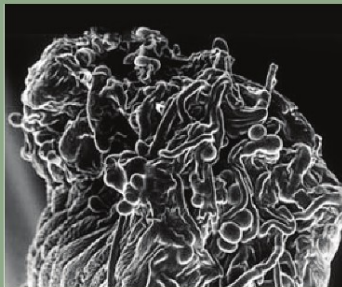
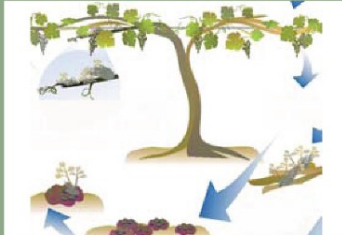
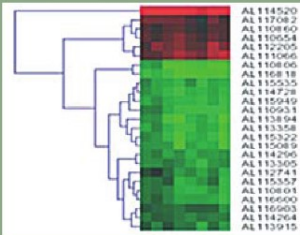
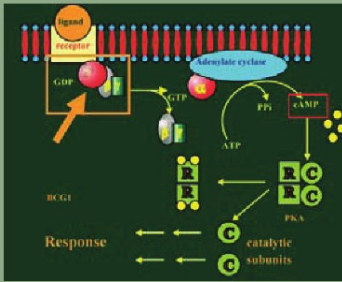


Botrytis: Biology, Pathology and Control

Edited by

Y. Elad, B. Williamson, P. Tudzynski and N. Delen



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Edited by

Y. Elad

*The Volcani Center,
Bet Dagan, Israel*

B. Williamson

*Scottish Crop Research Institute,
Dundee, U.K.*

Paul Tudzynski

*Institut für Botanik,
Münster, Germany*

and

Nafiz Delen

*Ege University,
Izmir, Turkey*



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Top row: Scanning electron microscopy (SEM) images of conidiophores and attached conidia in *Botrytis cinerea*, top view (*left*, Brian Williamson) and side view (*right*, Yigal Elad); hypothetical cAMP-dependent signalling pathway in *B. cinerea* (*middle*, Bettina Tudzynski).

Second row: Identification of a drug mutation signature on the *B. cinerea* transcriptome through macroarray analysis - cluster analysis of expression of genes selected through GeneAnova (*left*, Muriel Viaud et al., INRA, Versailles, France, reprinted with permission from 'Molecular Microbiology 2003, 50:1451-65, Fig. 5 B1, Blackwell Publishers, Ltd'); portion of Fig. 1 chapter 14, life cycle of *B. cinerea* and disease cycle of grey mould in wine and table grape vineyards (*centre*, Themis Michailides and Philip Elmer); confocal microscopy image of a *B. cinerea* conidium germinated on the outer surface of detached grape berry skin and immunolabelled with the monoclonal antibody BC-12.CA4 and anti-mouse FITC (*right*, Frances M. Dewey (Molly), Chapter 11).

Bottom row: SEM images of *B. cinerea* conidia germinated on a bean leaf (*left*, Y. Elad); on raspberry stigma (*centre*, B. Williamson) and on a rose petal (*right*, Y. Elad).

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Preface

There has been great progress in the science of *Botrytis* spp. and the diverse and complex interactions they make with plants, and the application of this science in agriculture and horticulture throughout the world. Therefore *Botrytis* spp. are of keen interest to scientists, crop consultants, farmers and students of agribusiness and plant protection. It is important to present this knowledge in one comprehensive volume that is a synthesis of this research endeavour. This book is being published on the occasion of the 2004 Thirteenth International *Botrytis* Symposium in Antalya, Turkey in the series following *Botrytis* symposia that took place in Invergowrie, Dundee, Scotland (1966); Siut-Truiden, Belgium (1968); Sweden (1971); Teresin, Skierniewice, Poland (1973); Gradignan, Bordeaux, France (1976); Amersfoort, The Netherlands (1979); Aberdeen, Scotland (1982); Alba, Torino, Italy (1985); Neustadt, Germany (1989); Gouves, Heraklion, Crete, Greece (1992); Wageningen, The Netherlands (1996); and Reims, France (2000).

The book is the result of intensive work of 43 authors, all of whom are leading scientists in the *Botrytis* sciences. Thanks to them the book is a comprehensive update of the subject and to all of them we owe our gratitude. The twenty interconnected chapters of the book are grouped according to three major themes: the fungus and its pathogenicity factors; plant reactions to infection; and epidemiology and management of important *Botrytis*-incited diseases. This book adopts a multidisciplinary approach to integrate the state-of-the-art knowledge in all key areas of common interest in the fungi and their plant interactions. The book includes detailed reviews of *Botrytis* spp. and the diseases they cause in plant systems and provides a comprehensive description of these fungal necrotrophs, including their diversity of response to the environment, their speciation and relatedness, sources of variation for evolution and molecular genetics and genomics. Aspects of *Botrytis*-host interactions, pathogenicity factors, the plant's reactions to infection, morphology and cellular organisation, signalling, key enzymes, reactive oxygen species and oxidative processes in disease on-set, secondary metabolites as plant defence substances and the role of phytohormones in such reactions are emphasized in the book. Several innovative approaches for disease management of this group of destructive pathogens and methods of detection, epidemiological studies and chemical and biological control are also discussed.

The number of publications concerning *Botrytis* spp. in international databases has increased steadily in the last three decades from c. 170 to more than 350 per year. Inevitably only a small selection of these publications is cited. During the compilation of this book the aim was to create a most comprehensive treatise on the rapidly developing science of *Botrytis* and to serve as a stimulus to future research for the benefit of agriculture and horticulture and all those who serve these industries.

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Y Elad
B Williamson
P Tudzynski
N Delen

21 April 2004

Contributors

- Radwan Barakat** – Department of Plant Production and Protection, College of Agriculture, Hebron University, P.O. Box 40, Hebron, Palestinian Authority; e-mail: rbarakat@netvision.net.il
- Ross E. Beever** - Landcare Research, Private Bag 92170, Auckland, New Zealand; e-mail: beeverr@landcare.cri.nz
- Trevor W. Bretag** - Victorian Department of Primary Industries, Private Bag 260, Natimuk Road, Horsham, Victoria, 3401, Australia; e-mail: trevor.bretag@dpi.vic.gov.au
- Sonja Coertze** - Department of Plant Pathology, University of Stellenbosch, Private Bag X1, Matieland (Stellenbosch), South Africa; e-mail: sc2@sun.ac.za
- Jenny A. Davidson** - South Australian Research and Development Institute, GPO Box 397, Adelaide, 5001, South Australia; e-mail: davidson.jenny@saugov.sa.gov.au
- Marjan de Boer** - Crop Protection and Diagnostics, Applied Plant Research (PPO), section Flowerbulbs, P. O. Box 85, 2160 AB Lisse, The Netherlands; e-mail: marjan.deboer@wur.nl
- Nafiz Delen** - Department of Plant Protection, Ege University, Faculty of Agriculture, Bornova, Izmir, Turkey; e-mail: delen@ziraat.ege.edu.tr
- Aleid J. Dik** - Applied Plant Research, Glasshose Horticulture, P.O. Box 8, 2670 AA Naaldwijk, The Netherlands; e-mail: aleid.dik@wur.nl
- Frances M. Dewey (Molly)** - Department of Viticulture and Enology, University of California at Davis, Davis CA95616, USA; e-mails: molly.dewey@plants.ox.ac.uk; fmdewey@ucdavis.edu
- Samir Droby** - Department of Postharvest Science, ARO, The Volcani Center, P.O. Box 6, Bet Dagan, 50250, Israel; e-mail: samird@volcani.agri.gov.il
- Yigal Elad** - Department of Plant Pathology and Weed Research, ARO, The Volcani Center, Bet Dagan 50250, Israel; e-mail: elady@volcani.agri.gov.il
- Philip A.G. Elmer** - HortResearch, Ruakura Research Centre, Private Bag 3132, Hamilton, New Zealand; e-mail: pelmer@hortresearch.co.nz
- Bernard A. Goodman** - ARC Seibersdorf research GmbH, A-2444 Seibersdorf, Austria; e-mail: bernard.goodman@arcs.ac.at
- Gustav Holz** - Department of Plant Pathology, University of Stellenbosch, Private Bag X1, Matieland (Stellenbosch), South Africa; e-mail: gh@sun.ac.za
- Iлона Kars** - Laboratory of Phytopathology, Wageningen University Plant Sciences, Binnenhaven 5, 6709 PD Wageningen, The Netherlands; e-mail: ilona.kars@wur.nl
- Gali Krishna-Kishore** - International Crop Research Institute for the Semi-Arid Tropics, Patancheru 502 324, Andhra Pradesh, India; e-mail: k.gali@cgiar.org
- John M. Labavitch** - Pomology Department, University of California, Davis, CA 95616; e-mail: jmlabavitch@ucdavis.edu
- Laurent Legendre** - University of Western Sydney, Centre for Horticulture and Plant Sciences, Locked Bag 1797, Penrith South DC, NSW 1797, Australia; e-mail: l.legendre@uws.edu.au
- Pierre Leroux** - INRA, Unité de Phytopharmacie et Médiateurs Chimiques, 78026 Versailles cedex, France; e-mail: lerouxp@versailles.inra.fr

- Amnon Lichter** - Department of Postharvest Science, ARO, The Volcani Center, P.O. Box 6, Bet Dagan, 50250, Israel; e-mail: vtlicht@volcani.agri.gov.il
- Kurt D. Lindbeck** - Victorian Department of Primary Industries, Private Bag 260, Natimuk Road, Horsham, Victoria, 3401, Australia; e-mail: kurt.lindbeck@dpi.vic.gov.au
- James W. Lorbeer** - Department of Plant Pathology, Cornell University, Ithaca, New York 14853, USA; e-mail: jw15@cornell.edu
- Gary D. Lyon** - Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK; e-mail: glyon@scri.sari.ac.uk
- Themis J. Michailides** - Department of Plant Pathology, University of California, Davis/Kearney Agricultural Center, 9240 South Riverbend Ave. Parlier, CA 93648, USA; e-mail: themis@uckac.edu
- Suresh Pande** - International Crop Research Institute for the Semi-Arid Tropics, Patancheru 502 324, Andhra Pradesh, India; e-mail: s.pande@cgiar.org
- Ann L.T. Powell** - Department of Vegetable Crops, University of California, Davis, CA 95616, USA; e-mail: alpowell@ucdavis.edu
- Christian Schulze Gronover** - Institut für Botanik und Botanischer Garten, Westfälische Wilhelms-Universität, Schlossgarten 3, 48149 Münster, Germany; e-mail: gronove@uni-muenster.de
- Alison M. Seyb** - Department of Plant Pathology, Cornell University, Ithaca, New York 14853, USA; e-mail: ams299@cornell.edu
- Amir Sharon** - Department of Plant Sciences, Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel; e-mail: amirsh@tauex.tau.ac.il
- Verena Siewers** - Institut für Botanik und Botanischer Garten, Westfälische Wilhelms-Universität, Schlossgarten 3, 48149 Münster, Germany; e-mail: siewers@uni-muenster.de
- Dani Shtienberg** - Department of Plant Pathology and Weed Sciences, ARO, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel; e-mail: danish@volcani.agri.gov.il
- Alison Stewart** - National Centre for Advanced Bio-Protection Technologies, P. O. Box 84, Lincoln University, Canterbury, New Zealand; e-mail: stewart@lincoln.ac.nz
- Henrik U. Stotz** - Department of Horticulture, Oregon State Univ., Corvallis, OR 7331; e-mail: stotzhe@science.oregonstate.edu
- Klaus B. Tenberge** - Institut für Botanik und Botanischer Garten, Westfälische Wilhelms-Universität, Schlossgarten 3, 48149 Münster, Germany; e-mail: tenberge@uni-muenster.de
- Bettina Tudzynski** - Institut für Botanik und Botanischer Garten, Westfälische Wilhelms-Universität, Schlossgarten 3, 48149 Münster, Germany; e-mail: Bettina.Tudzynski@uni-muenster.de
- Paul Tudzynski** - Institut für Botanik und Botanischer Garten, Westfälische Wilhelms-Universität, Schlossgarten 3 D-48149 Münster, Germany; e-mail: tudzyns@uni-muenster.de
- J. Ernst van den Ende** - Crop Protection and Diagnostics, Applied Plant Research (PPO), section Flowerbulbs, P. O. Box 85, 2160 AB Lisse, The Netherlands; e-mail: ernst.vandenende@wur.nl

Peter van Baarlen - Laboratory of Phytopathology, Wageningen University Plant Sciences, Binnenhaven 5, 6709 PD Wageningen, The Netherlands; e-mail: peter.vanbaarlen@wur.nl

Jan A.L. van Kan - Laboratory of Phytopathology, Wageningen University Plant Sciences, Binnenhaven 5, 6709 PD Wageningen, The Netherlands; e-mail: jan.vankan@wur.nl

Pauline L. Weeds - Landcare Research, Private Bag 92170, Auckland, New Zealand; e-mail: weedsp@landcareresearch.co.nz

Brian Williamson - Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, United Kingdom; e-mail: b.williamson@scri.sari.ac.uk

Jos P. Wubben - Applied Plant Research, Glasshouse Horticulture, P.O. Box 8, 2670 AA Naaldwijk, The Netherlands; e-mail: Jos.Wubben@wur.nl

David Yohalem - Horsekildevvej 38. 1 tv, Valby DK-2500, Denmark; e-mail: dsyohalem@hotmail.com

CHAPTER 1

BOTRYTIS SPP. AND DISEASES THEY CAUSE IN AGRICULTURAL SYSTEMS – AN INTRODUCTION

Yigal Elad¹, Brian Williamson², Paul Tudzynski³ and Nafiz Delen⁴

¹Department of Plant Pathology, ARO, The Volcani Center, Bet Dagan 50250, Israel; ²Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, United Kingdom; ³Institut für Botanik, Schlossgarten 3 D-48149 Münster, Germany; ⁴Department of Plant Protection, Ege University, Faculty of Agriculture, Bornova, Izmir, Turkey

Abstract. Some leading characteristics and historical notes on *Botrytis* spp. are described here. *Botrytis* spp. infect many host plants in all climate areas of the world, infecting mainly upper plant parts at pre- and post-harvest stages. Bulbs, seeds and other propagation material also suffer infection. Infection can occur in high humidity in the presence or absence of water films. Infection may be quiescent, aggressive, restricted or widely developing. The production of high numbers of conidia poses a long lasting threat to susceptible hosts. Genotypic and phenotypic variation is most important in the broad spectrum pathogen *B. cinerea*. Moreover, changes in populations in response to selection by exposure to xenobiotics, especially fungicides, are quite common in the genus and fungicide resistance has been recorded in *Botrytis* populations throughout the history of the modern fungicide era. Detailed studies on the precise conditions that promote infection, disease development and survival of inoculum have provided the essential epidemiological information required for design of control strategies. For example, cultural methods have been developed that increase aeration and drying of the plant canopy to reduce the risk of *Botrytis* epidemics. The increasing requirement for alternative approaches to reduce farmers' dependency on use of fungicides led to the evaluation and exploitation of potential biocontrol agents capable of substantial disease suppression in a commercial context, and within integrated crop management systems.

1. Introduction

It is almost a quarter of a century since a major textbook on *Botrytis* spp. was published (Coley-Smith et al., 1980). That erudite text was a milestone in plant pathology and much of the information it contained is still valid today. However, there have been many important scientific advances in the understanding of these interesting and often destructive fungi since that time and it is appropriate that a new volume is published to describe these findings. This book is a distillation of knowledge obtained about *Botrytis* species during the last 25 years. Each chapter describes a particular aspect of fungal biology and its impact on disease processes and host response. New technologies have arisen that have been most rewarding

when applied to long-standing problems or to test new hypotheses and many of these are covered in this book. Although the chapters cover specific topics and should stand-alone to some extent, inevitably there is some overlap. The editors have attempted to provide linkage between chapters where possible so that readers can follow associated material to better understand the practical implications of the advances made in fundamental science. In the following introductory text we provide some historical notes to make a bridge with the new information offered in later chapters.

Botrytis cinerea and other *Botrytis* species are important pathogens of nursery plants, vegetables, ornamental, field and orchard crops and stored and transported agricultural products (Chapters 14-17 and 19). Considerable effort is invested in protecting the agricultural produce against *Botrytis* before and after harvest. The market size for anti-*Botrytis* products have been US\$ 15-25 million in recent years. The intensity of anti-*Botrytis* measures taken by farmers continued unabated throughout the last 20 years but our understanding of the processes that govern *Botrytis* life cycles, pathogenicity and epidemiology have become comprehensive. MacFarlane (1968, cited in Jarvis, 1977) counted in the Review of Applied Mycology 235 host species belonging to a variety of families affected by *B. cinerea*. Other species of *Botrytis* are specific to certain hosts; they have restricted host range and usually affect single or a limited number of hosts. Interestingly, the more restricted host specificity in *Botrytis* spp. occurs on monocotyledonous plants.

Over the last 125 years, *Botrytis* spp. have been investigated by an increasing number of specialists in diverse fields including chemistry, biochemistry, molecular and cell biology, genetics, morphology and histology, taxonomy, host-parasite interaction, ecology and epidemiology (Jarvis, 1977; Coley-Smith et al., 1980; Verhoeff et al., 1992). They have been the subject of an immense number of published studies.

2. Geographical and ecological occurrence

In the introduction to the book 'The Biology of Botrytis' (1980) Coley-Smith referred to *Botrytis* spp. as temperate area pathogens perhaps because of the vast research that has been carried out in such areas or due to its importance on vineyard grapes. Nevertheless, species of the genus *Botrytis* occur wherever their hosts are grown, ranging from tropical and subtropical to cold areas. For example Anderson (1924) recorded *B. cinerea* in Alaska and Yunis and Elad (1989) dealt with this pathogen in warm and dry areas. A rapid rate of conidial germination, infection, mycelium growth and conidiation occur in many *Botrytis* spp. under a wide range of microclimate conditions and pose severe disease management problems all around the world.

The potent effect of near-ultraviolet light (320-400 nm) on induction of conidiation and the characterisation of potential photoreceptors was discussed fully by Epton and Richmond (1980). However, new research on the importance of light quality for infection with inoculated conidia is cited in Chapter 2. *Botrytis* spp. are regarded as high humidity pathogens (Chapter 2) and their conidia germinate at high

humidity (Snow, 1949). In many patho systems infection occurs in the presence of a film of water on the susceptible plant tissue. The role of water drops (Brown, 1916) and nutrients in germination and infection have been long recognised. However, it is interesting that the pathogen is also able to infect plants when no film of water exists on the plant surfaces (Williamson et al., 1995; Elad, 2000; Chapter 2). A change in spread, importance and range of hosts that are severely affected by *Botrytis* spp. is partly associated with the increasing importance of protected cropping in greenhouses or plastic tunnels (Chapter 17) and partly with change in the intensification and growth practices of open field crops. Although *Botrytis* spp. can be isolated from some soils (Lorbeer and Tichelaar, 1970) and are also present on seeds, bulbs and corms (Chapters 15 and 16), they are more commonly isolated from upper plant parts (leaves, flowers, fruits, buds and stems), and in some cases upper root parts and stem bases. Symptoms range from restricted lesions to dry or spreading soft rots, with or without the appearance of conspicuous sporulating colonies. *Botrytis* spp. are highly active at moderate temperatures, however, the ability of *B. cinerea* to be active at temperature as low as 0°C (Brooks and Cooley, 1917) makes it an important pathogen of stored products and a challenge for disease management during storage and shipment (Chapter 19). Most *Botrytis* spp. sporulate profusely and dry conidia are dispersed through the air making this group of pathogens a constant threat to susceptible crops. The limiting factor for epidemic outbreaks is usually the occurrence of the appropriate microclimatic conditions, rather than the amount of inoculum (Shtienberg and Elad, 1997).

3. Variability and adaptability

Botrytis has been recognized as a genus since Micheli erected it in 1729. In early times it was sometimes confused with *Sclerotinia* spp. but clarifications were made (Smith, 1900) and confusion was dispelled (Whetzel, 1945). The connection between *Botrytis* spp. and their *Botryotinia* teleomorphs was finally established during the 1940s and 1950s (Groves and Loveland, 1953). Four decades later, improved techniques for culture and spermatization (Faretra et al., 1988) allowed the mating of *Botrytis* strains for genetic analysis. Having multinucleate conidia and hyphal compartments, *Botrytis* isolates have a tendency to change constantly during successive generations *in vitro* and under field conditions. Genotypic and phenotypic variation is very common in *B. cinerea* (Chapter 3). Use of DNA population markers and sexual and vegetative compatibility studies have revealed limited evidence of clonality in *B. cinerea*. The roles of sexual reproduction and heterokaryosis in the determination of variation are still under study.

Changes in populations selected by xenobiotics are quite common in this species (Chapter 3). Development of resistance to fungicides has been recorded in *Botrytis* populations throughout the history of the modern fungicide era (Chapter 11). Reavill (1954) noted that *B. cinerea* could tolerate chlorinated nitrobenzene fungicides and when systemic benzimidazole fungicides were first used *Botrytis* spp. rapidly developed resistant isolates (Bollen and Scholten, 1971), later followed by resistance to the dicarboximides (Katan, 1982). A decade later the molecular basis of these

mutations was identified (Yarden and Katan, 1993). The management of *Botrytis* by chemical fungicides still poses a serious challenge to crop advisers (Chapter 11).

Development of complex strategies to cope with *Botrytis*-incited diseases has been necessary since the 1980s (Vincelli and Lorbeer, 1989). Following detailed studies on the precise conditions that promote infection and disease development, cultural methods were developed giving farmers a range of tools to assist in avoiding serious crop damage. Cultural methods that ensure ventilation and drying of plant canopy after rain, whilst maintaining adequate water supply to the roots, are the most effective means developed so far for prevention of *Botrytis* epidemics (Elad and Shtienberg, 1995). Rational warning systems based on conditions highly conducive to spore germination and host penetration for disease development have been developed for some crop systems (Chapter 18). Microorganisms on plant surfaces interact with *Botrytis* germination conidia (Newhook, 1951, 1957; Wood, 1951; Bhatt and Vaughan, 1962; Blakeman and Fraser, 1971; Blakeman, 1972) or conidiation (Köhl and Fokkema, 1993). Increasing public awareness of some potential drawbacks of chemical fungicides was addressed by the development of alternative control measures making use of microbial antagonists that are capable of disease suppression (Dubos, 1992); some of these agents were developed subsequently into commercial products (Elad and Freeman, 2002), but they are still commercially much less significant than the chemical measures (Chapter 13).

4. Quiescent, restricted and aggressive infection

One intriguing phenomenon associated with *Botrytis* infection is the ability of this pathogen to be quiescent in the host tissue for varying periods (Williamson, 1994; Elad, 1997). Originally this phase of infection was termed 'non-aggressive' as opposed to aggressive when lesions are expanding (Beaumont et al., 1936) and later the phenomenon was described as latent or quiescent infection and found to be common in many hosts (Jarvis, 1962; Verhooff, 1970; Chapter 2). The ultrastructure of *Botrytis*-plant interactions is described in Chapter 5.

Plants possess a range of pre-formed and induced defences for combating an infection. The antifungal activities of the induced phytoalexins, such as wyerone in *Vicia faba*, were described fully by Mansfield (1980). Many of these defences include secondary metabolites: stilbenes including resveratrol, saponins including α -tomatin, cucurbitacins, proanthocyanidins and tulipalin A, structural barriers, cell wall modifications, but also the pathogenesis-related (PR) proteins (Chapter 9). *Botrytis* species have evolved mechanisms to counteract some of these defence responses.

As a pathogen of grape berries, *B. cinerea* is economically extremely important. Its plant-pathogen interactions and epidemiology were thoroughly studied and reviewed (Jarvis, 1980; Ribéreau-Gayon et al., 1980). The pathogen may completely destroy grape berries, inflicting heavy crop losses as grey mould. Alternatively, under certain conditions, it may cause a slow decay permitting the berries to desiccate considerably. Such dry berries affected by 'noble rot' are harvested and processed into valuable sweet wines (e.g. the Sauternes of France, the

Trockenbeerenauslese of Germany, the Aszu of Hungary and Botrytized wines in other places). Grapes affected by the destructive grey mould are of low value for making wine not only because of the weight loss but also because of interference with fermentation and changing the flavour and colour of the wine. Among all the many *Botrytis* plant hosts, grey mould management in vineyards is therefore the most important target for agrochemical companies and researchers. The noble rots are not described in this book because they were covered extensively by Ribéreau-Guyon et al. (1980)

Scientists were fascinated by *Botrytis* conidial infection of plant tissues very early in plant pathology. Ward (1888) described the infection of lilies by germ tubes of a *Botrytis* spp. In early times penetration was regarded as a purely mechanical process (Blackman and Welsford, 1916). McKeen (1974) described enzymatic dissolution of faba bean cuticles that triggered three decades of research that has given a vast amount of information on secreted hydrolytic enzymes and their involvement in penetration and tissue maceration by *Botrytis* (Chapter 7). *Botrytis* spp. have turned out to be an important model for host cell wall enzymatic degradation, and before the turn of this century valuable molecular biological research uncovered some of the genes responsible for *Botrytis* pathogenicity (Ten Have et al., 2002; Chapters 4 and 7).

Over the last 25 years there have been substantial advances in methodologies for separation, quantification and identification of fungal and plant metabolites and other labile chemical species. Recent work provides evidence that *B. cinerea* exploits the production of active oxygen species (AOS) in colonising plant tissues (Chapter 8). Hydrogen peroxide and other AOS are produced by the fungus and interact with the plant-based antioxidant systems in determining the outcome of the infection process. Biochemical processes appear to be of importance for lesion development, and the perturbation of the free radical chemistry (Muckenschnabel et al., 2003). Transition metal redox processes (particularly those involving iron), the regulation of enzymes (of both plant and fungal origin), the production of toxic metabolites in the host, and host signalling and programmed cell death are all involved in these processes.

5. Molecular basis of host-parasite interactions

The availability of molecular genetic techniques since the late 1980s brought a revolutionary break-through in the understanding of the pathogenic strategies of *Botrytis*. It allowed for the first time the unequivocal identification of pathogenicity/virulence genes and hence an ability to define molecular targets for developing innovative fungicide and resistant host plants in the future (Chapters 4, 7, 8, 20). Since the first successful molecular transformation of *B. squamosa* (Huang et al. 1989), molecular tools and techniques have been rapidly adapted to the special requirements of *Botrytis*. Investigations using these tools have increased exponentially in the last few years, especially making it possible to perform targeted gene inactivation and functional analysis of all the putative pathogenicity factors identified in the wealth of biochemical, physiological and genetic data of the last

decades. As a consequence of these innovations, the science of molecular genetics underpins many of the chapters in this book. A list of 45 genes has so far been functionally identified (Chapter 4). However, only very few of the "classical" candidate genes have survived the rigorous molecular testing, which in some cases was unexpected. Recent work has also established that cyclic AMP (cAMP) and conserved MAP kinase signalling pathways play crucial roles during pathogenesis in *B. cinerea* (Chapter 6).

Molecular tools today offer many more possibilities for testing long-established hypotheses: the availability of "genomics" tools allows unbiased approaches which will give us a complete picture of the factors involved in the complex interaction processes of this potent and variable pathogen and assist the development of specific alternative defence strategies, including modified host resistance (Chapter 20). In combination with high-throughput screens it will be possible to develop new fungicides based on our detailed knowledge of the refined strategy of *Botrytis* to overcome its host's defence. However, due to the high variability of *B. cinerea* the fight probably never will be finally settled!

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CHAPTER 2

THE ECOLOGY OF *BOTRYTIS* ON PLANT SURFACES

Gustav Holz¹, Sonja Coertze¹ and Brian Williamson²

¹Department of Plant Pathology, University of Stellenbosch, Private Bag X1, Matieland (Stellenbosch), South Africa; ²Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK

Abstract. The initiation of disease by members of *Botrytis* species depends on a complex sequence of biological events involving host and environment sensing, chemical and physical interactions between the fungal propagules and the host surface and the microbial interactions on the surface of the host. The pathogen's inoculum is central to the understanding of this interaction. This chapter describes the inoculum ecology of *Botrytis* species on plant surfaces and relates this information to an understanding of disease initiation. *Botrytis* species deploy several propagules and survival structures. A knowledge of the precise behaviour of these propagules, especially the hydrophobic conidia, when dispersed and deposited on the host at high relative humidity in the presence or absence of water droplets is important for disease initiation and control. The responsiveness of propagules to the environment, and the diversity shown in attack strategies by these pathogens are discussed with examples of the infection pathways used. Special comment is made about suitable inoculation procedures to study grey mould in leaves and fruits.

1. Introduction

Botrytis species have a necrotrophic life style occurring as pathogens infecting a single specific host or closely related host, or as the broad spectrum pathogen *B. cinerea* infecting numerous host plants: after infection and death of host tissues all these fungi can survive and sporulate as saprophytes on the necrotic tissue, or produce long-term survival structures, such as sclerotia. These survival structures can be associated with living plants or with plant debris lying on or buried in soil. For species more specialized in their parasitism (*B. aclada*, *B. byssoidea*, *B. squamosa*, *B. gladiolorum*, *B. tulipae*, *B. elliptica*, *B. fabae*), the inoculum source will inevitably be within the crop, or debris from a previous crop in the vicinity. For *B. cinerea*, for which host range is extremely wide, the primary inoculum also is most likely generated within the crop (Johnson and Powelson, 1983), but the potential for incoming primary inoculum from a different crop or weed host is greater than for the host-specific pathogens, and will be affected by the phasing of crop growth and harvest within a district or region.

The fungus exists in the different habitats as mycelia, micro- and macro-conidia, chlamydospores, sclerotia, apothecia and ascospores and these are dispersed by

diverse means (Jarvis, 1980b). Although *B. cinerea* releases its macroconidia mainly in dry air currents, it is surprising that the majority of published work describes infection arising from suspensions of conidia in water droplets. This chapter summarises the new information available about the behaviour of *B. cinerea* and other *Botrytis* spp. and their responsiveness to different micro-environments, especially the effects of relative humidity (RH). It is particularly difficult to measure and maintain the RH of a host when inoculations are made and the host is incubated for periods to determine the outcome of the interaction. Harrison et al. (1994) reviewed these technical difficulties and devised specialised equipment that provides the best regulation of RH known to the authors. Results of work performed with dry-conidial inoculations, as well as the most recent achievements in inoculation with water droplets, are discussed.

2. Survival

The disease cycles of *Botrytis* species and the growth habit and phenologies of their host plants are often inextricably linked. Dormant or metabolically inactive fungal structures play a central role in each of these disease cycles. Each part of the fungus thallus can serve as a survival structure.

2.1. Sclerotia

All species of *Botrytis* form sclerotia which may, depending on isolate and cultural conditions, differ in size and shape. Sclerotia are generally considered to be the most important structures involved in the survival of *Botrytis* species. Sclerotia can survive adverse environmental conditions, can produce apothecia after a sexual process and possess a considerable capacity for producing successive crops of conidia in many *Botrytis* species (Coley-Smith, 1980). Under laboratory conditions, *B. cinerea* sclerotia continue to sporulate for about 12 weeks after the production of the first crop of conidia (Nair and Nadtotchei, 1987). Suppression of sporulation when the conidia were left on sclerotia and resumption of sporulation when the conidia were removed from the surface could extend the period of conidial production. Under natural conditions, rainfall would be expected to dislodge conidia from germinating sclerotia and initiate conidial production by removing the suppression in sporulation.

