustin MS, Lorson GB, Miller GC (1996) Microbial ecology, toxicology and chemical fate of methyl isothiocyanate in riparian soils from the upper Sacramento river. Envir Toxicol Chem 13:1694–1701

Triky-Dotan S, Austerweil M, Steiner B, Peretz-Alon Y, Katan J, Gamliel A (2007) Generation and dissipation of methyl isothiocyanate in soils following metam sodium fumigation: impact on Verticillium control and potato yield. Plant Dis 91:497–503

Triky-Dotan S, Austerweil M, Mintz D, Katan J, Gamliel A (2008) Accelerated degradation of metam sodium in soil: occurrence and possible mechanisms. Phytopathology 98(5 suppl):159

Triky-Dotan S, Austerweil M, Steiner B, Peretz-Alon Y, Katan J, Gamliel A (2009) Accelerated degradation of metam-sodium in soil and consequences on root-disease management. Phytopathology 99:(in press)

Turner NJ, Corden ME (1963) Decomposition of sodium *N*-Methyldithiocarbamate in soil. Phytopathology 53:1388–1394

van Dijk H (1980) Dissipation rates in soil of 1,2-dichloroporpane and 1, 3- and 2, 3-dichloropropenes. Pest Sci 11:625–632

Verhagen C, Smit E, Janssen DB, Van Elsas JD (1995) Bacterial dichloropropene degradation in soil: screening of soils and involvement of plasmids carrying the *dhlA* gene. Soil Biol Biochem 27:1547–1557

Verhagen C, Lebbink G, Bloem J (1996) Enhanced biodegradation of the nematicides 1, 3-dichloropropene and methyl isothiocyanate in a variety of soils. Soil Biol Biochem 28:1753–1756

Warton B, Matthiessen JN (2000) Enhanced biodegradation of metam sodium soil fumigant in Australia. Proc BCPC Conf – Pests & Diseases 4:377–380

Warton B, Matthiessen JN (2005) The crucial role of calcium interacting with soil pH in enhanced biodegradation of metam sodium. Pest Manag Sci 61:856–861

Warton B, Matthiessen JN, Roper MM (2001) The soil organisms responsible for the enhanced biodegradation of metam sodium. Biol Fertil Soils 34:264–269

Warton B, Matthiessen JN, Shackleton MA (2003) Cross-enhancement: enhanced biodegradation of isothiocyanates in soils previously treated with metham sodium. Soil Biol Biochem 35:1123–1127

Wilhelm SN, Shepler K, Lawrence LJ, Lee H (1996) Environmental fate of chloropicrin. In: Seiber JN (ed) Fumigant environmental fate: exposure and analysis. ACS Symp Ser 652:79–93. American Chemical Society, Washington, DC

Yarden O, Aharonson N, Katan J (1987) Accelerated microbial degradation of methyl benzimidazol-2-ylcarbamate in soil and its control. Soil Biol Biochem 19:735–739

Zheng W, Papiernick SK, Minxing G, Yates SR (2003) Competitive degradation between the fumigants chloropicrin and 1, 3 dichloropropene in unamended and amended soils. J Environ Quality 32:1735–1742

Chapter 23
Recent Developments in Disease Management

Behavior of Fumigants in soil

William Ntow and Husein Ajwa

Abstract Fumigants are vital agents in the production of many food crops, particularly the high-value crops such as strawberries, tomatoes, and grapes, which are susceptible to nematodes and other soil-borne pests. However, fumigants are highly volatile compounds that warrant exceptional safeguards to minimize environmental pollution and to ensure safety through improved application technology. It is therefore important to understand the transformation and behavior of fumigants in soil to better design optimum application techniques that would require minimal chemical input while maintaining adequate efficacy for controlling target organisms. This chapter examines the behavior mechanisms of soil fumigants that are considered potential replacements for methyl bromide, and that should lead to more informed decisions regulating fumigant use and application.

Keywords Fumigants • Methyl bromide alternatives • Iodomethane • Methyl isothiocyanate • Chloropicrin • 1,3-dichloropropene

23.1 Introduction

Soil-borne plant pathogens and parasitic nematodes can cause extensive damage to many crops, especially in intensive agriculture. Over the last few decades, soil fumigation has been the most widely used method for soil-borne pest control (Gan et al., 1999). The use of fumigants to control soil pests has, therefore, become a common agricultural practice to maximize the yield of various crops, especially in warm regions. Because of the broad range of pests controlled, soil fumigants are used as part of the production of a wide variety of crops and provide great benefits to many growers (http://www.epa.gov/oppsrrd1/reregistration/soil_fumigants/, accessed

W. Ntow and H. Ajwa (✉)
University of California, Davis, CA, USA
e-mail: haajwa@ucdavis.edu

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,*
Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_23,

August 27, 2008). In the US, particularly in California and Florida, fumigants are extensively used to grow strawberry (*Fragaria* × *ananassa* Rozier) and tomato (*Lycopersicon esculentum* Mill) (Dungan and Yates, 2003). Different fumigant application techniques are used depending on the formulation type, pest to be controlled, and timing of the application (Lembright, 1990). Granule formulations such as dazomet are applied to the soil surface then watered into the soil or mechanically incorporated. Liquid fumigants can be applied by directly injecting them into the soil or, in some cases, into the irrigation system. Usually the fumigants are injected anywhere from 30 to 60 cm below the soil surface (Dungan and Yates, 2003).

However, environmental concerns have been raised because of such negative attributes of fumigants as high volatility, toxicity or carcinogenicity (UNEP 1995; Baker et al., 1996). In particular, methyl bromide (MeBr) was found to contribute to stratospheric ozone depletion (Majewski et al., 1995; UNEP, 1995; Yagi et al., 1995; Yates et al., 1998). These concerns in combination with the frequent detection of fumigants in ambient air (van den Berg et al., 1994; Baker et al., 1996) mandate that the processes and factors that affect fumigant behavior in the environment be better understood. This chapter seeks to examine the behavior mechanisms of fumigants in soil, since behavior plays a large role in influencing the transport of these fumigants and, ultimately, their effectiveness against soil-borne pathogens.

Fumigants are pesticides which, when applied to soil, form a gas to control pests that live in the soil and can disrupt plant growth and crop production. The fumigants are either volatile chemicals that become gases at relatively low temperatures, around 40°F, or they are chemicals that react to produce such a gas (e.g., dazomet and metam-sodium converting to methyl isothiocyanate or MITC) (http://www.epa.gov/oppsrrd1/reregistration/soil_fumigants/, accessed August 27, 2008). Fumigants are usually heavier than air and commonly contain one or more halogen (Cl, Br, or F).

Although its use is now in decline because of environmental regulations, methyl bromide (MeBr) is the most heavily used of the fumigants; 68,424 metric tons were used worldwide in 1996, almost half of which were used in the United States (http://ipmworld.umn.edu/chapters/ware.htm, accessed July 29, 2008). The predominant use of MeBr is for preplant soil treatments, which accounted for 70% of that global total. Quarantine uses account for 5–8%, while 8% is used to treat perishable products, such as flowers and fruits, and 12% for nonperishable products, like nuts and timber. Approximately 6% is used for structural applications, such as for drywood termite fumigation of infested buildings (C&E News Nov. 9, 1998). With the changes to the Clean Air Act amendments of 1990, US production and importation must be reduced 25% from 1991 levels by 1999. A 50% reduction must be achieved by 2001, followed by a 70% reduction in 2003, and full ban of the product in 2005. Under the Montreal Protocol, developing countries have until 2015 to phase out methyl bromide production (C&E News Nov. 9, 1998). An update on the status of MeBr can be viewed at the following Environmental Protection Agency (EPA) website: http://www.epa.gov/ozone/mbr/.

Alternatives that can fully replace methyl bromide are unlikely to be available by the deadlines set for replacement. Its low cost and utility on a wide variety of pests are hard to match. Because the loss of methyl bromide has potentially large

economic consequences, EPA has made it a priority to find and register replacements. To this end some progress has been made. The chemical 1,3-dichloropropene (Telone®) was registered in 2001 for preplant soil fumigation in strawberries and tomatoes (http://ipmworld.umn.edu/chapters/ware.htm). Other chemicals, such as propargyl bromide and sodium azide were evaluated as alternatives to methyl bromide (Ajwa et al., 2003), but were not considered for registration in the USA.

Long before concerns regarding the use of methyl bromide surfaced, several chemical soil fumigants were already in use. In some cases those fumigants were used jointly with methyl bromide, in other cases they replaced MeBr, particularly in certain niche markets. Two such fumigants are 1,3-dichloropropene (1,3-D) and trichloronitromethane (chloropicrin).

Currently, there are only five registered chemical fumigants available: 1,3-D (marketed under the trade name Telone [DowAgroSciences LLC, Indianapolis, IN], which contains an equal ratio of *cis*-1,3-D and *trans*-1,3-D); MITC (primary a breakdown product of metam-sodium [sodium methyldithiocarbamate] or Basamid); Chloropicrin (trichloronitromethane, often formulated with Telone and metam-sodium); MeBr (Dungan and Yates, 2003); and Iodomethane (Methyl Iodide). In October 2007, the United States Environmental Protection Agency (USEPA) approved the registration of methyl iodide under highly restrictive provisions governing its use (http://www.epa.gov/pesticides/factsheets/iodomethane_fs.htm, accessed September 24, 2008). The fumigants 1,3-D and MITC are considered to be viable alternatives for MeBr. Although 1,3-D is effective against nematodes, it lacks herbicidal activity, and is often formulated with chloropicrin (e.g., Telone C-17 and C-35, which contain 17% and 35% chloropicrin, respectively) to provide control of some fungal pathogens. Methyl isothiocyanate is effective against nematodes and a variety of weeds and fungal pathogens (Dungan and Yates, 2003). Recently (May 2007), the USEPA has granted an Experimental Use Permit (EUP) for Arkema's new soil fumigant called Paladin™ (Dimethyl disulfide, DMDS) (http://www.arkema-inc.com/index.cfm?pag = 343&PRR ID = 713, accessed October 5, 2008). The permit will allow formulations of this new active ingredient to be evaluated by designated growers on commercial farms in several States in the USA (Florida, Georgia, and North Carolina). Arkema submitted dossier to the USEPA for registration of DMDS as a new active ingredient in February 2007. EUPs have been received for France, Israel and Morocco. The chemical structures of MeBr and chemical alternatives are summarized in Fig. 23.1. Following is a detailed description of the behavior of these five registered fumigants. Nevertheless, chemicals that may be registered in the future are mentioned.

23.2 Methyl Bromide

Methyl bromide (Fig. 23.1) is a colorless, non-flammable, low boiling point chemical with high vapor pressure (Table 23.1) and reasonable water solubility (13.4 g litre^{-1}) (Yates et al., 1996a). It is applied by shank injection as a liquid, but with a high

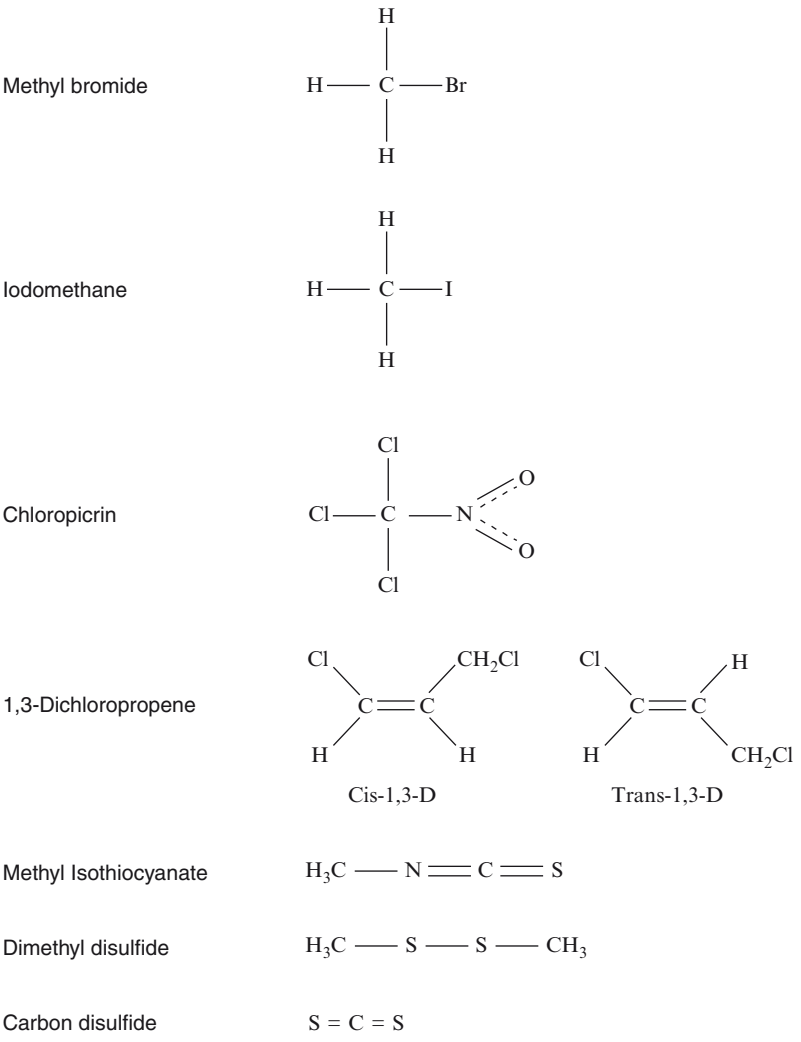


Fig. 23.1 Chemical structure of methyl bromide and selected alternative fumigants

vapor pressure of about 1600 mmHg at 20°C, the liquid MeBr quickly vaporizes and begins diffusing outward from the lines of injection through the soil air space. It has been reported that as much as 21% to 87% of the applied MeBr is released to the atmosphere (Yagi et al., 1993, 1995; Yates et al., 1997).

The Henry's law (K_H) constant for methyl bromide, a measure of its partition potential between air and water phases, is 0.24 at 20°C (Table 23.1). Generally, at 50% water saturation, the mobility of compounds of high K_H (>10^{-4}) is considered to be the result of diffusion in the vapor phase and therefore very fast.

Table 23.1 Physicochemical properties of soil fumigants

Fumigant	Boilingpoint (°C)	Density(g mL^{-1})	Water solubility (mg L^{-1})	Vapor pressure (mmHg)	K_H[a]	Rf[b]
Methyl bromide	3.6	1.73 (0°C)	13,400	1,600 (20°C)	0.24 (20°C)	2.37
Methyl iodide	42.4	2.28 (20°C)	14,000	398 (20°C)	0.21 (25°C)	n.a.
1.3-D (cis)	104	1.22 (20°C)	2,320	34.3 (25°C)	0.074 (25°C)	2.81
1.3-D (trans)	113	1.22 (20°C)	2,180	23.0 (25°C)	0.043 (25°C)	2.79
Chloropicrin	112	1.65 (20°C)	1,600	18.3 (20°C)	0.10 (20°C)	n.a.
MITC	119	1.05 (24°C)	8,200	19.0 (20°C)	0.01 (20°C)	1.34
Dimethyl disulfide	110	1.06 (16°C)	4,200	22.0 (20°C)	0.05 (20°C)	1.53
Carbon disulfide	45.5	1.26 (20°C)	2,940	352.6 (25°C)	0.078 (10°C)	0.90

[a]Henry's Law constant (dimensionless).
[b]Retention factor.
n.a., not available.