The internal structure and histochemistry of sclerotia of *B. cinerea* and *B. fabae* are similar; the rind walls contain melanic pigments, the medullary hyphae are surrounded by a continuous matrix of β -glucans, and the intracellular nutrient reserves are protein, glycogen, polyphosphate and lipid (Backhouse and Willets, 1984). The genetic control of the switch from rapid vegetative growth to production of sclerotia is not known. Recent work with the closely related species *Sclerotinia minor* suggests that β -carotene may be important for protection against oxidative stress when sugars and other nutrients decline in presence of light (Zervoudakis et al., 2003).

Formation of sclerotia in the field is generally associated with plant tissue. However, it also occurs in insects. Louis et al. (1996) demonstrated the ability of the vinegar fly, *Drosophila melanogaster*, to serve as vector for *B. cinerea*. Long-term *D. melanogaster*/*B. cinerea* relationships were found during the life of the insect. Conidia germinated in the insect foregut, developed into mycelium, and differentiated into microsclerotia, which can be carried by the flies during their entire life. Since the fly overwinters as an adult, it was concluded that it could play a role in winter conservation of *B. cinerea* inoculum.

2.2. Chlamydospores

Chlamydospores have been found in *B. cinerea*, *B. anthophila* and *B. fabae* (Coley-Smith, 1980). The chlamydospores of *B. cinerea* are hyaline cells of extremely variable form and size (Urbasch, 1983, 1986). They are generally found in ageing cultures and commonly occur in the stromatic sectors of cultures of the fungus which are contaminated by other organisms, and in association with sclerotia. Chlamydospores are formed as terminal or intercalary cells by transformation of vegetative mycelium parts and are liberated by hyphal disintegration. They were observed on and in tissue of naturally and artificially infected tomato and *Fuchsia hybrida* leaves and their numbers increased in older lesions (Urbasch, 1983, 1986). Under moist conditions and without added nutrients, the chlamydospores germinated on the leaves by microconidia which remained dormant. When fresh nutrients were supplied to the chlamydospores, they germinated with hyphae penetrating the host, or they produced a new crop of macroconidia. Histological studies of the infection process by *B. elliptica* show the formation of corresponding structures after conidium germination on oriental lily leaves (Hsieh et al., 2001). On tomato fruit, unsuccessful penetration was often associated with germ tubes which, after attachment to the host, differentiate into several cells (chlamydospores) at the point of attachment (Rijkenberg et al., 1980). On fruit of nectarine, plum and pear, germlings produced from dry airborne *B. cinerea* conidia formed chlamydospores on short germ tubes when fruits were subjected to intermittent dry periods, or were kept for 48 h at 5°C (Holz, 1999). Chlamydospores can therefore serve as short term survival structures which may help the fungus to overcome short unfavourable periods encountered on plant surfaces (Urbasch, 1983, 1986).

2.3. Conidia

Conidia of *Botrytis* are generally regarded as short-lived propagules in the field and their survival will largely be determined by temperature extremes, moisture availability, microbial activity and sunlight exposure. In the soil, *Botrytis* species are not particularly effective competitors and their conidia are subjected to fungistasis (Coley-Smith, 1980). Conidia of *B. cinerea* were able to survive on fruit surfaces of kiwifruit, remaining viable and infectious throughout the growing season (Walter et al., 1999b). Salinas et al. (1989) reported that conidia stored dry were able to survive at room temperature for up to 14 months, when some conidia were capable of

germinating *in vitro* and on ray florets of gerbera flowers to cause lesions. However, on the surface of Anjou pears, the viability of *B. cinerea* conidia after 7 weeks had declined to 10% germination (Spotts, 1985). In Scotland, conidia of *B. fabae* placed out of doors on cobwebs gradually lose their infectivity; only 15% of conidia were infective after 10 days exposure to ambient weather during summer (Harrison, 1983). When *B. cinerea* conidia were exposed to direct sunlight at midday in an Israeli summer, survival was only minutes (Rotem and Aust, 1991). In a New Zealand vineyard, mean percentages of conidia germinating after exposure to 4 h of sunlight ranged between 81 and 91% and between 49 and 50% after 8 h of sunlight exposure. Upon re-exposure on the second day, just 10 min of exposure to sunlight caused germination to drop between 26 and 27% for all isolates tested (Seyb, 2003). The UV spectrum of sunlight appeared to be the most important environmental factor influencing mortality of conidia (Rotem and Aust, 1991; Seyb, 2003).

Microconidia, which occur in all *Botrytis* species, provide an alternative microscopic propagule for these fungi when subjected to adverse conditions. In general they are found in ageing cultures of the fungus or those which are contaminated by other organisms, and in association with sclerotia. Microconidia develop from germ tubes produced by macroconidia, more mature hyphae, inside empty hyphal cells, and from appressoria and sclerotia (Jarvis, 1980a; Lorenz and Eichhorn, 1983). Germlings of *B. cinerea* form microconidia and chlamydospores in a corresponding manner on plant surfaces. On tomato plants, the dedifferentiation of *B. cinerea* appressoria proceeded by production of microconidia directly on appressoria, or by terminally and laterally outgrowing hyphae and their subsequent formation of microconidia (Urbasch, 1985a). The appressoria lost their function and the infection process at the site of interaction was interrupted. A similar process was infrequently observed on fruit surfaces of nectarine and plum that were subjected to intermittent dry periods, or were kept at 5°C after inoculation with dry, airborne *B. cinerea* conidia (Holz, 1999). Although their sole function is believed to be one of spermatization, they may also help the fungus to survive adverse conditions. The unicellular structures are generally produced in chains, but Urbasch (1984a) noted that after prolonged adverse conditions, *B. cinerea* formed clusters of microconidia bearing phialides and then embedded aggregates of these conidia in mucilage, which is then enclosed within a protective covering (hülle). Due to protection by this covering, the enclosed microconidial aggregates survived on dry agar plates without degeneration for up to 6 months and formed new mycelia when placed on fresh media. Urbasch (1984b) described a microcycle induced by nutritional deficiency that leads to production of microconidia and the oxygen concentration determined whether macro- or microconidia resulted, the latter being favoured by low O₂ concentrations. She also provides a good ultrastructural analysis of the differentiation of microconidia and comments on their rather thick outer wall (0.2 µm) suggestive of long-term survival (Urbasch, 1985b).

Macroconidia of *B. fabae* on agar films, buried in moist soil, germinated within a few days to produce short germ tubes which bore phialides and microconidia (Harrison and Hargreaves, 1977). After 29 days in moist soil, the macroconidia were dead and ruptured whereas the microconidia appeared to be quite healthy. Some

germination was observed amongst microconidia which had been left outside for 25-27 days during winter, suggesting that exposure to cold may be a factor in breaking the dormancy of microconidia. The ability of the microconidia to remain dormant under adverse conditions suggests that they may be important in the survival of *B. fabae* from one season to the next.

2.4. Mycelium

The survival of mycelium of *Botrytis* species under natural conditions has hardly been investigated and, unless particular care is taken, it is often difficult in practice to decide whether survival is by mycelium or whether microsclerotia or chlamydospores are involved. There is some evidence that the mycelium of certain *Botrytis* species, and especially those more specialized in their parasitism, can survive for considerable periods in bulbs, seeds and other vegetative plant parts (Coley-Smith, 1980). *B. cinerea* is considered to be a characteristic component of aerial surfaces of some species of plants whilst being absent or infrequently isolated from others. The frequency of isolation of the fungus tends to increase as the season progresses, reflecting an increasing ability to enter plant tissue as a weak parasite or as a saprophyte during senescence (Blakeman, 1980). Kobayashi (1984) observed that *B. cinerea* conidial masses developed throughout the year from mycelium in the fallen petals of 28 plant species belonging to 19 genera of 14 families.

3. Inoculum production and dispersal

It is generally assumed that for *B. cinerea*, inoculum is always present in the field and that production, liberation and dispersal of inoculum is an ongoing process (Jarvis, 1980b). This is clearly not always the case in all crops (Sosa-Alvarez et al., 1995; Seyb, 2003). There are various factors essential for high propagule numbers in the air: a viable, productive inoculum source, conditions favourable for propagule production, and for their dispersal at the source site. Correlations have been found between dispersal and conditions favourable for sporulation (usually surface wetness with moderate temperature) in many *Botrytis* species (Jarvis, 1980b). The frequency and duration of wetness events, and temperature, vary greatly during a growing season. It is anticipated that interrupted wetness periods, and temperature fluctuations, will affect the number of propagules produced (Rotem et al., 1978). A complicated relationship thus exists in the field between environmental conditions and propagule production and dispersal.

3.1. Dispersal and deposition

If it is to infect, the pathogen must conquer space (Zadoks and Schein, 1979), that is to move from the primary source and land on susceptible tissue. Each part of the fungus thallus can serve as a dispersal unit. These propagules are dispersed by wind, rain and insects.

3.1.1. Conidial dispersal by wind and rain

Conidia, which are dry and predominantly wind-dispersed, are generally considered to be the most important dispersal propagule of *Botrytis* species. Wind dispersal of conidia has three highly interdependent, yet distinct phases; liberation, transport and deposition (Aylor, 1990). Release of conidia in *Botrytis* species is caused by a twisting of the conidiophore which is brought about by changes in the relative humidity (Fitt et al., 1985) and their ejection by a mechanical shock. This mechanical shock has been considered to be caused by two forces, wind and splash (Fitt et al., 1985). Conidia of *Botrytis* species are typically found in highest concentration in the atmosphere during the daytime, often reaching a peak concentration near or shortly after mid-day (Jarvis, 1962a; Fitt et al., 1985) when wind speed and the level of turbulence near the ground are usually highest. A threshold wind speed has been demonstrated for their removal, and conidia of a given species are generally removed over a range of wind speeds. For these, the cumulative percentages of conidia removed increases rapidly with increasing speed. These curves tend to level off because a certain percentage of the conidia are difficult to remove from the source at any reasonable speed (Harrison and Lowe, 1987). Conidia of *Botrytis* species are released at different patterns from colonies, which can be ascribed to differences in spore size affecting the drying rate. Conidia of *B. cinerea* were consistently released at a faster rate from naturally infected broad bean leaflets than those of *B. fabae* (Harrison and Lowe, 1987). Conidia of *B. cinerea*, being smaller than those of *B. fabae*, may dry faster and consequently become more loosely attached than those of *B. fabae*.

Although average wind speeds in the lower part of closed crop canopies are typically a fraction of the speed above the canopy, gusts of wind with speeds several times faster than the local mean speed occur well inside plant canopies. These occur frequently enough to be important in the removal of conidia (Harrison and Lowe, 1987; Aylor 1990). After conidia are liberated from the source, some are transported within the canopy air space and some escape the canopy into the more freely moving air above. The number of conidia that escape the canopy depends largely on the balance between two competing forces, deposition and turbulent transport, and the vertical position of the inoculum source. In general, conidia produced on a source on the ground and lower in the canopy are exposed to slower wind speeds, less turbulence and rapid rates of sedimentation. They are thus transported over a short range (Fitt et al., 1985). In vineyards, 95% of *B. cinerea* conidia are deposited within 1 m from the ground source (Seyb, 2003). A similar pattern has been reported for *B. cinerea* dispersal in snap bean fields in which few conidia were detected beyond 2.5 m from the source (Johnson and Powelson, 1983).

The last phase in the dispersal of wind-borne conidia, deposition, is composed of two main processes, sedimentation and impaction (Aylor, 1978), both of which are influenced by wind strength. Sedimentation occurs in still air and is the process during which conidia descend under the influence of gravity with a certain terminal velocity. Air is rarely ever still; deposition is therefore a continuum of sedimentation and impaction (McCartney, 1994). Impaction is the process by which a conidium, because it is heavier than the surrounding air molecules, does not exactly follow the

fluctuations in the air and strikes an object even when air flows around it (McCartney, 1994). However, the effect of wind on deposition can be modified by attributes of the conidia. The rate of deposition, and therefore the steepness of deposition gradients, has been shown to be affected by whether the conidia are dispersed singly or in clusters. The greater the number of conidia clumped together, the faster the settling speed (Ferrandino and Aylor, 1984). Under simulated wind conditions, conidia of *Botrytis* species are released from different sources singly, and in clumps, consisting of c. three and five conidia per clump, for *B. cinerea* and *B. fabae*, respectively (Harrison and Lowe, 1987). Because similar proportions of conidia fell as clumps from undisturbed inverted cultures as from those blown by a strong wind and because the mean numbers of conidia per clump were similar, wind appears to have little effect on clumping (Harrison and Lowe, 1987).

Little is known about the deposition of airborne conidia under field conditions on different plant surfaces such as leaves, shoots and fruits. Fluorescence microscopy of leaves, berries and the inner bunch parts of grape (Coertze and Holz, 1999; Holz, 1999) and fruits of nectarine, plum and pear (Holz, 1999) dusted with dry *B. cinerea* conidia in settling chambers revealed that conidia were consistently deposited singly, and not in clumps or clusters. In these studies conidia were released from cultures or fruit with sporulating lesions in vacuum-operated settling chambers, or dispersed by air pressure into the top of the settling chamber.

Rain has been associated with large concentrations of airborne *Botrytis* conidia in both raspberry (Jarvis, 1962a) and grape (Vercesi and Bisiach, 1982), suggesting that it may be important in the release of conidia which are subsequently rain-dispersed. Investigations on the role of wind and rain in dispersal of *B. fabae* conidia in field bean plots, however, suggest that the majority of conidia are dispersed dry by wind, even during rain. Raindrops hitting leaves dislodge dry conidia from infected leaves, and experiments with simulated raindrops show puffs of dry conidia when the drops first hit dry leaves with sporulating lesions (Fitt et al., 1985). Laboratory experiments have shown that simulated raindrops can carry conidia within, or on the outside of splash droplets (Jarvis, 1962b; Hislop, 1969). Very few of the *B. cinerea* conidia dispersed by raindrops become wet enough to enter the droplets, and the majority are carried on the droplet surface as a dry coating (Jarvis, 1962b). Conidia of *B. cinerea* attach in two distinct stages to hydrophobic surfaces (Doss et al., 1993, 1995). The first stage, immediate adhesion, occurs upon hydration of freshly deposited conidia. Conidia of *B. cinerea* adhere to tomato fruit cuticle, grape berry epidermis, and leaves and petals of other hosts immediately upon hydration. Dry conidia of *B. cinerea* applied to wet fruit surfaces adhered to the same degree as conidia from liquid suspensions to the surface of plum and grape. The conidia adhere more strongly when applied in water suspension or to the wet surface of grape berries than when dry conidia are applied to a dry surface (Spotts and Holz, 1996). Raindrops may therefore deposit conidia carried on their surfaces as single cells on to plant surfaces during run-off. Data on washings made from grape berries in Californian (Duncan et al., 1995) and South African vineyards (G. Holz, University of Stellenbosch, South Africa, unpubl.) indicated that the number of *B. cinerea* conidia on berry surfaces was very low throughout the season, and *B. cinerea* occurred as single colony-forming units. These findings imply that infection

by solitary conidia, and not by conidial clusters, should play a prominent role in the epidemiology of *Botrytis* diseases.

3.1.2. Conidial dispersal by insects

Conidia of *Botrytis* species are also insect-dispersed. The conidia of *B. cinerea* are trapped in the ornamentations of segments, cuticle, body hairs and sculptured areas of the vinegar fly (*D. melanogaster*) (Louis et al., 1996), the grape berry moth (*Lobesia botrana*) (Fermaud and Le Menn, 1989), the New Zealand flower thrips (*Thrips obscuratus*) (Fermaud and Gaunt, 1995) and the Mediterranean fruit fly (*Ceratitis capitata*) (Engelbrecht, 2002). Ingested conidia also remain viable inside faeces of these insects. In the case of the Mediterranean fruit fly, digital photography and visual observations (Engelbrecht, 2002) of grape berries showed that the flies initially preferred to feed on the macerated tissue of the lesions that served as inoculum. However, they tended to feed on the sporulating colonies on the lesions. This was evident by the distinctive 'feeding paths' that appeared in the colonies as a result of their activities, and the disappearance of *B. cinerea* conidia from the colonies. Fluorescence microscopy revealed (Engelbrecht, 2002) that flies deposited conidia singularly, in feeding packages and in faeces on the surface of unblemished grape berries. Conidia in feeding packages were ensheathed by saliva and occurred in clusters of 10 to 50 conidia. An average of 60% of the conidia in feeding packages germinated under dry conditions (c. 56% RH).

3.1.3. Dispersal of other propagules

In some diseases, particularly those caused by *B. cinerea*, conidia seem of less importance than saprophytically-based mycelial inocula in establishing infections. It may well be that ascospores are more important than generally assumed; apothecia are easily overlooked in the field. Due to the ability of chlamydospores to germinate, they also represent dispersal units which can function as structures of infection. Urbasch (1984a) noted that in moist conditions the protective covering around microconidia aggregates became sticky and speculated that this may aid microconidia to adhere to surfaces of plants and insect vectors, which is indicative of their potential role in the survival and dispersal of the fungus.

B. cinerea can infect pollen grains and petals of strawberry (Bristow et al., 1986) and such floral organs can then be dispersed by wind, attach to other tissues for mycelial spread and infection and serve as a site for production of another generation of conidia. Colonised senescent blossoms of *Phaseolus vulgaris* lying on the moist soil surface beneath a bean crop produce large quantities of secondary inoculum (Johnson and Powelson, 1983).

4. Growth on plant surfaces

Germination and germ tube growth of *Botrytis* conidia on plant surfaces, host penetration, and duration of the incubation period are important stages in the process

of infection that can be used to investigate various aspects of host resistance, fungicide action and biological control. Current knowledge on the behaviour of conidia in most of these studies is based primarily on interpreting germling growth during artificial inoculation. For artificial inoculation, plant parts are sprayed with, dipped in, or injected with conidial suspensions, or suspension droplets are placed on to plant parts. Infection studies with conidial suspensions of *Botrytis* on different hosts have shown generally that the more conidia in the infection drop the more likely is aggressive infection. Therefore, to achieve symptom expression during inoculation, conidial suspensions usually contain a high number of conidia, 1×10^3 to 1×10^5 per millilitre of carrier, which is mostly sterile distilled water. In some cases, conidial suspensions are supplemented with nutrients to increase the possibility of penetration of tissue otherwise resistant to infection.

Microscopic observation of the sequence of events accompanying germination in conidia-bearing droplets on susceptible hosts revealed rapid germination with germ tubes protruding within 1-3 h after inoculation. Various penetration structures, ranging from simple to compound appressoria (see Emmet and Parbery (1975) for details) are formed prior to penetration of the cuticle. These structures form within 6 h after germination when germ tubes reach lengths of 10-15 μm . In *B. cinerea*, germ tubes commonly form protoappressoria (slightly swollen, hyaline germ tube apices adhering to the host and giving rise to an infection peg) and simple appressoria after 6 h (Clark and Lorbeer, 1976; Fourie and Holz, 1994, 1995). When exogenous nutrients are available, multicellular, lobate appressoria are formed after 12 h (Garcia-Arenal and Sagasta, 1980; Van der Heuvel and Waterreus, 1983). Continued growth in the presence of exogenous nutrients often leads to the formation of infection cushions (Backhouse and Willetts, 1987). In inocula with high conidial concentrations, a high proportion of germ tubes produce protoappressoria, whereas with lower conidial concentrations germ tubes produced predominantly multicellular, lobate appressoria and infection cushions (Van der Heuvel and Waterreus, 1983). Addition of exogenous nutrients to inoculum is a prerequisite for the formation of multicellular, lobate appressoria and for infection cushions on cucumber leaves (Akutsu et al., 1981), strawberry leaves and cucumber cotyledons (Shirane and Watanabe, 1985). However, these structures are all formed by *B. cinerea* conidia without the addition of exogenous nutrients on floral tubes, fragile petals and fruits of nectarine and plum (Fourie and Holz, 1994, 1995) and leaves and berries of grape (Holz, 1999; Coertze et al., 2001).

Infection studies suggest that conidial density and nutrient supplements may not only influence the pre-penetration activities in conidia-bearing droplets on plant surfaces, but also subsequent symptom expression. In water, *B. squamosa* induced symptoms on onion leaves. Addition of exogenous nutrients to inoculum increased the frequency of lesion formation (Clark and Lorbeer, 1976). Leaves of onion (Clark and Lorbeer, 1976), cucumber (Akutsu et al., 1981), strawberry (Shirane and Watanabe, 1985), broad bean (Harper et al., 1981), cucumber cotyledons (Shirane and Watanabe, 1985) and fruits of plum and nectarine (Fourie and Holz, 1998) remained asymptomatic when *B. cinerea* was inoculated in water. Addition of exogenous nutrients to inoculum was requisite for symptom induction by *B. cinerea* on these hosts. The pathogen could not induce symptoms on cucumber leaves when

conidial density in glucose or sucrose suspensions were low, but enhancing the conidial density caused rapid spreading lesions (Akutsu et al., 1981). Inoculations of primary leaves of French bean with conidia of *B. cinerea* suspended in glucose supplemented with KH_2PO_4 or Na-ATP as infection stimulants, yielded mostly spreading lesions (Van der Heuvel and Waterreus, 1983). Decreasing concentrations of conidia caused a delay of 1-4 days in the formation of spreading lesions. Although in most of these studies conidia suspended in nutrients allowed more extensive germ tube and hyphal growth and the development of a range of appressoria, only a small proportion of germlings of such inocula gave rise to penetrations (Williamson et al., 1995). The number of visible penetrations produced by inocula containing high conidial concentrations amounted to only c. 5-10% of all conidial germlings. These percentages were higher (20-80%) with lower conidial concentrations (Van der Heuvel and Waterreus, 1983). This agrees with Hill et al. (1981) studying unsupplemented conidial suspensions where from a total of 3500 conidia per 15.9 mm^2 cuticle surface, only 1-2 conidia were able to penetrate the cuticle layer of grape berries.

Use of glucose and phosphate supplements in small droplets ($5 \mu\text{l}$) as to ensure an adequate oxygen supply to conidia is now a standard method for host inoculation with *B. cinerea* in gene knock-out studies (e.g. Klimpel et al., 2002; Schouten et al., 2002) and for chemical studies on lesions (Muckenschnabel et al., 2002). Another significant factor affecting the success of inoculations made with conidial suspensions is the spectrum of light to which the host and pathogen is exposed. Islam et al. (1998) showed that near-UV and blue light (300-520 nm) induced negative phototropism in *B. cinerea* inoculated on to leaves of *V. faba*, and that red light (600-700 nm) induced positive phototropism and reduced the number of successful infections substantially.

Aggressive infection due to the addition of exogenous nutrients to inoculum may be ascribed to factors other than an increase in surface colonisation and successful penetration. Stimulation of infection by *B. cinerea* after addition of certain sugars to artificial inoculation is probably due to the active forms of oxygen formed (see Chapters 7 and 8), rather than to a nutritional effect (Edlich et al., 1989). Sugars act as substrates for the production of hydrogen peroxide and other forms of superoxide and hydroxyl radicals, which are highly toxic and may be capable of destroying relatively inert materials, such as cutin (see Chapter 5 for details). The addition of sugars also enables *B. cinerea* to overcome the inhibitory action of wyerone acid, an important phytoalexin produced by *Vicia faba* (Mansfield and Deverall, 1974). The mode of action of KH_2PO_4 or ATP in aggressive infection is unknown. Although phosphates might act by predisposing leaves to infection by *B. cinerea* (Van den Heuvel, 1981), they might also influence fungal metabolism, e.g. activity of cell wall-degrading enzymes, more directly.

The sequence of events accompanying germination of natural inoculum on plant surfaces, and how conidial density and substances occurring on these surfaces relate to infection and subsequent symptom expression has rarely been studied. The data from artificial inoculation studies mostly relate to detached plant parts kept in moist chambers, a situation that could lead to greater susceptibility to the pathogen than

that of parts attached to the host plant. The single-drop inoculation of plant parts with a high number of conidia in the laboratory also differs from inoculation in the field, where single, airborne conidia may be deposited intermittently at several sites on a fruit surface. In the event of rain, the frequent run-off of inoculum-containing raindrops would promote faster drying of host surfaces and a lower incidence of infection than might be expected from laboratory-inoculated material. In the latter instance, drops deposited on host surfaces remain undisturbed for longer periods, which could create highly localised zones of disease pressure and the collapse of host resistance (Fourie and Holz, 1995; Coertze and Holz, 1999; Coertze et al., 2001).

Dispersal by airborne conidia is an important mechanism by which a disease epidemic is perpetuated; its investigation requires a dry inoculation technique to simulate the natural dispersion pathway. Uncontrolled clouds of dry *B. cinerea* inoculum have been discharged over target hosts (Rijkenberg et al., 1980; Walter et al., 1999a). Alternatively, amounts of dry *B. cinerea* conidia have been directly brushed on to the host (Williamson et al., 1987). Settling chambers have been constructed to provide more controlled delivery of dry conidia (Salinas et al., 1989; Reifschneider and Boiteux, 1998). With this method, dry conidia are dusted in a settling chamber on to plant surfaces. The conidia can be subjected to conditions commonly encountered by the pathogen on plant surfaces: dry conidia on a dry surface under dry conditions, dry conidia on a dry surface under high relative humidity, and dry conidia exposed to a film of water on the host surface. Working with gerbera flowers, Salinas et al. (1989) observed that germ tubes of dry-inoculated conidia were mostly short; less than 1% of the germ tubes were longer than 20 μm . Dry-inoculated conidia of *B. cinerea* germinated in a similar fashion on fruits of tomato (Rijkenberg et al., 1980), plum and nectarine (Fourie and Holz, 1994), grape (Coertze et al., 2001), grape leaves (Holz, 1999) and rose petals (Pie and de Leeuw, 1991) held at high humidity. In fact, some germlings formed a protoappressorium underneath the conidium (Holz, 1999; Coertze et al., 2001). Although dry conidia were used in these studies, the plant material was held at high humidity in conditions where surface moisture may have formed. Williamson et al. (1995) describes the behaviour of dry and wet conidia of *B. cinerea* on the surface of rose petals held at precisely controlled humidities. Although conidia in all cases germinated with one or more germ tubes, the subsequent growth and behaviour of developing germ tubes varied considerably according to the mode of inoculation. Dry conidia germinated in the absence of surface water under humidities ranging from 94 to 100% RH, but germ tubes mostly remained shorter than the conidia. No extracellular material was visible on germ tubes arising from these conidia. On spray inoculated petals, conidia in water droplets germinated to produce long germ tubes. The morphogenesis of *B. cinerea* and *B. fabae* germ tubes was similarly affected whether conidia were inoculated dry or in the presence of aqueous glucose on to *Vicia faba* leaves (Cole et al., 1996). In the latter study, conidia and germ tubes grown in the presence of glucose were often encased by a sheath of fibrillar-like matrix material. Transmission electron microscopy revealed that a distinct amorphous pad of matrix material surrounded the short germ tubes on the bean leaf surface. The matrix material probably acts as an adhesive pad and thus serves to

secure the position of the germ tube at the site of penetration (see Chapter 5 for ultrastructural studies).

Studies with dry and wet *Botrytis* conidia provide evidence that the mode of inoculation may not only influence conidial growth on plant surfaces, but also subsequent symptom expression. On gerbera flowers, only inoculation with dry *B. cinerea* conidia led to the development of the typical necrotic lesions, as found in practice (Salinas et al., 1989). Inoculation with conidial suspensions led to the appearance of different types of symptoms: smaller and larger necrotic lesions, partial rotting of ray floret or of the whole flower, or even no symptoms at all. Working with conidial suspensions of *B. cinerea* in distilled water on mature berries, Nair and Allen (1993) showed that a 14-h wetness period is needed to give 63% symptomatic berries at 23°C. Berries at different phenological stages inoculated with single airborne conidia remained asymptomatic after extended periods (3-96 h) of moist, or wet incubation (Coertze and Holz, 1999; Coertze et al., 2001). This finding suggests that when high humidity (c. 93% RH) prevails in nature, airborne conidia will have an equal potential to infect dry and wet berry surfaces. This finding can have a major impact on the validation of disease prediction models.

5. Infection pathways on diverse plant organs

Botrytis pathogens are well known for their ability to form either spreading lesions in host tissues, or latent infections in young fruit and seeds. The route used by the pathogen to enter the host usually plays an important role in the establishment of the two types of infection.

5.1. Penetration through specialised host structures

Different routes have been described for the penetration and establishment of quiescent or latent infections by *B. cinerea* in flowers and developing fruit. In blackcurrant, the pathogen can grow through the style to the carpels (McNicol and Williamson, 1989). In pear (De Kock and Holz, 1992), as in strawberry (Bristow et al., 1986), styles might not be an important source of latent infection. On the other hand, infected stamens are important penetration sites in pear. Unlike the styles, hyphae in pear filaments grew without restriction and progressed, via vascular tissue, through sepals into tissues of the upper end of the flower receptacle, or of the mesocarp adjoining the sepals. *B. cinerea* has been associated with transmitting tissue of styles specialised to guide and nourish pollen tubes as they grow rapidly to the ovules in raspberry (McNicol et al., 1985), strawberry (Bristow et al., 1986) and blackcurrant (McNicol and Williamson, 1989).

Besides the specialised stigmatic fluids secreted by hosts for pollen germination, *B. cinerea* seems to have a remarkable ability to utilise other host fluids secreted for defence against insects. For example in chickpea (*Cicer arietinum*), dry-inoculated conidia of *B. cinerea* germinated in the malic acid-rich exudate released by stalked multicellular glands studied by low temperature scanning electron microscopy, and penetrated the gland cells to grow basipetally into the leaf lamina, as seen by

fluorescence microscopy; it is not clear if this is a major infection pathway but further studies are required (G. Senthil, G.H. Duncan and B. Williamson, Scottish Crop Research Institute, Dundee UK, pers. comm.)

Inoculation of immature grape berries with *B. cinerea* showed that the pathogen can enter styles and then become latent in the necrotic stylar tissue (McClellan and Hewitt, 1973). However, studies conducted on the occurrence of natural *B. cinerea* inoculum at various positions in grape bunches showed that styles might not be an important source of latent infection on grape. By use of a differential set of paraquat and freezing treatments on untreated and surface-sterilized berries, it was found that at all phenological stages the stylar end was virtually free of *B. cinerea* (Holz et al., 2003). The isolation studies showed that the pathogen seldom occurred on the surface or in the skin tissue near the proximal end, 'cheek' (equator) or stylar end of the berry. These findings indicate that *Botrytis* bunch rot was unlikely to be caused by colonisation of the pistil, and subsequent latency in the stylar end, as was observed elsewhere. Instead, berry rot consistently developed from the berry-pedicle attachment zone where micro-fissures in the epidermis may lead to exudation of nutrients.

5.2. Penetration through undamaged host tissue and natural openings

Direct penetration of the undamaged cuticle and natural openings by germlings in conidial suspensions has been observed in many *Botrytis*-host combinations (see Chapter 5). *B. elliptica* conidia germinated on both adaxial and abaxial surfaces of Oriental lily, but germ tubes failed to invade epidermal cells on the adaxial surface. On abaxial surfaces, germ tubes penetrated through stomatal openings, through the epidermis near guard cells, or directly through epidermal cells (Hsieh et al., 2001). *B. cinerea* penetrated fruits of plum and nectarine directly in the centre of the epidermal cells at the indentation above the anticlinal wall, at the indentation in the fruit surface adjacent to guard cells, through the guard cells, and through stomatal openings (Fourie and Holz, 1995). Sometimes more than one penetration occurred from the same or different conidia. Nelson (1951) found that *B. cinerea* penetrated grape berries directly through the cuticle. Others (Pucheu-Planté and Mercier, 1983) found the primary sites for penetration to be stomata and micro-fissures in the grape berry skin. The fungus entered French bean leaves directly and also through trichomes (Van der Heuvel and Waterreus, 1983). For *B. squamosa* on onion leaves, penetration occurred through stomata or the cuticle (Clark and Lorbeer, 1976). Histological studies with *B. cinerea* on fruit of plum, nectarine (Fourie and Holz, 1995), pear and grape (Holz, 1999) revealed that conidia suspended in droplets were inclined to settle in the centre of the droplet which caused an agglomeration of conidia. This action forced conidia to settle around or on stomata, and to enter these sites. They germinated and hyphal mats formed on the host surface in most droplets. It was also noted that micro-fissures, which acted as avenues for penetration by hyphal mats, developed with time in the cuticle under the droplet. Simulation of natural infection by dusting surfaces of these hosts with dry conidia in settling chambers indicated that conidia seldom landed on stomata or lenticels. In such an

event, the germ tubes formed by conidia on moist surfaces were too short to enter these structures. On wet surfaces, germ tubes or hyphae usually grew around the raised stoma or lenticel. Furthermore, attempted penetration was always direct, irrespective of germ tube length, number, or branching (Holz, 1999; Coertze et al., 2002).