The soil/water adsorption coefficient (K_d) for methyl bromide in three soil types was negligible. However, in soil of high organic content (potting mix) the adsorption was stronger (0.20). These results and the value for K_H indicate that more methyl bromide will be in the gas phase, and that it will diffuse very fast in the soil. Therefore, the predominant mechanism that induces the spreading of methyl bromide through the soil profile is vapor diffusion (Goring, 1962; Kolbezen et al., 1974; Reible, 1994). After injection, which may involve a short period where pressure-driven flow dominates, liquid methyl bromide vaporizes and methyl bromide moves throughout the soil in response to the phase-change expansion and the initially high gradients near the injection points. As this process continues, methyl bromide quickly approaches the surface where it can escape into the atmosphere.

Although diffusion is a primary spreading mechanism in soil, other mass flow processes have been suggested as potentially important in moving gases through the root zone (Yates et al., 1996a). For example, changes in barometric pressure caused from wind at the surface or density sinking may induce a mass flow. Along with volatilization at the soil surface, degradation is one of the principal factors removing methyl bromide from the treated area (Gentile et al., 1992; Gan et al., 1994). In soil, MeBr is mainly degraded chemically, by chemical hydrolysis and methylation through a S_N2 nucleophilic substitution with water and nucleophilic sites on soil organic matter (OM), respectively (Gan et al., 1994):

$$CH_3Br + H_2O \rightarrow CH_3OH + H^+ + Br^- \quad (1)$$

$$CH_3Br + OM \rightarrow CH_3OM + H^+ + Br^- \quad (2)$$

The addition of 5% composted manure to the top 5 cm of a packed soil column was reported to reduce MeBr emissions by 12% (Gan et al., 1998).

Bacteria have also been implicated by the oxidation of MeBr (Rasche et al., 1990; Oremland et al., 1994; Miller et al., 1997; Ou et al., 1997). This reaction is believed to be catalyzed by monooxygenase (Dungan and Yates, 2003):

$$CH_3Br + 1/2O_2 \rightarrow H_2CO + H^+ + Br^- \quad (3)$$

Rasche et al. (1990) found that two soil ammonia-oxidizing nitrifiers, *Nitrosomonas europaea* and *Nitrosolobus multiformis*, consumed MeBr only in the presence of ammonium chloride. Inhibition of biodegradation by allylthiourea and acetylene, specific inhibitors of the ammonia monooxygenase, suggests that the enzyme catalyzed MeBr degradation. Oremland et al. (1994) showed that a methanotrophic bacterium, *Methyloccus capsulatus*, was also capable of co-oxidizing MeBr when incubated in the presence of methane. Methyl bromide did not support growth of the methanotroph. Miller et al. (1997), however, isolated a Gram-negative aerobic bacterium that was able to utilize MeBr as a sole C and energy source (Dungan and Yates, 2003).

23.3 Methyl Iodide (Iodomethane)

Methyl iodide (MeI) was proposed as a potential candidate as a direct replacement for MeBr in soil fumigation (Becker et al., 1995; Sims et al., 1995; Ohr et al., 1996). In extensive laboratory and field-plot trials, MeI was shown to be equivalent to or more efficient than MeBr for controlling a wide variety of soil-borne pests including weeds, nematodes, and fungi (Becker et al., 1995; Sims et al., 1995; Ohr et al., 1996). Methyl iodide has also been tested on a small scale for controlling various pests in stored products (Muthu and Srinath, 1974). The main advantage of MeI over MeBr is that it degrades quickly in the troposphere via photolysis and therefore is unlikely to contribute to ozone depletion (Gan and Yates, 1996). The estimated lifetime for MeI in the atmosphere is only 4–8 days compared with 1.5–2 years for MeBr, and its estimated ozone depletion potential (ODP) is only 0.016 compared with 0.6–0.7 for MeBr (Gan and Yates, 1996).

Some of the basic physical–chemical properties of MeI are listed along with MeBr and other fumigants in Table 23.1. The emphasis on MeBr here is due to the great similarities between MeI and MeBr. Methyl iodide is structurally analogous to MeBr, and in comparison to MeBr, MeI has a similar water solubility and higher boiling point. However, MeI has a vapor pressure of 398 mmHg (20°C) which is less than that of MeBr (Table 23.1). Methyl iodide, therefore, has a safety advantage over MeBr because it is a liquid rather than gas at normal handling temperatures. However, it is sufficiently volatile and mobile in soil to be called a true fumigant (Eayre et al., 2000).

Because of the similarities to MeBr, after application into soil as a fumigant, MeI could be expected to behave similarly to MeBr. However, the distribution of MeI and MeBr between the soil–water–air phases (Gan and Yates, 1996) is slightly different, and the difference will result in slower movement of MeI in the soil profile and less volatilization from the soil surface on the same time scale.

As the only irreversible process, degradation of MeI in soil is important in determining the fraction available for volatilization to the air and/or diffusing to the groundwater (Gan and Yates, 1996). As found for MeBr and many other volatile compounds, limited degradation in soil consistently leads to significant volatilization or downward movement of the chemical in the soil (Wagenet et al., 1989; Yates et al., 1996a,b). The degradation of MeI is considerably similar to that of MeBr. However, the persistence of MeI in soil was found to decrease with increasing soil organic matter content (Gan and Yates, 1996).

Degradation rates of methyl iodide in soil are typically measured in days and do not appear to be drastically affected by sterilization. This implies that MeI mainly degrades chemically in soil, by chemical hydrolysis and methylation, like MeBr, through a S_N2 nucleophilic substitution with water and nucleophilic sites on soil organic matter (OM), respectively (Equations 4 and 5).

$$CH_3I + H_2O \rightarrow CH_3OH + H^+ + I^- \quad (4)$$

$$CH_3I + OM \rightarrow CH_3OM + H^+ + I^- \quad (5)$$

23.4 1,3-Dichloropropene

The chemical 1,3-dichloropropene, considered as one of the most viable alternatives to MeBr (Noling and Becker, 1994), has been widely used as a pre-plant control of parasitic nematodes and fungi. Commercial formulations are registered under the names Telone II (975 g AI kg^{-1}, Dow Agrosciences) and D–D (Shell). Both contain nearly equal concentrations of the corresponding *cis* and *trans* isomers. Typical application rates for Telone II soil fumigant for field crop use on mineral soils range from 130 to 195 kg ha^{-1} with 388 kg ha^{-1} being the maximum rate (Batzer et al. 1996). Because 1,3-D has relatively lower vapor pressure and a higher boiling point than MeBr (Table 23.1), application of this fumigant using emulsified formulation through drip irrigation system has been shown to be more effective and safer than traditional shank injection. During application, the irrigation water acts as a vehicle for pesticide distribution, which provides a more uniform distribution of the chemical in soil. The high vapor pressure of 1,3-D gas ensures a high degree of diffusion within the soil and, hence, has a desirably extensive pesticidal effect. However, this property of 1,3-D also facilitates its potential transfer from the soil into the air above the soil surface. Model simulations and laboratory experiments estimated that 2% to 77% volatilization loss would occur after subsurface injection (McKenry and Thomason, 1974; Leistra and Frissel, 1975; Basile et al., 1986; Chen et al., 1995).

With K_H (25°C) values of 0.074 (*cis*) and 0.043 (*trans*), 1,3-D movement in soil may be expected to be dominated by gas phase diffusion but at a slower rate than methyl bromide (K_H 0.24). Vapor sorption coefficients have been reported in a humus sand at 0.026–0.071 (*cis*) and 0.015–0.042 cm^{-3} g^{-1} (*trans*) at 2–20°C. Yates and co-workers have also calculated the retention factor (RF) for 1,3-D (Table 23.1). The RF is an indicator of the effect of vapor partitioning on a pesticide's mobility and it includes parameters of soil bulk density, water, and air content. It represents the relative time needed for a pesticide to move past a specified depth compared to a non-adsorbing tracer. 1,3-D isomers exhibit similar RF (2.8), which is somewhat greater than that of methyl bromide (2.4). In fact, the maximum depth of detectable residues in soil dissipation studies was less than 3 m. This movement has been considered to be the result of diffusion rather than leaching.

Extensive studies under laboratory and field conditions have indicated that environmental dissipation of 1,3-D also occurs by hydrolysis and metabolism (Batzer et al., 1996). Hydrolysis of 1,3-D is a major pathway for degradation which is independent of pH. The half-life of 1,3-D was 11 days in sterile buffer (pH 7) at 20°C (McCall, 1987; Batzer and Yoder, 1995). The *cis* and *trans* isomers degrade rapidly in aqueous solutions under both light and dark conditions with half-lives of 1–6 days. The hydrolytic dechlorination reactions yield 3-chloroallyl alcohols as the major products (*cis*/*trans*-3-chloroprop-2-en-1-ol), which are subsequently oxidized to the corresponding acids (Batzer et al., 1996).

The degradation of 1,3-D on aerobic soils has been examined by numerous investigators (Leistra, 1970; van Dijk, 1980; Leistra et al., 1991; Batzer and Yoder, 1995; Batzer et al., 1996; Jeffers and Wolfe, 1996; Roby and Melichar, 1996; Dungan et al., 2001; Dungan and Yates, 2003) with half-lives ranging from 2 days (silty clay) to about

6 days on clay soils and 17 days on sands at 20°C. In some cases, repeated applications of 1,3-D resulted in shorter half-lives on aerobic soils. The major metabolites are 3-chloroallyl alcohol, 3-chloroacrylic acid, and carbon dioxide.

The soil-mediated degradation rates of the 1,3-D isomers have been reported to be similar (van Dijk, 1980; van der Pas and Leistra, 1987; Smelt et al., 1989; Leistra et al., 1991). The rate of degradation increased with increasing temperature, and half-lives of the two isomers ranged from a few days to a few weeks. The increased degradation at higher temperatures has been attributed to increased microbial metabolism, in addition to increased chemical degradation (Gan et al., 1999; Dungan et al., 2001). Soil moisture content, which is also known to influence microbial activity and pesticide degradation, had little effect on 1,3-D degradation in a sandy loam soil. However, in a loamy sand soil, degradation was 2.3 to 2.6 times faster (*cis*- and *trans*-1,3-D, respectively) at a soil moisture content of 16% than at 1.8% (w/w).

In soil, the degradation of *cis*- and *trans*-1,3-D is a combination of biological and chemical mechanisms (Ou, 1998; Chung et al., 1999; Gan et al., 1999). Both cis- and trans-1,3-D are initially hydrolyzed to corresponding *cis*- and *trans*-3-chloroallyl alcohol, which is mainly attributed to chemical mechanisms. However, in enhanced soils, biological hydrolysis was the main factor in the initial degradation of 1,3-D, especially *cis*-1,3-D to 3-chloroallyl alcohol, according to Ou et al. (1995). The isomers of 3-chloroallyl alcohol are then oxidized to *cis*- and *trans*-3-chloroacrylic acid, which are subsequently degraded to succinic acid, propionic acid, and acetic acid. The aliphatic carboxylic acids are finally mineralized to CO_2, H_2O, and Cl^-. Dungan and Yates, (2003) have reviewed the biological degradation of 1,3-D in soil.

23.5 Methyl Isothiocyanate (MITC)

MITC is a commonly used agricultural fumigant. It is a broad-spectrum pesticide with activity against plant pathogenic nematodes, weeds, oomycota, and a variety of plant pathogenic fungi (Duniway, 2002). MITC is the primary breakdown product from metam sodium (Fig. 23.1), and is considered as the active ingredient. Metam sodium has been distributed under a variety of trade names since the 1950s (e.g., Vapam HL, 42% metam sodium, Amvac Chemical Corp., Newport Beach, CA). Metam sodium has been used on a limited scale as a stand-alone fumigant for strawberry production in California for a long time. Metam sodium is typically applied as a 37 wt% solution in water (Draper and Wakeham, 1993). MITC can also be generated in soil using the granular product dazomet (trade name Basamid; BASF Corp., Mount Olive, NJ). As is the case for metam sodium, dazomet is not likely to be used as a stand-alone fumigant for strawberry production, but may be a useful addition to other fumigants in sequential applications.

MITC is unstable and decomposes to methylamine in water, probably via thiocarbamic acid. Faster hydrolysis rates are obtained at lower pH levels. MITC is much less susceptible to acid catalysis in water than its oxo-analog but will react with a great variety of nucleophiles. Because of its high vapor pressure (19 mmHg at 20°C; Table 23.1) it is important to understand its photolysis in the vapor phase.

Photolysis of MITC in the gas phase proceeds with a half-life of 10 h using a xenon arc lamp and nearly 1 day under sunlight. This rapid rate stands in contrast to that in aqueous solutions, where the reaction is 20 times slower. Multiple products are observed including methyl isocyanide, sulfur dioxide, hydrogen sulfide, *N*-methyl formamide, methylamine, and carbonyl sulfide. Methyl isocyanide, in turn, degrades to methyl isocyanate (Ruzo, 2006).

MITC is weakly sorbed and because of its volatility and water solubility, it can partition into both the vapor and water phases. Thus, it comes into contact with soils through leaching and diffusion. MITC exhibits only moderate diffusive mobility when compared with other soil fumigants. Both metam-sodium and dazomet convert efficiently to MITC in moist soils with half-lives in the order of hours to days, depending on ambient conditions. Particularly, inclusion of organic amendments into the soil surface has served to enhance degradation. For instance, Dungan and Yates, (2003) have reported that in soil, the degradation rate of MITC was about six times higher when amended with 5% composted chicken manure. The addition of compost or manure adds more organic matter to the soil which can develop a new microbial population with enhanced degradation capacity for MITC (Zhang et al., 2005).

The degradation of MITC is also influenced by soil temperature and moisture content. In a sandy loam soil, the degradation rate of MITC was about three times higher at 40°C than at 20°C (Dungan et al., 2002). Changes in the soil moisture content below saturation had little influence on the degradation rate of MITC in this soil, but in contrast to 1,3-D, degradation of MITC was 2.6 times slower at a soil moisture content of 16% than at 1.8% in a loamy sand soil (Gan et al., 1999). Thus, MITC degradation decreased with increasing soil moisture content but increased with increasing soil temperature, so its effect would be magnified in the hot, dry surface layer of soil (Gan et al., 1999). This knowledge can be useful for designing strategies to minimize the hazardous effects of fumigants on the environment while sustaining their use in production agriculture. For example, several nonchemical approaches, including soil solarization, flooding and steaming, are currently under development as alternatives to MeBr fumigation.

Since degradation of MITC in sterile soil is significantly slower than in nonsterile soil, degradation of MITC can be attributed to biological and chemical mechanisms (Dungan and Yates, 2003). At 20°C, microbial degradation accounted for as much as 50% to 80% of the total degradation. The accelerated degradation of other carbamate pesticides by adapted microorganisms has also been reported (Rahman et al., 1979; Felsot et al., 1981). Repeated applications of MITC to soils also appear to enhance degradation as a result of increased populations of adapted microorganisms (Smelt et al., 1989).

23.6 Chloropicrin

The early use and development of chloropicrin as a soil fumigant is reviewed elsewhere (Wilhelm, 1966; Wilhelm and Paulus, 1980; Noling, 1996). Chloropicrin fumigation has been used for many decades to control soil-borne pests.