5.3. Penetration through wounds

Wound infection occurs when conidia enter a wound in the host tissue. In nearly all experiments with *Botrytis* species, especially *B. cinerea*, inoculations of fresh wounds with varying numbers of conidia in water suspensions result in establishment of infection. Little is known about the relationship between the inoculum dosage in air and incidence of wound infection, and how the relationship is influenced by environmental, wound and host factors. To better understand this relationship, information is needed on the period over which conidia have accumulated, the time they are able to survive and remain infectious, time of wounding in relation to conidium arrival at the infection court and host surface wetness. Different patterns of conidium and germling dieback were observed by microscopic observation amongst individuals on fruit and leaf surfaces (Holz, 1999; Coertze et al., 2001). On moist fruit, some conidia or germlings died, or only the conidium or short germ tube died on some germlings. A similar pattern of germling dieback was observed on wet fruit. Sections of long germ tubes, or branched germ tubes of some germlings, died, whereas on some germlings the conidium remained viable and the extended germ tube succumbed. Complete dieback was most pronounced in germlings without appressoria. Dieback of conidia and germlings occurred at a significantly higher rate on wet than on moist surfaces, and was more pronounced on immature than on mature fruits.

B. cinerea conidia or germlings adhering to the cuticle are not easily dislodged from fruit surfaces (Spotts and Holz, 1996). Therefore, to infect a wound in the host tissue, newly arrived conidia should alight in or near the wound and grow into the wound under the prevailing conditions. On the other hand, in the event of wounding, propagules of *B. cinerea* may occur in various growth stages at the wound site. Firstly, there may be conidia in a dormant state adhering to the skin. Secondly, there may be germlings that had penetrated the skin, but were localised by host defence. In the case of dormant conidia adhering on a dry surface, wounding should be near a conidium thereby breaking the cuticle and supplying the conidium with necessary moisture and nutrients to germinate and to infect. In the case of a germling that had penetrated the skin, but was localised by host defence, wounding should be near the germling, an action that should overcome the host resistance and supply the established pathogen with the necessary nutrients to escape the host defence barrier and cause the tissue to rot.

Coertze and Holz (2002) described infection of wounds on grape berries exposed to freshly deposited airborne conidia, and of wounds on berries carrying previously deposited conidia and germlings (latent infections). Fresh (immature and mature), and cold-stored grapes (mature), which are respectively highly resistant and highly

susceptible to infection and symptom expression by single airborne conidia of *B. cinerea* (Coertze and Holz, 1999; Coertze et al., 2001), were included in their study. In the case of berries inoculated at bunch closure and harvest stage, wounds were not infected by conidia deposited on berries 4 days prior to wounding. This finding indicated that, following adhesion and the first stages of growth, the pathogen did not survive for extended periods on surfaces of immature and mature grape berries. Freshly deposited dry conidia were needed to infect the wounds. The freshly deposited conidia furthermore needed free water, and not high humidity or wound exudates, to infect the fresh wounds. Proportions of wounds infected were extremely low. Fluorescence microscopy explained the inability of the conidia to infect the wounds by the phenomenon that conidia seldom landed at the wound periphery, or in the wound cavity. Nearly half the number of wounds on berries at bunch closure did not produce an exudate. When exudates were formed, it was produced on to a narrow margin of the surrounding skin. Germlings in the vicinity of wounds seldom had the capacity to reach the wound periphery and to enter the wound cavity.

5.4. The role of insects in wound infection

A synchronisation of a combination of events necessary for successful wound infection (Coertze and Holz, 2002), fresh wounds, freshly dispersed conidia and free water on the host surface, may not commonly occur in the field. Insects may play a very prominent role in this context and can be considered primary role players in disease outbreaks in the field. They can act both as suppliers of inoculum to wounds, and as initiators of the disease cycle under conditions generally unfavourable for disease development (Engelbrecht, 2002). Inocula packages consisting of clusters of conidia or/and mycelia, which might also be deposited into wounds, should be more aggressive than single conidia that land near the wound and that should grow into the wound under the prevailing conditions (Coertze and Holz, 2002; Engelbrecht, 2002). In both kiwifruit (Fermaud and Gaunt, 1995) and grape (Marroni et al., 2003), latent *B. cinerea* infection has been ascribed to the activities of the New Zealand flower thrips (*T. obscuratus*). The studies (Marroni et al., 2003) with thrips infested with a wild *B. cinerea* strain, and a marker strain deficient in nitrate reductase, showed that asymptomatic latent infections at flowering were always sited at the receptacle of the berry pedicel, the primary position for *B. cinerea* infection in grape bunches (Holz et al., 2003). Engelbrecht (2002) investigated the transport of *B. cinerea* by the Mediterranean fruit fly (*C. capitata*) on grape, and found a peculiar interaction between the pathogen, the fly and symptom expression at the berry-pedicel attachment zone. She monitored the activities on grape berries of flies using digital photography, and observed that visitations to the berry-pedicel attachment zone increased substantially from véraison to harvest, indicating the possibility of nutrient leakages at this site. The systematic explorative behaviour of the vinegar fly (*D. melanogaster*) on fruits and grapes at various maturity stages makes the fly a self-guided agent responsible for *B. cinerea* dispersal in vineyards and orchards (Louis et al., 1996). Vinegar flies were observed to emerge from

unblemished, surface disinfested grapes, nectarine and plum fruit incubated in moist chambers after 1 h freezing at -12°C (Holz, 1999).

6. Conclusions

It is important to recognize the distinction between the growth of *Botrytis* species and penetration of host tissue in artificial infection studies under ideal laboratory conditions and natural infection in the field. In the field, each part of the host plant is a potential target for deposition, growth and penetration of *Botrytis* inoculum. The inoculum consists of macro- and microconidia, ascospores, macro- and microsclerotia, chlamydospores and mycelia. The deposition site on the host will be determined by factors such as the position of the primary inoculum source, wind, rain, insect activity and feeding preferences. Growth on the host surface, and the pathway used to enter host tissue, will depend on factors such as inoculum type, the availability of free water and nutrients, cuticle characteristics, host natural exudates at floral organs or other glands, the abundance of natural openings and the size and age of wounds. Current knowledge on the options taken by different *Botrytis* species to enter a host is based primarily on interpreting germling growth during artificial inoculation of specific plant parts or organs with large numbers of macroconidia. There is little information about the behaviour of microconidia, ascospores, macro- and microsclerotia, chlamydospores and mycelia. It is therefore important to gain further knowledge on the ecology and behaviour of natural inoculum, and to simulate its behaviour in infection studies, as well as on host resistance, disease prediction models, timing of fungicide application and biological control.

7. References

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CHAPTER 3

TAXONOMY AND GENETIC VARIATION OF *BOTRYTIS* AND *BOTRYOTINIA*

Ross E. Beever and Pauline L. Weeds

Landcare Research, Private Bag 92170, Auckland, New Zealand

Abstract. The species of the anamorphic genus *Botrytis* and its associated *Botryotinia* teleomorphs are briefly assessed. Recent progress in understanding the genetics of variation in the polyphagous *B. cinerea* (teleomorph *Bt. fuckeliana*) is summarised, with emphasis on chromosome complement and extrachromosomal elements. Sexual and vegetative compatibility studies are reviewed in relation to the limited evidence of clonality revealed by DNA population markers. It is concluded that in contrast to the traditional view of this species, sexual reproduction plays a major role in determining variation whereas heterokaryosis plays only a limited role. Evidence supporting the existence of a second polyphagous species within *B. cinerea sensu lato* is discussed. The limited knowledge of the genetics of the host-restricted species is briefly described.

1. Introduction

Botrytis, especially the species *Botrytis cinerea*, has a reputation for unusual variability, although the underlying mechanisms involved are not well understood. Jarvis (1977, 1980) and Lorbeer (1980) provide comprehensive reviews of both the taxonomy and genetics, but these treatments are now over 20 years-old. This review examines recent advances with emphasis on evidence of variability and the mechanisms that give rise to it.

The generalised life cycle of *Botrytis* comprises various stages: a somatic (vegetative), mycelial system that produces asexual conidia (strictly macroconidia – the *Botrytis* anamorph stage), sclerotia and microconidia (spermatia). Sclerotia usually germinate to produce mycelium or conidia, but after appropriate pre-conditioning and fertilization, they may germinate to produce apothecia (the *Botryotinia* teleomorph stage), containing ascospores resulting from meiosis. For convenience, and because not all *Botrytis* species are known to have teleomorphs, we use *Botrytis* in the generic sense to include both *Botrytis* (abbreviated *B.*) and *Botryotinia* (abbreviated *Bt.*).

2. Taxonomy

The genus *Botrytis* comprises over 20 species, with a proportion linked to sexual stages (Table 1). Broadly speaking the group forms a coherent grouping, although Hennebert (1973), for example, segregated *B. ricini* into the genus *Arthrobotrys*. As well as the species listed in Table 1, additional host-specific species undoubtedly remain to be described, and a number of described species are poorly characterised. For example, a globose-spored species was described in the USA from *Vicia villosa* (not *Linaria* as listed by Farr et al., 1989) under the name *B. viciae* Green (an illegitimate homonym of *B. viciae* Berk. 1846). This fungus has also been reported from other *Vicia* species in Australia (Stovold and Walker, 1980; Backhouse et al., 1984) but further studies are needed. Furthermore, some species presently in *Sclerotinia*, such as *S. veratri*, may be species of *Botryotinia* (Kohn, 1979a).

Table 1. Some Species of *Botrytis* and *Botryotinia*

<i>Botrytis</i> sp. (anamorph)	<i>Botryotinia</i> sp. (teleomorph)	Mating system	Major Hosts	Refs ¹
<i>B. aclada</i> Fresen.	-	-	<i>Allium</i>	4, 7, 8, 16
<i>B. allii</i> Munn	-	-	<i>Allium</i>	4, 8, 16
<i>B. anthophila</i> Bondartsev	? ²	-	<i>Trifolium</i>	4, 8, 12
<i>B. byssoidea</i> J.C. Walker	? ³	-	<i>Allium</i>	4, 7, 8, 16
<i>B. calthae</i> Hennebert	<i>Bt. calthae</i> Hennebert & M.E. Elliott	-	<i>Caltha</i>	4, 6, 7, 8, 9
<i>B. cinerea</i> Pers.:Fr.	<i>Bt. fuckeliana</i> (de Bary) Whetzel	Heterothallic	Polyphagous	4, 7, 8, 9
<i>B. convallariae</i> (Kleb.) Ondřej	-	-	<i>Convallaria</i>	8
<i>B. convoluta</i> Whetzel & Drayton	<i>Bt. convoluta</i> (Drayton) Whetzel	-	<i>Iris</i>	4, 7, 8, 9
<i>B. croci</i> Cooke & Massee	-	-	<i>Crocus</i>	7, 8, 11
<i>B. elliptica</i> (Berk.) Cooke	? <i>Botryotinia</i> sp.	Heterothallic	<i>Lilium</i>	4, 7, 8, 13, 14
<i>B. fabae</i> Sardiña	<i>Bt. fabae</i> J.Y. Lu & T.H. Wu	-	<i>Vicia</i>	7, 8, 15
<i>B. ficararum</i> Hennebert	<i>Bt. ficararum</i> Hennebert	-	<i>Ficaria</i>	6, 7, 8, 9
<i>B. galanthina</i> (Berk. & Broome) Sacc.	-	-	<i>Galanthus</i>	4, 7, 8
<i>B. gladiolorum</i> Timmerm.	<i>Bt. draytonii</i> (Buddin & Wakef.) Seaver	-	<i>Gladiolus</i>	4, 7, 8, 9
<i>B. globosa</i> A. Raabe	<i>Bt. globosa</i> N.F. Buchw.	Homothallic	<i>Allium</i>	1, 7, 8, 9
<i>B. hyacinthi</i> Westerd. & J.F.H. Beyma	-	-	<i>Hyacinthus</i>	4, 7, 8

<i>Botrytis</i> sp. (anamorph)	<i>Botryotinia</i> sp. (teleomorph)	Mating system	Major Hosts	Refs ¹
<i>B. narcissicola</i> Kleb. ex Westerd. & J.F.H. Beyma	<i>Bt. narcissicola</i> (P.H. Greg.) N.F. Buchw.	-	<i>Narcissus</i>	4, 7, 8, 9
<i>B. paeoniae</i> Oudem.	-	-	<i>Paeonia</i> , <i>Allium</i>	4, 7, 8, 9
<i>B. pelargonii</i> Røed	<i>Bt. pelargonii</i> Røed	-	<i>Pelargonium</i>	7, 8, 9
<i>B. polyblastis</i> Dowson	<i>Bt. polyblastis</i> (P.H. Greg.) N.F. Buchw.	-	<i>Narcissus</i>	4, 7, 8, 9
<i>B. porri</i> N.F. Buchw.	<i>Bt. porri</i> (J.F.H. Beyma) Whetzel	Homothallic	<i>Allium</i>	3, 7, 8, 9
<i>B. ranunculi</i> Hennebert	<i>Bt. ranunculi</i> Hennebert & W.H. Groves	Heterothallic	<i>Ranunculus</i>	4, 6, 7, 8, 9
<i>B. ricini</i> N.F. Buchw.	<i>Bt. ricini</i> (G.H. Godfrey) Whetzel	Homothallic	<i>Ricinus</i>	4, 5, 7, 8, 9
<i>Botrytis</i> sp.	<i>Bt. fritillarii-pallidiflori</i> Q.T. Chen & J.L. Li	-	-	10
<i>Botrytis</i> sp.	<i>Sclerotinia spermophila</i> Noble ²	Homothallic	<i>Trifolium</i>	4, 8, 9, 12
<i>B. sphaerosperma</i> N.F. Buchw.	<i>Bt. sphaerosperma</i> (P.H. Greg.) N.F. Buchw.	-	<i>Allium</i>	7, 8, 9
<i>B. squamosa</i> J.C. Walker	<i>Bt. squamosa</i> Vienn.-Bourg.	Heterothallic	<i>Allium</i>	2, 4, 7, 8, 9
<i>B. tulipae</i> Lind	-	-	<i>Tulipa</i> , <i>Lilium</i> , <i>Allium</i>	4, 7, 8, 9

¹1=Buchwald, 1953; 2=Bergquist and Lorbeer, 1972; 3=Elliott, 1964; 4=Farr et al., 1989; 5=Godfrey, 1923; 6=Hennebert and Groves, 1963; 7=Hennebert, 1973; 8=Jarvis, 1980; 9=Kohn, 1979a; 10=Li and Chen, 1987; 11=Moore, 1959; 12=Noble, 1948; 13=Van den Ende and Pennock, 1996; 14=Van den Ende and Pennock-Vos, 1997; 15=Wu and Lu, 1991; 16=Yohalem et al., 2003.

²It has been assumed (e.g. Farr et al., 1989) that *Sclerotinia spermophila* is the teleomorph of *B. anthophila* but Noble (1948), although discussing this possibility, concluded that the linkage needed confirmation. Although *S. spermophila* may belong to *Botryotinia* (Kohn, 1979a), the combination of *Bt. spermophila* used by Jarvis (1977) has not been formalised.

³It has been assumed (e.g. Jarvis, 1980) that *Bt. allii* (Sawada) Yamam. is the teleomorph of *B. byssoidea*. However, Kohn (1979b) provided evidence that Yamamoto was in error in concluding Sawada's species produces a *Botrytis* anamorph and she recombined *Sclerotinia allii* (Sawada) as *Ciborina allii* (Sawada) Kohn. Nevertheless, Yamamoto (1959), who worked on Japanese isolates, determined the anamorph as *B. byssoidea*, in which case the teleomorph he describes is that of *B. byssoidea*, albeit as yet unnamed. However, the relationships of the Japanese fungus needs further study (Hennebert, 1963; Neilsen et al., 2001).

Botryotinia is closely related to *Sclerotinia* and indeed findings with the rDNA internal transcribed spacer (ITS) region challenge the generic delimitation and provide support for a broad concept for *Sclerotinia* (Kohn, 1979a; Holst-Jensen et al., 1998). The association of host-specific taxa with primarily northern hemisphere temperate hosts indicates *Botrytis* is a northern hemisphere genus that has probably been widely distributed by human activity including movement to southern temperate regions. Knowledge of sexual breeding systems is critical to understanding variation, but the rarity of apothecia of most species in the field, and

the patience needed to produce the sexual stage in the laboratory, mean information is incomplete.

Three species concepts have been emphasised in fungi, albeit with different shades of interpretation: the morphological based on appearance, the biological based on interbreeding, and the phylogenetic based on common descent, although with plant pathogens host specificity is also emphasised (Brasier, 1997; Harrington and Rizzo, 1999; Taylor et al., 2000). Species of *Botrytis* have to date been delimited primarily on the basis of morphological and cultural characteristics coupled with host specificity (Hennebert, 1973; Jarvis, 1977, 1980). Features such as sclerotial size and form and conidium size are useful in delimiting some species, but many species are morphologically similar and growing conditions significantly influence variation. No key to all recognised species is available and identification of species based on traditional criteria can be fraught (Nielsen et al., 2001). Use of the biological species concept based on conducting sexual crosses *in vitro* is limited and sexual crosses have been largely confined to elucidation of the sexual system, although Bergquist and Lorbeer (1972) reported crosses were unsuccessful between *B. squamosa* and *B. cinerea*. Most field isolates of *B. cinerea* can be readily crossed in the laboratory producing highly viable ascospores, suggesting the existence of one inter-breeding population (Sect. 3.2). Nevertheless, recent evidence suggests the existence of a group of *B. cinerea*-like isolates unable to cross with *B. cinerea* tester strains, at least in parts of Europe (Sect. 3.6).

Another approach to defining biological species involves determining the distribution of allelic variation to define populations that are inter-breeding. This approach has been applied to *B. cinerea* using DNA markers in particular, and supports the proposal that *B. cinerea*, as currently recognised, does indeed comprise two distinct inter-breeding populations (Sect. 3.6). The phylogenetic approach to systematics has been boosted by the advances in DNA sequencing, but for *Botrytis* this approach is in its infancy. The ITS rDNA region has been widely used for species-level discrimination of fungal species, but variation in the ITS region within *Botrytis* is low, limiting its use in this genus (Nielsen et al., 2001). The intergenic spacer region (IGS) rDNA region may offer better prospects (Giraud et al., 1997), although its usefulness may be limited by recombination. The value of using multiple genes to recognise species on the basis of concordance between independent gene phylogenies has been emphasised by Taylor et al. (2000). A preliminary phylogenetic analysis for four nuclear genes (Leroux et al., 2002b) demonstrates the existence of two phylogenetically distinct groups in *B. cinerea sensu lato* and also confirms a close relationship between these species and *B. calthae*, *B. convoluta* and *B. fabae*. Indeed, the analysis suggests *B. calthae* and *B. convoluta* are segregates within the *B. cinerea* 'phylogenetic' species. These latter species are all in the *B. cinerea* complex recognised by Hennebert and Groves (1963), and have been distinguished primarily by host range coupled with subtle differences in morphology.

An exemplar for the application of modern methods to species recognition in *Botrytis* has been provided for the neck-rotting species of onion. Universal-primed polymerase chain reaction (UP-PCR) fingerprinting, coupled with restriction analysis of ITS rDNA regions, allowed five groups to be distinguished: *B. cinerea*,

B. squamosa, *B. byssoidea*, and two groups in *B. aclada* (AI and AII) (Nielsen et al., 2001). The two groups of *B. aclada* could also be distinguished on spore size and chromosome number (AII has 32 compared with 16 in the other species), and comparison of sequence-characterised DNA fragments provided strong evidence that AII arose as an interspecific hybrid between AI and *B. byssoidea* (Nielsen and Yohalem, 2001). Subsequently, Yohalem et al. (2003) proposed that the name *B. aclada* be reserved for the small-spored subgroup (A1) and the previously synonymised *B. allii* name be applied to the larger-spored group (AII). The species concept resulting from this study is essentially the operational species unit (OSU) of Brasier (1997), defined as population units “sharing a common gene pool and exhibiting a common set of physiological, ecological and morphological attributes”.

3. *Botrytis cinerea*

Most genetic studies in *Botrytis* have been carried out on *B. cinerea*. The two single ascospore mating type tester strains SAS56 and SAS405 provide a common focus in many studies and are referred to where appropriate. A recurrent theme of variation in *B. cinerea*, indeed perhaps the main reason for its reputation as an unusually variable fungus, is the existence of morphologically distinct cultural types. Usually they are grouped as mycelial, sporulating (conidial) and sclerotial (Jarvis, 1977; Lorbeer, 1980), but sub-types can be recognised (Faretra et al., 1988; Martinez et al., 2003). Typically, such morphotypes are essentially stable when subcultured using mass inoculum, but subcultures from single conidia often differ from the parents and each other (Sect. 3.4.2). It is obvious that mycelia, sclerotia and conidia have different abilities for survival and dispersal, and the relative roles of these structures will vary greatly depending on ecosystem and season. Perhaps their dual occurrence reflects a major role of disruptive selection with, for example, sclerotial types being favoured on perennial hosts over winter and conidial types on annual hosts with abundant susceptible flowers.

3.1. Nuclear number and chromosomes

It has been long established that both hyphal cells and conidia are multinucleate with numbers for conidia usually in the range 3-6 (Grindle, 1979; Lorenz and Eichorn, 1983; Shirane et al., 1988). Microconidia are, on the other hand, uninucleate, seldom germinate on laboratory media, and apparently function primarily as male gametes in sexual crosses. Ascii initially contain one diploid nucleus that undergoes meiosis generating a tetrad of nuclei that divide again producing nuclei around which the eight ascospores are formed; subsequently, mitotic divisions occur resulting in about four nuclei in the mature single-celled ascospores (Lorenz and Eichorn, 1983; Faretra and Antonacci, 1987).

The small size of fungal chromosomes makes their cytological karyotyping difficult. Nevertheless, Shirane et al. (1988), using an elegant method to release chromosomes from the tips of germlings, provided clear evidence for the presence in mitotic metaphase of 16 chromosomes, and 16 chromosomes have also been reported in developing asci (Faretra and Grindle, 1992).

The small size of fungal chromosomes is an advantage for pulsed-field gel electrophoresis, which has revealed that fungi in general exhibit a high degree of chromosome-length polymorphism with homologous chromosomes differing in length (Zolan, 1995), and in addition supernumerary chromosomes ("B" chromosomes) are common (Covert, 1998). Studies with *B. cinerea* provide evidence for up to 13 major bands (in the range 1.8-4.6 Mbp), corresponding to one or more large chromosomes, along with up to three minor bands (in the range 220-580 Kbp), corresponding to small chromosomes and mitochondrial DNA (Van Kan et al., 1993; Faretra et al., 1996; Vallejo et al., 1996, 2002). Vallejo et al. (2002) reported seven different profiles amongst 22 field strains with from five to eight bands per strain, and found individual karyotypes were highly reproducible following repeated subculturing. Estimates of minimal genome size based on the assumption of only one chromosome per band, range from 13.19-22.64 Mbp (Vallejo et al., 2002). Higher estimates of 33.9-39.7 Mbp were calculated by Van Kan et al. (1993), assuming intense bands correspond to two chromosomes. These latter estimates equate to a chromosome complement of 12-14 chromosomes that, given the cytological evidence of 16 chromosomes, indicates some bands may correspond to more than two chromosomes. The chromosome profiles of ascospore progeny in some instances match one or other parent, but other siblings may differ from either parent and show novel profiles (Vallejo et al., 2002). These authors conclude that generation of new chromosomal bands, and loss of others, is a result of meiotic crossing-over between homologous chromosomes having heterologous regions, thus generating homologous chromosomes of different length. Use of an rDNA probe showed the rDNA gene cluster was located in a single high molecular weight band, which varied in size depending on the strain. Faretra et al. (1996) reported some small chromosomes showed anomalous segregation, indicating they are supernumerary.

The ploidy level of various strains was examined by Büttner et al. (1994) using fluorescent microscopy to estimate DNA content of individual nuclei relative to the single ascospore strain SAS56. They treated SAS56 with benomyl under conditions that induce haploidisation of diploid strains in 'model' fungi, and recovered a series of strains that showed reduced DNA content, including one with only one-third of that of the parental strain. They suggested strain SAS56 is polyploid, perhaps triploid, with the derived strains representing euploid, or more likely aneuploid, derivatives. Single ascospore strain SAS405 showed a DNA content of 0.69x that of SAS56, consistent with it perhaps being diploid, and a series of field strains showed values both lower and higher than strain SAS56. Büttner and Tudzynski (1996), in a preliminary cytological report, detected differences in chromosome number both within and between strains. In addition, individual nuclei in a given strain showed a spectrum of values over a 3-fold range some of which were 'haploid' (P. Tudzynski, Institut für Botanik, Münster, Germany, pers. comm.). Various forms of heteroploidy have been suggested for a range of fungi (Tolmsoff, 1983), and a variety of mechanisms including failure of mitosis or fusion of nuclei, coupled with chromosome loss or even gain may be involved. A small amount of the variation in DNA content of the benomyl-induced variants may be a result of the elimination of supernumerary chromosomes during vegetative growth (Covert, 1998). These

findings must be considered in light of the knowledge that strains SAS405 and SAS56 in particular, and most strains in general, are able to cross sexually producing viable progeny (Sect. 3.2). It may be that the sexual crossing process acts to restrict participation to haploid nuclei, whereas somatic cell function encourages heteroploidy. If this is so, ascospores may initially be strict haploids, but give rise to heteroploid colonies as they grow.

3.2. The sexual cycle in nature and in the laboratory

B. cinerea apothecia (Figure 1) are seldom found in nature (Lorbeer, 1980), although it is salutary to reflect that Anton de Bary described *Peziza (Botryotinia) fuckeliana* and *B. cinerea* from grapevine in Switzerland well over a century ago (Gregory, 1949). While lack of searching and confusion with apothecia of *Sclerotinia* and *Monilinia* may account in part for their apparent rarity, we suspect apothecia are genuinely uncommon, at least when compared with these other genera. Sclerotia have not been found, or are rare in the field in regions with warm dry summers, including Almeria, Spain (Raposo et al., 2001) and Israel (Yunis and Elad, 1989), and it is thus unlikely apothecia will be found in these regions.



Figure 1. Apothecia of *Botryotinia fuckeliana* the teleomorph of *Botrytis cinerea* on a peach mummy collected in the field in Hawke's Bay, New Zealand (left and centre) along with a cluster of apothecia arising from a sclerotium produced in the laboratory (right)

Despite the rarity of apothecia in nature, they can be readily obtained in the laboratory following protocols refined by Faretra and Antonacci (1987) and Faretra et al. (1988). Critically, they confirmed that most strains are heterothallic, carrying one or other allele of the mating type gene, and designated standard single ascospore tester strains for each mating type; SAS56 for *MATI-1* and SAS405 for *MATI-2*. No DNA sequence information is available for these genes in *B. cinerea*, but it is likely they will comprise alleles of very different sequence (idiomorphs) as has been found with other filamentous ascomycetes (Coppin et al., 1997). Crossing involves preconditioning sclerotia of the female (sclerotial) parent in the cold, before fertilizing them with a suspension of microconidia and vegetative cells derived from the male (fertilizing or spermatial) parent. Strains are usually able to act as female or

male parents, although strains incapable of sclerotial production cannot act as female parents.

In studies involving numerous isolates sourced from around the world most strains from the field were heterothallic, crossing successfully with one or other tester strain (Lorenz and Eichorn, 1983; Faretra et al., 1988; Beever and Parkes, 1993; Faretra and Pollastro, 1993; Van der Vlugt-Bergmans et al., 1993; Delcán and Melgarejo, 2002). The percentage of *MATI-1* strains was slightly higher than *MATI-2* strains in most populations. In addition some strains behaved in homothallic fashion producing fertile apothecia without spermatization and/or with both tester strains. Faretra et al. (1988) concluded such *MATI-1/2* strains (16% in their study) are heterokaryotic for the mating type genes, that is, they are pseudohomothallic. Some single ascospore progeny were also homothallic, but as the ascospore nuclei derive ultimately from one haploid nucleus, such strains cannot readily be explained by heterokaryosis. Reports of the incidence of homothallic single ascospore strains vary from 4-6% (Faretra et al., 1988; Faretra and Pollastro, 1991, 1996), although Lorenz and Eichorn (1983) reported five of six strains behaved in this manner. Faretra and Pollastro (1996) further showed some pairs of ascospores dissected in order from eight-spored asci behaved in homothallic fashion, and these 'homothallic' ascospores occupied the positions where *MATI-2* would have been expected, suggesting a process of unidirectional mating type switching similar to that reported for some other ascomycetes including *Sclerotinia trifoliorum* (Uhm and Fujii, 1983). The molecular basis of this instability is not yet understood, but may result from the presence of both mating type idiomorphs in 'switching' strains and the deletion of one of these during switching (Raju and Perkins, 2000). The *B. cinerea* data are consistent with the proposal that in a few asci, one or two of the four meiotic nuclei are epigenetically modified as a result of which switching occurs in the following mitotic divisions and a mycelium heterokaryotic for mating type is produced. The observation that *MATI-1* strains typically exceed *MATI-2* strains in field samples is consistent with switching during meiosis, although switching during mitotic growth cannot be excluded.

3.3. Extrachromosomal elements

Fungi in general, and *B. cinerea* in particular, possess a variety of extrachromosomal genetic elements including the chromosomes of mitochondria, viruses, plasmids and transposable elements (Rosewich and Kistler, 2000). While mitochondria are clearly essential to cell growth, the others are generally considered dispensable and to behave as 'selfish genetic elements', although transposons integrate into chromosomes and may play an important long-term role in evolution (Kidwell and Lisch, 2001). Apart from encoding genes involved in their own existence, such 'genomic parasites' may influence their hosts in various ways. One critical question is the nature of the inheritance and loss of such elements. It is probable such elements are readily transmitted via conidia, but transmission into ascospore progeny may in some cases be limited or occurs only via the maternal parent. Transmission via somatic cell fusion may be restricted to a greater or lesser extent by vegetative incompatibility (Sect. 3.4.1). In *Neurospora crassa* it is suggested the

primary role of various gene 'silencing' processes such as the meiosis-associated processes of 'repeat-induced point' mutation (RIP) and 'meiotic silencing by unpaired DNA' (MSUD), as well as the somatic cell process of quelling, may act to restrict the spread of 'genomic parasites' (Shiu et al., 2001). It is not known whether such processes are active in *B. cinerea*.

3.3.1. Mitochondria and mitochondrial plasmids

Mitochondrial DNA (mtDNA) provides a distinct non-nuclear source of genetic variation especially suitable for the study of intraspecies variation in fungi (Typas et al., 1998). The ascomycete mitochondrial genome is typically circular and the full sequences of *N. crassa* (Griffiths et al., 1995) and some other species are available, providing a rich resource for future investigation in *B. cinerea*. Preliminary studies indicate that *B. cinerea* has a circular genome of about 25.8 Kbp (Vallejo et al., 1996). Holst-Jensen and Schumacher (1994) found no polymorphisms amongst seven isolates using a *Neurospora* mitochondrial rDNA probe, whereas all were distinguishable by RFLP using a nuclear rDNA probe. Both linear and circular mitochondrial plasmids are known in filamentous ascomycetes (Griffiths, 1995), and linear plasmids of 2-3 Kb have been found in *B. cinerea* (Hiratsuka et al., 1987). Such plasmids are generally considered to confer no selective advantage or disadvantage on their host, merely encoding genes needed for their own replication, although some may be associated with mitochondrion-induced 'senescence' (Griffiths, 1995). Gene transfer via cytoplasmic contact between both compatible and incompatible strains has been invoked to explain patterns of distribution of plasmids within other fungal species, and direct horizontal transfer has been demonstrated (Rosewich and Kistler, 2000).

3.3.2. Transposable elements

Transposable elements in general can be divided into Class I and Class II types (Daboussi, 1996; Kidwell and Lisch, 2001). Class I elements transpose by reverse transcription of an RNA intermediate, whereas Class II elements transpose at the DNA level by excising from one site and reintegrating at another site. Two transposable elements have been found in some strains of *B. cinerea*. *Boty* is a 6-kb putative Class I retrotransposon, characterised by a long terminal repeat (LTR), present in multiple copies in different regions of the genome (Diolez et al., 1995; Giraud et al., 1999). *Flipper* is a 1842 bp Class II element, present in up to 20 copies per genome, and is known to be mobile by its insertion into nitrate reductase during spontaneous mutant selection (Levis et al., 1997). Initially dot blot methods were used to detect these elements, but more recently PCR methods have been developed (Muñoz et al., 2002). When first reported, both elements were found in the same strain (*transposa* strains), whereas *vacuma* strains lack both elements. However, strains containing only the *Boty* element have now been detected in Europe (Giraud et al., 1999; De Miccolis et al., 2004) and Chile (Muñoz et al., 2002), and strains containing only the *Flipper* element have been detected in Europe (Albertini et al.,

2002; De Miccolis et al., 2004). The nine copies of *Flipper* in strain SAS405 each segregated in Mendelian fashion in a cross with SAS56, which lacks the element (Levis et al., 1997). The copies segregated independently, indicating they were unlinked, and copy number did not change during the cross. This observation suggests *Flipper* at least will spread rapidly through a population if sexual crossing is occurring.

3.3.3. Mycoviruses

Mycoviruses, including those characterised by encapsulated genomes and others that lack protein capsids, are common in fungi but unlike viruses in other organisms, are not infectious *per se* (Buck, 1998; Ghabrial, 1994). They are typically readily transmitted into asexual progeny, but transmission to sexual progeny is often inefficient or absent (Coenen et al., 1997; Chun and Lee, 1997). Most mycovirus genomes are double-stranded RNA (dsRNA) and their presence can be readily detected by gel electrophoresis. Such dsRNAs are common in *B. cinerea*. Howitt et al. (1995) reported that over 70% of 200 isolates were infected. The dsRNA profiles observed in this survey varied widely in number (1-8 bands) and size (c. 0.8-1.5 Kbp) and few of the 143 profiles were identical. This complexity may reflect mixed infections, the presence of satellite viruses, or defective dsRNAs derived by deletion. Isometric, bacilliform and filamentous virus-like particles have been found in *B. cinerea* (Howitt et al., 1995), and two isometric dsRNA mycoviruses have been partially characterised (Vilches and Castillo, 1997; Castro et al., 1999). In both cases, the viruses were located in the cytoplasm and associated with some cellular degeneration. Apart from this observation, there is no evidence that dsRNA viruses have any major phenotypic effect (Howitt et al., 1995). Transmission studies indicate they are not passed to ascospore progeny (F. Faretra, Università di Bari, Bari, Italy, pers. comm.).

As well as dsRNA mycoviruses, two single-stranded RNA (ssRNA) mycoviruses have been characterised and indeed fully sequenced from *B. cinerea* (Howitt, 1998; Howitt et al., 2001). Both were associated in the same isolate with flexuous rod-shaped particles resembling plant 'potex-like' viruses. Botrytis virus F (BVF) contains a genome of 6827 nucleotides, and Botrytis virus X (BVX) a genome of 6966 nucleotides both with poly(A) tracts. They each differ sufficiently from existing viruses, and from each other, to warrant recognition as new viral genera. The similarity between these mycoviruses and plant viruses, including, a remarkable 73% amino acid identity between the putative RNA-dependent RNA polymerase of BVX and that of the allexivirus *Garlic virus A*, suggests some form of horizontal gene transfer between plants (specifically *Allium*) and *B. cinerea*.

Hypovirulence-associated dsRNA mycoviruses, which reduce the pathogenicity of their host fungi, have attracted particular interest because of their possible use in biocontrol (Buck, 1998; Dawe and Nuss, 2001). Such viruses are known from *Sclerotinia* spp. (Boland, 1992; Deng et al., 2002) and have recently been identified in *Botrytis* (Castro et al., 2003).

3.4. Somatic compatibility and heterokaryosis

3.4.1. Somatic compatibility

Characterisation of groups of somatically compatible individuals provides a powerful approach to subdividing fungal species (Correll and Gordon, 1999; Glass et al., 2000). Evidence for the existence of vegetative compatibility groups (VCGs) has usually been obtained using auxotrophic mutants derived from the strains being tested. Different classes of chlorate resistant, nitrate non-utilising (Nit) mutants have been widely used for this purpose because spontaneous mutants are often readily selected in target fungi, they can be easily classified into different nitrogen usage phenotypes on minimal medium amended with various nitrogen sources, and complementation is easily scored on minimal medium. Typically, a range of mutants are recovered, but the most reliable are usually those deficient in nitrate reductase apoenzyme (*nit1*) and those defective in synthesis of the molybdenum containing cofactor needed for nitrate reductase and xanthine dehydrogenase activity (NitM).

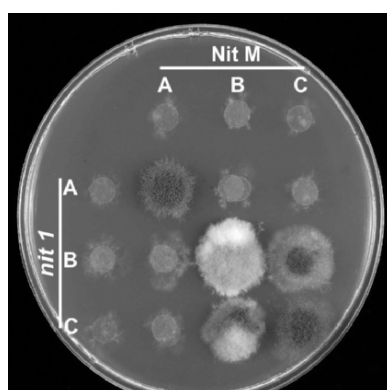


Figure 2. Complementation matrix demonstrating existence of VCGs in *B. cinerea*. Spore suspensions of *nit1* (rows) and NitM (columns) mutants derived from three field strains (A,B,C) have been superimposed on nitrate medium + Triton (Beever and Parkes, 2003). Mutant pairs from the same parent all complement as do the mutants from strains B and C, indicating they are in the same VCG

Beever and Parkes (2003) devised a method facilitating recovery of both *nit1* and NitM pairs of *B. cinerea* mutants and confirmed the existence of multiple VCGs, with all six field strains examined being in different groups. These strains all crossed successfully with SAS56 or SAS405 tester strains, which were themselves in different VCGs. Subsequent studies have shown a large number of VCGs exist within the *B. cinerea* population, and to date we have recognised 45 amongst 57 field strains, with few isolates being in the same group (Figure 2) (P. Weeds and R. Beever, unpubl.). Selenate resistant mutants have also been used to demonstrate complementation (Korolev et al., 2003).

The genetic basis of vegetative incompatibility in *B. cinerea* is not known, but evidence to date suggests it conforms to the system found in other ascomycetous fungi (Glass et al., 2000). In these, vegetative incompatibility is typically determined by a series of vegetative incompatibility (*vic* or *het*) genes that exist in two or occasionally more allelic states. Strains that carry identical alleles at all loci are compatible, those that differ at one or more loci are incompatible. VCGs are determined by unique combinations of *vic* genes, such that if six *vic* loci with two alleles per locus are segregating in a population, 64 (2^6) groups are theoretically possible. Sexual crossing has been shown to generate new VCGs in *B. cinerea* (Beever and Parkes, 2003). Considering both field and single ascospore isolates we have identified over 66 distinct VCGs consistent with the presence of at least seven *vic* genes in the *B. cinerea* population (P. Weeds and R. Beever, unpubl.). The existence of homothallic strains of *B. cinerea* heterokaryotic for mating type (Faretra et al., 1988) indicates *MAT1* does not act as a *vic* gene in this species, as it does in *N. crassa*. The large number of VCGs, and the limited occurrence of isolates in the same VCG, suggests sexual recombination plays an important role in the field in *B. cinerea*. The diversity of dsRNA profiles seen in field strains (Howitt et al., 1995) suggests hyphal fusion is uncommon, a proposal consistent with the existence of multiple VCGs. The homologue (Bc-*hch*) of the *N. crassa* *het-c* gene has been cloned and sequenced in a number of *B. cinerea* strains, but it does not appear to act as a *vic* gene in this species (Fournier et al., 2003).

Numerous fungi, including *Sclerotinia* and *Monilinia*, form distinctive interaction lines when paired on agar media (Kohn et al., 1990; Free et al., 1996). While such “barrage” lines indicate the existence of incompatibility, caution should be exercised in assuming that mycelial compatibility groups (MCGs) recognised in this manner match VCGs described above. All tester isolates for the nine VCGs recognised by Beever and Parkes (2003) formed strong interaction zones with other tester strains, an observation consistent with the congruence of these two systems. However, classifying field strains into distinct groups using the mycelial compatibility test is often difficult, with the number and intensity of dark lines varying depending on strain combination. Delcán and Melgarejo (2002) examined the interactions between numerous strains and found few were compatible, although a mycelial-free space, rather than a dark interaction line, was observed in some interactions indicating that more than one phenomenon may be involved. R. Beever and S.L. Parkes (unpubl.) obtained single ascospore strains after a series of backcrosses that complemented using Nit mutants but nevertheless still produced a dark interaction line. Ford et al. (1995) likewise reported the lack of a direct correlation between these two systems in *S. sclerotiorum*, and Micali and Smith (2003) concluded the two phenomena are distinct in *N. crassa*. We conclude it is premature to equate mycelial and vegetative incompatibility in *B. cinerea*.

3.4.2. Heterokaryosis

Despite the longstanding interest in heterokaryosis in *B. cinerea* (Hansen, 1938; Menzinger, 1966; Jarvis, 1977; Grindle, 1979; Lorbeer, 1980), evidence for its

existence is rather limited, although the formation of 'laboratory' heterokaryons between mutants from the same parent is clearly established (Weeds et al., 1998; Beever and Parkes, 2003). Broadly speaking early work showed monoconidial isolates derived from a single parent often differ morphologically. For example, Hansen (1938) recognised so-called mycelial (M), conidial (C) and intermediate (M/C) types, and found M and C types ('homotypes') generated only M and C types respectively, whereas the M/C types ('heterotypes') gave rise to all three types. He concluded the M/C types were natural heterokaryons, comprising a mixture of C and M nuclei. Menzinger (1966) obtained broadly similar results, although in some instances finding more than two 'homotypes', and concluded *B. cinerea* isolates are frequently heterokaryotic (18 of 29 isolates examined). Grindle (1979) also studied variation of monoconidial progeny, and found that while five strains showed little variation, one strain generated a diversity of morphotypes and in addition some monoconidia from this isolate were non-viable, perhaps carrying a lethal gene. He concluded that while heterokaryosis as interpreted by Hansen (1938) might account for his findings, the possible role of cytoplasmic elements needed consideration.

The major question about heterokaryosis is to what extent it accounts for biologically significant variation of field strains. The most direct evidence for significant heterokaryosis in field strains comes from mating type gene studies. Faretra et al. (1988) found that monoconidial isolates of homothallic field isolates either remained homothallic (i.e. were presumably heterokaryotic *MATI-1* + *MATI-2*) or behaved as heterothallic (i.e. were presumably either *MATI-1* or *MATI-2* homokaryons). Some evidence on heterokaryosis has come from the study of fungicide resistance. Summers et al. (1984) demonstrated that a dicarboximide resistant field isolate was a heterokaryon and they were able to resolve it using monoconidial isolates into homokaryotic sensitive and resistant types. Furthermore, they showed the relative balance of fungicide-resistant and fungicide-sensitive nuclei in the heterokaryon responded to the presence of fungicide in the medium. Faretra and Pollastro (1993), working with field and laboratory dicarboximide-resistant isolates, and Pollastro et al. (1996), working with field dichlofluanid-resistant isolates, found some isolates did not always transmit their resistant character to sexual progeny, and resistance was also lost from some asexual progeny, indicating the parent isolates were heterokaryons between resistant and sensitive nucleotypes. Low-level anilinopyrimidine-resistant strains from the field behave as heterokaryons in which the resistant nucleotype is lethal in the homokaryotic state (De Miccolis Angelini et al., 2002).

Findings on population structure (Sect. 3.6), sexual crossing (Sect 3.2) and somatic compatibility (Sect. 3.4.1) allow an explicit interpretation of how heterokaryosis may operate in *B. cinerea*. Population and VCG studies indicate sexual reproduction plays a major role in determining population variation, and sexual crossing studies indicate most strains are heterothallic and capable of crossing. It is reasonable to propose that ascospore progeny will be initially

homokaryotic, carrying one or other mating type gene, and mostly be in different VCGs. The large number of VCGs observed in the field, and the diversity of their dsRNA profiles, suggest fusion of genetically different strains in the same VCG is uncommon. Thus the question can be asked as to how heterokaryons comprising genetically different nuclei do arise in the field. As for mating type, it is reasonable to suggest that it arises by 'switching' after meiosis within a single ascospore lineage (Sect. 3.2), and thus the heterokaryotic nuclei will be homozygous for other loci including the *vic* loci, a prediction that can be tested. This special case apart, we suggest the main mechanism for heterokaryon generation is likely to be mutation within somatic lineages. The apparently infrequent occurrence of apothecia in the field implies such lineages may be long-lived, in which case it is axiomatic that mutations will occur and, if recessive in particular, might persist for long periods. It is probable that many such mutations will affect morphological properties such as conidiation and sclerotium production and be essentially recessive in the heterokaryotic state. And of course long-lived lineages would be expected to produce multiple nuclear types within the same lineage; the recognition of 'dual' heterokaryons comprising just two nucleotypes may be an over-simplification. Such an explanation might account, for example, for highly variable strains such as Strain 9 studied by Grindle (1979) and Strain 16 studied by Menzinger (1966).

It is possible that a form of the parasexual cycle (Debets, 1998) implying at least transient diploid nucleus formation, aneuploid formation and gene recombination, may operate in *B. cinerea*. The heteroploidy observed by Büttner et al. (1994) is consistent with, but by no means evidence for, the existence of such a process. Parasexual recombination could at least in theory provide an alternative to sexual recombination in generating diversity (Sect. 3.6). However, the scarcity of VCGs with multiple members suggests it is unlikely to play a significant role.

3.5. Linkage studies

Although a method to routinely cross *B. cinerea* has been available for over a decade, few studies involving sexual crossing have been published. Nevertheless, these are sufficient to establish that the essential features conform to those of model fungi such as *N. crassa*. Single gene markers encoding resistance to benzimidazole fungicides (*Mbc1*) and dicarboximide fungicides (*Daf1*) have been identified and shown to be loosely linked to each other, but not to *Mat1*, using random ascospore analysis (Faretra and Pollastro, 1991; Beever and Parkes, 1993). Tetrad analysis demonstrated *MAT1* is located about 12 map units from its centromere, and *Mbc1* may be loosely linked to its centromere (Faretra and Pollastro, 1996). Two single genes (*Dic1* and *Dic2*) encoding resistance to the fungicide dichlofluanid have been identified, showing loose linkage to *Daf1* in some crosses (Pollastro et al., 1996). Weeds et al. (1998) showed neither *Sel1* (encoding resistance to selenate) nor *nit1* was linked to *Daf1* or *Mbc1*. As well as their intrinsic value in beginning a framework for a genetic map of *B. cinerea*, a major use of these various marker genes is to confirm whether crossing has indeed occurred in laboratory crosses.

3.6. Population studies using molecular markers

The development of molecular techniques has revolutionised and energised fungal population genetics by providing numerous readily available genetic markers (Chapter 4; Bridge et al., 1998). Multilocus techniques such as RAPDs and AFLPs are convenient and allow ready scoring of numerous polymorphic loci, but are limited by features including difficulties of reproducibility and the assumption that co-migrating bands are identical. Single-locus techniques such as RFLPs are highly reproducible and allow greater precision for estimating genetic parameters, but are more labour intensive (McDonald, 1997). Microsatellite markers, which offer numerous polymorphisms coupled with high reproducibility and convenience, have been developed for *B. cinerea* (Fournier et al., 2002), but as yet not widely applied.

Studies using RAPDs and AFLPs with *B. cinerea* have usually recognised 50 or more polymorphic markers. Van de Vlugt-Bergmans et al. (1993) studied eight Dutch field isolates, as well as SAS56 and SAS405, and found all could be differentiated, although two of the eight field isolates, recovered 4 years apart from different hosts, differed by only one marker. Crossing studies showed most markers segregated independently in Mendelian fashion and were unlinked. Kerssies et al. (1997) studied 29 isolates collected from inside and outside Dutch glasshouses and found that for all 70 markers scored, only two were identical, although a few others showed high similarity. Cluster analysis recognised three groups, but no pattern relating to biology of the groups was detected. Alfonso et al. (2000) studied 40 strains from Spain and found the population as a whole was highly heterogeneous, with little differentiation of the subpopulations between different greenhouses or regions; nor was significant differentiation detected when isolates from other countries (Israel, Italy, Holland) were included in the analysis. Moyano et al. (2003), working with 44 Spanish isolates from six greenhouses, found only two haplotypes (multilocus genotypes) had more than one member, one comprised of three isolates, the other of two. Six isolates showing the benzimidazole-sensitive/procymidone-resistant fungicide resistance phenotype clustered together, but no other biological correlations were detected. Thompson and Latorre (1999), studying isolates from various hosts in Chile, also found high genotypic diversity and, based on only 15 isolates, speculated that there was some clustering based on host. Yourman et al. (2000) found all 56 isolates from greenhouses in South Carolina (USA) were different, but found some clustering in relation to fungicide sensitivity. Muñoz et al. (2002) found all of 69 isolates from Chile, including two from the same kiwifruit, had different haplotypes indicating the absence of clonal lineages; however, the data indicated some clustering of isolates by host. In summary, these findings all indicate the *B. cinerea* population is genetically very diverse with no indication of widespread clonal lineages, even in relation to fungicide resistance that might have imposed a genetic bottleneck on some populations.

The most comprehensive population studies of *B. cinerea* have been conducted by Y. Brygoo and colleagues (Brygoo et al., 1998; Chapters 4 and 12) using a range of markers including presence or absence of the transposable elements *Boty* and *Flipper*, a suite of PCR-RFLP markers (based on the rDNA intergenic spacer region, nitrate reductase, ATP synthase and ADP-ATP translocase, and some unidentified

sequences) as well as single gene-encoded fungicide resistance to benzimidazole and dicarboximide fungicides. Giraud et al. (1997), using 16 such markers, identified 134 haplotypes in a sample 259 isolates collected from grape in Champagne, France, with the most common accounting for only 5% of isolates. Isolates collected from the same plant always had different haplotypes and up to five different haplotypes were found in spores isolated from a single berry. Even isolates identical for all 16 markers could be further differentiated on the number and location of copies of the transposable elements. This extensive genotypic diversity indicates limited clonal propagation and a significant role for recombination. Additionally they found highly significant differences in allelic frequencies between *transposa* isolates, carrying the transposable elements *Flipper* and *Boty*, and *vacuma* isolates that lack them, and four alleles were restricted to one or other population. Linkage disequilibrium estimates suggested 17.9% of pairs of loci for *vacuma*, and 5% for *transposa*, were in linkage disequilibrium, low values consistent with limited clonal reproduction.

In a subsequent study, Giraud et al. (1999) used the previous markers as well as some additional ones, including resistance to the fungicide fenhexamid, to examine 107 field isolates from various host plants growing around the vineyards where the grape isolates were recovered. Their findings mirrored those of the grape study, with 74 haplotypes being found – with the most common constituting only 8% of strains. Genotypic diversity (number of haplotypes/ number of isolates x 100) was similar in both studies, with *transposa* values of 55% (56% in grapes) and *vacuma* of 57% (70% in grapes). Giraud et al. (1997, 1999) concluded their data indicated the existence of two ‘sibling species’ *vacuma* and *transposa*, a conclusion consistent with the restriction of transposons to one population. The two groups recognised in this way correlated with a slight but statistically significant difference in spore size, spores of *vacuma* being slightly larger than *transposa* isolates. Subsequent studies have extended and modified these conclusions (Albertini et al., 2002; Fournier et al., 2003). While two distinct populations are still recognised, they are not fully coincident with those previously recognised. The new groups are differentiated unequivocally by fixed amino acid level polymorphisms in two genes, *Cyp51* (14 α -demethylase gene) and *Bc-hch* (*B. cinerea* het-c homolog), as well as by their response to the fungicide fenhexamid: Group I isolates are resistant to fenhexamid, whereas Group II isolates are sensitive to fenhexamid. Group I isolates were all *vacuma* type, whereas Group II isolates included both *vacuma* and *transposa* types. Fournier et al. (2003) re-analysed part of the data of Giraud et al. (1997) and confirmed the genetic distinctiveness of Groups I and II. Leroux et al. (2002a, b) provide additional data on the nature of Groups I and II. Fertile crosses have been obtained between strains within each group (including both *vacuma* and *transposa* types in the case of Group II), but not between the groups, indicating they are reproductively isolated.

Mycelium compatibility tests suggest that Group I isolates comprise one MCG, as distinct from Group II isolates, which encompass numerous MCGs. Group I strains also have longer conidia and faster mycelium growth rate than Group II strains. Furthermore, combined phylogenetic analysis of four nuclear genes, including *Bc-hch*, and *Cyp51*, clearly support the distinction between Group I and II

strains. In summary, while some data are still to be published in full, these findings provide sound support for recognition of a second polyphagous *Botrytis* species reproductively and genetically distinct from *B. cinerea*. Leroux et al. (2002a, b) name but do not formally describe Group I isolates as *B. 'pseudocinerea'*. This species is so far known only from Europe (France, Germany, UK), where it occurs on a number of hosts (Leroux et al., 2002a; Fournier et al., 2003). In many ways the situation here resembles that of *B. fabae*, which also closely resembles *B. cinerea* but can be differentiated morphologically by its slightly larger conidia (Harrison, 1988).

Excluding the *B. 'pseudocinerea' vacuma* group from consideration leaves questions about the relationship of *vacuma* and *transposa* subpopulations within *B. cinerea sensu stricto*. In particular, if sexual reproduction is common, why have the transposons not infected all strains in the population? One possibility is that they are still spreading through the populations (Muñoz et al., 2002), a possibility supported by the rarity of apothecia in the field. Another is that *B. cinerea* possesses a mechanism to resist infection or to remove transposons from their genome. Martinez et al. (2003) report on phenotypic differences between *transposa* and *vacuma* groups in a sample of 121 isolates from near Bordeaux (France). Only two were *B. 'pseudocinerea'*, and thus the findings apply essentially to *B. cinerea sensu stricto*. Pathogenicity of both groups on grape and tobacco leaves was similar, but mycelial growth rate of *transposa* strains was slightly lower than *vacuma* strains at favourable temperatures.

3.7. *Botrytis cinerea* - a synthesis

A changing picture is emerging of the genetic structure of *B. cinerea*. Traditionally, sexual reproduction has been considered to play a minor role, but most strains retain the ability to intercross, producing fully fertile progeny (Sect. 3.2). On the other hand, heterokaryosis has traditionally been considered very important, but the numerous VCGs in the population, presumably resulting from sexual recombination, suggest this process plays only a limited role (Sect. 3.4.2). Molecular markers indicate high levels of recombination (Sect. 3.6) which, given the limitations imposed on heterokaryosis by vegetative incompatibility, probably results from sexual rather than parasexual processes. The rarity of identical haplotypes, even when hundreds are examined, suggests the total number of variants in populations is very large. The effective size of such populations is unknown, but *B. cinerea* is recognised as present in appreciable numbers in the air spora (Jarvis, 1977). It is possible that migration over considerable distances is more important than generally appreciated, especially in increasing variation in dry climates where apothecia are unlikely to occur.

The infrequent discovery of apothecia in the field, the large proportion of field strains infected by mycoviruses and the observation that many strains do not carry transposons, all suggest that, despite its critical role, sexual reproduction is nevertheless infrequent and lineages may be relatively old. It is probable that mutations conferring morphological variation and fungicide resistance will arise in such lineages and, depending on selection, increase. The extent mutation plays in

generating haplotype variation within ascospore lineages needs clarification. Recent findings indicate that other processes such as heteroploidy (including aneuploidy) may be important but exactly how is still unclear (Sect. 3.1), as is the role of extrachromosomal determinants (Sect. 3.3). The impact of agricultural practice on *B. cinerea* genetics has yet to be clarified, but has likely been very significant both in providing large susceptible monocultures and protected environments encouraging proliferation in otherwise non-conducive climates. It remains to be seen whether a 'natural' population of *B. cinerea* can be located, but if so it would likely provide fresh insights into its genetics. In summary, we conclude the *B. cinerea* population comprises a very large number of different ascospore lineages that are both widespread and highly mobile. The ability of most strains to intercross with standard tester strains suggests *B. cinerea*, excluding *B. 'pseudocinerea'*, comprises a single species.

4. Genetics of other species of *Botrytis*

In contrast to *B. cinerea*, relatively little is known about the genetics of other species of *Botrytis*. Shirane et al. (1989) reported chromosome numbers of 16 for *B. byssoidea*, *B. squamosa* and *B. tulipae*, while two chromosome numbers were found in *B. allii sensu lato*; 16 for what is now recognised as *B. allii sensu stricto* and 32 for the slightly larger spored *B. aclada sensu stricto* (Sect. 2). Additionally, they noted significant differences in nuclear number per conidium with mean values for *B. allii* and *B. aclada* of 1.3-1.5, of *B. byssoidea* of 5.0-5.1 (similar to *B. cinerea* with values of 4.0-5.1) and *B. squamosa* of 18.4.

Horizontal gene transfer may play a greater role in fungal evolution than in the evolution of other eukaryotes, nevertheless remaining 'difficult to prove beyond reasonable doubt' (Rosewich and Kistler, 2000). The possibility of such transfer has been explored in relation to *B. porri* and *B. elliptica*, as part of a study on possible horizontal gene transfer between species of Sclerotiniaceae (Holst-Jensen et al., 1999). Specifically, these authors proposed that a nuclear rDNA intron might have been transferred from a hypothetical *Myriosclerotinia*-like ancestor to these *Botrytis* species, as well as to species in other genera, including *S. sclerotiorum*. The intron is not present in *B. cinerea*, *B. calthae* or *B. convoluta*. They suggest that the transfer may have been mediated by an RNA or DNA mycovirus moving between the species, and speculate that such gene transfer may be on-going.

4.1. *Botrytis elliptica* and *Botrytis tulipae*

The population genetics of *B. elliptica* has been studied by examining 69 isolates from the USA and Taiwan using 22 polymorphic RAPD markers (Huang et al., 2001). A total of 43 unique haplotypes were identified divided into two clusters, one restricted to Taiwan, the other to the USA. Only a few haplotypes were represented by more than one isolate, mostly but not exclusively from samples collected within one nursery block. Provided that these presumptive clones were excluded, there was little indication of gametic disequilibrium. The findings suggest that sexual reproduction plays a significant role in determining the population structure in this

species, although apothecia have never been observed in the field in the USA or Taiwan. Nevertheless, apothecia have been reported from the field in The Netherlands, albeit with scant details available (Van den Ende and Pennock, 1996; Van den Ende and Pennock-Vos, 1997). Unpublished results with Dutch isolates using multiple gene sequence information and AFLP data confirm *B. elliptica* is highly variable (M. Staats and J. van Kan, Wageningen University, The Netherlands, pers. comm.). In contrast, Dutch isolates of *B. tulipae* showed little variation, suggesting this species is primarily clonal, consistent with the lack of a sexual stage.

4.2. *Botrytis* species from onion

Morphological mutants have been recovered in *B. squamosa* using chemical mutagenesis as well as mutants resistant to the fungicide botran, the latter segregating as a single gene (Bergquist and Lorbeer, 1973). The existence of heterokaryosis in this species, based on the morphology and stability of single-spored conidial cultures (Sun, 1989) has been suggested but, as with *B. cinerea* (Sect. 3.4.2), other interpretations are possible.

Variation of a number of onion-associated species has been investigated using UP-PCR (Nielsen et al., 2001). *B. squamosa* showed high diversity with 10 of 11 isolates showing unique haplotypes, consistent with its known heterothallic sexual reproduction. In contrast, *B. aclada* and *B. allii* (*B. aclada* subgroup AII) showed little variation consistent with a high degree of clonality and the absence of known teleomorphs for these species. While only three isolates of *B. byssoidea* were examined, they were all identical, despite being from the USA, The Netherlands and the UK, suggesting the population may be mainly clonal. Its presumed teleomorph, *Bt. allii*, is only known from Japan where it is associated with a leaf blight rather than neck rot. The precise relationship between these entities remains to be elucidated (Table 1).

4.3. *Botrytis fabae*

This species closely resembles *B. cinerea* but is a specialised pathogen of *Vicia* bean, distinguished by higher pathogenicity, somewhat larger spore size, tendency to produce small sclerotia in culture and protein electrophoresis patterns (Backhouse et al., 1984; Harrison, 1988). Hutson and Mansfield (1980) explored the pathogenicity of 15 different macroconidial lineages from one parent, and found a two-fold difference in lesion diameter hinting at the possibility the original strain was a heterokaryon or heteroplasmon.

5. The future

Botrytis genetics is poised for rapid advances in the next few years, following elucidation of the full nuclear genome of at least one selected strain of *B. cinerea*. While this information will help resolve outstanding questions such as the number of chromosomes, it will leave unanswered many questions in taxonomy, population genetics and ecological and host specialisation. Progress in both taxonomy and genetics of *Botrytis* depends on availability of characterised strains and we make a

plea for workers to deposit strains in recognised culture collections, and where appropriate to include well-studied strains in their work.

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CHAPTER 4

APPROACHES TO MOLECULAR GENETICS AND GENOMICS OF *BOTRYTIS*

Paul Tudzynski and Verena Siewers

Institut für Botanik und Botanischer Garten, Westfälische Wilhelms-Universität, Schlossgarten 3, 48149 Münster, Germany

Abstract. Molecular genetic techniques have revolutionized the detailed analysis of infection strategies and pathogenicity of *Botrytis*. Based on the availability of all necessary molecular tools, an impressive (and rapidly growing) number of genes has been functionally analysed by targeted inactivation approaches. The result of these studies, taken together with the new opportunities arising from "genomics" of *B. cinerea*, has developed into one of the model systems for molecular phytopathology. The methodologies and tools available for the cloning of genes and their functional analysis will be discussed and a compilation given of the deletion mutants obtained so far (dealt with in detail in other chapters). Current trends and perspectives of this rapidly developing field are discussed.