It is typically applied together with MeBr as a warning agent or with 1,3-D as a fungicide to achieve broad-spectrum control. For instance, mixtures of MeBr with 2% chloropicrin and 1,3-D with 17% chloropicrin (i.e., Telone C-17) or 35% chloropicrin (i.e., Telone C-35) are used (Gan et al., 2000). Chloropicrin and its combinations with 1,3-D or MITC have been identified as effective replacements for MeBr in many field studies (Moldenke and Thies, 1996; South et al., 1997; Freitas et al., 1999; Porter et al., 1999; Trout and Ajwa, 1999). Therefore, it can be expected that the use of chloropicrin will increase (Gan et al., 2000).

Chloropicrin (Fig. 23.1) is a clear, colorless, nonflammable liquid with moderate vapor pressure and boiling point (Table 23.1). The physicochemical parameters for chloropicrin transport in soil have been reported (Wilhelm et al., 1996). Typically, chloropicrin is injected into the soil approximately 15–25 cm below the surface about 2 weeks before planting. Chloropicrin moves rapidly by diffusion in soils within 30 cm of injection but may diffuse much further to a maximum depth of 120 cm in the sandiest soils.

Degradation of chloropicrin in soil follows first-order kinetics (Gan et al., 2000). In three soil types (Arlington sandy loam, Carsitas loamy sand, and Waukegan silt loam), the half-life of chloropicrin is 1.5, 4.3, and 0.2 days, respectively, under aerobic conditions (Dungan and Yates, 2003). After sterilization of these soils, the degradation half-life of chloropicrin increased to 6.3, 13.9, and 2.7 days, respectively, which, suggests that soil microorganisms play an important role in the degradation of chloropicrin. On the basis of the difference in degradation rates between sterile and nonsterile soils, it was estimated that microbial degradation accounted for 68% to 92% of the overall chloropicrin degradation. The major metabolic pathway occurs through three successive reductive dehalogenations to nitromethane:

$$Cl_3CNO_2 \rightarrow Cl_2CHNO_2 \rightarrow ClCH_2NO_2 \rightarrow CH_3NO_2 \quad (6)$$

A small portion (about 4%) of the chloropicrin was also converted to CO_2 (Dungan and Yates, 2003). In an anaerobic aquatic (soil) environment, chloropicrin degraded very rapidly with a half-life of 1.3 h. Nitromethane was the major product. In both aerobic and anaerobic environments significant binding of radiocarbon to soil fulvic and humic fractions was observed. As with other fumigants, competitive degradation between chloropicrin and 1,3-D has been reported in amended and unamended soils (Zheng et al., 2003; Desaeger et al., 2004).

23.7 Dimethyl Disulfide

Dimethyl disulfide (DMDS) is a new pre-plant soil fumigant being developed by Arkema on a worldwide basis for the treatment of nematodes, weeds and soil-borne plant pathogens. Arkema is the world's largest producer of DMDS for use in the petroleum industry for hydro-desulfurization and other industrial applications. DMDS is a ubiquitous natural product, common in the global sulfur cycle and is detected as a metabolite in numerous biological processes. DMDS is not only

malodorous, but also very toxic for all organisms. It exerts a complex mode of action through mitochondria dysfunction and activation of ATP sensitive potassium channels and it has a powerful inhibition of the cytochrome oxydase (Auger et al., 2002 cited in Fritsch, 2005). The product is being evaluated in the USA and other countries for the control of weeds, nematodes, and fungal pathogens. The properties of DMDS are similar to the properties of MITC (Table 23.1). However, the behavior of DMDS in soil is not well documented. Recent research indicated that the half life of DMDS in soil is two to three times greater than the half life of MITC.

23.8 Sodium Tetrathiocarbonate

Sodium tetrathiocarbonate (STTC) is formulated as Enzone®, was first manufactured by Unocal Corp., Chemical Division, and currently is the product of Arysta Lifescince, Inc. Enzone® is a deep amber-colored, nonexplosive liquid formulation of sodium tetrathiocarbonate that breaks down in the soil to carbon disulfide (CS_2) gas, the active moiety. Enzone is registered both as a preplant and postplant fumigant but is used primarily postplant in established orchards or vineyards in California. Additional registrations outside the United States include vegetables, raspberries and strawberries. Sodium tetrathiocarbonate is not as volatile as other fumigants, e.g., methyl bromide, chloropicrin, 1,3-D, and does not move as easily with the soil air. It moves through the soil profile to the target pests more efficiently with soil moisture (Rf value~0.9) (Adaskaveg, 1999; http://mbao.org/1999airc/97philli.pdf, accessed October 11, 2008). Based on manufacturer's recommendations, STTC can be used at high concentrations as a preplant fumigant or at low concentrations as a postplant treatment possibly without causing phytotoxicity to growing plants at application sites.

The dissociation of STTC occurs by dilution or hydrolysis as described below:

$$Na_2CS_4 + 2H_2O \rightarrow CS_2 + H_2S + S + 2NaOH$$

The hydrolysis reaction is very fast and occurs within 2 h at water pH values of less than 9. Our research indicated that the generation of CS_2 in soil after the application of STTC is instantaneous and complete generation of CS_2 occurs within 1 h after application to sandy loam and clay loam soils. Therefore, the distribution of CS_2 depends of the application method that controls the distribution of the water soluble formulation (Enzone®).

23.9 Concluding Remarks

Effective control of soil-borne pathogens requires an understanding of the behavior of the fumigant in the soil in order to tailor the optimal application. This includes all the aspects of the physical–chemical characteristics of the chemical (listed in

Table 23.1), the soil conditions (especially soil temperature, soil moisture, pH, soil organic matter, soil texture and soil microorganisms), and their behavior in soil under field conditions. The fumigants described in this chapter differ in the pests they control, their ability to reach and penetrate the target pest, and their potential to move off-site. Fumigant applicators must be attentive to all these parameters to assure the best balance of field performance and environmental stewardship. Broad-spectrum soil fumigants, such as MeBr, MeI, MITC, chloropicrin and SSTC, provide growers reliable and excellent disease and pest control, increased yields, better product quality, extended crop seasons and more reliable economic returns.

Because of its high vapor pressure, MeBr fills the soil air space rapidly. This fumigant moves easily downwards and laterally during the first stage after application. Behavior of the gas in the soil is influenced strongly by simultaneously occurring sink processes, such as chemical and physical absorption, degradation, and escape into the atmosphere. Being highly volatile, MeBr dissipates from the soil after a short period (this behavior enables the planting of a new crop within a short time after the fumigation process). Chloropicrin, 1,3-D, MITC and STTC require a longer waiting period for re-planting to prevent damage due to phytotoxicity. MeI is considered to occupy a middle stand regarding this property.

Unlike MeBr, 1,3-D and chloropicrin, which are present at more or less constant concentration (the dosage of the active ingredient is given by the product formulation) of the active ingredient when the fumigant is applied to the soil, the application of metam sodium and dazomet involve a chemical and microbial degradation of the commercial molecule to generate the active ingredient MITC. Metam sodium is chemically hydrolyzed in the soil to generate MITC, reaching the peak of its concentration after a few hours. In contrast, application of dazomet involves microbial degradation and a slower rate of MITC generation, reaching the peak after 24–48 h (Gamliel, 2005). These results indicate that short exposure to metam sodium and dazomet (for only a few hours after application) may not be sufficient for efficient pest control, due to low concentration of MITC.

Although the dissipation of pesticides is environmentally desirable, rapid degradation has a negative consequence. The degradation of chloropicrin, 1,3-D, MITC and STTC and their dissipation from the soil last a few days, compared to MeBr and/or MeI. For instance, chloropicrin vaporizes and disperses in the soil more slowly than either MeBr or MeI because of its lower vapor pressure. It is considered nonpersistent in soil, degrading rapidly at a rate estimated at 20% per day at 25°C. The vapor pressure of 1,3-D is nearly twice that of chloropicrin, thus providing adequate dispersion through the soil principally as a gas. The fumigant has a short persistence and dissipates in the soil at a rate of about 12% a day at 25°C. Because of its lower vapor pressure and strong affinity for soil water, MITC disperses only a few centimeters through the soil when it is in its vapor phase. Therefore, the product must be distributed throughout the soil while still in the form of a MITC generator (e.g., metam sodium) that move more effectively. MITC has a short persistence and dissipates in soil at a rate of about 20% per day at 25°C. STTC has a very short half-life in soil (about 1 d at 25°C) and is considered nonpersistent. The degradation of fumigants should be taken into consideration when developing alternatives to MeBr.

The successful efforts spent in the past 17 years to understand the behavior of fumigants in soils and to develop and make available molecules which can successfully be applied to soil fumigation has had a positive impact on growers' concerns. In particular, combinations of metam with 1,3-D and chloropicrin, and of MeI with chloropicrin applied by drip irrigation, are showing excellent results in mitigating the impact of emissions on outdoor air quality. The USEPA, USDA, and the University of California, along with farmers, have been especially proactive in examining the interactions between the components of combinations of chemicals, and developing exposure mitigation strategies and the concept of buffer zones as applied to the emerging MeBr alternatives. Further research on the compatibility and/or incompatibility of 'cocktails' of fumigants is required to improve their application in order to achieve optimal soil pest control results. Combination of fumigants with different spectrum of target pests can extend the spectrum of control of these fumigants. In addition, combination of fumigants enables significant reduction of dosages and can make such application more environmentally acceptable.

References

Adaskaveg JE (1999) Efficacy of sodium tetrathiocarbonate and propiconazole in managing armillaria root rot of almond on peach rootstock. Plant Dis 83:240–246

Ajwa HA, Klose S, Nelson SD, Minuto A, Gullino ML, Lamberti F, Lopez-Aranda JM (2003) Alternatives to methyl bromide in strawberry production in the Unites States of America and the Mediterranean region. Phytopathol Mediterr 42:220–244

Baker LW, Fitzell DL, Seiber JN, Parker TR, Shibamoto T, Poore MW, Longley KE, Tomlin RP, Propper R, Duncan DW (1996) Ambient air concentrations of pesticides in California. Environ Sci Technol 30:1365–1368

Basile M, Senesi N, Lamberti F (1986) A study of some factors affecting volatilization losses of 1,3-dichloropropene (1,3-D) from soil. Agric Ecosyst Environ 17:269–279

Batzer FR, Balcer JL, Peterson JR, Wolt JD (1996) Fate of 1,3-dichloropropene in aerobic soils, in Fumigants: environmental fate, exposure and analysis, ACS Symp Series 652, Ch 7, pp 60–78

Batzer FR, Yoder RN (1995) Degradation of 1,3-dichloropropene in dilute aqueous solutions, in 210th ACS national meeting, Chicago, IL

Becker JO, Grech NM, Ohr HD, McGriffin M, Sims JJ (1995) Efficacy of methyl iodide against root-knot nematodes in bucket and field trials. In: Proceedings of the second annual international research conference on methyl bromide alternatives and emissions reductions, San Diego (6–8 November 1995), Methyl Bromide Alternatives Outreach, Fresno, CA, p 47

Chen C, Green RE, Thomas DM, Knuteson JA (1995) Modeling 1,3-dichloropropene fumigant volatilization with vapor-phase advection in the soil profile. Environ Sci Technol 29:1816–1821

Chung KY, Dickson DW, Ou L-T (1999) Differential enhanced degradation of *cis*- and *trans*-1, 3-D in soil with a history of repeated field application of 1, 3-D. J Environ Sci Health Part B B34:749–768

Desaeger JAJ, Eger JE, Csinos AS, Gilreath JP, Olson SM, Webster TM (2004) Movement and biological activity of drip-applied 1, 3-dichloropropene and chloropicrin in raised mulched beds in the Southeastern USA. Pest Manag Sci 60:1220–1230

Draper WM, Wakeham DE (1993) Rate constants for metam sodium cleavage and photodecomposition in water. J Agric Food Chem 41:1129–1133

Dungan RS, Yates SR (2003) Degradation of fuimigant pesticides: 1, 3-dichloropropene, methyl isothiocyanate, chloropicrin, and methyl bromide. Vadose Zone J 2:279–286

Dungan RS, Gan J, Yates SR (2001) Effect of temperature, organic amendment rate and moisture content on the degradation of 1, 3-dichloropropene in soil. Pest Manag Sci 57:1107–1113
Dungan RS, Gan J, Yates SR (2002) Accelerated degradation of methyl isothiocyanate in soil. Water Air Soil Pollut 142:299–310
Duniway JM (2002) Status of chemical alternatives to methyl bromide for pre-plant fumigation of soil. Phytopathol 92:1337–1343
Eayre CG, Sims JJ, Ohr HD, Mackey B (2000) Evaluation of methyl iodide for control of peach replant disorder. Plant Dis 84:1177–1179
Felsot A, Maddox JV, Bruce W (1981) Enhanced microbial degradation of carbofuran in soils with histories of Furadan use. Bull Environ Contam Chem 26:781–788
Freitas L, Dickson DW, Mitchell DJ (1999) Methyl bromide and chloropicrin effects on *Pasteuria penetrans*, In Proceedings of annual international research conference on methyl bromide alternatives and emissions reductions, San Diego (1–4 November 1999), Agricultural Research Consulting, Fresno, CA, p 24:1–5
Fritsch J (2005) Dimethyl Disulfide as a new chemical potential alternative to methyl bromide in soil disinfestations in France, in Proceedings VIth IS on chemical and non-chemical soil and substrate disinfestation, Acta Hort 698, ISHS, p 71–76
Gamliel A (2005) Behavior of fumigants in soil, In Proceedings VIth IS on chemical and non-chemical soil and substrate disinfestation, Acta Hort 698, ISHS, pp 41–50
Gan J, Yates SR (1996) Degradation and phase partition of methyl iodide in soil. J Agric Food Chem 44:4001–4008
Gan J, Yates SR, Anderson MA, Spencer WF, Ernst FF (1994) Effect of soil properties on degradation and sorption of methyl bromide in soil. Chemosphere 29:2685–2700
Gan J, Yates SR, Crowley D, Becker JO (1998) Acceleration of 1, 3-dichloropropene degradation by organic amendments and potential application for emissions reduction. J Environ Qual 27:408–414
Gan J, Papiernik SK, Yates SR, Jury WA (1999) Temperature and moisture effects on fumigant degradation in soil. J Environ Qual 28:1436–1441
Gan J, Yates SR, Ernst FF, Jury WA (2000) Degradation and volatilization of the fumigant chloropicrin after soil treatment. J Environ Qual 29:1391–1397
Gentile IA, Ferraris L, Sanguinetti M, Tiprigan M, Fisichella G (1992) Methyl bromide in natural fresh waters: hydrolysis and volatilization. Pestic Sci 34:297–301
Goring CAI (1962) Theory and principles of soil fumigation. Adv Pest Control Res 5:47–84
Jeffers PM, Wolfe NL (1996) Hydrolysis of methyl bromide, ethyl bromide, chloropicrin, 1,4-dichloro-2-butene and other halogenated hydrocarbons, in Fumigants: environmental fate, exposure and analysis, ACS Symp Series 652, Ch 4, pp 32–41
Kolbezen MJ, Munnecke DE, Wilbur WD, Stolzy LH, Abu-El-Haj FJ, Szuszkiewicz TE (1974) Factors that affect deep penetration of field soils by methyl bromide. Hilgardia 42:465–492
Leistra M, Frissel MJ (1975) Computations on the material balance of pesticides in soil, in Pesticides: environmental quality and safety, supplement vol 3. Thieme, Stuttgart, Germany, pp 817–828
Leistra M, Groen AE, Crum SJH, van der Pas LJT (1991) Transformation rate of 1,3-dichloropropene and 3-chloroallyl alcohol in top soil and subsoil material of flower-bulb fields. Pestic Sci 31:197–207
Leistra M (1970) Distribution of 1,3-dichloropropene over the phases in soil. J Agric Food Chem 18:1124–1126
Lembright HW (1990) Soil fumigation: principles and application technology. Suppl J Nematol 22:632–644
Majewski MS, McChesney MM, Woodrow JE, Prueger JH, Seiber JN (1995) Aerodynamic measurements of methyl bromide volatilization from tarped and nontarped fields. J Environ Qual 24:431–444
McCall PJ (1987) Hydrolysis of 1,3-dichloropropene in dilute aqueous solution. Pestic Sci 19:235–242
McKenry MV, Thomason IJ (1974) 1,3-Dichloropropene and 1,2-dibromoethane compounds: I. Movement and fate as affected by various conditions in several soils. Hilgardia 42:393–421