1. Introduction

Though the first report of a successful transformation of a *Botrytis* strain appeared in 1989 (*B. squamosa*, Huang et al., 1989), it took some years before the molecular genetics of *Botrytis* was approached on a broad scale. In the last monograph on *Botrytis* (Verhoeff et al., 1992) there is only limited reference to molecular data and few research groups had initiated molecular work. Today more than a dozen teams are working intensively on molecular genetics of *Botrytis* spp., and relevant molecular tools e.g. transformation protocols, genomic and cDNA libraries, are available. As a consequence of this expansion and success the genus *Botrytis* has become one of the model systems for molecular phytopathology. The great economic importance of this work and hence the strong industrial interest (i.e. Syngenta and Bayer) helped to hasten this development. Indeed, the inputs from agribusiness companies initiated the genomics approach for *Botrytis*. Though the genomic sequence of *B. cinerea* is so far not publicly available, cloning of any gene of interest is possible, due to high homology to other available fungal genomic sequences and with the help of e.g. public expressed sequence tags (EST) data (see below). Expression of individual genes can be easily monitored *in planta* by

classical northern analysis (Rolke et al., 2003), or by reverse transcription-PCR (RT-PCR; e.g. Choquer et al., 2003).

The high efficiency of targeted gene inactivation allows a rapid functional analysis of proposed pathogenicity-related genes. The list of functionally analysed genes is growing (see below). These analyses yielded several surprising results, both positive and negative, i.e. supporting or challenging hypotheses based on "classical" analyses. The successful application of these techniques is presented in several chapters of this book. In combination with "classical" approaches of biochemistry, genetics and cytology, these molecular genetic techniques have led to a breakthrough in our understanding of the complex biology of important pathogens including *Botrytis*.

Various techniques that are available for cloning *B. cinerea* genes and for generation of transgenic strains of *Botrytis* will be reported in this chapter, focusing on functional analyses.

2. Generation of transgenic *Botrytis* strains

2.1 Transformation systems

Huang et al. (1989) were the first to report successful transformation of a *Botrytis* species, *B. squamosa*. They used a standard *Aspergillus nidulans* vector, pDH25, containing a bacterial gene for hygromycin phosphotransferase (*hph*), and a standard transformation protocol via protoplast generation. They obtained stable transformants carrying ectopic (partly multiple copy) integrations of the vector, at a rate of < 0.1 transformants/ μ g DNA. The *hph* selection and protoplast transformation system is still the method of choice for *Botrytis*, though the vectors and the techniques have been refined. Hamada et al. (1994) used the standard vector pAN7-1 for the transformation of *B. cinerea*; they obtained up to 10 transformants/ μ g. The resulting transformants proved to be stable without selection pressure after several rounds of sub-culturing. This vector was used by several groups. However, the *trpC* terminator and the *gpd* promoter from *A. nidulans* was later found to be suboptimal for *Botrytis* (J. van Kan, Wageningen Univ., The Netherlands, pers. comm.). Therefore, the Wageningen group designed a new vector (pLOB1) based on the *oliC* promoter of *A. nidulans* and the tubulin terminator from *B. cinerea*. Using this vector and a special recipient strain, B05.10 (Quidde et al., 1999), transformation rates of up to 100/ μ g DNA were routinely achieved. As an alternative to protoplast transformation, biolistic transformation was applied by Hilber et al. (1994). However, the transformation rate was too low; since the protoplast technique was efficient, the biolistic approach has not been used widely.

Alternative selection systems were also developed: the bacterial *ble* gene conferring resistance to bleomycin (and its derivative phleomycin) was successfully used by Santos et al. (1996) and Quidde et al. (1999). This selection system is effective, but there often is high background and a higher level of spontaneous resistance than with the *hph* system. Recently, an attractive alternative selection system based on resistance to the non-toxic antibiotic nourseothricin was developed.

The vector pNR1 carrying the bacterial *nat1* gene conferring resistance to nourseothricin, with the same promoter/terminator sequence as in pLOB1 (B. Tudzynski, unpubl.), yields high transformation rates without background. Several groups who had worked with the bialaphos resistance system in *Magnaporthe grisea* found that this selection system also was quite effective in *B. cinerea* (e.g. Viaud et al., 2003). In addition, Levis et al. (1997) demonstrated the option to use the nitrate reductase system for selection of transformants. Taken together, advances provide selection systems that allow the generation of multiple knock-out mutants in *B. cinerea*.

2.2. Targeted gene-inactivation

An important specific feature of fungal molecular genetics is the possibility to create knock-out mutants by homologous integration of DNA fragments provided by vectors. The degree of homologous integration varies between different fungal systems, e.g. 1-2 % in *Claviceps purpurea* (Oeser et al., 2002) to more than 50 % in *Ustilago maydis* (Banuett, 2002). In most of the analyses reported so far, homologous integration rates in *B. cinerea* are at the upper end of the scale. In principle, two different approaches are possible for a targeted gene inactivation: 1) gene disruption, involving integration of an internal gene fragment (in a circular vector) by a single crossover event; and 2) gene replacement, involving integration (by two simultaneous crossover events) of a replacement fragment consisting of two flanking regions of the gene separated by the selection cassette (usually transformed as a linear DNA fragment, without the *E. coli* vector component). Since the former approach yielded only infrequent inactivation rates (experience in P. Tudzynski's laboratory, Münster and J. van Kan, pers. comm.), gene replacement has become the method of choice for the generation of knock-out mutants in *Botrytis*.

When designing a functional replacement vector sufficiently long flanking regions (normally 0.5 – 1 kb) are required, and the replacement cassette (flanking sequences + selection cassette) should be excisable from the vector with two different restriction enzymes. Otherwise, re-circularisation of the fragment can occur yielding complex integration patterns without the desired knock-out effect (J. van Kan, pers. comm.; experience in P. Tudzynski's laboratory). Though long flanks facilitate homologous integration, smaller flanking regions can also be used, allowing application of knock-out strategies developed as for yeast transformation. J. van Kan and I. Kars (pers. comm.) optimised a PCR-based technique for the first generation of replacement cassettes. To circumvent the need of plasmid cloning procedures, a gene replacement construct can be engineered with overlap-extension PCR. Four amplification reactions are required: 1) amplification of the selection marker gene; 2) and 3) amplification of two 500 base pair (bp) fragments of the target gene with a primer containing a 5' extension complementary to the 5' or 3' end of the selection marker gene; and 4) amplification of the total construct. The total construct is a fusion product of a selection marker gene flanked by two 500 bp fragments of the target gene produced in a single amplification. It is advisable to choose the primers such that the ends of the fragment perfectly match the genomic sequence. No additional sequence stretches (e.g. restriction sites) should be added to

the fragment extremities. The fragment is purified from agarose gel before being used in protoplast transformation. The fragment can be integrated directly into the *Botrytis* genome by homologous recombination. The efficiency of homologous recombination varies between target loci. In the first dozen genes that were mutated using this strategy, at least 10% of the transformants were proper homologous recombinants. It remains to be determined whether the length of the flanking fragments can be reduced even further than 500 bp without drastically affecting the homologous recombination frequency.

Most of the knock-out mutants reported so far (Table 1) were derived from strain B05.10 (Quidde et al., 1999). This strain was obtained from a benomyl treatment programme intended to yield haploid strains (Büttner et al., 1994; Chapter 3); it has a low nuclear DNA content, as determined by 4,6-diamino-2-phenyl-indole (DAPI) staining, is highly virulent on several host plants and is genetically stable. Since it constantly yields high transformation rates, it is used as the standard recipient strain in several laboratories. The original assumption that it was derived from the standard strain SAS56, however, is now thought to be most unlikely because recent molecular analyses have shown considerable sequence variation between SAS56 and B05.10 (J. van Kan, pers. comm.). Nevertheless, it has become one of the standard strains used in molecular genetics of *B. cinerea*, and it was also used for the first genome project (Catlett et al., 2003, see below).

Table 1 gives a compilation of the knock-out mutants described so far. Several of the reported genes are discussed in other chapters. There are, however, some apparent general trends:

1) The majority of the knock-outs (26 of 43) had no obvious effect on virulence; this could of course be due to the artificial test systems, but it also supports the concept that the necrotroph *Botrytis* attacks with a broad arsenal of weapons, and that loss of a single trait rarely has significant impact. Within this group of genes are found excellent candidates for pathogenicity/virulence genes identified on the basis of non-molecular analyses, e.g. the cutinase/lipase genes, or *bcgod 1* (Chapters 2 and 8). On the other hand, deletion of one of five endo-polygalacturonase (PG) genes had an unexpected effect (*bcpgl*), and deletion of the laccase gene *bclcc2* even identified it as an avirulence gene (Chapter 7). Thus the 'targeted inactivation approach' yielded both positive and negative results, and has become a powerful technique for functional analysis of the pathogenic potential of gene products and of *Botrytis* attack strategies.

2) So far only four knock-outs yielded a drastic reduction of virulence, identifying the respective genes as pathogenicity genes in a classical sense. In two cases signal chain components were involved: *bmpl*, encoding a MAP kinase, and *bcg1*, encoding an α -subunit of a heterotrimeric GTP-binding protein. The availability of such mutants allows the identification of the target genes of the respective signal chains obviously involved in pathogenicity (Chapter 6). The third essential gene to be identified is *bcp1s1*, encoding a tetraspanin-like membrane protein involved in appressorial function. The reason for the drastic effect of the deletion of *bcglyox1*, encoding a glyoxal oxidase, is not yet clear.

3) In several cases mutants showed varying degrees of reduced virulence, i.e. the respective genes can be considered as virulence genes. They code for a widespread

set of enzymes/proteins: three pectin-degrading enzymes (*bcp1*, *bcp2*, *bcpme1*), a chitin synthase (*bcchs1*), an ABC transporter (*bcatrB*), a superoxide dismutase (*besod1*), two enzymes probably involved in secondary metabolism (*cnd5*, *bctri5*) and three other signal chain components (*bcg2*, *bacl*, *bcp1*). For two of these genes divergent effects of knock-outs were reported from different groups. The *Bcpme1* deletion mutants obtained by Valette-Collet et al. (2003) showed reduced virulence, whereas I. Kars and J. van Kan (pers. comm.) found no effect of deletion of the same gene. The difference in outcome may be due to the use of different recipient strains (Bd90 versus B05.10). In addition, deletion of the *bcP450-12* gene (equivalent to *cnd5*) yielded different results in the three strains SAS56 and ATCC58025 (no effect, Siewers et al., unpubl.) and T4 (reduced virulence, M. Viaud and J.-M. Pradier, pers. comm.). These data emphasize the importance of the choice of strain to be used in such experiments and the urgent need to standardize these parameters in the *Botrytis* research community.

3. Unbiased gene cloning systems

Most of the genes listed in Table 1 have been cloned by standard direct approaches from genomic lambda libraries (heterologous probes, reverse genetics, etc.) with an expectation that isolated genes will have a known mode of action during pathogenesis. Since the classical "candidate genes" more or less have been functionally analysed in *Botrytis*, unbiased cloning approaches are gaining much more interest because they offer the perspective of identification of novel genes with new functions. Two general strategies are available for "unbiased" approaches. First, screening for genes that are differentially expressed, e.g. specifically *in planta* or in certain developmental stages, so that these genes can be analysed for their specific contribution/impact as evaluated by targeted inactivation. Techniques which have been successfully applied in this area in *Botrytis* range from "classical" differential screening of cDNA libraries, the PCR-based techniques [differential display (DD) RT-PCR; suppression subtractive hybridization (SSH)] to macroarrays. Second, insertional mutagenesis, which has the distinct advantage that the genetic analysis begins from a known phenotype. However, technical difficulties and the inordinate labour involved in the method have limited its broad application so far.

3.1 Random insertional mutagenesis

In several phytopathogenic fungi, the restriction enzyme mediated integration (REMI) technique has been successfully used to generate insertional mutant libraries and to identify new and interesting pathogenicity genes, e.g. in *M. grisea* (Balhadere et al., 1999) and *Ustilago maydis* (Bölker et al., 1995). In *B. cinerea*, a joint approach by J. van Kan and P. Tudzynski's groups to establish the REMI technique failed to yield satisfactory results, a major problem being the fact that most of the transformants obtained had multiple integration sites, and the link between the phenotype of a given mutant and the integration of a vector molecule could not be established. Recently, Kunz et al. (2002) reported successful use of the REMI technique for the isolation of pathogenicity mutants of *B. cinerea*. However, they

also failed to establish genetic linkage between the tag and the phenotype. Probably, the technique induces a high mutation rate and genetic instability; comparable problems have been faced in other fungal systems (Tudzynski and Tudzynski, 2002). Therefore, alternative strategies were developed.

Agrobacterium-mediated transformation has been established successfully in two laboratories for *B. cinerea* (Rolland et al., 2003; N. Segmüller and P. Tudzynski, unpubl.). It could be shown that this system, which had been used for transformation of various fungi (e.g. Covert et al., 2001), fulfils two major criteria that are essential for use in insertional mutagenesis. The criteria are firstly most of the transformants carry single-copy integrations, and secondly the integration sites appear to be random, i.e. no "hot spots" of integration are observed, based on genomic Southern analyses. In our hands, from a total of 1350 transformants tested 30 showed significant reduction of virulence on tomato leaves. Recovery of the tagged sequence can be achieved by thermal asymmetric interlaced (TAIL) PCR (Rolland et al., 2003). The link between integrated T-DNA and phenotype, which can be tested by targeted inactivation of the "tagged" gene in the wild type, has not yet been tested. However, this technique has the advantage over REMI that it does not involve the physiological stress of protoplast generation (which *per se* can be mutagenic), and no mutagenic restriction enzymes enter the nucleus.

In the near future, a large number of insertional mutants will be available for evaluation by the *Botrytis* research community. The availability of the *B. cinerea* genomic sequence will facilitate considerably the identification of the tagged genes and allow rapid detection of chromosomal rearrangements caused by insertions.

3.2 Screening systems based on differential gene expression

Several techniques are available for the identification of genes differentially expressed in various life stages or under specified culture conditions. Benito et al. (1996) used the differential display of reverse transcribed RNA (DDRT) PCR approach to isolate genes of *B. cinerea* induced *in planta*. This technique uses sets of random primers to amplify subsets of mRNA populations to allow a comparison in an acrylamide gel electrophoresis system. Benito et al. (1996) used mRNA samples from axenic culture and from tomato leaves infected with *B. cinerea*. As controls, uninfected tomato leaves and – to identify also plant genes responding to pathogen attack – tomato leaves infected with *Phytophthora infestans* or tobacco necrosis virus. They could identify three *in planta*-induced genes in *B. cinerea* and their expression pattern was confirmed by northern analysis. None of these genes showed homology to any known sequence. One of the differentially expressed genes, Bdc47, was inactivated by gene replacement. Interestingly, the mutant showed increased virulence (Arranz et al., 2003).

Schulze Gronover et al. (2004) used an alternative approach to identify genes of *B. cinerea* induced *in planta*. They applied suppression subtractive hybridization (SSH) which yields a stepwise enrichment of cDNAs derived from differentially expressed genes. In a first experiment, they enriched a cDNA sample for fungal cDNAs from *B. cinerea* infected bean leaves by subtracting cDNAs from uninfected bean leaves, and were able to show that the few plant clones were derived from

pathogen-inducible genes. In their main experimental line, they compared mRNA from plant material infected with the wild-type strain and the $\Delta bcg1$ mutant, that is almost non-pathogenic, in both directions. They identified a whole set of differentially expressed genes (up- or down-regulated in the mutant), which obviously are targets of the signal chain(s) directed by *bcg1*. By comparing these data with a second signalling mutant, $\Delta bac1$, they could discriminate different pathways downstream of *bcg1* (Chapter 6). Thus, the SSH technique proved to be a powerful tool for the analysis of complex signalling and regulation networks in *Botrytis*.

3.3 Genomics

The first and so far only public available data base of *B. cinerea* is the EST library established by Genoscope (<http://www.genoscope.cns.fr>). The library contains 6598 sequences (2839 genes) obtained from a cDNA library of *Y. Brygoo* established from strain T4 grown under nitrogen limitation. Although this library is not normalized and is highly redundant, it has promoted molecular research in *Botrytis* considerably, because it allowed rapid isolation of a whole range of genes.

Recently, Viaud et al. (2003) used macroarrays of these clones to perform differential hybridizations (Chapter 6) for the identification of genes controlled by the Ca^{++} pathway. In our lab, we used the Genoscope data and an EST library prepared from strain ATTC 58025 [an abscisic acid (ABA) producer] for a "semi-direct" approach to identify genes involved in ABA synthesis. A putative NADPH:cytochrome P450 oxidoreductase gene (contained in our EST library) was knocked out, leading to a significant reduction of ABA biosynthesis. This strongly suggested participation of P450 monooxygenases in the ABA biosynthetic pathway. Therefore, all 28 putative P450 monooxygenase genes contained in both libraries were spotted on filters and differentially hybridized with cDNA preparations from ABA producing and non-producing mycelia. This led to the identification of the first ABA-biosynthetic gene, *bcabal* (Siewers et al., 2004).

The first genomic sequence of *B. cinerea* was established by a commercial company and is not publicly available at present. Recently, Catlett et al. (2003) presented the first results derived from these sequence data. They compared histidine kinase genes from several fungi, including *B. cinerea*, and identified altogether 20 putative histidine kinase genes in the *B. cinerea* genome, in contrast to only 11 in the non-pathogenic fungus *Neurospora crassa*. A European initiative has been set up for the establishment of a publicly available genome sequence of *B. cinerea*, centred around the French *B. cinerea* research groups and Genoscope.

4. Perspectives

The data presented have clearly demonstrated that *B. cinerea* has developed into a model system of great value in molecular phytopathology. There are still very few fungi where so many genes have been functionally analysed, and the number is expected to increase tremendously in the next few years, due to fast and simple knock-out procedures (described above). New techniques, like RNA interference

(RNAi), are already in development (J. van Kan, pers. comm.) and will speed the analyses. Important factors affecting progress will be the public availability of a genomic sequence of sufficient quality, an increasing number of EST datasets (especially from parasitic cultures), and corresponding macroarrays. Together with growing collections of insertional mutants available within the *Botrytis* research community, a more detailed and full-scale view of the complex interaction of fungal pathogen and its host plants can be expected.

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Table 1. Mutants of *Botrytis cinerea* obtained by targeted inactivation

Gene	Symbol	Recipient strain	Size of flanks	Selection system	No. ¹ _a	Impact on pathogenicity ²	Other phenotype	Cit. ³
Cutinase	<i>cutA</i>	B05.10	1 kb/ 0.6 kb	hygromycin	3	-		1
Lipase	<i>lip1</i>	B05.10	1.2 kb/ 1.4 kb	hygromycin	1	-	no lipolytic activity	2
Tetraspanin	<i>bcp1s1</i>	T4	0.4 kb	bialaphos	1	non-pathogenic on bean and tomato leaves	unable to penetrate onion epidermis	3
Fructose/H ⁺ symporter	<i>frt1</i>	B05.10	1.85 kb/ 1.9 kb	hygromycin	1	-		4
Chitin synthase	<i>bcc1s1</i>	Bd90	disruption	hygromycin	6	about 32 % reduced lesion diameter on vine leaves	cell wall more sensitive to enzymatic degradation	5
Endopolygalacturonase (endoPG)	<i>bcp1</i>	B05.10	1 kb	hygromycin	2	reduced lesion growth rate on tomato leaves, tomato fruits and apple fruits		6
endoPG	<i>bcp2</i>	B05.10	c. 3 kb/ c. 4.5 kb	hygromycin	3	reduced virulence on multiple hosts		7
endoPG	<i>bcp3</i>	B05.10	c. 500 bp	nourseothricin	1	reduced virulence on multiple hosts		7
endoPG	<i>bcp3</i>	B05.10	c. 500 bp	hygromycin	5	-		7
endoPG	<i>bcp5</i>	B05.10	c. 500 bp	nourseothricin	3	-		7
Pectin methyl-esterase (PME)	<i>bcpme1</i>	Bd90	disruption	hygromycin	1	reduced virulence on apple fruits, grapevine and <i>A. thaliana</i> leaves		8
PME	<i>bcpme1</i>	B05.10	831 bp	nourseothricin	1	-		7
PME	<i>bcpme1</i>	B05.10	c. 500 bp	hygromycin	4	nt		7
endoPG + PME	<i>bcp1+</i>	B05.10	c. 500 bp	nourseothricin	10	reduced virulence, similar to $\Delta bcp1$ mutant		7
Aspartic protease	<i>bcpme1</i>	B05.10	c. 500 bp	+ hygromycin	2	nt		9
Aspartic protease	<i>bcp1</i>	B05.10	c. 500 bp	nourseothricin	1	-		9
Aspartic protease	<i>bcp3</i>	B05.10	c. 500 bp	nourseothricin	1	-		9

Table 1 (cont.)

Table 1 (cont.)

Gene	Symbol	Recipient strain	Size of flanks	Selection system	No. ¹	Impact on pathogenicity ²	Other phenotype	Cit. ³
Aspartic protease	<i>bcap4</i>	B05.10	c. 500 bp	hygromycin	?	nt		9
Aspartic protease	<i>bcap4</i>	B05.10	c. 500 bp	hygromycin	?	nt		9
Aspartic protease	<i>bcap5</i>	B05.10	c. 500 bp	hygromycin	6	-		9
ABC transporter	<i>bcatrA</i>	B05.10	?	hygromycin	?	-		10
ABC transporter	<i>bcatrB</i>	B05.10	c. 1.8 kb/ c. 2 kb	hygromycin	2	reduced virulence on grapevine leaves	increased sensitivity to resveratrol and fenpiclomidil	11
ABC transporter	<i>bcatrD</i>	B05.10	1.3 kb/ 2.5 kb	hygromycin	2	-	increased sensitivity to DMI fungicides	12
MFS transporter	<i>bemfsI</i>	B05.10	c. 1 kb/ c. 2 kb	hygromycin	2	-	increased sensitivity to camptothecin and cercosporin	13
ABC transporter + MFS transporter	<i>bcatrD</i> + <i>bemfsI</i>	B05.10	1.3kb/2.5k b	hygromycin nourseothricin	1	-	higher sensitivity to DMI fungicides than Δ BcatrD	13
ABC transporter + MFS transporter	<i>bcatrB</i> + <i>bemfsI</i>	B05.10	1.5 kb/3 kb 1.8 kb/2 kb	hygromycin nourseothricin	1	like BcatrB	no alteration to single replacements	13
Saponinase (avenacinase)	<i>sapI</i>	B05.10	c. 0.5 kb/ c. 0.6 kb	phleomycin	3	-	increased sensitivity to avenacin	14
Laccase	<i>belcI</i>	B05.10	1.1 kb/ 1.5 kb	hygromycin	3	-	-	15
Laccase	<i>belc2</i>	B05.10	1.6 kb	hygromycin	6	-	no growth inhibition on resveratrol	15
Glutathione S-transferase	<i>bcgstI</i>	B05.10	disruption -> 698 bp	hygromycin	3	-		16
Catalase	<i>becat2</i>	B05.10	1.1 kb/ 2.1 kb	hygromycin	2	-	increased sensitivity to H ₂ O ₂	17
Glucose oxidase	<i>begodI</i>	B05.10	0.7 kb/1 kb	hygromycin	4	-		18

Table 1 (cont.)

Table 1 (cont.)

Gene	Symbol	Recipient strain	Size of flanks	Selection system	No. ¹	Impact on pathogenicity ²	Other phenotype	Cit. ³
Superoxide dismutase	<i>bcsod1</i>	B05.10	0.9 kb/ 1 kb	hygromycin	3	reduced lesion size on bean leaves	increased sensitivity to paraquat	18
MAP kinase	<i>bmp1</i>	A-1-3/ B05.10	1 kb/ 2.6 kb	hygromycin	1/2	non-pathogenic on carnation and rose flowers, tomato leaves		19
Cyclophilin A	<i>bcp1</i>	T4	0.3 kb	bialaphos	1	reduction of lesion development on bean and tomato leaves	resistant to cyclosporin A	20
G protein α subunit	<i>beg1</i>	B05.10	1.7 kb/ 3 kb	hygromycin	3	drastically reduced virulence, no soft rot formation on bean leaves		21
G protein α subunit	<i>beg2</i>	B05.10	1.1 kb/ 1.6 kb	hygromycin	7	up to 80 % reduced lesion diameter on bean leaves		21
Adenylate cyclase	<i>bac1</i>	B05.10	1 kb/ 4 kb	hygromycin	2	up to 80 % reduced lesion diameter on bean leaves		22
Protein kinase	<i>bpk2</i>	B05.10	0.6kb/0.7kb	nourseothricin	5	-		23
Protein kinase	<i>bpk3</i>	B05.10	0.4 kb/1 kb	hygromycin	4	-		23
Protein kinase	<i>bpk4</i>	B05.10	1 kb/ 0.5 kb	hygromycin	3	-		23
Transmembrane protein	<i>btp1</i>	B05.10	1.1kb/0.6kb	nourseothricin	3	-		23
<i>ent</i> -copalyl diphosphate synthase / <i>ent</i> -kaurene synthase	<i>becps/ks1</i>	B05.10	1.5 kb / 1.4 kb	hygromycin	4	-		24
FPP cyclase	<i>bctri5</i>	T4	0.5 kb/1kb	bialaphos	4	reduced lesion development on bean leaves		25
NADPH:cytochrome P450 reductase	<i>becpr-1</i>	K1 ATTC	0.5 kb/1kb 0.4 kb /	bialaphos hygromycin	5 3	-	reduced growth rate	24
Cytochrome P450 monooxygenase	<i>bcp450-12</i>	58025 ATTC	0.42 kb 1 kb /	hygromycin	2	-		24
Cytochrome P450 monooxygenase	<i>bcp450-12</i>	58025 SAS56	1.5 kb 1 kb /	hygromycin	3	-		24

Table 1 (cont.)

Table I (cont.)

Gene	Symbol	Recipient strain	Size of flanks	Selection system	No. ¹	Impact on pathogenicity ²	Other phenotype	Cit.
Cytochrome P450 monooxygenase	<i>cmd5</i>	T4	0.4 kb / 0.5 kb	bialaphos	1	reduced lesion development on bean leaves		25
Cytochrome P450 monooxygenase	<i>bcaba1</i>	G3	0.57 kb / 0.49 kb	hygromycin	3	-	no abscisic acid production	24
Polyketide synthase	<i>bcpks1</i>	B05.10	c. 500 bp	nourseothricin	2	-		7
Glyoxal oxidase	<i>beglyox1</i>	B05.10	1.5 kb	hygromycin	10	non-pathogenic	pleiotropic conditional growth defect	26

¹ no. of mutants characterized

²; -: no effect, nt: not tested

³ 1 = Van Kan et al., 1997; 2 = H. Reis and M. Hahn, pers. comm.; 3 = Gourgues et al., 2004; 4 = G. Döhlemann, F. Molitor and M. Hahn, pers. comm.; 5 = Soulié et al., 2003; 6 = Ten Have et al., 1998; 7 = I. Kars and J.A.L. van Kan, pers. comm.; 8 = Valette-Collet et al., 2003; 9 = A. ten Have, pers. comm.; 10 = G. Del Sorbo and M.A. de Waard, pers. comm.; 11 = Schoonbeek et al., 2001; 12 = Hayashi et al., 2002a; 13 = Hayashi et al., 2002b; 14 = Quidde et al., 1999; 15 = Schouten et al., 2002a; 16 = Prins et al., 2000; 17 = Schouten et al., 2002b; 18 = Rolke et al., 2004; 19 = Zheng et al., 2000; 20 = Viaud et al., 2003; 21 = Schulze Gronover et al., 2001; 22 = Klimpel et al., 2002; 23 = C. Schulze Gronover and B. Tudzynski, pers. comm.; 24 = V. Siewers and P. Tudzynski, 2004 25 = M. Viaud, A. Brunet-Simon, Y. Brygoo, J.-M. Pradier and C. Levis, pers. comm.; 26 = F.L. Stefanato and J.A.L. van Kan, pers. comm.

CHAPTER 5

MORPHOLOGY AND CELLULAR ORGANISATION IN *BOTRYTIS* INTERACTIONS WITH PLANTS

Klaus B. Tenberge

Institut für Botanik und Botanischer Garten, Westfälische Wilhelms-Universität, Schlossgarten 3, 48149
Münster, Germany

Abstract. Besides the ability of *Botrytis* species to survive saprophytically or endophytically these pathogens rapidly cause grey mould and other diseases in hundreds of mono- and dicotyledonous plants. The broad habitat range of one of these species, *B. cinerea*, should be perceived as having tremendous flexibility, not restricted regarding hosts and tissues. This potential is put into action by means of different infection strategies that vary along with conditions. The physical exploitation of host tissue can be best investigated by combined cytological techniques ranging from light microscopy to confocal laser video and high resolution electron microscopy. These techniques were applied to early infection phases and fungal infection organs, eventually further elucidated by molecular biological approaches. Emphasis is placed on hyphal tip swellings proven to be elaborated infection structures. Although constituting real appressoria, they might not mediate mechanical penetration in the first place. In addition, other infection mechanisms are reviewed, including enzymatic attack, and new evidence based on electron microscopy and cytochemistry is discussed, indicating chemical penetration mechanisms of cuticles not yet found in other fungal-plant interactions.

1. Introduction

Do pathogenic fungi penetrate plant cuticles mechanically or by means of cutinases? This fundamental question was formulated by Blackman and Welsford as early as 1916, providing a far-sighted hypothesis because cutinolytic enzymes were not then known. They addressed the analysis of this phytopathological problem and applied it to interactions of *Botrytis cinerea* and broad bean, at times when *Botrytis* was regarded as the best investigated fungus (Epton and Richmond, 1980). Considering their introductory research paradigm is worthwhile:

“It seemed then advisable to make a careful study of the early stages of infection by *Botrytis cinerea*, paying particular attention to the phenomena to be observed in connexion with the penetration of the cuticle” (Blackman and Welsford, 1916).

Nowadays, this message would naturally be perceived in an ultrastructural sense, as electron microscopic techniques have evolved, allowing resolution surely beyond

the belief of Blackman and Welsford. Electron microscopy (EM) was quickly applied and after effective fixation techniques were developed in the late 1960s, high quality ultrastructural research was performed on *B. cinerea*.

Extensive *Botrytis* research has been summarised for many aspects (Jarvis, 1977; Coley-Smith et al., 1980; Verhoeff et al., 1992), but paying little attention to cytology and fine structure of plant-microbe interaction phases. Morphology and surfaces have been precisely visualised by scanning EM (SEM) (Richmond and Pring, 1971a, b; Elad, 1989; Pie and De Leeuw, 1991; Williamson et al., 1995), whereas comprehensive transmission EM (TEM) analysis is limited. Electron micrographs of the early phases of infection are particularly rare in the literature. This chapter describes progress over a 30-year period of *Botrytis* research and comments on some early publications, because some are milestones in ultrastructural research, or must now be re-evaluated in the light of new results, e.g. conidial surfaces and hydrophobins. Considerable advances have been achieved in methodologies such as the affinity-gold technique, allowing analytical *in situ* research by EM, and in molecular genetics, allowing targeted gene deletion.

Much effort has been placed on understanding cuticle penetration by the pathogen. While, at least in some plant-fungus systems, the contribution of mechanical pressure to penetration is proven, e.g. in *Magnaporthe* (Howard, 1997), the alternatively suggested cutinase-mediated invasion became one of the most controversially discussed topics in phytopathology (see Tudzynski and Tudzynski, 1996). Hence, the introductory question posed early last century is still open in phytopathology and remains among the most important ones in *B. cinerea* research. As *Botrytis* is known to use a wide range of infection strategies (Chapter 2), its penetration strategies appear complex and crucial to its success (Chapter 7). The interpretation of ultrastructure depends critically on the chemical and physical changes imparted by the processes used in sample preparation for electron microscopy. There have been great advances in the understanding of such methods and effects on specimen ultrastructure. This review deals mainly with the ultrastructure of *Botrytis*-plant interactions, laying special emphasis on the early infection phases.