Miller LG, Connell TL, Guidetti JR, Oremland RS (1997) Bacterial oxidation of methyl bromide in fumigated agricultural soils. Appl Environ Microbiol 63:4346–4354

Moldenke AR, Thies WG (1996) Application of chloropicrin to control laminated root rot-research design and seasonal dynamics of control populations of soil arthropods. Environ Entomol 25:925–932

Muthu M, Srinath D (1974) Toxicity of methyl iodide to the adults of five beetles from stored products. J Stored Prod Res 10:65–66

Noling JW (1996) Role of soil fumigants in Florida Agriculture. In: Fumigants: environmental fate, exposure, and analysis, ACS Symp Series 652, Ch 2, pp 14–24

Noling JW, Becker JO (1994) The challenge of research and extension to define and implement alternatives to methyl bromide. J Nematol 26:573–586

Ohr HD, Sims JJ, Grech NM, Ole Becker J, McGiffen ME Jr (1996) Methyl iodide, an ozone-safe alternative to methyl bromide as a soil fumigant. Plant Dis 80:731–735

Oremland RS, Miller LG, Culbertson CW, Connell TL, Jahnke L (1994) Degradation of methyl bromide by methanotrophic bacteria in cell suspensions and soils. Appl Environ Microbiol 60:3640–3646

Ou L-T (1998) Enhanced degradation of the volatile fumigant-nematicides 1, 3-D and methyl bromide in soil. J Nematol 30:50–64

Ou L-T, Chung K-Y, Thomas JE, Obreza TA, Dickson DW (1995) Degradation of 1, 3-dichloropropene (1, 3-D) in soils with different histories of field applications of 1, 3-D. J Nematol 27:249–257

Ou L-T, Joy PJ, Thomas JE, Hornsby AG (1997) Stimulation of microbial degradation of methyl bromide in soil during oxidation of an ammonia fertilizer. Environ Sci Technol 31:717–722

Porter IJ, Brett RW, Nicholls JW, Rae JE, Mattner SW (1999). Will fumigants, biofumigants or IPM, adequately replace MB in Australia? In: Proceedings of annual international research conference on Methyl Bromide alternatives and emissions reductions, San Diego (1–4 November 1999), Agricultural Research Consulting, Fresno, CA, p 17:1–2

Rahman A, Atkinson C, Douglas JA, Sinclair DP (1979) Eradicane causes problems. NZ J Agric 139:47–49

Rasche M, Hyman MR, Arp DJ (1990) Biodegradation of halogenated hydrocarbon fumigants by nitrifying bacteria. Appl Environ Microbiol 56:2568–2571

Reible DD (1994) Loss of methyl bromide to the atmosphere during soil fumigation. J Hazard Mat 37:431–444

Roby DM, Melichar MW (1996) 1,3-Dichloropropene regulatory issues, in Fumigants: environmental fate, exposure and analysis, ACS Symp Series 652, Ch 3, pp 25–31

Ruzo LO (2006) Review: physical, chemical and environmental properties of selected chemical alternatives for the pre-plant use of methyl bromide as soil fumigant. Pest Manag Sci 62:99–113

Sims JJ, Grech NM, Ole Becker J, McGiffen M. Jr, Ohr HD (1995) Methyl iodide: a potential alternative to methyl bromide. In: proceedings of annual international research conference on methyl bromide alternatives and emissions reductions, San Diego (6–8 November 1995), Methyl Bromide Alternatives Outreach, Fresno, CA, p 46

Smelt JH, Crum SJH, Teunissen W (1989) Accelerated transformation of the fumigant methyl isothiocyanate in soil after repeated application of metam sodium. Environ Sci Health B24:437–455

South DB, Carey WA, Enebak SA (1997) Chloropicrin as a soil fumigant in forest nurseries. For Chron 73:489–494

Trout T, Ajwa H (1999) Strawberry response to fumigants applied by drip irrigation systems. In: proceedings of annual international research conference on methyl bromide alternatives and emissions reductions, San Diego (1–4 November 1999), Agricultural Research Consulting, Fresno, CA, p 10:1–3

United Nations Environment Programme (1995) The Montreal protocol on substances that deplete the ozone layer: 1994 report of the Methyl Bromide Technical Option Committee. UNEP, Nairobi, Kenya

Van den Berg F, Ross AH, Tuinstra LGMTh, Leistra M (1994) Measured and computed concentrations of 1, 3-dichloropropene and methyl isothiocyanate in air in a region with intensive use of soil fumigants. Water Air Soil Pollut 78:247–264

Van der Pas LJT, Leistra M (1987) Movement and transformation of 1,3-dichloropropene in the soil of flower-bulb fields. Arch Environ Contam Toxicol 16:417–422

Van Dijk H (1980) Dissipation rates in soil of 1,2-dichloropropene and 1,3- and 2,3-dichloropropenes. Pestic Sci 11:625–632

Wagenet RJ, Hutson JL, Biggar JW (1989) Simulating the fate of a volatile pesticide in unsaturated soil: a case study with DBCP. J Environ Qual 78:247–264

Wilhelm S (1966) Chemical treatments and inoculum potential of soil. Annu Rev Phytopathol 4:53–78

Wilhelm S, Paulus AO (1980) How soil fumigation benefits the California strawberry industry. Plant Dis 64:264–270

Wilhelm SN, Shepler K, Lawrence LJ, Lee H (1996) Environmental fate of chloropicrin. In: Fumigants: environmental fate, exposure, and analysis, ACS Symp Series 652, Ch 8, pp 79–93

Yagi KJ, Williams J, Wang NY, Cicerone RJ (1993) Agricultural soil fumigation as a source of atmospheric methyl bromide. Natl Acad Sci USA 90:8420–8423

Yagi KJ, Williams J, Wang NY, Cicerone RJ (1995) Atmospheric methyl bromide (CH_3Br) from agricultural soil fumigations. Science 267:1979–1981

Yates SR, Gan JY, Ernst FF, Mutziger A, Yates MV (1996a) Methyl bromide emissions from a covered field. I. Experimental conditions and degradation in soil. J Environ Qual 25:184–192

Yates SR, Ernst FF, Gan JY, Gao F, Yates MV (1996b) Methyl bromide emissions from a covered field. II Volatilization. J Environ Qual 25:192–202

Yates SR, Wang D, Ernst FF, Gan J (1997) Methyl bromide emissions from agricultural fields: bare-soil deep injection. Environ Sci Technol 31:1136–1143

Yates SR, Wang D, Gan J, Ernst FF, Jury WA (1998) Minimizing methyl bromide emissions from soil fumigation. Geophys Res Lett 25:1633–1636

Zhang Y, Spokas K, Wang D (2005) Degradation of methyl isothiocyanate and chloropicrin in forest nursery soils. J Environ Qual 34:1566–1572

Zheng W, Papiernik SK, Guo M, Yates SR (2003) Competitive degradation between the fumigants chloropicrin and 1, 3-dichloropropene in unamended and amended soils. J Environ Qual 32:1735–1742

Part V
Plant Breeding and Resistance Strategies

Chapter 24
Worldwide Monitoring Systems: The Need for Public and Private Collaboration

Roger D. Magarey, William E. Dolezal, and Thomas J. Moore

Abstract In recent years, invasive plant pathogens such as *Phytophthora ramorum* and *Puccinia graminis* (TTKS) have emerged as global threats that move rapidly across international boundaries. To counter such threats, the accurate monitoring of global plant health concerns necessitates the involvement of all agricultural sectors: governmental agencies, universities and the agricultural industry. Many members of industry conduct (i) scouting of research and seed production fields for pests, (ii) phytosanitary field inspections, and (iii) provide disease diagnostics testing for their customers. The Pest Information Platform (PIPE) proved to be an excellent vehicle for fostering collaborative efforts between the public and private sector in monitoring for Soybean Rust (*Phakopsora pachyrhizi*) in North America. The American Seed Trade Association's Phytosanitary Committee has developed a draft proposal for an industry PIPE platform for a narrow list of new and emerging pests of corn, soybeans and watermelon. Combining private industry and public pest data into a central database can provide (i) useful pest distribution information for state extension specialists; (ii) improved monitoring for emerging and exotic pest threats; and (iii) improved pest distribution records for state and federal regulators to enhance their decision making. Expanding PIPE like systems beyond national borders has potential to provide additional information for agencies to prepare and respond to exotic pest threats. Many members of industry with international operations have the capacity to jump start the development of a global PIPE. However, a global PIPE would require a comprehensive data sharing policy that protects national, state and corporate interests.

R.D. Magarey (✉)
Center for Integrated Pest Management, North Carolina State University,
1730 Varsity Drive, Suite 300, Raleigh, NC, 27606, USA
e-mail: Roger_Magarey@ncsu.edu

W.E. Dolezal
Johnston, Pioneer Hi-Bred International, IA, USA

T.J. Moore
Enza Coastal Seeds, Salinas, CA, USA

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,*
Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_24,

Keywords Decision support systems • Phytosanitary

24.1 Monitoring Invasive Pathogens

The economic and environmental costs of pest invasions sometimes indicate the need for improved plant biosecurity. It has been estimated that annual losses on agricultural crops – including the cost of control – are $14.1 billion for exotic arthropods and $21.5 billion for exotic plant pathogens (Pimentel et al., 2000). International trade has been the most important pathway for the accidental or purposeful introduction of invasive species into the United States (Mack et al., 2002; Mack, 2003; Le Maitre et al., 2004; Taylor and Irwin, 2004; Baker et al., 2005).

To counter such threats, the accurate monitoring of global plant health concerns necessitates the involvement of all agricultural sectors: governmental agencies, universities and the agricultural industry. In 2004, the United States Department of Agriculture (USDA) initiated the Integrated Pest Management Pest Information Platform for Extension and Education (*ipm*PIPE) to warn farmers of the threat of Asian soybean rust. The PIPE was an outstanding example of the success of an information platform. One of the limitations of the platform is the cost of data collection, mainly through an extensive network of sentinel plots in over 30 states. For example, the USDA *ipm*PIPE spends approximately $900,000 of federal funds to collect data for just two soybeans pests (Livingston et al., 2004). While this type of expenditure can be justified for an important exotic pest, it does not provide an effective model for expansion. This is where industry can provide assistance by supplementing public data collection.

Industry has extensive data gathering capabilities that have not yet been widely incorporated into pest monitoring systems (Dolezal, 2007). For example, seed companies routinely collect data from activities including (i) pest scouting of research and seed production fields, (ii) phytosanitary field inspections, and (iii) disease diagnostics testing for their customers. Another potential source of field survey data would be voluntary contributions from crop consultants. In the United States, there are thousands of crop consultants who routinely scout agricultural fields. Data could potentially be accessed through third party record keeping software. The reliability of different sources could be verified by cross checking with public or industry-certified diagnostic laboratories.

24.2 Potential Benefits of Data Sharing

The combination of private industry and public pest data in a central database can provide (i) useful pest distribution information for state extension specialists, (ii) improved monitoring for emerging and exotic pest threats, and (iii) improved pest distribution records for state and federal regulators to enhance their decision making.

In the United States the university extension system provides recommendations to farmers and industry. However, extension specialists often do not have access to privately held data and consequently, they may be unaware of the complete distribution of a pest. We believe that extension specialists could make more accurate recommendations with access to more complete pest distribution data. The distribution data may also be useful for epidemiological research (Christiano and Scherm, 2007).

Data sharing will also be useful to USDA Animal Plant Health Inspection Service, Plant Protection and Quarantine (APHIS-PPQ), the agency charged with plant regulatory responsibilities in the United States. In the event of a pest emergency, APHIS-PPQ needs a mechanism to quickly gather a pest's observed distribution from both public and industry sources. Improved regulatory decision making is the third reason to combine public and private datasets. In the United States, pest distribution data is useful for phytosanitary export certification and pest permitting. There is potential to create an online tool for addressing multiple requests or reporting requirements from regulatory authorities.

24.3 The Proposed Industry Pipe

The American Seed Trade Association (ASTA) is an industry body representing over 800 seed companies. The ASTA Phytosanitary Committee has developed a draft proposal for an industry Pest Information Platform (iPIPE) platform for a narrow list of new and emerging pests of corn, soybeans and watermelon (Table 24.1). Watermelon was included to engage horticultural seed companies. The incentive for ASTA companies to share data is to provide Federal and State regulators with better information for regulatory decision making. In particular, the industry data could be useful for proving that a pest is widespread (a permitting issue for interstate movement of plant pests) or proving that a pest is absent (export certification).

The proposed *i*PIPE will be based upon the same architecture as the USDA *ipm*PIPE for the information management of soybean rust (Isard et al., 2006). The PIPE components include data collection, modeling, integration, interpretation and dissemination (Fig. 24.1). Data collection begins with ASTA member companies voluntarily uploading data to the *i*PIPE. Data can be uploaded by an on-line form, an uploadable spreadsheet or by file transfer protocol. Other groups, such as crop consultants, may also contribute data. The collection of data may also involve cooperation between multiple organizations. For example, crop inspection data collected for phytosanitary export certification is usually held by individual state departments of agriculture. However, these data cannot be shared without permission from the involved companies. The *i*PIPE provides a mechanism to handle such complex data sharing relationships. As *i*PIPE expands there are plans to link it with other data sources such as the National Plant Diagnostic Network (NPDN) (Stack et al., 2006).