2. Cytology and ultrastructure of *Botrytis*

2.1. Conidia

The *Botrytis* spp. are typical ascomycetes as the micrographs of sectioned cells exhibit characteristic structural elements common in this taxon. Conidia constitute the main propagules of fungus (Chapters 2 and 3). In sections they appear round to elliptical with a short neck and a scar created during external abscission from conidiophores. The conidia of *B. cinerea* measure about 10 x 8.5 μm (Pezet and Pont, 1990). A dormant conidium contains a protoplast, which has been analysed thoroughly regarding various aspects and mostly in conidia grown in axenic culture (Hawker and Hendy, 1963; Gull and Trinci, 1971; Richmond and Pring, 1971a, b; Epton and Richmond, 1980). Inside the plasma membrane of *B. cinerea* or *B. fabae*, a set of organelles typical of ascomycetes is present: several nuclei each surrounded

by a nuclear envelope with nuclear pores, round to ovoid mitochondria with many cristae, few small spherical vacuoles (prevacuoles) delimited by single membranes, multivesicular bodies (vesicles with membranous inclusions), microbodies, sparse endoplasmic reticulum (ER), dictyosomes, vesicles, storage bodies in the conidium periphery, lipid bodies, ribosomes and polysomes. With regard to secretion and signalling events, the organisation of the plasma membrane and internal membranes is of great importance. Detailed views of internal and external surfaces of the *B. fabae* plasmalemma provided by freeze-etching and TEM display several distinct types of particles and branched invaginations (Richmond and Pring, 1971a), the latter probably corresponding to the undulating outline found in sectioned conidia. Small vesicles pass through the plasmalemma into the cell wall. Importantly, the freeze-etched samples revealed considerable number of ER cisternae. Vesicles were seen to originate from ER strands but not from Golgi compartments, which were thought to be absent in *B. fabae* (Richmond and Pring, 1971a) and are ill-defined in ascomycetes (Mendgen et al., 1995). In chemically fixed samples of *B. cinerea*, vacuoles have irregular, indented outlines and often incorporate parts of the cytoplasm or electron-dense material and similar observations have been made in *B. fabae*. However, according to freeze-etching, these organelles reveal smooth surfaces (Richmond and Pring, 1971a).

After glutaraldehyde-osmium fixation with uranyl-lead contrast (Standard-TEM), or after use of other fixatives, conidia of *B. cinerea* have a two-layered cell wall tightly connected to the plasma membrane, a thick electron-transparent inner layer and a thinner electron-dense outer layer (Hawker and Hendy, 1963; Gull and Trinci, 1971). This layering was not detected in replicas of cross-fractured samples, indicating a similar basic construction of both layers apart from the electron-dense material (Richmond and Pring, 1971a). Both layers are formed of microfibrils, probably chitin, with specific orientation in a granular matrix. Wheat germ agglutinin (WGA)-gold labelling and TEM showed that chitin is evenly distributed throughout the cell wall of vegetative hyphae. β -1,3-Glucan, which is visualised by β -1,3-glucanase-marker molecules, is similarly distributed (El Ghaouth et al., 1997). The basic importance of β -glucans in cell wall construction in *B. cinerea* is clearly evident because inhibition of glucan synthesis (Goody, 1993) or exo- β -1,3-glucanase treatment result in bursting of hyphae and leakage of cytoplasm from germ tubes (Jijakli and Lepoivre, 1998). Chitin is linked via glucans to glycoproteins in the wall and appears to influence its shape, rigidity and physical strength. During searches for means to control *Botrytis*, class I chitin synthase mutants have been generated recently and the chitin content of these *Bcchs1* mutants was reduced by 30%. Although this chemical change did not alter fluorescence staining with Calcofluor White, protoplasts were released more easily than in controls when the glucanase cocktails were applied. These results indicate that the chitin-depleted cell walls of the mutant give greater accessibility to enzyme substrate and may account for the reduced virulence of mutants when challenged by PR proteins generated by the host (Soulié et al., 2003). The electron-density of the outer layer was attributed to melanin (Richmond and Pring, 1971a), the presence of which has only recently been established (Doss et al., 2003).

The surface of the hydrophobic conidia is altered by contact with the substratum

(Doss et al., 1993) indicating an adhesive function probably mediated by hydrophobins, proteins conferring hydrophobic interactions. According to standard SEM, the surfaces of conidia of *B. fabae* and *B. cinerea* are very similar, being nearly smooth with no conspicuous ornamentation, spines or a distinctive pattern (Richmond and Pring, 1971a; Epton and Richmond, 1980). When viewed by low temperature scanning electron microscopy (LTSEM), *B. cinerea* conidia exhibit a finely granular surface (Williamson et al., 1995), but freeze-etched replicas and TEM revealed a dimpled wall covered with small particles in parallel lines between numerous small ridges, many aligned longitudinally (Richmond and Pring, 1971a). The surface of dry conidia was rough with numerous 200 to 250 nm short protuberances that disappeared upon hydration and re-drying. Although TEM of replicas and high resolution SEM visualised the rodlet pattern typical of hydrophobins in *Aspergillus* controls, no such rodlets were detected on conidia of *Botrytis* spp., but this does not exclude the presence of non-rodlet-forming hydrophobins (Doss et al., 1997).

2.2. Germination and germinated conidia

2.2.1. Germ tube structure

If certain requirements that include availability of nutrients and water are fulfilled during inoculation, conidia swell and germinate (Verhoeff, 1980; Salinas and Verhoeff, 1995; Chapter 2), usually with one or two germ tubes. After dry-inoculation of petals, up to five germ tubes emerge under 100% relative humidity (Salinas and Verhoeff, 1995; Williamson et al., 1995).

During conidial germination the outer wall ruptures, and the inner layer extends from this conidial wall into those of the germ tube (Hawker and Hendy, 1963). Using improved TEM preparation techniques, this early mis-representation was corrected. Rather than decreasing due to swelling of the conidium, its conidial wall thickens by apposition and newly formed layers are localised inside the original conidial wall 4 h after incubation. Inside these layers, another layer is affixed at the germination site, which is continuous with the emerging germ tube wall (Gull and Trinci, 1971; Richmond and Pring, 1971b). A septum, typical of ascomycetes, is laid down centripetally at the base of the germ tube and Woronin bodies are closely associated with its single central pore (Hawker and Hendy, 1963; Gull and Trinci, 1971; Richmond and Pring, 1971b). In the emerging germ tube no large vacuoles are present, whereas in the conidium a central vacuole is formed while prevacuoles have gone. A polysome-rich cytosol and organelles, nuclei and many mitochondria, move forward into the emerging germ tube, exhibiting a streaming motion towards the tip (Hawker and Hendy, 1963; Richmond and Pring, 1971b). The movement of cytosol can clearly be seen by light microscopy (LM) (B. Hoppe and K.B. Tenberge, unpubl.). Importantly, ER cisternae increase in number and sometimes are connected to the nuclear envelope. As shown by different techniques, multivesicular bodies, not seen in preceding phases, tubular-vesicular complexes and numerous cytoplasmic vesicles occur (Gull and Trinci, 1971; Richmond and Pring, 1971b). Within the conidium, storage bodies and glycogen granules break down, prevacuoles disappear and vacuoles containing inclusions of electron-dense material (Richmond

and Pring, 1971b) are present which thought to be the final destination of substances transported by the endocytotic pathway. TEM suggested that such vacuoles in *B. cinerea* are involved in lytic processes, because incorporated heterogeneous material is possibly digested here. Acid phosphatase activity has been located in larger vacuoles, up to 2.5 μm , that stained positively with neutral red (Weber et al., 1999). These authors found that small osmiophilic organelles, below 0.5 μm , are lipid-rich spherosomes and precursors of lipid bodies, rather than being equivalent to mammalian lysosomes because acid phosphatase activity was too low.

An apical corpuscle was observed in *B. cinerea* and in *B. fabae* near the apex of emerging germ tubes, but not earlier in dormant conidia. It is a hemispherical structure of electron-dense material, sometimes having a membranous appearance, surrounded by cell wall material and occasionally connected to the cytoplasm (Gull and Trinci, 1971; Richmond and Pring, 1971b). A similar structure was found in *Phytophthora* germlings and it is suggested that it may be involved either in conidial wall dissolution or in germ tube protrusion (Bartnicki Garcia, 1969). However, such corpuscles have not been found in other fungi (Girbardt, 1969).

2.2.2. Tip growth and Spitzenkörper

Germ tube elongation is affected by turgor-driven protrusion of the hyphal tip accompanied by cell wall fabrication. Arrangement of microfibrils in the wall was assumed to be connected with the particles clearly organised in a geometrical pattern on the outer plasmalemma surface of *Botrytis* (Richmond and Pring, 1971b). In actively growing hyphae, the tip zone fluoresces intensely with Calcofluor White or with WGA-fluorescein isothiocyanate (FITC) probes for presence of chitin (Held et al., 2002). In this tip zone the plasma membrane is wavy (Epton and Richmond, 1980), most likely due to fusion of numerous vesicles carrying cell wall material and enzymes or due to transfer of other components. In this respect, tip growth in *Botrytis* is connected with the apical body (Spitzenkörper) that is well known in filamentous fungi. The first direct confirmation and three-dimensional view of apical vesicles comprising the Spitzenkörper was gained recently by confocal-laser scanning microscopy (CLSM) of whole-mount hyphae after freeze substitution, using FITC-conjugated concanavalin A (Con A) for specific labelling. In apices of *B. cinerea*, the endo-membrane system, including the Spitzenkörper and the plasma membrane, were intensely labelled, whereas the prominent Spitzenkörper core region was rich in actin and free from vesicles (Bourett et al., 1998). Acid phosphatase activity was located in apical secretory vesicles of *B. cinerea* (Weber et al., 1999). Golgi-equivalent organelles, the presence of which was previously uncertain, were visualised in *B. cinerea* sub-apical regions by Con A fluorescence (Bourett et al., 1998). These Golgi-like organelles were arranged in linear arrays parallel to the longitudinal axis of the hypha along with mitochondria and vesicles. Immunolabelling and CLSM visualised actin filaments in *B. cinerea* (Bourett et al., 1998), which together with other cytoskeletal elements, are likely to be involved in trafficking of organelles. Membrane recycling occurring simultaneously with endocytosis is regarded as crucial in tip growth as it should compensate for the

massive membrane flow to the plasmalemma along with polarised exocytosis of cell wall material and enzymes at the apex. However, concrete evidence for the occurrence of endocytosis in filamentous fungi has been lacking. Recently, CLSM of a membrane-selective dye FM4-64 has been successfully used to demonstrate endocytosis in several fungi, including *Botrytis* (Fischer-Parton et al., 2000). The multi-component Spitzenkörper was stained by FM4-64 in living *B. cinerea* with a distinct central area of reduced FM4-64-mediated fluorescence matching with the Con A-negative core region described above in fixed samples. In general, the overall structure of the *Botrytis* Spitzenkörper fitted very well with those in other fungi; satellite Spitzenkörper were most frequently observed in *B. cinerea* (Fischer-Parton et al., 2000). As the dynamics of the endo-membrane system, including the Spitzenkörper, is best visualised with FM4-64, the effect of the new botryticide fenhexamid was analysed by video CLSM during uptake and localisation at its target. Shortly after feeding living hyphae with fenhexamid, it was observed that endocytosis, vesicle trafficking and membrane turn-over ceased as the cells died (Held et al., 2002). Subsequently it was shown that fenhexamid inhibits sterol biosynthesis in *Botrytis* and alters its membrane composition (Debieu et al., 2001), thus explaining the lethal effects of the fungicide seen in our video microscopy. Understanding basic mycology and cell biology is as important as knowledge of interaction-specific mechanisms to discover new methods of disease control.

Not only the number, but also the length, of germ tubes varies (Chapter 2). Molecular control of these processes is under investigation but not yet clarified. Presence of water droplets or nutrients led to longer germ tubes (Williamson et al., 1995; Cole et al., 1996). Longer germ tubes were seen after targeted mutagenesis but could not yet be attributed to an induced defect because they were also formed by wild-type strains probably because conditions or penetration sites were inappropriate (K.B. Tenberge and P. Tudzynski, unpubl.). Germ tube development and direction of *B. cinerea* appears to be light-sensitive and negative phototropism is important during infection (Islam et al., 1998).

2.2.3. Mucilage

Soon after the germ tube starts to emerge from the conidium, it becomes covered by a mucilaginous sheath (Gull and Trinci, 1971). In 1916 this sheath had been stained by colloidal silver or gentian violet and detected throughout the inoculum drop, where it connected to all surfaces (Blackman and Welsford, 1916). The sheath has been visualised since by LM, differential interference contrast microscopy, SEM, TEM and is 25 to 60 nm in thickness as measured by atomic force microscopy (Doss et al., 1995). After dry inoculation, no extracellular adhesive material is visible by LTSEM or LM on short germ tubes (shorter than conidia) on broad bean leaves, rose petals or glass (Williamson et al., 1995; Cole et al., 1996). However, using a monoclonal antibody and TEM, a thin sheath is detectable even around dry-inoculated conidia on bean and in aqueous glucose around germ tubes that tightly adhered even to glass (Cole et al., 1996). After conidia at first had only weakly adhered upon hydration (Doss et al., 1993, 1995), germ tubes firmly attach to either

hydrophobic or hydrophilic surfaces by this secreted ensheathing film (Doss et al., 1995), probably based on hydrophobic interactions. As it is possible to physically remove germlings without removing the sheath, an adhesive function of the sheath is evident.

Germlings are resistant to removal by boiling or by treatment with a number of hydrolytic enzymes, 2 M periodic acid, or 1 M sulphuric acid. Only 1.25 N NaOH was capable of chemically removing germlings together with the sheath. Alkaline-soluble material, composed of c. 30% glucose, 3% galactosamine and 30-44% protein, was also found adhering to walls of flasks containing liquid cultures of *B. cinerea* (Doss et al., 1995). In liquid cultures, this fungus secretes cinerean, a β -(1,3)(1,6)-D-glucan, of which 40% remained in solution but 60% was attached to hyphae in an extracellular capsule and slime. Beside adhesion and water storage, this capsule serves as an extracellular polysaccharide reserve, because *B. cinerea* is able to degrade it by action of three out of four secreted exo-glucanases that do not cause cell lysis (Stahmann et al., 1992, 1993), but the composition of the mucilage appears to be intricate (see also Chapter 2). Its polyanion content is indicated by ruthenium red-mediated fixation that preserves the capsules in TEM (Cook et al., 2000). Some surface fibrils of *B. cinerea* are proteinaceous (Gardiner and Day, 1988) and fibrillar proteins have been stained by colloidal gold (Jones et al., 1995). In the extracellular matrix isolated from glass slides, lipids, including fatty acids and wax esters, were identified (Cooper et al., 2000). Recently, the electron-dense granules incorporated into the wall matrix of *B. cinerea* germlings were shown by electron paramagnetic resonance (EPR) spectroscopy to be melanins (Doss et al., 2003). The melanin content within and on the surface of walls has previously been assumed and suggested to protect fungi against UV irradiation, desiccation and temperature extremes (Bell and Wheeler, 1986).

Monoclonal antibodies (MAbs) raised to *B. cinerea* antigens in the extracellular matrix (ECM) (Meyer and Dewey, 2000; Meyer et al., 2000; Chapter 11) were used to observe (FITC-conjugated MAbs) and study the nature of *Botrytis* ECM binding to surfaces. Two MAbs had antigenic binding sites on respectively, rhamnose (BC-12.CA4) (Meyer et al., 2000) and mannose (BC-5.AD6). Rhamnose and mannose are principal sugars in important structures in the plant cell wall, like rhamnogalacturonans in pectic polysaccharides and glucomannans and galactoglucomannans in hemicelluloses (Cooper, 1983; Kobayashi et al., 1999). The antigens were capable of binding to various plant components; the rhamnose antigen bound strongly to pectin and carboxymethyl cellulose while the mannose antigen bound strongly to soluble plant tissue components that are likely to be hemicellulose fractions. The glycoconjugate antigens produced in the ECM of *B. cinerea* play a role in interactions between the fungus and the plant tissue with attachment as an important feature (U. Meyer, Adelaide Univ., Australia, unpubl.), however the antigens are involved in other functions because blocking them led to 50-90% reductions in disease severity. BC-5.AD6 seems important for the ability of the fungus to penetrate, as blocking the antigen at inoculation leads to an extensive hyphal growth together with a reduction in attempted penetrations. BC-12.CA4 affects germination and germ tube elongation on glass, did not affect penetration of bean leaves, but affected lesion development (Meyer et al., 2000).

3. Imaging of infection

3.1. Infection sites and infection structures

Virulence of *Botrytis*, usually recorded by lesion number or diameter, depends greatly on inoculum conditions (Chapter 2), e.g. inoculum density and presence of glucose enhanced the establishment of *B. cinerea* infection on cucumber (Elad, 1988). However, an increasing number of germinated conidia does not necessarily lead to a higher number of penetration sites since identical sites are frequently used for later penetration (Verhoeff, 1980). As verified by environmental SEM during fixation for standard TEM most of a dense inoculum applied as droplets was washed off; only a few adherent structures remained, leaving masses of non-germinated conidia for later inoculations (K.B. Tenberge, unpubl.). Hence, initial penetration events are crucial and, therefore, attention will be focused on those. Reports in the literature on infection structures may be confusing as several different interaction systems, different *Botrytis*-host systems and various organs e.g. flowers, leaves and roots have been analysed at pre- and post-harvest stages with varying inoculation and investigation techniques, not always in great detail. Obviously, penetration strategies vary with conditions, which might reflect the pathogens' flexibility. Infection sites can arise from conidia, mycelium and ascospores (Jarvis, 1977). The host may be entered by germ tube extension via natural openings or after wounding (Chapter 2), or via direct penetration of petals, sepals, stigmas, leaves and young fruits (Clark and Lorbeer, 1976; Verhoeff, 1980; Elad, 1988). Following germination of conidia, direct penetration through the intact cuticle and cell wall, rather than via stomata, appears to be the rule (Blackman and Welsford, 1916; Elad, 1989). After inoculation of French bean hypocotyls with mycelium, *B. cinerea* differentiated a set of infection structures from single lobate appressoria up to dome-shaped infection cushions, that present a complex of hyphal tips with and without swellings and originating from a single highly branched hypha (Garcia-Arenal and Sagasta, 1980). In all, germ tube apices, appressoria, and infection cushions constitute three types of penetration structures formed by *Botrytis* (Elad, 1989). The long-lasting debate as to whether penetration occurs with or without appressoria (Blackman and Welsford, 1916; McKeen, 1974) has not been settled completely.

3.2. Appressorium-mediated penetration

According to definition, appressoria are fungal attachment organs that mediate penetration through host surfaces and usually are conceived as swollen terminal compartments. Although direct penetration by *Botrytis* spp. without such swellings has been noticed (Sect. 3.3), appressoria depicted as swollen tips have been found frequently, e.g. on broad bean leaves (Blackman and Welsford, 1916), tomato fruits (Rijkenberg et al., 1980) or French bean leaves (Tenberge et al., 2002; Figure 1). *B. cinerea* also develops swollen tips on artificial surfaces such as glass (Blackman and Welsford, 1916; Doss et al., 1995) or host surface replicas prepared with fingernail varnish (amyl acetate) (Clark and Lorbeer, 1976), indicating that the cellular developmental programme appears to be independent of host surface interaction.

The supply of nutrients was noted to be indispensable for appressorial development on either glass or host surfaces; in pure water no appressoria formed and no penetration of onion were recorded (Clark and Lorbeer, 1976). On petals, swollen hyphal tips of *B. cinerea* were seen by SEM after inoculation by spraying (Hammer and Evensen, 1994), but not after dry inoculation of rose (Williamson et al., 1995) or gerbera (Salinas and Verhoeff, 1995), indicating that water is needed during apical swelling.

The movement of cytosol and organelles into the swelling is visible by LM (B. Hoppe and K.B. Tenberge, unpubl.). The appressoria differ in structure from those formed by *Magnaporthe*, e.g. no septum forms to seal off the germ tube. During its formation, the cell wall of the inflating terminal region fluoresced intensely when treated with Calcofluor White or FITC-WGA for detection of chitin (S. Koch, S. Krümberg and K.B. Tenberge, unpubl.). The outer cell wall layer of this region is melanised, as inferred from its electron-density (Richmond and Pring, 1971b) and as shown by chemical analysis (Doss et al., 2003). Masses of melanised matrix material often connect appressoria to the host surface (Figure 1). The appressorial function of terminal swellings is clear when infection is initiated; connected infection vesicles are found in the epidermal cell wall beneath the swollen tip as seen in thin sections of tomato fruits (Rijkenberg et al., 1980) or bean leaves (Figure 1). Contrary to some reports (e.g. Zheng et al., 2000), convincing evidence that *B. cinerea* develops melanised appressoria has now been presented.

To unravel the morphogenetic control of the formation of infection structures, calcineurin, a highly conserved calcium/calmodulin-regulated phosphatase known to be involved in *Magnaporthe* appressoria formation, was inhibited by cyclosporin A. An increase in branching, followed by growth cessation on the host surface, was found, rather than direct penetration of tomato or bean leaves. This important result indicated a role for calcineurin in early penetration processes in *B. cinerea* and was confirmed by a failure to isolate calcineurin null mutants (Viaud et al., 2003).

3.2.1. Breaching the host cuticle

The cuticle forms the primary host barrier and is thought to be impermeable as long as hyphae do not reach the underlying polysaccharide cell wall layers (Rijkenberg et al., 1980). Hence, secreted non-diffusible material at first is confined to the outer host surface. Accordingly, alteration of the subcuticular cell wall was not observed prior to cuticle penetration (Blackman and Welsford, 1916).

A penetration peg through the cuticle and outer epidermal wall of *Vicia faba* is formed by *B. cinerea* (McKeen, 1974). Hence, a pore is produced beneath the appressorium that is visible after removal of the infection structure (Rijkenberg et al., 1980). The cell wall of the fungal infection vesicle extends from the appressorial wall (Figure 1). It was reported that the fungal wall of the penetration peg was absent and that the fungal plasma membrane was in direct contact with the host

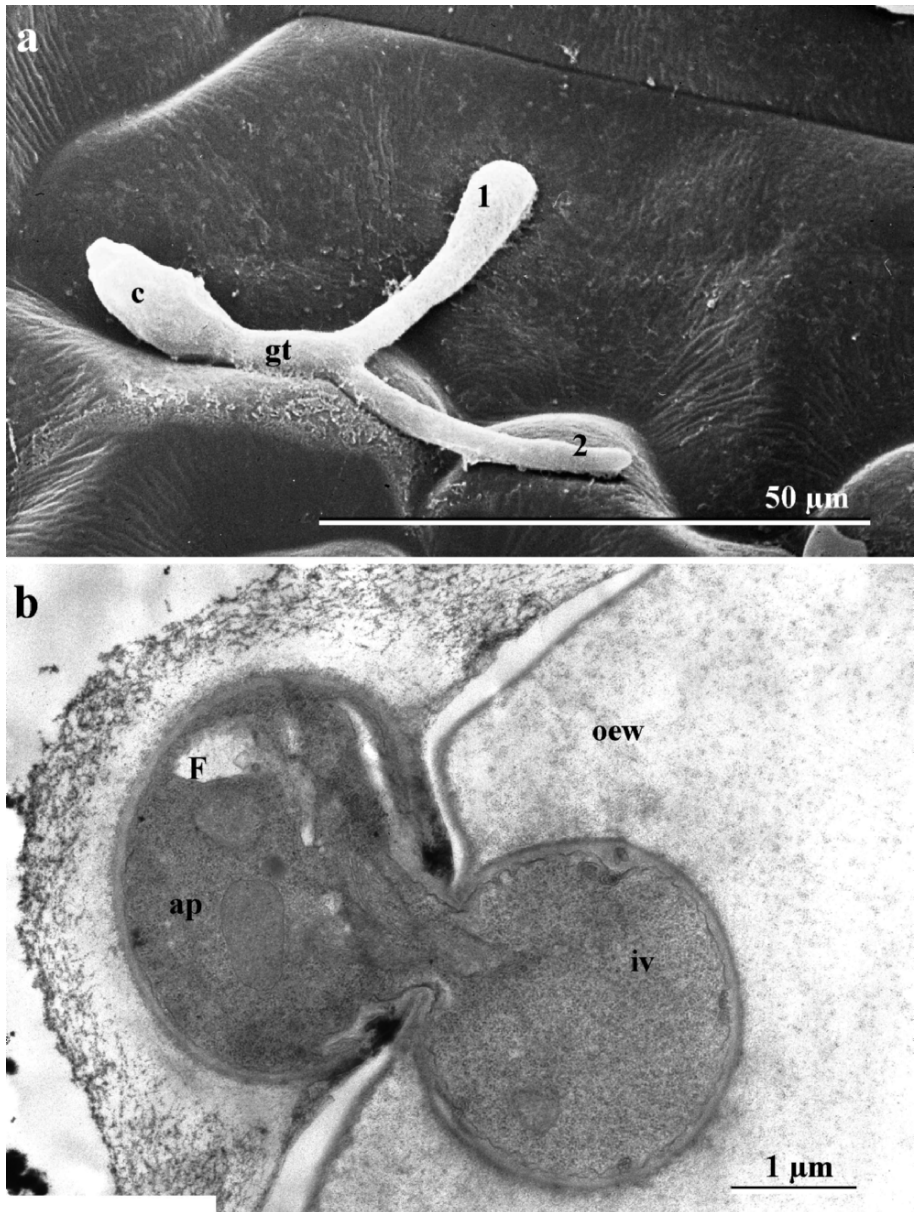


Figure 1. Infection structures of *Botrytis cinerea* on bean leaves. a - germ tube (gt) branches with (1) and without (2) tip swelling; and b - appressorium (ap)-mediated penetration of *B. cinerea* (F) into the outer epidermal wall (oew) of the leaf at 12 h post inoculation, iv= infection vesicle

(McKeen, 1974; Rijkenberg et al., 1980). However, this interpretation appears not to be supported by their published micrographs.

It has been repeatedly debated whether the pathogen directly penetrates undamaged leaves by means of enzymes or mechanical processes (Verhoeff, 1980; Chapter 7). Using various staining reactions in LM, no evidence was found that the cuticle of *Vicia faba* was altered chemically; therefore, Blackman and Welsford (1916) concluded that it must have been ruptured mechanically by pressure applied by *B. cinerea*. This view was supported by the observation that *B. cinerea* can penetrate artificial surfaces, including gold layers (see Verhoeff, 1980). To my knowledge, reports of direct penetration that exclude a role of pressure are still lacking; this mechanism appears to have been neglected recently in favour of enzymatic action. According to EM analysis, this pathogen produces a small hole with sharp and clean edges in the cuticle of a bean leaf, which clearly was interpreted as enzymatic action because esterase activity was demonstrated at germ tube tips (McKeen, 1974). According to SEM and TEM studies, the thick cuticular membrane of tomato fruits was judged to be dissolved enzymatically rather than being ruptured mechanically, as considerable cuticle erosion was associated with germ tube and penetration peg but not with the conidia (Rijkenberg et al., 1980). On rose stems, a small pore might be formed in the cuticle by enzyme activity (Elad, 1989).

If penetration of the cuticle is an enzymatic process, then cutin-degrading enzymes must be found (reviewed by Prins et al., 2000). *Botrytis* spp. often proliferate subcuticularly, causing the cuticle to separate from the underlying cell wall layers in later infection phases (Clark and Lorbeer, 1976). In such case cuticles remain intact although remaining in close contact with hyphae for days (Rijkenberg et al., 1980). Usually no alteration of cuticles distant from penetration sites is recorded (Cole et al., 1996). These observations may indicate that cutinolytic activity is confined to penetrating tips. Anti-lipase antibodies prevented infection of tomato leaves by *B. cinerea* (Comménil et al., 1998). Breaching of the cuticle by secreted cutinases is substantiated by the 80% suppressive effect that monoclonal anti-cutinases applied to the host exerted on lesion formation (Salinas, 1992; see Van Kan et al., 1997), but cutinase A-deficient mutants of *B. cinerea* are not altered in penetration ability on undamaged tissue (Van Kan et al., 1997). This apparent discrepancy may point to separate cutinolytic processes, probably mediated by appressoria, the development of which is enhanced by glucose that represses *cutA* expression (see Van Kan et al., 1997). A redox-responsive cutinase has been described recently (Wang et al., 2002), suggesting that active oxygen species (AOS) might be involved in cutinase control.

Recently, a small pore was visualised in the cuticle beneath appressoria by TEM of serial sections (Figure 2). Hydrogen peroxide of fungal origin was localised above, in and below this pore and diffused into the epidermal wall where a halo was extending in a concentric manner. This newly discovered event might resemble the initial process leading to cuticle penetration, because it was observed early and in advance of any cellular structure punching the surface (Tenberge et al., 2002). By cytochemical staining in LM and TEM, secretion of AOS by *B. cinerea* in axenic culture and during pathogenesis was discovered (Tenberge et al., 2002). By specific

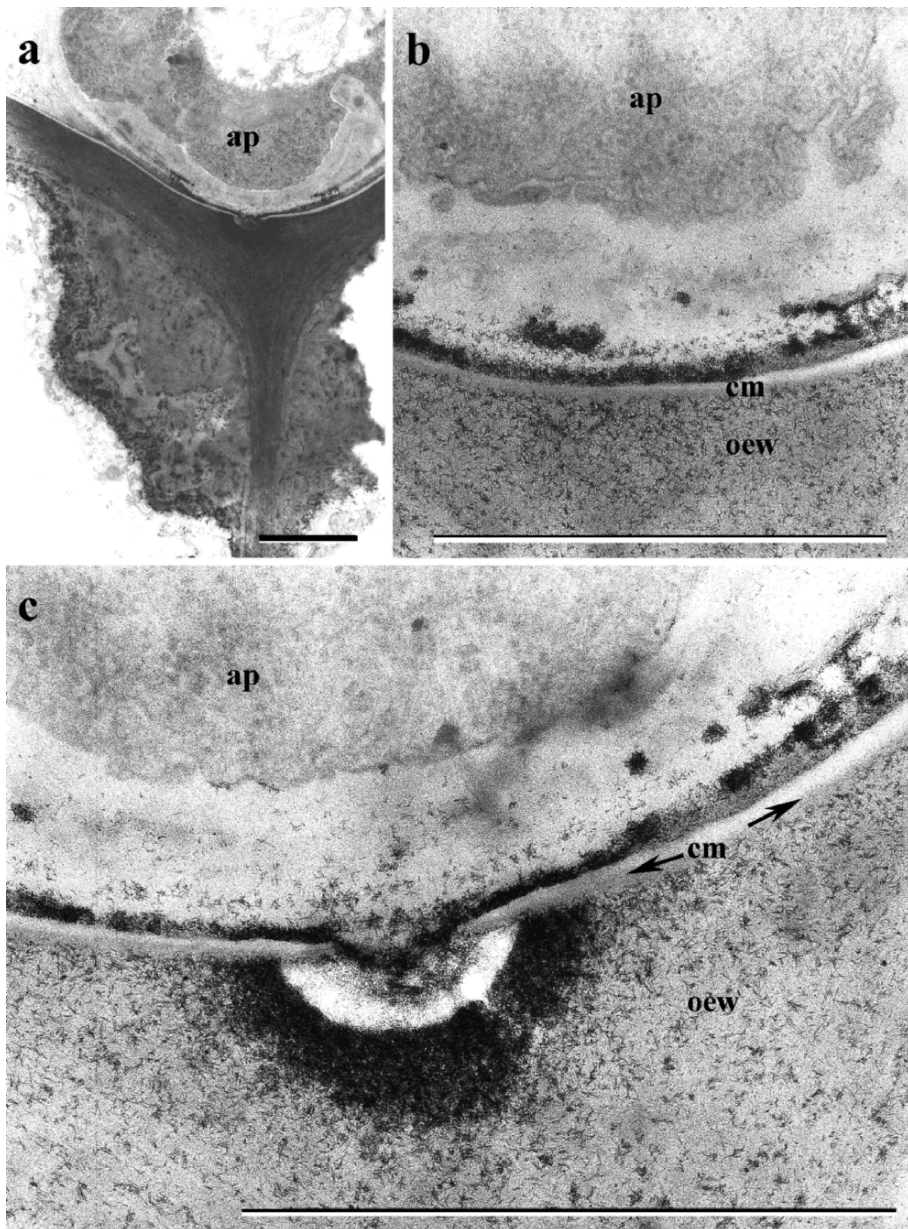


Figure 2. Localisation of H_2O_2 by cerium chloride at the interface of a *B. cinerea* appressorium (ap) and the outer epidermal wall (oew) of a tomato leaf at 12 h post inoculation. Overview (a) and detail (c) of a small pore in the cuticle (cm), which is continuous in following sections of a series (b). Scale bars = 1 μ m.

inhibitors it was confirmed that fungal superoxide dismutases are involved in this process (Tenberge et al., 2002; Rolke et al., 2004).