The next component after data collection is modeling. The PIPE system can combine model output and pest observations. In the *ipm*PIPE, models were used to predict the dispersal of Asian soybean rust and also its rate of epidemic development

Table 24.1 Suggested pests of concern for the proposed industry PIPE

Commodity Pest common name (scientific name)
Watermelon
Bacterial fruit blotch (*Acidovorax avenae* subsp. *citrulli*)
Gummy stem blight (*Didymella bryoniae*)
Fusarium wilt of watermelon (*Fusarium oxysporum* f. sp. *niveum and Fusarium oxysporum* f. sp. *melonis*) Race 1 and 2
Cucumber vein yellowing virus
Squash vein yellowing virus
Cucumber green mottle mosaic virus
Groundnut (peanut) bud necrosis virus
Soybean
Soybean aphid (*Aphis glycines*)
Asian soybean rust (*Phakopsora pachyrhizi*)
Frogeye leaf spot (*Botryosphaeria obtuse*)
Sudden death syndrome (*Fusarium solani* (Mart.))
Bean pod mottle virus
Corn
Southern corn rust (*Puccinia polysora*)
Goss's wilt (*Clavibacter michiganensis* subsp. *nebraskensis*)
Bacterial stalk rot (*Erwinia chyrsanthemi pv. zeae*)
Diplodia leaf blight (*Diplodia macrospora*)
Black bundle (*Cephalosporium acremonium*)

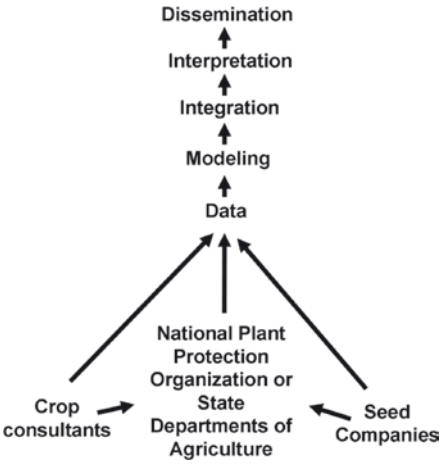


Fig. 24.1 Simplified information architecture for the industry Pest Information Platform

(Isard et al., 2007). In the industry PIPE, it is planned to provide model output by linkages to third party modeling tools such as NAPPFAST – the plant pest forecasting system developed by North Carolina State University and USDA-APHIS

(Magarey et al., 2007). NAPPFAST is a series of biological templates linked to North American and global weather databases. The interactive templates provide an easy method to develop models such as those for infection or day degree accumulation.

The next component of the PIPE is integration. Integration is complicated by the need to combine data from multiple sources. For example, there are a several pest identification codes (ID) in use in North America including those maintained by APHIS, NPDN, the European Plant Protection Organization and the International Society of Horticultural Science (ISHS). This integration requires a cross tabulation to translate a given pest ID from one format to another. The user is unaware of these complexities but enjoys an online interface that includes a map for spatial navigation and a calendar for moving in time (Fig. 24.2). The user also may see various types of model output, geographic data or images and commentary depending upon the pest or commodity selected.

Interpretation is the fourth component of the PIPE. In the *ipm*PIPE, soybean specialists provide commentary for their state and there is also a national commentary. Users can see the commentary by zooming on selected states. Interpretation, including the writing of pest commentaries and developing management guidelines, is a time consuming process and often underappreciated contribution to the success of pest information systems. While specialists benefit from having access to state or national

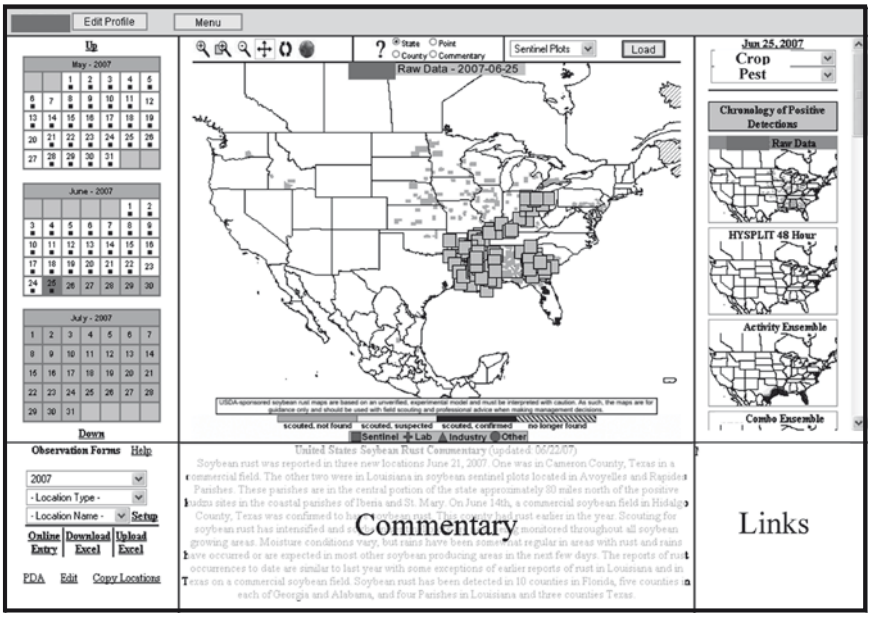


Fig. 24.2 Conceptual interface of a Pest Information Platform showing the calendar, observation upload tool, commentary and model thumbnails

observations, providing adequate compensation or professional recognition for specialists is a barrier to the development of PIPE systems.

The final and most important part to the PIPE is dissemination. There are many dangers associated with the dissemination of data and products. The misuse of data may result in (i) the disclosure of proprietary information, (ii) the imposition of trade barriers at the national or state level, and (iii) threats to national security. There are several design elements within the *i*PIPE that will provide protection from these dangers. Individual companies will not be identified in any products or data obtained by users through the *i*PIPE. Except under special circumstances, data will only be displayed at the county scale. All users of the *i*PIPE will have role based access. Role based access limits what a user can see and do by their organization (e.g. university, government, industry), their geographical responsibility (e.g. state, national, international) and their job role (e.g. inspector, researcher, extension, manager) (Sandhu and Coyne, 1996). In addition to the role based access, an administrator for each organization will control sharing (e.g., the movement of data in and out) with other organizations. Each organization will retain ownership of its data and may change data sharing preferences at anytime.

24.4 Towards a Global Pest Information Platform

The creation of a pest information platform on a global scale is a much more challenging undertaking than building a national system. Two examples of international data sharing initiatives include the NAPPO Phytosanitary Alert system and the *ipm*PIPE, both of which involve Canada, Mexico and the United States. One constraint on international data sharing is the need to cooperate with National Plant Protection Organization (NPPO). Although many seed companies have international laboratories and staff, the data they collect cannot be shared internationally without the permission of the NPPO. The NPPO is rightfully concerned about false reports which could negatively impact trade. The solution is to build into the PIPE data sharing mechanisms that allow an NPPO official to control data sharing of industry data collected within their jurisdiction.

We see slow progress towards a global monitoring system unless it becomes a higher priority for countries to share pest data. The *ipm*PIPE was developed within a matter of months following the introduction of Asian soybean rust into the United States. The system included the USDA, states, universities and industry. Without such a crisis, the development of such a system would have been more problematic. At present, trade sensitivities create a bigger incentive for NPPO to withhold data rather than to share. However, there is cooperation between countries that are part of the same trade blocs or regional plant protection organizations. We expect to see international data sharing efforts emerge for high priority pests on a regional basis first and expanded to additional pests and countries later.

Another important driver of the need for data sharing is the increasingly complex nature of what constitutes an exotic pest threat. Many emerging pest threats are not

new exotic species but rather new races, biotypes or pathotypes with virulence on previously resistant genotypes. An example, is TTKS (formerly UG99) – a new virulent race of wheat stripe rust (Jin et al., 2007). For the benefit of policy makers, we suggest terming these 'asymmetric' pest threats, and like terrorism, they can easily be missed by existing surveillance activities. The knowledge to detect asymmetric pest threats will increasingly rely upon industry collected data that includes details such as crop cultivar and phenology. We believe that in the next few years, global monitoring from industry will become essential to recognizing and combating these emerging asymmetric pest threats.

Acknowledgements We thank Joe Russo for reviewing the manuscript and for providing a preliminary version of Fig. 24.1.

References

Baker R, Cannon R, Bartlett P, Barker I (2005) Novel strategies for assessing and managing the risks posed by invasive alien species to global crop production and biodiversity. Ann Appl Biol 146:177–191

Christiano RSC, Scherm H (2007) Quantitative aspects of the spread of Asian soybean rust in the Southeastern United States, 2005 to 2006. Phytopathology 97:1428–1433

Dolezal W (2007) Field and diagnostic data collection: (Part 2) industry sources. Phytopathology 97:S140

Isard SA, Russo JM, DeWolf ED (2006) The establishment of a national pest information platform for extension and education. Plant Health Progress DOI.1094/PHP-2006-0915-01-RV

Isard SA, Russo JM, Ariatti A (2007) The Integrated Aerobiology Modeling System applied to the spread of soybean rust into the Ohio River valley during September 2006. Aerobiologia 23:271–282

Jin Y, Singh RP, Ward RW, Wanyera R, Kinyua M, Njau P, Fetch T, Pretorius ZA, Yahyaoui A (2007) Characterization of seedling infection types and adult plant infection responses of monogenic Sr gene lines to race TTKS of *Puccinia graminis* f. sp. *tritici.* Plant Dis 91:1096–1099

Le Maitre DC, Richardson DM, Chapman RA (2004) Alien plant invasions in South Africa: driving forces and the human dimension. South African J Sci 100:103–112

Livingston M, Johansson R, Daberkow S, Roberts M, Ash M, Breneman V (2004) Economic and policy implications of wind-borne entry of Asian soybean rust into the United States: USDA ERS Outlook Report No. (OCS04D02), April 2004

Mack RV (2003) Global plant dispersal, naturalization, and invasion: pathways, modes and circumstances. In: Ruiz GM, Carlton JT (eds) Invasive species, vectors and management strategies. Island, Washington, DC, pp 3–30

Mack RN et al (2002) Predicting invasions of nonindigenous plants and plant pests Washington. National Academy, DC

Magarey RD, Fowler GA, Borchert DM, Sutton TB, Colunga-Garcia M, Simpson JA (2007) NAPPFAST an internet tool for the weather-based modeling of plant pathogens. Plant Dis 91: 336–345

Pimentel D, Lach L, Zuniga R, Morrison D (2000) Environmental and economic costs of nonindigenous species in the United States. BioScience 50:53–64

Sandhu RS, Coyne EJ (1996) Role-based access control models. Computer 2:38–47

Stack J et al (2006) The national plant diagnostic network. Plant Dis 90:128–136

Chapter 25
Identification of New Sources of Resistance to Soybean Rust

Shuxian Li

Abstract Soybean rust, caused by *Phakopsora pachyrhizi*, is one of the most destructive diseases of soybean (*Glycine max* (L.) Merr.). Yield losses caused by soybean rust were reported from 13% to 80%. Chemical treatment with fungicide has been used as the first line of defense to minimize the impact of soybean rust. However, breeding for resistance to soybean rust is one of the most effective long-term strategies for controlling the disease. In the following chapter, the general information about soybean rust disease and its causal pathogens, reported resistance genes and the approaches for evaluation and identification of sources of resistance are presented and discussed.

Keywords Soybean • Soybean rust • *Phakopsora pachyrhizi* • Disease resistance

25.1 Introduction

25.1.1 Discovery of Soybean Rust in the World

Soybean rust is one of the most important foliar diseases of soybean (*Glycine max* (L.) Merr.). It was reported for the first time in 1902 in Japan (Hennings, 1903). Torama Yoshinaga collected the soybean rust fungus on soybean in September of 1902 at Inabu-mura, Tosa Province (now Kochi Prefecture), Shikoku island (Kitani and Inoue, 1960). In other documents (Bresadola and L'abbé, 1891; Zhuang, 1991), it was reported that Moller found the rust on *Vignea litea* leaves in Sao Thome Island, Africa in 1887, and the fungus was named *Uredo vignae* Bres (Bresadola and L'abbé, 1891). Later, the fungus was referred to the synonymy of *Phakopsora*

S. Li (✉)
United States Department of Agriculture–Agricultural Research Service, Crop Genetics and Production Research Unit, Stoneville, MS38776, USA
e-mail: Shuxian.li@ars.usda.gov

U. Gisi et al. (eds.), *Recent Developments in Management of Plant Diseases,* Plant Pathology in the 21st Century 1, DOI 10.1007/978-1-4020-8804-9_25,

pachyrhizi (Hiratsuka, 1935; Bromfield, 1984; Ono et al., 1992). Since then, the pathogen has spread from Asia (Hennings, 1903; Hiratsuka, 1935; Sharma and Mehta, 1996; Tan et al., 1996a), to Australia in 1934 (Kochman, 1977), South America in 2001 (Rossi, 2003; Yorinori et al., 2005) and North America in 2004 (Schneider et al., 2005). The widespread distribution and potential for severe yield losses makes soybean rust one of the most serious foliar diseases of soybean worldwide.

25.1.2 Distribution of Soybean Rust in the United States

The first discovery of soybean rust in the United States was in Hawaii in 1994 (Killgore et al., 1994), and the disease was first detected in the continental United States in Louisiana in November 2004 (Schneider et al., 2005). Since soybean rust was observed at approximately 5° latitude in South America before several hurricanes impacted the continental Unites States in September 2004, the introduction of soybean rust might be associated with at least one of those tropical storms, especially hurricane Ivan that carried rust spores and deposited then in the Gulf Coast and the Southeastern United States (Schneider et al., 2005). In 2004, following the first discovery in Louisiana, soybean rust also was confirmed in eight additional southern states – Alabama (Mullen et al., 2006), Arkansas, Florida (Harmon et al., 2005), Georgia, Mississippi (Li et al., 2007), Missouri, South Carolina, and Tennessee. In 2005, it was found for the first time in Kentucky (Hershman et al., 2006), North Carolina (Koenning et al., 2006), and Texas (Isakeit et al., 2006). In 2006, Illinois (Hartman et al., 2007), Indiana, and Virginia (Phipps et al., 2007) reported their finding of soybean rust for the first time. In 2007, soybean rust was confirmed in Iowa (Li et al., 2008), Kansas (Jardine, 2007), Nebraska, and Oklahoma. By the end of 2007, soybean rust had occurred in 250 countries in 19 states in the United States. Information about the occurrence of soybean rust in the United States can be obtained from the website at www.sbrusa.net.

25.1.3 Causal Organisms and Its General Biology

Soybean rust is caused by either of two obligate fungal species: *Phakopsora meibomiae* (Arthur) Arthur or *Phakopsora pachyrhizi* Sydow and P. Sydow. *P. meibomiae* has only been found in limited areas in the Western Hemisphere and is not known to cause severe yield losses in soybean (Sinclair and Hartman, 1999). *P. meibomiae* is referred to as the tropical, Latin American or New World rust strain, while *P. pachyrhizi* is also called Asian soybean rust (ASR), one of the major diseases of soybean in many Asian countries and causes severe year losses. *P. pachyrhizi* is a much more virulent pathogen of soybean than *P. meibomiae*. To date, only *P. pachyrhizi* has been found in the United States. Physiological specialization of *P. pachyrhizi* in soybean has been known for several decades (Bromfield, 1984). Variations of isolates on soybean have been reported (Bromfield

et al., 1980; Hartman et al., 2004). These two pathogens shared only 80% nucleotide sequence similarity within the ribosomal internal transcribed space region (Frederick et al., 2002).

Morphology of the two soybean rust causal species have been well-studied and described (Bonde and Brown, 1980; Ono et al., 1992). Urediniospores of *P. pachyrhizi* (15–24 × 18–34 μm) are obvoid to broadly elliptical with walls that are 1.0 μm thick, minutely and densely echinulate, and colorless to pale yellowish brown (Sinclair and Hartman, 1999). The sizes of urediniospores were measured 18–34 × 15–24 μm. Paraphyses were cylindric toclavate, surrounding the margin of a uredinium (Fig. 25.1; Li et al., 2007). Urediniospores of *P. meibomiae* (12–24 × 16–31 μm) are ellipsoid, densely echinulate, and colorless to pale yellow brown (Sinclair and Hartman, 1999).