In addition, melanins present in the appressorial wall presumably contribute to this process, because they constitute a pool of stable free radicals and may have the potential to generate superoxide, and H_2O_2 , in the presence of superoxide dismutase (SOD) (Bell and Wheeler, 1986). In summary, the appressoria probably do not primarily transmit pressure, but mediate a direct oxidative attack which may be manifest as the separate cutinolytic process proposed above. Involvement of AOS in pathogenicity is described in chapters 7 and 8.

In the appressorium above the pore, an intricate cell wall structure is developed (Figure 2). Serial and longitudinal sections revealed that it is not a septum but a hemispherical structure, facing the penetration site of the swelling and incorporating electron-dense material positively stained for H_2O_2 . One might speculate that this structure is connected with the apical corpuscle described above.

3.2.2. Breaching the outer epidermal cell wall beneath the cuticle

When the cuticle is breached, an intermediate swelling is formed in the sub-cuticular cell wall layers (Figure 1) as has been seen earlier (Clark and Lorbeer, 1976). Once the cutinaceous barrier is ruptured, secreted cell wall degrading enzymes (CWDE) and other compounds may diffuse into the host (Chapter 7). Alteration and disintegration of the cell wall is obvious in TEM micrographs, indicating that CWDE were active. As has been discussed recently (Prins et al., 2000; Ten Have et al., 2002), especially polygalacturonases are required for full virulence. This suggests that *Botrytis* has the potential to release high levels of calcium, usually bound to pectic substances in the cell wall (Kaile et al., 1991). Calcium may be involved in hyphal tip growth, signalling (Chapter 6) and morphogenetic control of the formation of infection structure as discussed above.

3.3. Germ tube tip-mediated penetration

There are many reports on direct penetration of germ tube tips especially after dry inoculation without supply of nutrients (Elad, 1989; Pie and De Leeuw, 1991; Salinas and Verhoeff, 1995; Williamson et al., 1995). In these cases, the infection hyphae are regarded as a direct elongation of the germ tube and the penetration is assumed to occur without special infection structures, since no hyphal tip swellings have been observed. As outlined above, direct penetration is the result of sensing and signalling events, triggering a morphogenetic switch from elongation to penetration. Similar to *Magnaporthe*, where *PMK1* encoding for a MAP kinase was shown to be indispensable for appressorium formation and infection, a *B. cinerea* mutant lacking the homologous *BMP1* gene was non-pathogenic on carnation petals; neither tip swellings nor penetration by mutants were seen in SEM (Zheng et al., 2000). However, in the wild-type controls no appressoria developed on this host which shows that it is not the swelling that seems to be important, but a morphogenetic switch in the programme allowing a redirection of tip growth

towards the host. This process may also occur in non-swollen tips. The shape of appressoria varies widely from simple swollen tips to digitate structures (Verhoeff, 1980). If exclusively based on SEM, a lack of the swelling does not necessarily mean that no specialised infection structures developed; the internal tip structure should also be analysed. So far, the function of PMK1 is unknown and Zheng et al. (2000) speculated that PMK1 is involved in many of the processes that could mediate penetration.

3.4. Tissue invasion and colonisation

Subsequent to penetration of the outer epidermal wall, the fungus invades the sub-epidermal tissue inter- and intra-cellularly to establish the infection. *B. cinerea* kills host cells before they are invaded (Clark and Lorbeer, 1976). Some of the mechanisms involved in colonisation and spreading, e.g. CWDE, toxins, oxalic acid, have been discussed (Verhoeff et al., 1992; Prins et al., 2000; Chapters 7 and 9). In TEM micrographs, structural disintegration of cell walls upon colonisation is clear. Accordingly, polygalacturonases and recently pectin methylesterase were shown to be virulence factors (Ten Have et al., 2002; Valette-Collet et al., 2003). Cellulose is another target polysaccharide because cellulase-gold labelling was altered in swollen cell walls of apple after penetration by *B. cinerea* (El Ghaouth et al., 1998). Most of the hyphae appear to be healthy (El Ghaouth et al., 1998) as judged by their ultrastructure and melanin, present in outer cell wall layers, is likely serving as a protective coating against hydrolytic enzymes (Bell and Wheeler, 1986). However, depending on conditions, *Botrytis* has to survive a quiescent phase and restriction of post-penetration growth frequently occurs, e.g. in rose petals (Volpin and Elad, 1991), grape berries (Chapter 14) or on bean leaves. This indicates that there are crucial events in cross-talk that determine post-penetration phases. Cyclophilins are involved in post-penetration development of *B. cinerea* on tomato or bean because null mutants showed normal penetration but reduced virulence (Viaud et al., 2003). Mutants lacking *bcg1* encoding G protein α subunits were found inside leaves, but spreading lesions were never observed (Schulze Gronover et al., 2001).

4. Host response

The host response to infection has been summarised recently by Elad (1997). One very early reaction is the collapse of epidermal cells upon successful penetration (Clark and Lorbeer, 1976; Cole et al., 1996; see Verhoeff, 1980). Defence reactions of the structural type, especially papillae, were not often observed (Hammer and Evensen, 1994; see Pie and De Leeuw, 1991). After inoculation of bean and tomato leaves, papilla formation occurs but does not block *B. cinerea* infection, although AOS of host origin are present (Chapter 8) and are likely to be involved in cross-linking of internal papilla compounds (Figure 2). Chloronaphthol and nitro blue tetrazolium (NBT) staining visualize H_2O_2 and superoxide, respectively (Tenberge et al., 2002). The primary lesion area stained positively for AOS and most intensely at its border zone. The host oxidative burst is the source of a wave-like AOS

production, that can be visualized by LM and TEM. H_2O_2 detected as precipitate is present at the interface of *B. cinerea* and host cells and was also present in the periplasmic space, in the host cell wall and on the outer surface of the host cell as well as at the outside of the fungal wall. Consequently, H_2O_2 is produced in host cells at the plasma membrane and diffuses through the host cell wall into the intercellular space (Prins et al., 2000; Schouten et al., 2002). However, these defence reactions do not seem to block *B. cinerea* infection.

Chitosan treatment of *Capsicum* induced defence reactions including papilla formation, cell wall appositions containing pectin, occlusion of intercellular spaces and deposition of electron-dense material in cell walls that damaged *B. cinerea* hyphal structure (El Ghaouth et al., 1994). Upon infection hemispherical protuberances are deposited outside apple cell walls (El Ghaouth et al., 1998).

5. Conclusions

Infection success and with it the commercial importance of the genus *Botrytis* is thought to be based on its high flexibility and a rather broad habitat range, making it difficult to present a generalised picture of the interaction profile. *B. cinerea* actually may be regarded as a sub-species complex in which distinct populations seem to be adapted to different hosts (see Viaud et al., 2003). Nevertheless, considerable progress has been made in understanding the ultra-structure and function of penetrating hyphae and *Botrytis* can serve as a model system in phytopathology and mycology. *B. cinerea* produces functional appressoria that are obviously different from those developed by the well studied fungus *Magnaporthe*. However, the initial question about the precise cuticle penetration strategies remains to be answered. Modern cytological analyses combined with mutational techniques are required to clarify our understanding of phytopathogenic interactions. Affinity-gold labelling and TEM could provide much insight into processes at the host-pathogen interface, e.g. secretion of CWDE and the intended alteration of the cell wall, which have not been applied extensively. Video CLSM and new specific fluorescent probes will help to overcome the limitation of stationary micrographs. However, even standard TEM eventually combined with cytochemistry still has the potential to unravel critical phenomena at the dynamic interface between pathogen and host in this necrotroph. The recent discovery of small pores in the cuticle and localisation of pathogen-derived AOS at this site indicative of an oxidative attack, sheds new light on cuticle penetration mechanisms. These results are of wide interest not only in *Botrytis* research but in general phytopathology.

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CHAPTER 6

SIGNALLING IN *BOTRYTIS CINEREA*

Bettina Tudzynski and Christian Schulze Gronover

Institut für Botanik und Botanischer Garten, Westfälische Wilhelms-Universität, Schlossgarten 3, 48149 Münster, Germany

Abstract. The cellular environment plays an important role in growth and differentiation of fungi. Signal transduction cascades mediate communication between environmental signals and the cellular machinery regulating developmental programmes. Fungal pathogens of plants have to 'recognize' their susceptible hosts, penetrate any physical barriers, overcome host defences and proliferate in the invaded tissues. Recent work has established that cyclic AMP (cAMP) and conserved MAP kinase signalling pathways play crucial roles during pathogenesis in several plant-infecting fungi, including *Botrytis cinerea*. In all fungal pathogens analyzed so far, it has been demonstrated that the knock-out of genes whose products encode components of signaling cascades interferes with pathogen development. This chapter summarizes the recent progress in studying the function of genes that code for signalling components in *B. cinerea*.

1. Introduction

Botrytis cinerea affects nearly all species of dicotyledons including most vegetable and fruit crops, flowers, woody ornamentals and greenhouse-grown crops (Chapters 14-19). Thus, the fungus must have evolved strategies to 'recognize' suitable hosts, penetrate and invade plant tissues and overcome host defences. To perform these tasks, the fungus is capable of perceiving chemical and physical signals from different host plants and responding with the appropriate metabolic activities required for pathogenic development. In general, such metabolic adaptations include adhesion of conidia to the plant surface, directed germ-tube growth, differentiation of infection structures and secretion of lytic enzymes and phytotoxins (Knogge, 1996). All of these responses require a network of signal transduction pathways, such as the activation of G proteins (Bölker, 1998), cyclic adenosine monophosphate (cAMP) signalling (Mitchell and Dean, 1995) and mitogen-activated protein kinase (MAPK) cascades (Xu, 2000) to communicate the perceived external signal to the fungal genome so that the appropriate gene, or sets of genes, can be activated to build the developmental response required by the pathogen.

Enormous progress was made in recent years in the study of single components of signalling pathways, their functional analysis and interaction with other

components of the same or different signalling cascades. Some model filamentous fungi, *Aspergillus nidulans* and *Neurospora crassa*, and plant pathogens, such as *Magnaporthe grisea*, *Ustilago maydis* and *Cryphonectria parasitica*, have been most extensively studied. These studies reveal a high degree of conservation between different fungi even between divergent organisms, and illustrate conserved basic principles in the molecular determination of life (see Lengeler et al., 2000). However, despite the high degree of sequence conservation, signalling components can have different functions. Thus, the replacement of the highly homologous (98% amino acid identity) *Ga* subunits CPG1 of *C. parasitica* and GNA1 of *N. crassa* resulted in the loss of conidiation in *C. parasitica* (Gao and Nuss, 1996), but had no effect on conidiation in *N. crassa* (Ivey et al., 1996). Furthermore, CPG1 inhibits and GNA1 activates the activity of the adenylate cyclase. These examples clearly demonstrate that the complicated networks of signalling cascades, and their functions and cross-talks, must be studied in each fungus to gain a clear insight to their structures and performance. In *B. cinerea* some progress has been made recently in studying signalling components and their role in plant-fungus interaction; the identified components are summarized in Table 1.

2. *Gα* subunits of heterotrimeric G proteins

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are involved in regulating a variety of cellular functions in eukaryotic cells. They act as transducers between activated cell-surface receptors and intracellular effectors. In *B. cinerea*, two *Gα* subunit genes, *bcg1* and *bcg2*, have been identified (Schulze Gronover et al., 2001). The deduced amino acid sequence of BCG1 has the highest level of identity with the *Gα* subunits from other phytopathogenic fungi, such as CPC1 from *C. parasitica* (Gao and Nuss, 1996), CTG1 from *Colletotrichum trifolii* (Truesdell et al., 2000), CGA1 from *Cochliobolus heterostrophus* (Horwitz et al., 1999) and MAGB from *M. grisea* (Liu and Dean, 1997). All these *Gα* subunits are homologous to the mammalian $G_{i\alpha}$ family. On the other hand, BCG2 is quite similar to GNA2 from *N. crassa* (Turner and Borkovich, 1993) and MAGC from *M. grisea* (Liu and Dean, 1997). RT-PCR experiments showed clearly that both genes are expressed *in planta* at very early stages of infection. Characterization of *bcg1* and *bcg2* deletion mutants revealed that both *Gα* subunits affect growth and fungal pathogenicity in different ways. BCG1 controls multiple functions, including vegetative growth, pigmentation, proteolytic activity and pathogenicity. On media with up to 1% sucrose $\Delta bcg1$ mutants grow slowly and form small, more compact colonies similar to *cgal* mutants of *C. heterostrophus* and *cpg1* mutants of *C. parasitica*. However, on media with higher sucrose concentrations, the phenotype is comparable to the wild-type (WT) (Schulze Gronover et al., 2001). Interestingly, 2 mM cAMP fully restored the WT colony morphology suggesting a stimulatory affect of BCG1 on adenylate cyclase, similar to GNA1 in *N. crassa* and MAGB in *M. grisea*. Apart from effects on vegetative growth and morphogenesis, BCG1 was shown to have a major role in the process of colonization of host tissue. Germination of conidia and penetration ceases after formation of primary lesions. After 48 h, on leaves infected

with WT conidia, rapidly enlarging spreading soft rot lesions are formed, whereas for *Abcg1*-infected leaves spreading lesions were never observed. Scanning electron microscopical (SEM) analysis of *Abcg1*-induced primary lesions clearly showed that the hyphae of the mutant penetrate the plant surface in a manner comparable to the WT. These observations demonstrate that protein BCG1 seems to play a major role in the process of invasion of plant tissue after penetration, whereas e.g. *magB* mutants of *M. grisea* cannot penetrate host tissue due to the loss of its ability to form specialised appressoria (Liu and Dean, 1997).

Table 1. Signalling components identified in *Botrytis cinerea*

Gene	Protein	Function	Reference ¹
<i>bcg1</i>	Gα subunit	affects pathogenicity, no spreading lesions	1
<i>bcg2</i>	Gα subunit	delay in symptom development	1
<i>bac</i>	adenylate cyclase	delay in symptom development no conidiation on plant tissue	2
<i>bcpka</i>	catalytic subunit of PKA	?	3
<i>bpk2</i>	Sch9-like protein kinase	no obvious phenotype, expression induced by H ₂ O ₂	4
<i>bmp1</i>	MAPK (PMK1 homologue)	loss of pathogenicity, no penetration of plant tissue	5
<i>bmk3</i>	MAP kinase (Hog-homologue)	?	6
<i>bcras2</i>	Rab subfamily of Ras proteins	vesicular transport? protein secretion?	7
<i>bcras3</i>	Ras subfamily	morphogenesis, conidiation and pathogenicity	7, 8 (sequence)
<i>BCP1</i>	cyclophilinA	reduced virulence	9
<i>Btp1</i>	putative Gα-coupled receptor	no obvious phenotype	10
<i>BOS1</i>	histidine kinase	Osmo-sensing, fungicide resistance	11
<i>bpk3</i>	CLK1-like Ser/Thr protein kinase	no obvious phenotype expression induced by CuSO ₄	12
<i>bpk4</i>	ran1-like protein kinase	no obvious phenotype	12
<i>BcSNF</i>	SNF-subgroup of Ser/Thr protein kinases	involved in carbon repression of gene expression?	13

¹1=Schulze Gronover et al., 2001; 2=Klimpel et al., 2002; 3=B. Tudzynski and S. Richter, unpubl. ; 4=C. Schulze Gronover and B. Tudzynski, unpubl.; 5=Zheng et al., 2000; 6=U. Ellendorf, B. Tudzynski and P. Tudzynski, unpubl.; 7=U. Ellendorf, P. Hantsch, C. Schulze Gronover and B. Tudzynski, unpubl.; 8=Park et al., 1997; 9=Viaud et al., 2003; 10= C. Schulze Gronover, P. Hantsch and B. Tudzynski, unpubl.; 11=Cui et al., 2002; 12= C. Schulze Gronover, B. Tudzynski, and P. Tudzynski, unpubl.; 13=J. Schumacher and B. Tudzynski, unpubl.

In contrast to *Abcg1* mutants, *Abcg2* mutants show WT colony morphology in axenic culture and still produce and secrete a set of proteases visible as a halo around the colonies on milk agar (Schulze Gronover et al., 2001). The infection process is comparable to the WT, except that the lesions caused by conidia from *Abcg2* mutants spread more slowly.

Recently, suppression subtractive hybridization (SSH) was used to identify fungal genes, which are specifically expressed on the host plant in the WT, but very low or not expressed in *bcg1* mutants (Schulze Gronover et al., 2004). Among the 22 differentially expressed genes were found several encoding unknown proteases,

some enzymes involved in secondary metabolism and others encoding cell wall-degrading enzymes. Most of the genes controlled by BCG1 in the signal cascade are still expressed in adenylate cyclase (*bac*) mutants *in planta*. This result suggests that BCG1 is involved at least in one additional signalling cascade beside the cAMP-dependent pathway (Figure 1).

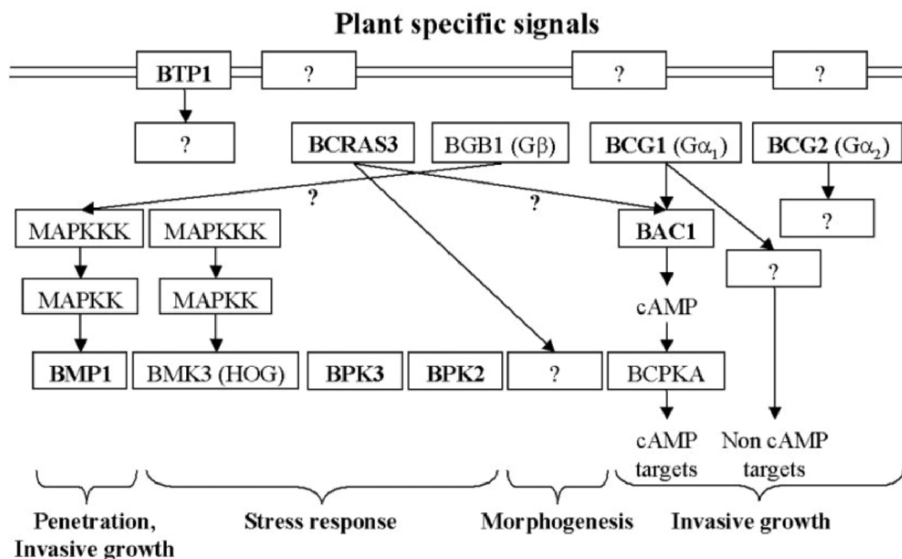


Figure 1. Hypothetical scheme of signalling pathways in *Botrytis cinerea*

3. cAMP signalling pathway

cAMP-dependent signalling pathways regulate several important processes in plant pathogenic fungi, such as morphogenesis, differentiation and virulence. In *M. grisea*, a cAMP signalling pathway is involved in appressorium formation. As a consequence, adenylate cyclase (MAC1) mutants fail to penetrate and are non-pathogenic (Choi and Dean, 1997). In *U. maydis*, adenylate cyclase (*uac1*) mutants show constitutive filamentous growth, are not pathogenic and fail to induce pheromone expression in the presence of an activating pheromone (Barrett et al., 1993; Gold et al., 1994). In addition to the adenylate cyclase gene, others involved in cAMP signalling also have been characterized in several fungi. Disruption of the gene encoding the catalytic subunit of the cAMP-dependent protein kinase (CPKA) in *M. grisea* resulted in mutants defective in appressorium formation that failed to cause lesions on a susceptible rice cultivar (Mitchell and Dean, 1995). Mutations in the gene encoding the regulatory subunit of the *M. grisea* PKA (*sum*) restore growth, sexual and asexual morphogenesis as well as appressorium formation.

In *B. cinerea*, the *bac* gene encoding adenylate cyclase was cloned and characterized (Klimpel et al., 2002). The deduced protein sequence (2139 amino acids) shows a high degree of identity with other fungal adenylate cyclases (66% and 63% amino acid identity to the adenylate cyclases of *Blumeria graminis* and *M. grisea*, respectively). The BAC protein consists of functional domains typical for adenylate cyclases, such as the “Ras association” (RA) motif, the middle leucine-rich repeat regions, the catalytic domain and the C terminus with a putative binding site for the cyclase-associated protein (CAP). RT-PCR studies revealed *bac* expression already occurring from the beginning of necrosis development (12 hours post inoculation, hpi) till at least 48 hpi, when spreading soft rot lesions start to grow out from primary necrotic spots (Klimpel et al., 2002). Interestingly, Δbac mutants show similar colony morphology to the $\Delta bcg1$ mutants on potato sucrose agar (PSA) with 0.5-1.0% sucrose. They grow slowly as small compact colonies (diam. 2-3 cm), whereas the WT strain B05.10 grows rapidly and reaches the margins of the Petri dish 7 days after inoculation. An exogenous supply of cAMP (2 mM) was optimal for partial restoration of both the WT colony morphology and growth rate.

Based on the suggestion that both BAC and BCG1 positively influence the production of cAMP, intracellular cAMP levels were measured of WT, $\Delta bcg1$, $\Delta bcg2$ and Δbac mutant strains grown on PSA for three or six days. The deletion of *bac* resulted in the most significant reduction (about 85%) of intracellular cAMP level, which remained constantly low for up to six days. The $\Delta bcg1$ mutants showed about 50% reduction of cAMP level after three days of cultivation, but it increased to the levels found in the WT after six days. Infection assays were performed on bean plants. For a better comparison, $\Delta bcg1$ and $\Delta bcg2$ mutants (Schulze Gronover et al., 2001) were included in these bioassay experiments. The aggressiveness of Δbac mutants was significantly reduced and comparable with that of $\Delta bcg2$ mutants. The development of spreading lesions four days after inoculation was much slower on leaves infected with conidia produced by Δbac mutants than on leaves infected with WT conidia. After seven days, leaves infected by the WT had already wilted and the tissue was totally macerated, whereas Δbac -infected leaves showed slowly expanding lesions with a diameter only of about 2-3 cm. In addition to slower spreading-lesion formation, no conidia developed on the surface of Δbac -infected leaves. In contrast, conidia were produced prolifically in the area of secondary lesions infected with the WT within four days of incubation (Klimpel et al., 2002).

These data show that in *B. cinerea* adenylate cyclase plays an important, but not essential, role in vegetative development and aggressiveness. The much stronger effect of mutations in the *bcg1* mutants (which can penetrate plant tissue but are not able to develop spreading secondary lesions) than those in the *bac* gene are evidence that *bcg1* controls at least one additional signalling pathway involved in pathogenicity beside the cAMP pathway. This suggestion was confirmed by SSH experiments in which mRNA derived from $\Delta bcg1$ -infected leaves was subtracted from that of WT-infected leaves. Only four out of 22 BCG1-controlled genes were regulated via the cAMP signalling pathway (Figure 1).

To gain better insight into the cAMP pathway, a gene encoding a catalytic subunit of PKA, *bcpka*, was cloned recently (B. Tudzynski and S. Richter, unpubl.). The gene replacement experiments for functional analysis are underway. Beside

bcpka, a homologue of the genes *Sch9* and *SchA* from *Saccharomyces cerevisiae* and *Aspergillus nidulans*, respectively, were also cloned (Toda et al., 1988; Fillinger et al., 2002). These serine/threonine protein kinases are known to suppress defects resulting from cPKA mutations; however the nature of the link between *Sch9*-like kinases and cAMP signalling is still unclear. The characteristic feature of BPK2 and all other Sch9-like protein kinases is the occurrence in their terminal moiety of amino acid residues typical of phospholipid-binding domains also found in phospholipases and protein C kinases. It is still not known if other proteins can bind to this part of the enzyme. Expression studies with *bpk2* revealed a significant up-regulation by H₂O₂. However, the implications of this result for the gene function are not clear. Deletion of *bpk2* did not affect vegetative growth, conidiation or the pathogenicity of *B. cinerea*. A combination of null mutations in the *bcpka* and *bpk2* genes will give a new insight into the functional relationship between these two protein kinases.

4. MAP kinase pathways

In addition to the G protein α subunits and components involved in the cAMP signalling pathway, several MAP kinase genes were found to be essential for vegetative and sexual development, and for osmoregulation and pathogenicity in different fungi (see Lengeler et al., 2000; Xu, 2000; Tudzynski and Sharon, 2003). In *M. grisea*, three MAP kinase genes, *pmk1*, *mps1* and *osm1* were characterized, which play a role in pathogenicity, sporulation and penetration, and osmoregulation, respectively (Xu and Hamer, 1996; Xu et al., 1998; Dixon et al., 1999).

The *pmk1*, *mps1* and *osm1* deletion mutants are all viable and the genes are not essential for growth. The Hog-homologous kinase OSM1 is the only *M. grisea* MAP kinase that is dispensable for fungal pathogenicity. *Pmk1*-homologous genes have been cloned from several phytopathogenic fungi, including *C. heterostrophus* (Lev et al., 1999), *Colletotrichum lagenarium* (Takano et al., 2000), *Claviceps purpurea* (Mey et al., 2002), *Fusarium oxysporum* (Di Pietro et al., 2000), and *U. maydis* (Mayorga and Gold, 1999; Müller et al., 1999). All these MAP kinase genes were found to be essential for pathogenicity. The PMK1 pathway is conserved in many, if not all, fungal pathogens for regulating appressorium formation and/or other infection processes. Despite the differences in life cycle and mechanisms of plant infection, the *pmk1* homologues can complement infection defects of other fungal pathogens (Mey et al., 2002).

In *B. cinerea*, the *pmk1* homologue, *bmp1*, was cloned and shown to be essential for pathogenesis (Zheng et al., 2000). The Δ *bmp1* mutants grow and sporulate as well as the WT, but they are non-pathogenic on tomato leaves, carnation and rose flowers and fail to elicit plant defence responses (Zheng et al., 2000). Conidia from Δ *bmp1* mutants still germinated on plant surfaces, but lost their ability to penetrate and macerate plant tissue. Wounding of plants does not overcome the penetration defect. Therefore, as in most of the plant pathogenic fungi, both the cAMP and MAP kinase pathways are involved in infection processes. For *U. maydis* it was shown that both pathways act synergistically during plant infection and may be connected

by “cross talks” at the level of the transcription factor Prf1 (Mayorga and Gold, 1999; Müller et al., 1999). In *B. cinerea*, a similar connection has not yet been shown, and transcription factors such as Prf1, which are under control of both signalling cascades, have not been found.

Though the Hog-homologue in *M. grisea*, OSM1, is not essential for pathogenesis in that system, a role for the corresponding MAP kinase cascade in *B. cinerea* is possible. Recently Lara-Ortiz et al. (2004) demonstrated that the Hog-homologue of *A. nidulans*, Sak A, influences differentiation and active oxygen species (AOS) status. Since AOS play an important role in pathogenesis of *B. cinerea* (Chapter 8), a functional analysis of the HOG MAPK cascade was initiated recently. The *hog* homologue of *B. cinerea*, *bmk3*, was cloned and characterized; its derived amino acid sequence shows highest homology to the corresponding stress-activated MAP kinase from *A. nidulans* (U. Ellendorf, B. Tudzynski and P. Tudzynski, unpubl.). A functional analysis by gene replacement is under way.

5. Genes of the Ras superfamily

Ras proteins are a superfamily of small GTP-binding proteins which are highly conserved in all eukaryotic organisms and which are involved in several processes of morphogenesis, differentiation, nutrient sensing and pathogenicity. Previous studies in *S. cerevisiae* have shown that two Ras proteins, Ras1 and Ras2, sense changes in nutrient environment and regulate cAMP synthesis and cell cycle progression (Kataoka et al., 1984; Toda et al., 1985; Jiang et al., 1998). In filamentous fungi, not many data are available on the function of Ras homologues and their interaction with other signalling components. In *Colletotrichum trifolii*, the expression of a constitutively activated form of Ras resulted in aberrant hyphal morphology, no conidiation and failure to produce appressoria when the fungus was grown on minimal medium; addition of proline can restore the WT phenotype (Truesdell et al., 1999; Memmot et al., 2002). In *U. maydis*, disruption of *ras2* resulted in loss of pathogenicity and dramatic changes in cell morphology. It was shown that RAS2 interacts with the cAMP as well MAPK pathways (Lee and Kronstad, 2002).

Dumas et al. (2001) and Siriphutthaiwan et al. (2003) could also demonstrate that small G proteins belonging to the Rab subfamily of the Ras superfamily can affect pathogenicity of the fungus *Colletotrichum lindemuthianum*. In yeasts, members of the Rab subfamily regulate intracellular vesicular transport and protein secretion. Expression of the dominant-negative gene copy of the *C. lindemuthianum* Rab-encoding gene, *CLPT1*, resulted in large amounts of vesicles randomly distributed in the fungal cell, but the strains were unable to secrete proteins and were non-pathogenic. In *B. cinerea*, seven small G proteins of the Ras superfamily have been cloned so far (P. Hantsch and B. Tudzynski, unpubl.) (Table 1). Most of the sequences were found in the public ESTs database (<http://www.genoscope.Cns.fr/>), and the sequence of one of the genes has already been published (Park et al., 1997). Among them there is one Ras-encoding gene homologous to *ras2* of *S. cerevisiae* (*bcras3*), and one gene homologous to *CLPT1* (*bcras2*). Both genes were deleted in

the *B. cinerea* WT strain B05.10 and shown to be involved in morphogenesis, conidiation and pathogenicity (U. Ellendorf, P. Hantsch, C. Schulze Gronover and B. Tudzynski, unpubl.). The detailed characterization of *bcras2* and *bcras3* mutants as well as transformants carrying a dominant active gene copy of *bcras3* are underway.