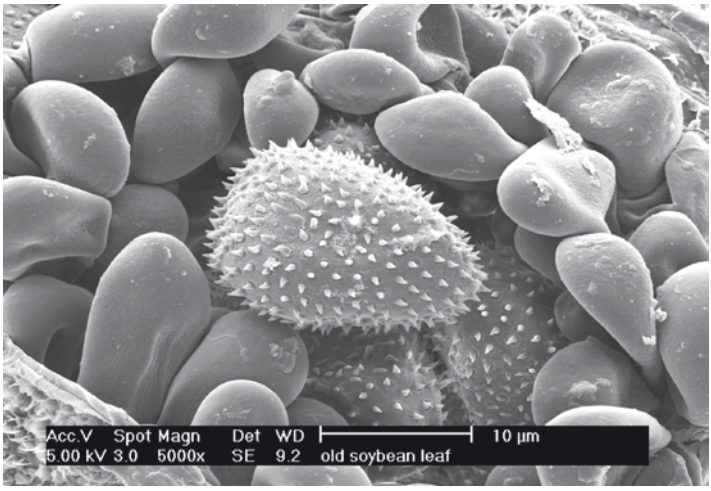


Fig. 25.1 Scanning electron micrograph of urediniospores of a *Phakopsora pachyrhizi* isolate (MS06-1) from soybean

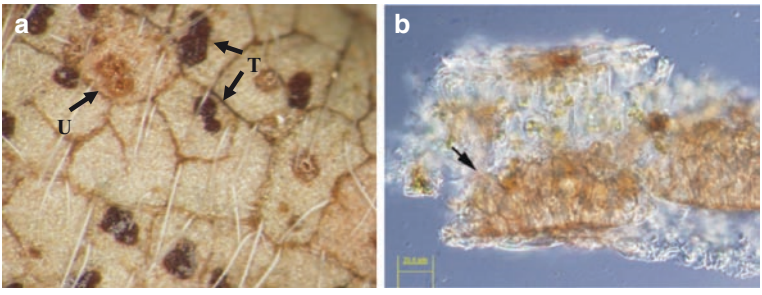


Fig 25.2 (**a**): Telia (T) and uredinia (U) of *Phakopsora pachyrhizi* on the abaxial leaf surface of kudzu; (**b**): Telia and teliospores (*arrow*) of *P. pachyrhizi* in a cross section of a kudzu leaf (courtesy T. Isakeit)

The two soybean rust causal species differ morphologically in the number of cell layers present in the telium. Telia of *P. pachyrhizi* have 2–7 spore layers (Fig. 25.2). Teliospores are yellow-brown to hyaline, and the teliospore walls are 1–3 μm, while telia of *P. meibomiae* contains teliospores arranged in layers of 1–4. Teliospores are cinnamon to chestnut brown, and teliospore walls are 1.5–6 μm (Sinclair and Hartman, 1999).

Environmental effects on the urediniospores germination have been studied. It was reported that urediniospores germinated in the dark within 1–2 h on inoculated leaves at 20°C in dew chambers (Bonde et al., 1976). The optimum temperature for germination in the dark is about 15–20°C, and that minimum and maximum temperatures for germination were 8°C and 33°C, respectively (Keogh, 1974). No germination was observed at 5°C or 35°C (Keogh, 1974). The effects of solar irradiance on the mortality of *P. pachyrhizi* urediniospores also were studied. Urediniospores exposed to doses of solar and ultraviolet (UV) radiation ≥27.3 and ≥1.2 MJ/m^2, respectively, did not germinate (Isard et al., 2006). In addition, appressorium formation, penetration, colonization, and sporulation are influenced by biotic (pathogen and host) and abiotic factors (environment).

Teliospores of *P. pachyrhizi* have been reported to occur naturally on soybean and other legumes in different locations (Bromfield, 1984), including in the United States (Harmon et al., 2006; Isakeit and Jo, 2008). Production of telia of soybean rust can be induced by regulation of the temperature (Saksirirat and Hopp, 1991). Telia were observed on soybean plants within 30 days when they were kept at 15°C overnight (Yeh et al., 1981).

Soybean rust has a broad host range (Rytter et al., 1984; Sinclair and Hartman, 1999; Lynch et al., 2006). *P. pachyrhizi* is currently reported to occur on approximately 150 species in 53 genera of the legume family Fabaceae. Approximately 120 of the known host of *P. pachyrhizi* grow in North America (Slaminko et al., 2008). For example, kudzu (*Pueraria lobata)*, widespread in the United States, is one of the hosts of *P. pachyrhizi* and may play an important role in the epidemiology of the disease as overwintering host or source of inoculum to soybean.

25.1.4 Disease Symptoms and Impacts

The most common soybean rust symptoms are gray green, tan to dark brown or reddish brown lesions with one to many erumpent, globose uredinia, particularly on the undersides of the leaflets (Sinclair and Hartman, 1999). The color of a lesion depends on the virulence of the pathogen, host genotype, and the interaction between the host and the pathogen. Lesions can be formed not only on leaf (Fig. 25.3a, b), but also on cotyledon (Fig. 25.3c) and petioles and small stems (Fig. 25.3d). Three infection types are caused by *P. pachyrhizi* on soybean (Bromfield, 1984; Bromfield and Hartwig, 1980): TAN, RB, and Immune (Fig. 25.4a, b, c). In general, "TAN" refers to the tan lesion type, a susceptible reaction that is associated with high rust

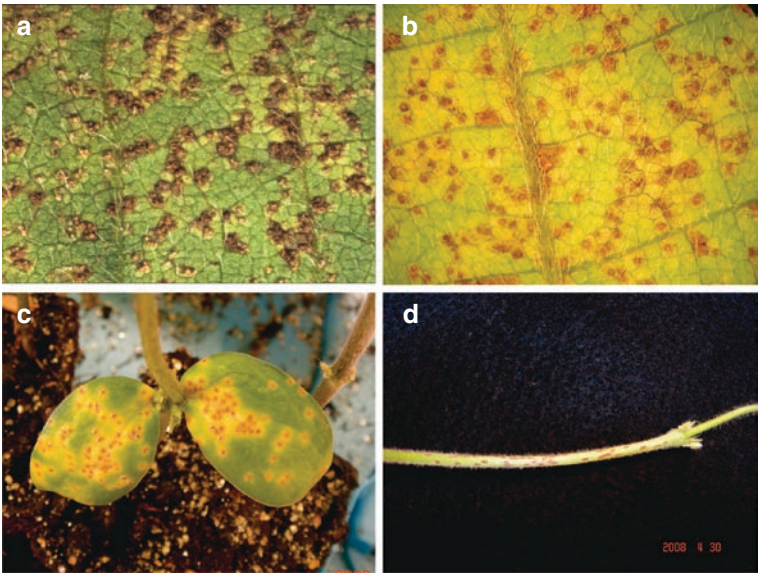


Fig. 25.3 Asian soybean rust lesions. (**a**): Affected soybean leaf that was collected from Stoneville, Mississippi in October 2006; (**b**) Affected kudzu leaf that was collected from Jefferson County in August, 2006; (**c**) Lesion on cotyledon of soybean; and (**d**) Lesion on soybean stems. Pictures were taken by S. Li at the USDA-ARS Stoneville Research Quarantine Facility, Mississippi, USA

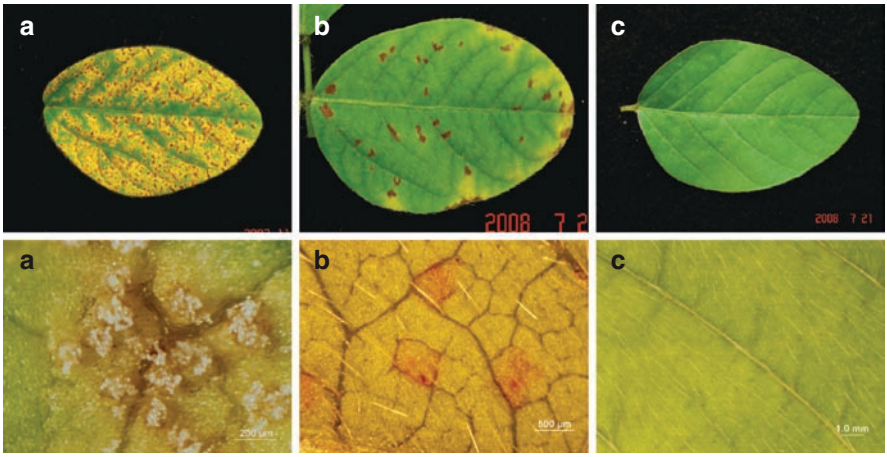


Fig. 25.4 Infection types of soybean caused by *Phakopsora pachyrhizi* by visual (*upper row*) and microscopic (*lower row*) observations. (**a**): TAN lesion type; (**b**): RB lesion type; (**c**): Immune reaction. Pictures were taken by S. Li at the USDA-ARS Stoneville Research Quarantine Facility, Mississippi, USA

severity with abundant sporulation, while "RB" refers to the reddish brown lesion color, considered a resistant reaction with lower severity and reduced sporulation. Some RB lesions can be well-sporulated (Li, 2008, unpublished). The "Immune" means lack of visible infection.

Soybean is susceptible to *P. pachyrhizi* at any stage of development (Melching et al., 1989). Severely infected plants show premature defoliation and reduced green leaf area that correlates negatively with yield. Plants that are heavily infected have fewer pods and smaller seeds that are of poor quality. Yield losses caused by soybean rust were reported from 13% to 80% (Ogle et al., 1979; Yang et al., 1990, 1991; Sinclair and Hartman, 1995). Soybean rust has the potential to cause significant yield losses and major economic damage to United States soybean production (Grau et al., 2004; Livingstone et al., 2004), although the current impact of soybean rust on soybean production in North America has been limited since it arrival in 2004 to the southern United States. However, substantial yield losses were reported in some fields in Alabama and Georgia in 2005 and in Louisiana in 2006, as well as in some research plots in those states and Florida and South Carolina (Sikora and Hershman, 2007).

25.1.5 Disease Management

Several strategies have been used to manage soybean rust and to reduce the impact of the disease: including chemical approaches with fungicide; culture practices such as modification of planting date, control of wild weed hosts, and selection of planting sites (Pupipat, 1997); biological control (Blakeman and Fokkema, 1982); and genetics approaches, such as breeding for resistance (Bromfield, 1984).

Chemical treatment with fungicide has been used as the first line of defense to minimize the impact of soybean rust (Levy, 2005). Successful control of soybean rust using fungicide is the result of skillful utilization of appropriate fungicide and timely application. Early detection of soybean rust in a region is key to successful management of the disease (Dorrance et al., 2007). Although there are significant benefits, using fungicide for controlling soybean rust increases production costs. It was estimated that the costs to apply fungicide for soybean rust control ranged from $10 to $35 per acre per application (Dorrance et al., 2007). Application of high levels of fungicides to soybean field is not an environmental friendly approach and can raise a concern about the pollution.

25.2 Reported Soybean Rust Resistance Genes

25.2.1 Identification of Soybean Rust Resistance Genes

Breeding for resistance to soybean rust is one of the most effective long-term strategies for controlling soybean rust (Hartman et al., 2005; Monteros et al., 2007). Specific resistance to *P. pachyrhizi* has been found, four single dominant genes

were identified as *Rpp1* (Bromfield, 1984), *Rpp2* (Bromfield et al., 1980), *Rpp3* (Bromfield and Hartwig, 1980; Bromfield et al., 1980; Hartwig and Bromfield, 1983), and *Rpp 4* (Hartwig, 1986). The *Rpp1* locus was mapped between SSR markers BARC_Sct_187 and BARC_Sat_064 on linkage group G (Hyten et al., 2007), and the *Rpp2* and *Rpp4* loci were mapped on the linkage groups J and G, respectively (Silva et al., 2008). The red-brown lesion resistance gene for ASR from soybean 'Hyuuga', a Japanese cultivar, was mapped and confirmed on linkage group C2 (Monteros et al., 2007). Marker-assisted selection with the SSR markers on linkage group-C2 can be used to select lines with the Hyuuga RB lesion type that is associated with reduced soybean rust severity and lower sporulation (Monteros et al., 2007). Recently, *Rpp*5 gene (Garcia et al., 2008) and two major recessive soybean genes conferring soybean rust resistance (Calvo et al., 2008) were identified.

The name of reported single genes, linkage groups, original sources, *P. pachyrhizi* isolates used for the original inheritance studies and gene identification, and related references are summarized in Table 25.1.

Table 25.1 A list of reported soybean rust resistance genes, linkage group, original sources, *Phakopsora pachyrhizi* isolates used in the original inheritance studies, and the references

Reported single gene	LG[a]	Original source[b]	Isolate[c]	Plant reaction	References for *Rpp* gene identification	References for molecular mapping
Rpp1	G	PI200492 (Komata)	IN 73-1	Resistant	Hartwig and Bromfield (1983)	Hyten et al. (2007)
			TW 72-1	Susceptible		
			TW 80-2	Susceptible		
Rpp2	J	PI230970	AU 72-1, IN 73-1	Resistant	Bromfield and Hartwig (1980)	Silva et al. (2008)
			PH 77-1, TW 72-1	Resistant	Hartwig and Bromfield (1983)	
			TW82-2	Susceptible		
Rpp3	C2	PI462312 (Ankur)	IN 73-1	Resistant	Hartwig and Bromfield (1983)	Hyten et al. (2008, submitted)
			TW 72-1	Susceptible		
			TW 80-2	Susceptible		
		PI506764 (Hyuuga)	Georgia	Resistant	Monteros et al. (2007)	Monteros et al. (2007)
Rpp4	G	PI459025 (Bing Nan)	IN 73-1	Resistant	Hartwig (1986)	
			TW 72-1	Resistant		
			TW 80-2	Resistant		
Rpp5	N	PI200526	Brazil	Resistant	Garcia et al. (2008)	Garcia et al. (2008)

(continued)

Table 25.1 (continued)

Reported single gene	LG[a]	Original source[b]	Isolate[c]	Plant reaction	References for *Rpp* gene identification	References for molecular mapping
		PI200487	Brazil	Resistant		
		PI471904	Brazil	Resistant		
rpp5		PI200456	Brazil	Resistant		

[a]Linkage group.
[b]Accession number and cultivar name (in parentheses) of the original sources.
[c]Isolates of *Phakopsora pachyrhizi* used in the original inheritance studies. AU: Australia; Brazil: Collected from naturally infected greenhouse plants in Brazil, 2004; Georgia: Collected from field-grown soybean plants and surrounding kudzu plants in Georgia, USA, 2005; IN: India; PH: Philippines; and TW: Taiwan.

25.2.2 Evidences of Known Rpp Genes Were Defeated

Four reported genes condition resistance to a limited set of *P. pachyrhizi* isolates, but the ability of *P. pachyrhizi* to overcome single-gene resistance has also been reported. For example, PI200492 (cultivar Komata, *Rpp1*) was identified as resistant in germplasm evaluations during 1961 to 1963 (Bromfield, 1984). By 1966, susceptible lesions were found on plants of this line in field trials (Hartman et al., 2005). Similarly, PI230970 (*Rpp2*) was identified as resistant in field evaluation from 1971 to 1973, but in 1978, most of the lesions found on plants in the field were of the susceptible TAN type (Hartman et al., 2005).

Another case happened in Brazil. When soybean rust occurred for the first time there in 2001, all four of the resistance genes were at first effective against the disease, but in the following year, only *Rpp2* and *Rpp4* conferred resistance while *Rpp1* and *Rpp3* were defeated (Yorinori et al., 2005; Garcia et al., 2008).

25.3 Evaluation and Identification of New Sources for Resistance to Soybean Rust

Currently, no US soybean cultivars have been reported being resistant to soybean (Hartman, 1991). There is a need to identify new sources of soybean rust resistance. Scientists have been using different approaches to evaluate soybean genotypes and identify new sources for resistance to soybean rust.

25.3.1 Examples of Germplasm Evaluation

United States: Over 16,000 soybean accessions in the United States Department of Agriculture-Agricultural Research Service (USDA-ARS) germplasm collection were evaluated for rust resistance using a mixture of four *P. pachyrhizi* isolates that

were originally from Brazil, Paraguay, Thailand, and Zimbabwe (Miles et al., 2006). Experiments were performed under controlled conditions in the USDA-ARS Foreign Disease-Weed Science Research Unit (FDWSRU) Biosafety Level 3 (BSL-3) containment greenhouse at Ft. Detrick, Frederick, MD. Accessions that had low levels of disease severity were further evaluated in Africa and Paraguay against local soybean rust populations (Bandyopadhyay et al., 2006; Miles et al., 2008).

Since the arrival of soybean rust in North America in November 2004, field evaluations of accessions from the USDA soybean germplasm collection have been conducted at multiple locations in the southern USA (Walker et al., 2008b). In 2007, the reactions of 209 to 403 Plant Introductions (PIs) from Maturity Groups (MGs) 000-X were evaluated in seven locations in five states in the southeastern USA. Late planting date and artificial lighting in some sites were used to synchronize plant maturity with fall rust epidemic. Approximately 85 of the PIs that showed high to moderate resistance to rust at two or more locations were identified in 2007 (Walker et al., 2008b). It was also reported that the *Rpp1* gene conferred immunity to the local isolate at all locations, while *Rpp2*, *Rpp3*, and *Rpp?*(Hyuuga) reduced severity and sporulation at most locations. The reactions of other accessions with unknown resistance genes ranged from apparent immunity to reduced severity and/or sporulation relative to susceptible cultivars with similar maturities (Walker et al., 2008b). In addition, variations in soybean rust reactions in a set of resistant germplasm accessions were analyzed with microscopic observation (Walker et al., 2008a). This research involved collaboration with several different research institutions.

China: Soybean rust was reported for the first time in 1935 in Taiwan (Hiratsuka, 1935) and in 1936 in Shaanxi (Tan et al., 1996a). The disease can reduce soybean yield by up to 80%. Collaborative research was carried out to identify sources of field resistance to soybean rust in early-maturing Chinese soybean germplasm. More than 2,700 accessions of Chinese soybean germplasm with maturities II and earlier were evaluated for resistance to soybean rust in the field at two locations, Sanming and Nanjing, in China between 2004 and 2006 (Wang et al., 2006). Soybean plants in Nanjing were inoculated several times during the V3, R1, and R2 stages with urediniospores of *P. pachyrhizi*. The plants in Sanming were evaluated under high natural disease pressure. Four accessions that were resistant in both locations were identified. In addition, one of these accessions, GD0518, also showed the most resistance in the greenhouse test in Georgia, USA (Wang et al., 2006). In Taiwan, there were at least eight Chinese varieties reported to be resistant to soybean rust including Qiudou 1 and Pingnan Dou (Tan et al., 1996b).

Nigeria: Soybean rust, caused by *P. pachyrhizi* was observed for the first time on soybean in Nigeria during the 1999 main soybean growing season (Akinsanmi et al., 2001). The disease is now endemic in most soybean-producing areas (Adeleke et al., 2006; Bandyopadhyay et al., 2006), which is the largest producer of soybean in Africa (Sanginga et al., 2003). Fungicide treatment in commercial soybean planting is not a viable option in most developing countries in Africa because it increases production cost, therefore, development of resistance cultivars is the most important strategy to soybean rust. Twizeyimana et al., (Twizeyimana et al., 2008)

evaluated soybean germplasm for resistance to soybean rust in Nigeria. One hundred and seventy-eight soybean breeding lines from the International Institute of Tropical Agriculture and 101 accessions from the USDA-ARS and National Agriculture Research Organization (Uganda) were evaluated under field conditions from 2002 to 2006 and 2005 to 2006, respectively. Three breeding lines (TGx, 1835-10E, TGx 1895-50F, and TGx 1903-3F) and three accessions (PI 594538A, PI 417089A, and UG-5) were identified as useful sources of soybean rust resistance for incorporation into high-yielding and adapted cultivars (Twizeyimana et al., 2008).

25.3.2 *Evaluation of Resistant Lines Identified from Foreign Countries with US Domestic Isolates*

Before 2004, soybean rust was not present in the continental USA, and evaluation of US soybean lines for resistance was conducted only with foreign isolates. Since the discovery of *P. pachyrhizi* in North America in 2004, evaluation of soybean lines with US isolates has become an important objective to identify resistance sources to the pathogen. One of the strategies was to evaluate soybean resistant lines that were identified from foreign countries with US domestic isolates.

In a study performed at the USDA-ARS Stoneville Research Quarantine Facility, ten plant introductions (PIs) that were previously identified as ASR-resistant in Paraguay were evaluated using a single-uredinium isolate of *P. pachyrhizi* from Mississippi (Li, 2007, 2008). Those ten PIs were further evaluated with three single-uredinium isolates from Mississippi. Soybean line PI567102B was identified as the most resistant line (Fig. 25.5). It had the lowest severity, no sporulation, and immune reaction to one isolate and the red-brown reactions to other two isolates (Li, 2008, unpublished). In another study (Li and Young, 2008), two sets of plant introductions (PI) were evaluated using a bulk population of the ASR isolates from

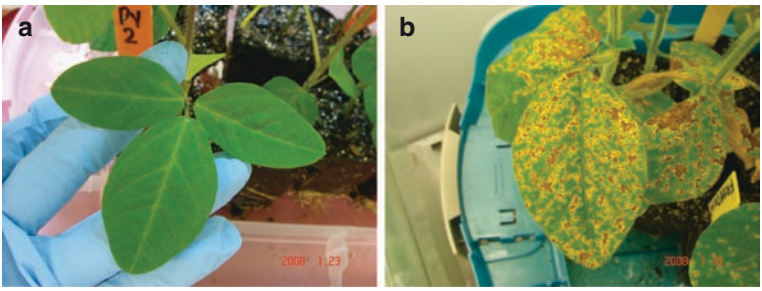


Fig. 25.5 Reaction of selected resistance lines identified in Paraguay to the infection of *Phakopsora pachyrhizi* isolate MS07-2. (**a**): PI567102B (Immune) and (**b**): PI594754 (TAN). Pictures were taken by S. Li at the USDA-ARS Stoneville Research Quarantine Facility, Mississippi, USA

Mississippi. The first set of PIs contained 10 PIs previously identified as resistant in Paraguay and the second set had 17 lines that were selected based on information from Germplasm Resources Information Network (GRIN). Replicated experiments were conducted in growth chambers at the Stoneville Research Quarantine Facility from 2007 to 2008. Seven soybean lines had RB resistant reactions. PI567102B was still identified as the most resistant line. Collaborative research is underway to evaluate a segregating population with PI567102B as a parent for molecular mapping and genetics studies. Soybean lines having resistant reactions to both United States and foreign isolates may be important for developing elite cultivars with broad resistance to soybean rust.

25.3.3 Evaluation of Other Soybean Disease Resistant Lines

Development of dual or multiple disease resistance cultivars is beneficial to disease management and soybean production. Smith et al. (2007) reported finding Phytophthora resistant soybean germplasm with high potential for Asian soybean rust resistance. In their experiments, Phytophthora resistance of soybean seedling was identified in greenhouse studies following hypocotyl inoculations with selected *Phytophthora sojae* race isolates. Of 46 soybean line tested, 22 lines were resistant to *P. sojae* based on inoculation with an Indiana isolate of *P. sojae* (*Rps*7 virulence). Thirteen of *P. sojae* resistant lines identified were among 30 soybean rust resistant lines identified by R. Boerma et al., as having a disease rating of <3.0 for *Phakopsora pachyrhizi* in field tests in 2006 (http://edge.cropsoil.uga.edu/soylab/rustresistance.html). Evaluation of soybean lines with known resistance to other soybean diseases for resistance to soybean rust is of a useful approach to identify soybean lines with multiple disease resistance.

25.3.4 Evaluation of Glycine Soja and Perennial Glycines

Glycine soja and perennial *Glycines* are potential sources of genes for resistant to *P. pachyrhizi.* Bromfield (1984) found that the *G. soja* accession PI 339871 from Korea had hypersensitive reaction to the *P. pachyrhizi* isolate TW 80-2. Progeny of resistance plants were uniformly resistant. In addition, extensive screening of 189 accessions of six Australian native perennial species with an Australian isolate of *P. pachyrhizi* for resistance was reported by Burdon and Marshell (Burdon and Marshall, 1981). Those perennial species included *G. canescens* F. J. Herm, *G. clandestina* Willd., *G. falcata* Benth., *G. latrobeana* (Meissn.) Benth., *G. tabacina* (Labill.) Benth., and *G. tomentella* Hayata. Reactions varied with the species, but some of the species showed potential resistance to soybean rust. In another test, resistance to *P. pachyrhizi* was identified in accessions of *G. argyrea*, *G. canescens, G. clandestina, G. latifolia, G. microphylla,* and *G. tomentella,* but not in accessions

of *G. arenaria, G. cyrtoloba, G. curvata,* and *G. falcate* (Hartman and Wang, 1992). Soybean rust resistance derived from *G. tomentella* in amphiploid hybrid lines was identified (Patzoldt et al., 2007). The resistance gene(s) may be moved from *G. tomentella* to the cultivated soybean *G. max* for resistance to soybean rust.

25.3.5 Evaluation of Other Hosts for Resistance to Soybean Rust

The soybean rust pathogen *P. pachyrhizi* has a very broad host range of leguminous crops (Slaminko et al., 2008). Some of these hosts appear to be much less susceptible to *P. pachyrhizi* than soybeans and their resistance may offer an opportunity to control the soybean rust pathogens.

Soybean rust has been reported on common bean (*Phaseolus vulgaris*) in Argentina (Ivancovich et al., 2007), Brazil, South Africa (DuPreez et al., 2005), and the United States (Lynch et al., 2006). A collaborative research, coordinated by ARS Plant Pathologist Pastor-Corrales, evaluated the response of common bean cultivars for resistance to soybean rust. Among 16 common beans that were inoculated with six *P. pachyrhizi* isolates from Taiwan, Brazil, Paraguay, Thailand, and Zimbabwe, the cultivar Compuesto Negro Chimaltenango(CNC) was the most resistant to all six isolates, with little or no sporulation, low severity, and consistent reddish brown lesions (Fig. 25.6) which are associated with resistance in soybean to *P. pachyrhizi* (Miles et al., 2007; Pastor-Corrales and Frederick, 2008b). The inheritance of soybean rust resistance in CNC was further studied by crossing CNC to the susceptible bean cultivar Mexico 309 (Fig. 25.6b). They concluded that soybean rust resistance in CNC is controlled by the interaction of two genes with complete dominance at both gene loci; one dominant allele of each two genes is necessary to produce the resistant phenotypes but either recessive homozygote is epistatic to the effects of the other gene (Pastor-Corrales and Frederick, 2008a,b).

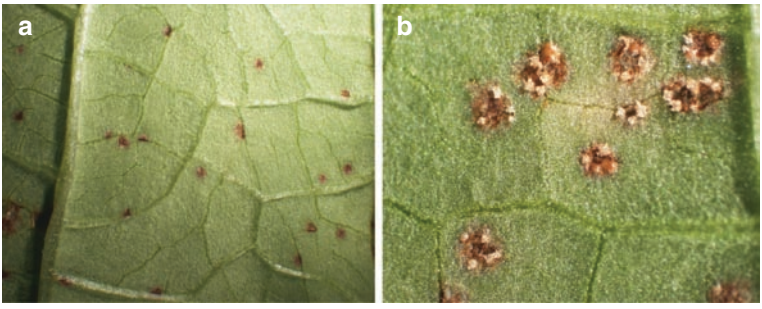


Fig. 25.6 Representative symptoms on infected leaves of common bean 14 to 21 days after inoculation with a US *Phakopsora pachyrhizi* isolate AL04-3. (**a**): Lesions on resistant cultivar CNC without sporulation; (**b**): Lesions on susceptible cultivar Mexico 309 with perfuse sporulation (Courtesy M.A. Pastor-Corrales)

Acknowledgments Author is really indebted to Dr. L. Young for his effort to establish a laboratory in the USDA-ARS Stoneville Research Quarantine Facility for soybean rust research. Special thanks to Drs. D. Walker, M. A. Pastor-Corrale, and T. Isakeit for sharing their unpublished data and provide disease photos; and my appreciation to Drs. W. Moore, D. Wang, P. Chen, J. Andrews, T. Muller, G. Hartman, J. Ray and R. Smith, Y. Xu, and J.Y. Zhuang, D. Eastburn for valuable discussions in dealing with soybean rust research. Author also would like to thank the organizer of the 9th International Congress of Plant Pathology for the invitation and the opportunity for writing this book chapter.

Trade and manufacturers names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of products to the exclusion of others that may also be suitable.

References

Adeleke RA, Asafo-Adjei B, Twizeyimana M, Ojiambo PS, Paul C, Hartman GL, Dashiell K, Bandyopadhyay R (2006) Breeding for rust resistance in soybean at the International Institute of Tropical Agriculture, Nigeria. In: National soybean rust symposium, St. Louis. http://www.plantmanagement network.org/infocenter/topic/soybeanrust/2006/posters/41.pdf

Akinsanmi OA, Ladipo JL, Oyekan PO (2001) First report of soybean rust (*Phakopsora pachyrhizi*) in Nigeria. *Plant Dis*, 85:97

Bandyopadhyay R, Paul C, Twizeyimana M, Adeleke R, Miles MR, Hartman GL (2006) Identification and development of resistance to soybean rust in Nigeria. Phytopathology 96:S8

Blakeman JP, Fokkema NJ (1982) Potential for biological control of plant diseases on phylloplane. Annual Rev Phytopathol 20:167–192

Bonde MR, Brown MF (1980) Morphological comparison of isolates of *Phakopsora pachyrhizi* from different areas of the world. Can J Microbiol 26:1443–1449

Bonde MR, Melching JS, Bromfield KR (1976) History of the suscet-pathogen relationship between Glycine max and *Phakopsora pachyrhizi*, the cause of soybean rust. Phytopatholgy 66:1290–1294

Bresadola P, L'abbé J (1891) Contributions à la Flore Mycologique de l'Ile de St. Thomé. Revue Mycologique 13:65–69

Bromfield KR (1984) Soybean Rust. Mongr. No. 11, American Phytopathlogical Society, St. Paul, MN

Bromfield KR, Hartwig EE (1980) Resistance to soybean rust and mode of inheritance. Crop Sci 20:254–255

Bromfield KR, Melching JS, Kingsolver CH (1980) Virulence and aggressiveness of *Phakopsora pachyrhizi* isolates causing soybean rust. Phytopathology 70:17–21

Burdon JJ, Marshall DR (1981) Evaluation of Australian native species of *Glycine* for resistance to soybean rust. Plant Dis 65:44–45

Calvo ES, Kiihl RAS, Garcia A, Harada A, Hiromoto DM (2008) Two major recessive soybean genes conferring soybean rust resistance. Crop Sci 48:1350–1354

Dorrance A, Hershman D, Draper M (2007) Economic importance of soybean rust. In: Dorrance A, Hershman D, Draper M (eds) Using fungicides to manage soybean rust. The Ohio State University, Columbus, OH, pp 11–19

DuPreez ED, van Rij NC, Lawrence KF, Miles MR, Frederick RD (2005) First report of soybean rust (*Phakopsora pachyrhizi*) on dry beans in South Africa. Plant Dis 89:206

Frederick RD, Snyder C, Peterson GL, Bonde MR (2002) Polymerase chain reaction assays for the detection and discrimination of the soybean rust pathogens *Phakopsora pachyrhizi* and *P. meibomiae*. Phytopathology 92:217–227

Garcia A, Calvo ÉS, de S Kiihl RA, Harada A, Hiromoto DM, Vieira LGE (2008) Molecular mapping of soybean rust (*Phakopsora pachyrhizi*) resistance genes: discovery of a novel locus and alleles. Theor Appl Genet 117:545–553

Grau CR, Dorrance AE, Bond J, Russin JS (2004) Fungal diseases. In: Boerma HR, Specht JE (eds) Soybeans: improvement, production, and uses. 3rd edition. Agron, Monogor, 16, ASA, CSSA, and SSSA, Madison, WI, pp 679–763

Harmon PF, Momol MT, Marois JJ, Dankers H, Harmon CL (2005) Asian soybean rust caused by *Phakopsora pachyrhizi* on soybean and kudzu in Florida. Online. Plant Health Prog . doi:10.1094/PHP-2005-0613-01-RS

Harmon CL, Harmon PF, Mueller TA, Marois JJ, Hartman GL (2006) First report of *Phakopsora pachyrhizi* telia on kudzu in the United States. Plant Dis 90:380

Hartman GL (1991) Soybean rust development and the quantitative relationship between rust severity and soybean yield. Plant Dis 75:596–600

Hartman GL, Wang TC (1992) Sources of resistance to soybean rust in perennial *Glycine* species. Plant Dis 76:396–399

Hartman GL, Bonde MR, Miles MM, Frederick RD (2004) Variation of *Phakopsora pachyrhizi* isolates on soybean. In: Moscardi F, Hoffmann-Campo CB, Saraiva OF, Galerani PR, Krzyzanowski FC, Carrão-Panizzi MC (eds) Proceedings of VII world soybean research conference, Londrina, PR, pp 440–446

Hartman GL, Miles MR, Frederick RD (2005) Breeding for resistance to soybean rust. Plant Dis 89:664–666

Hartman GL, Hines RA, Faulkner CD, Lynch TN, Pataky N (2007) Late season occurrence of soybean rust caused by *Phakopsora pachyrhizi* on soybean in Illinois. Plant Dis 91:466

Hartwig EE (1986) Identification of a fourth major gene conferring resistance to soybean rust. Crop Sci 26:1135–1136

Hartwig EE, Bromfield KR (1983) Relationships among three genes conferring specific resistance to rust in soybeans. Crop Sci 23:237–239

Hennings P (1903) Some new Japanese Uredinale. IV. Hedwigia (Suppl.) 42:107–108

Hershman DE, Bachi PR, Harmon CL, Harmon PF, Palm ME, McKemy JM, Zeller KA, Levy L (2006). First report of soybean rust caused by *Phakopsora pachyrhizi* on Kudzu (*Pueraria montana* var. *lobata*) in Kentucky. Plant Dis 90:834

Hiratsuka N (1935) *Phakopsora pachyrhizi* of Japan I. Botanical Magazine 49:781–788

Hyten DL, Hartman GL, Nelson RL, Frederick RD, Concibido VC, Narvel JM, Cregan PB (2007) Map location of the Rpp 1 locus that confers resistance to soybean rust in soybean. Crop Sci 47:837–840

Isakeit T, Jo Y (2008) Occurrence of telia of *Phakopsora pachyrhizi* on kudzu in Texas. Plant Dis 92:1369

Isakeit T, Miller ME, Saldaña R, Barnes LW, McKemy JM, Palm ME, Zeller KA, DeVries-Paterson R, Levy L (2006) First report of rust caused by *Phakopsora pachyrhizi* on soybean and kudzu in Texas. Plant Dis 90:971

Isard SA, Dufault NS, Hartman GL, Russo JM, De Wolf ED, Morel W (2006) The effects of solar irradiant on the mortality of *Phakopsora pachyrhizi* urediniospores. Plant Dis 90:941–945

Ivancovich AJ, Botta G, Rivadaneira M, Saieg E, Erazzú L, Guillin E (2007) First report of soybean rust caused by *Phakopsora pachyrizi* on *Phaseolus* spp. in Argentina. Plant Dis 91:211

Jardine D (2007) Soybean rust confirmed for first time in Kansas. Plant Health Progress. Online http://www.agriculture.com/ag/story.jhtml?storyid=/templatedata/ag/story/data/1161202461745.xml&catref=ag1001

Keogh RC (1974) Studies on *Phakopsora pachyrhizi* Syd.: the causal agent of soybean rust. MS thesis, University of Sydney, p 95

Killgore E, Heu R, Gardner DE (1994) First report of soybean rust in Hawaii. Plant Dis 78:1216

Kitani K, Inoue Y (1960) Studies on the soybean rust and its control measure. Part 1. Studies on the soybean rust. Shikoku Agric Exp Stn (Zentsuji, Japan) Bull 5:319–342 (Japanese text, English summary)

Kochman JK (1977) Soybean rust in Australia. In: Ford RE, Sinclair JB (eds) Rust of soybean – the problem and research needs, International Agricultural, Manila, pp 44–48

Koenning SR, Moore AD, Creswell TC, Abad GZ, Palm ME, McKemy JM, Hernández JR, Levy L, DeVries-Paterson R (2006) First report of soybean rust caused by *Phakopsora pachyrhizi* in North Carolina. Plant Dis 90:973

Levy C (2005) Epidemiology and chemical control of soybean rust in Southern Africa. Plant Dis 89:669–674

Li S (2007) Reaction of soybean rust resistant lines identified in Paraguay to a Mississippi isolate of *Phakopsora pachyrhizi.* In: 2007 National Soybean Rust Symposium, St. Louis. http://www.plantmanagementnetwork.org/infocenter/topic/soybeanrust/2007/posters/27.pdf

Li S (2008) Identification of new potential sources of resistance to soybean rust. J Plant Pathol 90:S2.64

Li S, Young YD (2008) Evaluation of selected soybean genotypes for resistance to *Phakopsora pachyrhizi*. Phytopathology 98:S89

Li S, Moore WF, Spinks BL, Wells BC, Sciumbato GL, Robinson SJ, Libous-Bailey L (2007) Occurrence of Asian soybean rust caused by *Phakopsora pachyrhizi* in Mississippi. Plant Health Prog, doi:10.1094/PHP-2007-0917-02-BR

Li X, Engelbrecht CJ, Mueller DS, Yang XB (2008) First report of soybean rust caused by *Phakopsora pachyrhizi* in Iowa and its statewide occurrence. Plant Dis 92:975

Livingstone MR, Johanson S, Daberkow M, Roberts Ash M, Breneman V (2004). Economic and policy implications of wind-borne entry of Asian soybean rust into the United States, Electronic outlook report from the Economic Research Service, 2004, N0. OCS-04D-02. US Department of Agriculture

Lynch TN, Marois JJ, Wright DL, Harmon PF, Harmon CL, Miles MR, Hartman GL (2006). First report of soybean rust caused by *Phakopsora pachyrhizi* on *Phaseolus* spp. in the United States. Plant Dis 90:970

Melching JS, Dowlwer WM, Koogle DL, Royer MH (1989) Effects of duration, frequency, and temperature of leaf wetness periods on soybean rust. Plant Dis 73:117–122

Miles MR, Frederick RD, Hartman GL (2006) Evaluation of soybean germplasm for resistance to *Phakopsora pachyrhizi.* Online. Plant Health Prog . doi:10.1094/ PHP-2006-0104-01-RS

Miles MR, Pastor-Corrales MA, Hartman GL, Frederick RD (2007) Differential response of common bean cultivars to *Phakopsora pachyrhizi*. Plant Dis 91:698–704

Miles MR, Morel W, Ray JD, Smith JR, Hartman GL, Frederick RD (2008) Adult plant evaluation of soybean accessions for resistance to *Phakopsora pachyrhizi* in the field and greenhouse in Paraguay. Plant Dis 92:96–105

Monteros MJ, Missaoui AM, Phillips DV, Walker DR, Boerma HR (2007) Mapping and confirmation of the "Hyuuga" RB lesion resistance gene for Asian soybean rust. Crop Sci 47:829–834

Mullen JM, Sikora EJ, McKemy JM, Palm ME, Levy L, DeVries-Paterson R (2006) First report of Asian soybean rust caused by *Phakopsora pachyrhizi* on soybean in Alabama. Plant Dis 90:112

Ogle HJ, Byth DE, McLean RJ (1979) Effect of rust (*Phakopsora pachyrhizi*) on soybean yield and quality in south-eastern Queensland. Aus J Res 30:883–893

Ono Y, Buritica P, Hennen JF (1992) Delimitation of *Phakopsora*, *Physopella* and *Cerotelium* and their species on Leguminosae. Mycol Res 96:825–850

Pastor-Corrales MA, Frederick RD (2008a) Resistance to the soybean rust pathogen (*Phakopsora pachyrhizi*) in common bean cultivar CNC. Annual Rep Bean Improv Coop 51:20–21

Pastor-Corrales MA, Frederick RD (2008b) Inheritance of soybean rust resistance in common bean. Phytopathology 98:S122–S123

Patzoldt ME, Tyagi RK, Hymowitz T, Miles MR, Hartman GL, Frederick RD (2007) Soybean rust resistance derived from *Glycine tomentella* in amphiploid hybrid lines. Crop Sci 47:158–161

Phipps PM, Holshouser DL, Rideout SL, Stromberg EL, Bush EA, Hansen MA, Palm ME (2007) Asian soybean rust confirmed on soybeans in 18 countries of eastern Virginia in 2006. Phytopathology 98:S185

Pupipat U (1997) Soybean rust research in Thailand. Soybean Rust Newsletter 1:7–10

Rossi RL (2003) First report of *Phakospora pachryhizi*, the causal organism of soybean rust in the Provence of Misiones, Argentina. Plant Dis 87:102

Rytter JL, Dowler WM, Bromfield KR (1984) Additional alternative hosts of *Phakopsora pachyrhizi*, causal agent of soybean rust. Plant Dis 68:818–819

Saksirirat W, Hopp HH (1991) Teliospore germination of soybean rust fungus (*Phakopsora pachyrhizi* Syd.). J Phytopathol 132:339–342

Sanginga N, Dashiell KE, Diels J, Vanlauwe B, Lyasse O, Carsky RJ, Tarawali S, Asafo-Adjei B, Menkir A, Schulz S, Singh BB, Chikoye D, Keatinge D, Ortiz R (2003) Sustainable resource management coupled to resilient germplasm to provide new intensive cereal-grain-legume livestock systems in the dry savanna. Agri Ecosystem Environ 100:305–314

Schneider RW, Hollier CA, Whitam HK, Palm ME, McKemy JM, Hernández, Levy L, DeVries-Paterson R (2005) First report of soybean rust caused by *Phakopsora pachyrhizi* in the continental United States. Plant Dis 89:774

Sharma ND, Mehta SK (1996) Soybean rust in Madhya Pradesh. Acta Botanica Indica 24:115–116

Sikora E, Hershman D (2007) Soybean rust in review: 2004–2007. In: Dorrance A, Draper M, Hershman D (eds) Using fungicides to manage soybean rust. The Ohio State University, Columbus, OH, pp 20

Silva DCG, Yamanaka N, Brogin RL, Arias CAA, Nepomuceno AL, Di Mauro AO, Pereira SS, Nogueira LM, Passianotto ALL, Abdelnoor RV (2008) Molecular mapping of two loci that confer resistance to Asian rust in soybean. Theor Appl Genet 117:57–63

Sinclair JB, Hartman GL (1995) Proceedings of the soybean rust workshop, Urbana, IL. 9–11, Aug 1995. Natl Soybean Res Lab Pul. 1. Urbana-Champaign, IL

Sinclair JB, Hartman GL (1999) Soybean rust. In: Hartman GL, Sinclair JB, Rupe JC (eds) Compendium of soybean diseases. 4th edition. American Phytopathological Soceity, St. Paul, MN, pp 25–26

Slaminko TL, Miles MR, Frederick RD, Bonde MR, Hartman GL (2008) New legume hosts of *Phakopsora pachyrhizi* based on greenhouse evaluations. Plant Dis 92:767–771

Smith DA, Abney TS, Westphal A (2007) Phytophthora resistance of soybean germplasm with high potential for Asian soybean rust resistance. Proceedings of the Southern soybean disease workers, Thirty-fourth Annual Meetings, February 18–19, 2007, St. Louis, MO

Tan Y, Yu Z, Yang CY (1996a) Distribution and damage. In: Tan Y, Yu Z, Yang CY (eds), Soybean rust, China Agriculture, Beijing (in Chinese) pp 4–11

Tan Y, Yu Z, Yang CY (1996b) Soybean host genetic characters for resistance to rust. In: Tan Y, Yu Z, Yang CY(eds), Soybean rust, China Agriculture, Beijing (in Chinese) pp 44–51

Twizeyimana M, Ojiambo PS, Ikotun T, Ladipo JL, Hartman GL, Bandyopadhyay R (2008) Evaluation of soybean germplasm for resistance to soybean rust (*Phakopsora pachyrhizi*) in Nigeria. Plant Dis 92:947–952

Walker DR, Narváez D, Marois JJ, Wright DL (2008a) Variation in soybean rust reactions in a set of resistant germplasm accessions. Abstracts of the Soy2008 – Molecular and cellular biology of the soybean conference, July 20–23, Indianapolis, IN

Walker DR, Nelson RL, Hartman GL, Buckley B, Moore S, Schneider RW, Weaver D, Shipe E, Mueller J, Boerma HR (2008b) Identification and characterization of soybean rust resistance in Plant Introductions from the USDA's soybean germplasm collection. Phytopathology 98:S195

Wang D, Chen H, Luo Y, Wang S, Hammerschmidt R, Boerma R (2006) Screening soybean germplasm for field resistance to soybean rust. http://www.plantmanagementnetwork.org/infocenter/topic/soybeanrust/2006/posters/46.asp

Yang XB, Royer MH, Tschantz AT, Tsia BY (1990) Analysis and quantification of soybean rust epidemics from seventy-three sequential planting experiments. Phytopathology 80:1421–1427

Yang XB, Tschantz AT, Dowler WM, Wang TC (1991) Development of yield models in relation to reduction of components of soybean infected with *Phakopsora pachyrhizi*. Phytopathology 81:1420–1426

Yeh CC, Tschanz AT, Sinclair JB (1981) Induced teliospore formation by *Phakopsora pachyrhizi* Syd. On soybean and other hosts. Phytopathology 71:1111–1112

Yorinori JT, Pavia WM, Frederick RD, Costamilan LM, Bertagnolli PF, Hartman GL, Godoy CV, Nunes JJ (2005) Epidemics of soybean rust (*Phakopsora pachyrhizi*) in Brazil and Paraguay from 2001 to 2003. Plant Dis 89:675–677

Zhuang JY (1991) The causal organism, host ranges, and distribution of soybean rust in China. Oil Crops China 3:67–69 (in Chinese)

Index