6. Calcineurin/cyclophilin A signalling

Calcineurin is a heterotrimer Ca^{2+} -calmodulin-activated phosphatase composed of a catalytic subunit (calcineurin A), a regulatory subunit (calcineurin B) and calmodulin (Dickman and Yarden, 1999). In the rice blast fungus *M. grisea*, calcineurin is involved in formation of the appressorium (Lee and Lee, 1998; Viaud et al., 2002). In yeasts, there is some evidence that calcineurin interacts with cyclophilin A, the cellular primary target of the immuno-suppressive drug cyclosporin A (Marks, 1996). It was suggested that cyclophilin A might regulate calcineurin assembly or activity (Wang et al., 2001). In *B. cinerea*, among 6598 Expressed Sequence Tags (ESTs), putative sequences encoding calcineurin subunits and cyclophilin A were identified (C. Levis and Y. Brygoo, unpubl.). The cyclophilin A gene, *BCPI*, was characterized in more detail (Viaud et al., 2003). A $\Delta BCPI$ strain was selected and shown to be less virulent than the WT on tomato and bean leaves. Full pathogenicity was restored after complementing the mutant strain with the WT gene copy (Viaud et al., 2003). In contrast to the WT, the $\Delta BCPI$ mutant grew normally in the presence of 100 $\mu\text{g/ml}$ cyclosporin A, indicating that the *BCPI* is the target of cyclosporin A. The effect of both cyclosporin A and *BCPI* disruption on the expression of genes was analyzed by using macroarrays spotted with the 2839 unique ESTs. Among the cyclosporin A-affected genes are those with high homology to two P450 monooxygenase genes and one sesquiterpene cyclase gene from the trichothecene biosynthesis pathway in *Fusarium sporotrichioides*, which are organized as a gene cluster in *B. cinerea*. Furthermore, the endopolygalacturonase 1 gene, *bcpgl*, which is required for full virulence of *B. cinerea* (Ten Have et al., 1998) was also shown to be down-regulated by cyclosporin A. The signature of *BCPI* inactivation on the same macroarrays allowed the identification of only three cyclophilin (*BCPI*)-dependent genes that were different from cyclosporin A-affected ones (Viaud et al., 2003). The relationship between the calcineurin and other signalling pathways has yet to be determined.

7. Putative transmembrane receptor proteins

In *S. cerevisiae*, a G protein-coupled receptor protein, Gpr1, was shown by a two-hybrid analysis to be identical to the $\text{G}\alpha$ subunit Gpa2 (Xu et al., 1998). This is one of the few examples in which an integral membrane protein was shown to interact directly with one of the $\text{G}\alpha$ subunits. The only transmembrane protein so far shown to affect the virulence of a plant pathogenic fungus has been identified in *M. grisea*. In contrast to $\text{G}\alpha$ -coupled receptor proteins with their characteristic seven transmembrane domains, PTH11 consists of nine membrane-spanning domains. The

PTH11-encoding gene was identified by screening for non-pathogenic mutants that develop functional appressoria and thus penetrate rice leaves. It was suggested that PTH11 may function as an upstream effector of appressorium differentiation in response to surface cues (DeZwaan et al., 1999).

In *B. cinerea*, a gene, *btp1*, encoding a transmembrane protein with significant homology to PTH11, has been found by an SSH approach, where mRNAs from non-infected bean leaves were subtracted from mRNA obtained from infected bean leaves (C. Schulze Gronover, P. Hantsch and B. Tudzynski, unpubl.). The BTP1 protein contains seven transmembrane domains suggesting that it could be a member of the family of *Ga* protein-coupled receptors. However, the $\Delta btp1$ mutants were not deficient in pathogenicity and therefore the BTP1 protein does not interact with BCG1 during pathogenic development.

8. Two-component signal transduction genes in *Botrytis cinerea*

“Two-component” histidine kinase (HK) phosphorelay signalling systems are a major mechanism by which organisms sense and adapt to their environment. In response to a specific signal, the HK autophosphorylates a conserved histidine residue. The phosphate is then transferred to a conserved aspartic acid residue in a response regulator (RR) protein resulting in changed transcription or regulation of e.g. a MAP kinase cascade (Wolanin et al., 2002). To date, only a few HK genes from filamentous fungi have been characterized. The *N. crassa* NIK1/OS-1 is implicated in osmotic response (Schumacher et al., 1997). The homologous osmo-sensing HK, BOS-1, was cloned in *B. cinerea* and shown to mediate dicarboximide fungicide resistance (Cui et al., 2002). *B. cinerea* is one of the few recently sequenced genomes in filamentous fungi (Catlett et al., 2004), following the sequencing of the genomes of *N. crassa* (Galagan et al., 2003), *C. heterostrophus* and *Gibberella moniliforme*. The genome sequence data revealed 20 HKs for *B. cinerea* and 11 (*N. crassa*), 21 (*C. heterostrophus*) and 16 (*G. moniliforme*) HKs for the other fungi. The function of these HKs is still unknown. However, the number of downstream two-component signalling genes is rather low in contrast to bacteria. It was suggested that all HKs signal through the same downstream histidine phosphotransfer (HPt) and RR proteins, combining multiple inputs into a single signalling pathway (Catlett et al., 2004).

9. Further protein kinase encoding genes with unknown function

In differential cDNA screening approaches, two other genes with high homology to protein kinases have been identified, *bpk3* and *bpk4* (C. Schulze Gronover, P. Tudzynski and B. Tudzynski, unpubl.). *bpk3* revealed a high homology to *ckl1* from *C. lindemuthianum*, the causal agent of bean anthracnose (Dufresne et al., 1998). *ckl1* was identified in an insertional mutagenesis screen for non-pathogenic mutants. The gene encodes a serine/threonine protein kinase that is involved in pathogenicity. The *ckl1* mutants are not able to penetrate bean leaves, possibly because of the differentiation of defective appressoria. In order to show if *bcpk3* is a functional

homologue of *clk1*, we deleted the gene in *B. cinerea* B05.10. However, three independent knock-out mutants were not affected in pathogenicity, or vegetative growth and conidiation. Expression analysis showed that *bpk3* is strongly induced by CuSO_4 . Addition of H_2O_2 had no effect on *bpk3* expression demonstrating the specificity of the copper effect. *bpk3* mutants are not affected in growth when exposed to high concentrations of CuSO_4 , but it still has not been shown whether copper transport is affected in these mutants.

bcpk4 revealed the highest homology to the Ran1 protein kinases of *Schizosaccharomyces pombe* that regulate the cell transition between mitosis and meiosis. As expected from the need for nutrient limitation and pheromone signalling, many signal transduction pathways converge to regulate differentiation. However, each phase of fission yeast life cycle can be governed by the activity of Ran1 kinase (McLeod et al., 2000). Deletion of *bpk4* in *B. cinerea* resulted in no obvious phenotype: the mutant strains grow and sporulate like the WT, and the pathogenicity is not affected. Expression studies showed that the kinase is constitutively expressed. For identification of putative functions for these signalling components, macroarrays differentially hybridized with cDNA from the WT and the corresponding mutant will be used in the near future.

Recently we cloned the *SNF* homologue in *B. cinerea*, *bcSNF* (J. Schumacher and B. Tudzynski, unpubl.). *SNF* proteins are members of a highly conserved subfamily of serine/threonine kinases found in fungi, plants and animals (Hardie et al., 1998). In *Cochliobolus carbonum* and *F. oxysporum* it has been shown that *SNF* protein kinases are essential for expression of glucose-repressed genes, such as genes encoding cell wall-degrading enzymes. As a result, *SNF* mutants are less virulent than the corresponding WT (Vacher et al., 2003; Ospina-Giraldo et al., 2003). For *B. cinerea* the gene replacement experiments are underway.

10. Conclusions

It is becoming clear that *B. cinerea* has numerous genes encoding signalling components that function in a complicated network of signalling cascades. So far, we are still at the level of gene cloning and characterization. The investigation of “cross talks” between signalling pathways, as well as searching for transcription factors and corresponding target genes on one hand, and for receptors receiving and transducing specific signals on the other hand, are only at the beginning.

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CHAPTER 7

EXTRACELLULAR ENZYMES AND METABOLITES INVOLVED IN PATHOGENESIS OF *BOTRYTIS*

Ilona Kars and Jan A.L. van Kan

Laboratory of Phytopathology, Wageningen University Plant Sciences, Binnenhaven 5, 6709 PD Wageningen, The Netherlands

Abstract. The infection of host plants by *Botrytis* spp. is mediated by numerous extracellular enzymes and metabolites. Each of these compounds may play a role in different stages of the infection process. Cutinases, lipases and some cell wall-degrading enzymes may facilitate the penetration of the host surface, while toxins, oxalate and reactive oxygen species may contribute to killing of the host cells. Several cell wall-degrading enzymes contribute to the conversion of host tissue into fungal biomass, but also other enzymes, such as laccases and proteases are potentially involved in pathogenesis. The cloning of the corresponding genes in recent years has facilitated studies on gene expression and targeted mutagenesis. This chapter gives an updated overview of the research performed on these secreted enzymes and metabolites and the role they play in pathogenesis.

1. Introduction

Botrytis cinerea is able to infect a wide spectrum of host plant species, whereas other *Botrytis* species are confined to a single host species (Chapters 1 and 3). All *Botrytis* species, whether specific or not, are necrotrophs implying they are able to kill host cells during the infection process. De Bary (1886) observed that carrot cells were killed in advance of invading hyphae of the soft rot fungus *Sclerotinia*. He also noted that fluid from rotten tissue could degrade healthy host tissue, while boiled fluid had no effect. This led to his conclusion that the fungus produced heat-labile enzymes and toxins that kill and degrade plant cells. The same is true for *Botrytis* species. They are equipped with a set of enzymes and/or metabolites that enable the pathogen to invade host tissue, kill host cells and eventually convert host tissue into fungal biomass. Many of these enzymes and metabolites act extracellularly at the plant-fungus interface, or even in the host tissue at some distance from the growing hyphae. This chapter will deal with pathogenicity factors, i.e. effector molecules that cause damage to the host thereby enabling the pathogen to complete its disease and life cycle. The emphasis is placed on fungal extracellular enzymes, but we will also

discuss the biosynthetic pathways of metabolites secreted during pathogenesis. This chapter discusses neither differentiation of infection structures nor the production of phytohormones (covered in Chapters 5 and 10, respectively). Most data originate from the research on *B. cinerea*, but we will also discuss other *Botrytis* species where relevant information is available.

2. Penetration of the host surface

The disease cycle starts with a conidium landing on the host surface. Upon attachment, it germinates on the host surface and produces a germ tube that develops into an appressorium that facilitates penetration of the host surface. Invasion of host tissue can be achieved by active penetration or passive ingress. *B. cinerea* is an opportunist that can initiate infection at wound sites, or at sites previously infected by other pathogens. Nevertheless, *Botrytis* spp. are perfectly able to penetrate intact host surfaces. Only direct, active penetration of the epidermal surface is discussed in this section. For reasons of simplicity the penetration of dead or wounded tissue is regarded as an expansion process rather than a penetration process and is dealt with in sections 4 and 5.

The first barrier to breach is the host cuticle covering all aerial parts of the plant. The cuticle consists of cutin, a polyester of hydroxylated and epoxidised C₁₆- and C₁₈-fatty acids, in many cases covered with a hydrophobic wax layer consisting of fatty alcohols. Physical damage or brute mechanical penetration of the cuticle by *B. cinerea* is not usually observed (Williamson et al., 1995; Cole et al., 1996) indicating that enzymatic activity is involved in penetrating intact host surfaces (Salinas and Verhoeff, 1995).

2.1. The role of lipase in wax layer penetration and surface adhesion

The wax layer does not seem to pose a serious barrier, although removal of the wax layer by abrasion was reported to increase the infection incidence (Sutton et al., 1984). No correlation was observed between the wax layer dry weight of rose or gerbera petals and their susceptibility to *B. cinerea* (Kerssies and Frinking, 1996).

It is conceivable that *B. cinerea* produces surfactants: proteins or metabolites that reduce surface hydrophobicity and “dissolve” the wax layer, thereby providing access to the underlying cutin polymer. The polysaccharide cinerean, covering *B. cinerea* germ tubes (Chapter 5) might fulfil a role as surfactant. Alternatively, the reduction of host surface tension may be achieved enzymatically. Cutinases, serine esterases, lipases and other non-specified esterases are reported to be involved in the adhesion of several plant pathogenic fungi: *Alternaria brassicola* (Köller et al., 1995; Yao and Köller, 1995; Berto et al., 1997; Fan and Köller, 1998), *Colletotrichum gramimicola* (Pascholati et al., 1993), *Erysiphe graminis* (Pascholati et al., 1992), *Uromyces viciae-fabae* (Deising et al., 1992; Clement et al., 1993a, b).

B. cinerea produces an extracellular triacylglycerol lipase, with a molecular mass of 60 kDa during culture in the presence of a fatty acid ester. The enzyme is able to hydrolyse unsaturated long chain fatty acid esters (Comménil et al., 1995), known to

be components of cutin and waxes. Lipase production *in vitro* was induced by wax esters and free fatty acids (Comménil et al., 1999). The lipase possesses cutinolytic activity, although its kinetic properties (Comménil et al., 1998) are clearly distinct from those of a 'typical' cutinase that will be discussed below. It was proposed that the enzyme plays a role in modifying the waxes and cuticle, and in adhesion of conidia to the plant surface (Comménil et al., 1997, 1998). Studies with polyclonal antibodies blocking the active site suggested that the lipase plays an important role in the infection process. When antibodies were applied on to intact tomato leaves prior to inoculation with *B. cinerea* conidia, the fungal germ tubes were unable to penetrate the cuticle (Comménil et al., 1998). The antibodies did not affect germination of *B. cinerea* conidia nor did they inhibit the infection of wounded tissue, suggesting a role for the lipase specifically during host surface penetration. These antibodies also inhibited a lipase purified from *Alternaria brassicicola* and they were able to reduce by 90% the occurrence on intact cauliflower leaves of blackspot lesions caused by *A. brassicicola*. The antibodies did not prevent *A. brassicicola* infection on dewaxed cauliflower leaves, again indicating a crucial role for lipase in the penetration phase of the infection (Berto et al., 1999).

The *B. cinerea* triacylglycerol lipase was partially sequenced (Comménil et al., 1999). The corresponding gene (*Lip1*) was cloned and targeted mutants were made by insertion of a hygromycin resistance cassette. *Lip1*-deficient *B. cinerea* mutants did not produce extracellular lipase under inducing conditions, but remained able to infect intact primary leaves of *Phaseolus vulgaris* (H. Reis and M. Hahn, Univ. Kaiserslautern, Germany, pers. comm.), indicating that the lipase is not essential in host surface penetration.

2.2. Penetration of the cutin network by cutinase

Below the wax layer lies cutin, a highly complex three-dimensional network of chemically heterogeneous esterified hydroxylated, partly unsaturated fatty acids. How does *B. cinerea* breach the cutin network? In the 1980s and early 1990s several groups studied the role of cutinases of a number of plant pathogenic fungi in penetration (reviewed by Köller et al., 1995). Evidence was presented that cutinase was important for cuticle penetration by *Nectria haematococca* (*Fusarium solani* f.sp. *pisi*; Rogers et al., 1994), although this conclusion was firmly rebutted by others (Stahl and Schäfer, 1992; Stahl et al., 1994).

Salinas (1992) purified a 18-kDa cutinase from *B. cinerea* and raised monoclonal antibodies against the protein. Application of the antibody to gerbera flowers prior to inoculation reduced the number of lesions formed, suggesting that indeed the 18-kDa cutinase was important for penetration. This conclusion was however rejected on the basis of molecular-genetic studies. The *BccuA* gene, encoding the 18-kDa cutinase, was cloned (Van der Vlugt-Bergmans et al., 1997). The gene was expressed during pathogenesis from the onset of germination on the host surface onwards (Van Kan et al., 1997). Gene replacement mutants were made that lacked the enzyme activity. The mutants were equally virulent as the wild type isolate both on gerbera flowers and tomato fruits, and the fungus remained able to penetrate

intact cuticle surfaces (Van Kan et al., 1997). Further experiments on other host species and different tissues have never revealed any reduction of virulence of the *BccutA*-deficient mutant (J. van Kan et al., unpubl.). Although the results of Salinas (1992) remain to be explained, it is evident that this cutinase is not essential in penetration.

2.3. The role of pectinases in penetrating the anticlinal epidermal wall

Over the years many studies have dealt with the secretion of cell wall-degrading enzymes (CWDEs) by *B. cinerea* during the early stages of infection. Swelling of the anticlinal epidermal cell wall (Mansfield and Richardson, 1981) suggested active involvement of CWDEs in penetration. Enzymes that attack pectic substances in the plant cell wall are thought to play a major role in pathogenicity (Clark and Lorbeer, 1976; Collmer and Keen, 1986; Cole et al., 1998). Endopolygalacturonase activity was detected in ungerminated *B. cinerea* conidia (Verhoeff and Warren, 1972) and two polygalacturonase isozymes were associated with the penetration stage of the infection process (Van den Heuvel and Waterreus, 1985). It was suggested that the early, constitutive production of polygalacturonases enables fast penetration of the host tissue (Kapat et al., 1998), although no evidence was presented to support this hypothesis. Conclusions can only be drawn by studying mutants in pectinolytic genes during early stages of infection. In all of the mutants generated in the course of our work on *B. cinerea* CWDEs, we have never observed a mutant incapable of penetrating intact host tissue (I. Kars et al., unpubl.). In section 4, we discuss the role of CWDEs in later stages of infection.

3. Killing of host cells

Once through the cuticle, *B. cinerea* kills underlying epidermal cells before they are invaded by hyphae (Clark and Lorbeer, 1976). Invasion of plant tissue by *B. cinerea* triggers processes indicative of programmed cell death at a distance from the hyphae (Govrin and Levine, 2000), implying that diffusible factors have a direct or indirect phytotoxic activity. Also *B. elliptica* triggers programmed cell death in its host plant lily (P. van Baarlen and J. van Kan, unpubl.). The inducing factors may be proteins or low molecular weight compounds secreted by the fungus into its environment. The induction of (programmed) cell death facilitates *B. cinerea* invasion and may in fact be essential for successful infection (Govrin and Levine, 2000). Most studies on the induction of cell death were performed with *B. cinerea*.

3.1. Toxins

Culture filtrates of *B. cinerea* may be phytotoxic when applied to plant tissue (Rebordinos et al., 1996). Compounds with phytotoxic potential were identified as botcinolide, a highly substituted lactone (Cutler et al., 1993) and botrydial, a tricyclic sesquiterpene (Colmenares et al., 2002). Several compounds related to botcinolide and botrydial have been identified that may either be precursors or

conversion products, but these compounds generally have a lower toxicity (Durán-Patrón et al., 2000; Colmenares et al., 2002). Botrydial requires light for phytotoxic activity (Colmenares et al., 2002), but the reason for light dependence remains to be clarified. The observation that botcinolide and botrydial types of secondary metabolites were only secreted by *B. cinerea* in medium with high glucose levels initially raised doubts about their production *in planta*. However, with analytical chemical methods it was demonstrated that botrydial accumulates in infected tissue (Deighton et al., 2001) at concentrations that are presumably physiologically relevant. No evidence has yet been presented for production of botcinolide *in planta*. The role of botrydial in the infection of host plants needs to be evaluated by constructing mutants in the botrydial biosynthetic pathway. Resolution of this pathway is in progress, but the relevant genes have not been identified.

The *B. cinerea* toxins described above have a general phytotoxic activity. There is no evidence for production by *B. cinerea* of host-specific toxins, i.e. molecules produced by a pathogen that are specifically and exclusively toxic to its host and essential for the pathogen to achieve successful infection (Walton, 1996). This may not be surprising in view of the broad host range of *B. cinerea*. Recently, however, a protein was identified that is secreted by *B. elliptica* and able to trigger programmed cell death in its host plant lily, but not in non-host plant species (P. van Baarlen and J. van Kan, unpubl.). This protein meets the criteria of a host-selective toxin and may turn out to be a determinant of compatibility for *B. elliptica*. By analogy, it is worth considering the possibility that the other, specialised *Botrytis* species may also be equipped with host-selective toxins, but this remains to be studied.

3.2. Oxalic acid

Secretion of oxalic acid (OA) occurs in fungi from various taxonomic classes (reviewed by Dutton and Evans, 1996). A key role has been postulated for OA in pathogenesis of *Sclerotinia sclerotiorum* (Godoy et al., 1990), a close relative of *Botrytis* spp. Mutants of *S. sclerotiorum*, deficient in OA production, were unable to infect *Arabidopsis* plants (Dickman and Mitra, 1992) and the deficiency could be restored by supplementing inoculum with OA. The oxalate-mediated acidification facilitates induction of gene expression by the ambient pH-dependent regulator *pac1*, which is required for virulence of *S. sclerotiorum* (Rollins, 2003).

B. cinerea produces OA both *in vitro* (Gentile, 1954; Germeier et al., 1994) and *in planta* (Verhoeff et al., 1988). OA forms calcium oxalate crystals within the host tissue (Figure 3 in Prins et al., 2000b). The sizes of lesions induced by different strains of *B. cinerea* on grapevine and bean leaves correlated with the amount of OA that these strains secreted *in vitro* (Germeier et al., 1994). It remains unclear whether the levels of OA produced *in planta* are sufficient to cause host cells to directly collapse. OA may in fact be a co-factor in pathogenesis rather than the primary phytotoxic agent. Culturing *B. cinerea* in low ambient pH resulted in the enhanced production of secreted endopolygalacturonase, aspartic protease and laccase activity (Manteau et al., 2003). Fungal endopolygalacturonases (Section 4.1.2), aspartic proteases (Section 5.1) and laccases (Section 5.2) have an optimal activity at low pH

and are therefore stimulated by the simultaneous secretion of OA (Ten Have et al., 2002; Manteau et al., 2003). Moreover, OA may stimulate pectin degradation resulting from endopolygalacturonases action by sequestering the Ca^{2+} ions from (intact or partially hydrolysed) Ca-pectates in the cell walls. The removal of Ca^{2+} ions disturbs intermolecular interactions between pectic polymers and disrupts the integrity of the pectic backbone structure. Consequently, the pectic structure absorbs water and swells, as described by Mansfield and Richardson (1981).

The oxalate biosynthetic pathway in *B. cinerea* remains to be established. Several metabolites may serve as precursors of oxalate: glyoxal, oxaloacetate, erythroascorbic acid. Glyoxal oxidase is an enzyme that converts glyoxal into OA and H_2O_2 . In the white rot fungus *Phanerochaete chrysosporium* this enzyme serves to generate H_2O_2 , required as substrate for lignin peroxidases involved in lignin degradation (Kersten and Kirk, 1987). *B. cinerea* contains a gene encoding a glyoxal oxidase homologue, which is expressed *in vitro* and *in planta* at a constitutive level (J. van Kan et al., unpubl.). The gene product is predicted to possess a secretion signal peptide, suggesting extracellular localisation. However, mutants in the glyoxal oxidase gene retained the ability to produce OA (J. van Kan et al., unpubl.), ruling out an important role of this enzyme in OA production. A second candidate enzyme that can generate OA is oxaloacetate hydrolase, converting oxaloacetate into acetate and OA. An oxaloacetate hydrolase gene was cloned from *Aspergillus niger* (Pedersen et al., 2000) and *B. cinerea* expresses a homologous gene. Its role in OA production and pathogenesis is under investigation (J. van Kan et al., unpubl.). Finally, several compounds were identified as potential OA precursors in *Sclerotinia sclerotiorum*: L-ascorbic acid, D-erythroascorbic acid, and D- and L-arabinose (Loewus et al., 1995; Keates et al., 1998; Loewus, 1999). The enzymes involved in this pathway remain to be identified. It seems plausible to speculate that *B. cinerea* possesses a similar biosynthetic pathway, but this question remains to be addressed. Chapter 8 describes the role of OA in oxidative processes in more detail.

3.3. Induction of Active Oxygen Species

Recent studies have focussed on Active Oxygen Species (AOS) production in relation to *B. cinerea* pathogenicity (Von Tiedemann, 1997; Govrin and Levine, 2000; Patykowski and Urbanek, 2003). AOS is the joint term for the superoxide anion, hydroxyl radical and hydrogen peroxide. An oxidative burst and enormous perturbances in the redox status are observed at the host-fungal interface, as well as in plant tissue at some distance from the infection front (Chapter 8). A large part of the H_2O_2 production seems to occur in plasma membranes of host cells adjacent to fungal hyphae (Govrin and Levine, 2000; Schouten et al., 2002a). There are a number of fungal enzymes that potentially contribute to the H_2O_2 production. Fungal extracellular sugar oxidases (Edlich et al., 1989; Liu et al., 1998) or superoxide dismutase (SOD) were considered as candidate enzymes potentially responsible for generating the H_2O_2 . Genes encoding an extracellular glucose oxidase and SOD were cloned. The targeted deletion of the SOD gene significantly reduced virulence

and extracellular H₂O₂ accumulation at the host-fungus interface whereas deletion of the glucose oxidase gene did not affect virulence (Rolke et al., 2004).

4. Conversion of host tissue into fungal biomass

Plant cell walls function as barriers to biotic and abiotic agents. The strength and flexibility of cell walls depend on their composition. Plant cell walls are made up of different types of polysaccharides: the primary cell wall consists of cellulose and hemi-cellulose, while the middle lamella has a high pectin content. Pectin, a complex network of various polygalacturonans, also extends into the primary wall.

Once it has penetrated the anticlinal epidermal cell wall, *Botrytis* grows through the middle lamella and produces a range of CWDEs. Enzymatic breakdown of the plant cell wall releases carbohydrates which form a major carbon source for consumption. Cell wall degradation by *Botrytis* is mediated by pectinases, cellulases and hemicellulases. These CWDEs each have specific features and are involved in different steps of host tissue colonisation and maceration. In the next sections we review research performed in recent decades on different types of *Botrytis* CWDEs.

4.1. Pectinases

Pectin is a major component of the plant cell wall and consists of three main types of polygalacturonans: homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II. Homogalacturonans are made of α -1,4-linked chains of D-galacturonic acid that can be methylated and/or acetylated. Highly methylated homogalacturonan is referred to as pectin, while homogalacturonan with a low degree of methylation is called pectate. Enzymes that are able to degrade pectic components are denoted as pectinases. During infection *Botrytis* produces various types of pectinases, each having distinct roles in pectin degradation.

4.1.1. Pectin methylesterase

Most pectinolytic enzymes cannot degrade highly methylated pectin. Therefore, the action of pectin methylesterase, which de-methylates pectin to pectate, is required. The biochemical characteristics were described of two *B. cinerea* pectin methylesterase (BcPME) isozymes with different molecular mass and identical pI values (Marcus and Schejter, 1983). In contrast, Reignault et al. (1994) identified two isozymes in a different *B. cinerea* strain (Bd90), which were of identical molecular mass (42 kDa) but had different pI values (pI 7.0 and 7.4, respectively). Levels of BcPME activity in liquid cultures were neither enhanced by pectic derivatives nor subject to glucose repression. In *B. fabae*-infected broad bean tissue, the content of methyl-esterified homogalacturonan decreased in the vicinity of hyphae, indicative of either pectin methylesterase action or pectin degradation (Cole et al., 1998). Pectin methylesterase activity was detected in *B. cinerea*-infected bean leaves and its level was correlated with disease severity (Zimand et al., 1996).

Disruption of the *B. cinerea* pectin methylesterase gene *Bcpme1* in strain Bd90 revealed that this gene encodes the isozyme with pI 7.4, and confirmed that this strain possesses more than one pectin methylesterase-encoding gene. Pectin methylesterase activity in the *Bcpme1* mutant was reduced by 75% and the mutant was less virulent on apple, grapevine and *Arabidopsis* (Valette-Collet et al., 2003). Only a single mutant was obtained; complementation of the mutant with the wild type gene restored virulence. In our laboratory, however, multiple *Bcpme1* mutants were generated in strain B05.10 using a gene-replacement strategy (Chapter 4). None of these mutants showed a reduction in virulence on five different host plants tested, including apple (I. Kars et al., unpubl.). The mutants did not grow differently on plates containing 75% methyl-esterified pectin, as compared to B05.10. The differences in virulence between *Bcpme1* mutants in the two different strains must be further investigated. Zymograms of pectin methylesterase activity in plant tissues infected by either Bd90 or B05.10 were similar (Reignault et al., 2000). No differences were detected between the two strains by Southern analysis of genomic DNA (Valette-Collet et al., 2003). This raises the possibility of the involvement of another pectin methylesterase isozyme. A second BcPME isozyme (pI 7.1) was detected in strain Bd90 (Valette-Collet et al., 2003), but was proposed to play a less prominent role than *Bcpme1*. This isozyme could be encoded by the recently identified gene *Bcpme2*. *Bcpme2* might be more important for pathogenicity of strain B05.10 than of Bd90. It will be of interest to generate mutants lacking both genes and study the effect on pathogenesis.

4.1.2. Endopolygalacturonase

Endopolygalacturonases are endo-acting enzymes catalysing the hydrolysis of homogalacturonan, resulting in substrate fragmentation. These enzymes are not able to hydrolyse highly methylated pectin, but first require the action of pectin methylesterase to de-methylate pectin to pectate. Over the years, many researchers have tried to unravel the complex mechanism of action of *Botrytis* endopolygalacturonases (BcPGs). Research mainly focused on the production, purification and characterisation of *Botrytis* endopolygalacturonase isozymes *in vitro* and *in planta* (Hancock et al., 1964a, b; Verhoeff and Warren, 1972; Drawert and Krefft, 1978; Marcus and Schejter, 1983; Urbanek and Zalewska-Sobczak, 1984; Van den Heuvel and Waterreus, 1985; Leone, 1990; Leone et al., 1990a, b; Movahedi and Heale, 1990b; Johnston and Williamson, 1992a, b; Tobias et al., 1993, 1995; Van der Cruyssen et al., 1994; Zimand et al., 1996; Chilosi and Magro, 1997; Kapat et al., 1998; Reignault et al., 2000; Pashkoulov et al., 2002; Manteau et al., 2003). Nevertheless, these studies have not revealed the exact function of individual endopolygalacturonase isozymes in pathogenesis. In the late 1990s the first BcPG encoding genes were cloned and characterised (Wubben et al., 1999). A gene family was discovered which encoded six isozymes, each having different characteristics. The expression pattern of each gene family member was studied and indicated how these genes are regulated *in vitro* and *in planta* (Wubben et al., 2000; Ten Have et al., 2001). Characterisation of five heterologously expressed, pure BcPGs revealed

that each isozyme differs in specific activity, protein stability, substrate preference and end-products (G. Krooshof et al., unpubl.).

The availability of *Bcpg* genes facilitated the analysis of function of individual isozymes in pathogenesis. Disruption of *Bcpg1* resulted in a reduction in virulence on tomato leaves and fruit, as well as on apple (Ten Have et al., 1998). Disruption of other members of the endopolygalacturonase gene family was achieved and mutants were characterised. Assays on various host species showed that *Bcpg2* also plays an important role in virulence (I. Kars et al., unpubl.).

It was recently reported that BcPG1 displays elicitor activity, triggering an oxidative burst in grapevine cell suspensions (Poinssot et al., 2003). Mild heat treatment drastically reduced enzyme activity without strongly affecting elicitor activity, suggesting that defence responses result from BcPG1 protein recognition rather than from its enzyme activity. It was hypothesised that BcPG1 triggers a defence reaction through a gene-for-gene interaction, in which *Bcpg1* acts as an *avr* gene (Poinssot et al., 2003). This novel hypothesis, however, seems to contradict the proposed role of endopolygalacturonases in tissue colonisation and maceration (Ten Have et al., 2002). Unfortunately, Poinssot et al. (2003) did not present evidence that the elicitor activity of BcPG1 (i.e. the amount of H₂O₂ produced upon incubation of grapevine cells with BcPG1) was linearly correlated with the amount of protein added, or demonstrate that enzyme activity (i.e. the amount of reducing sugar ends released from the model substrate polygalacturonic acid) was linearly correlated with the amount of protein added. It can be envisaged that grapevine cell suspensions still mount an equally strong oxidative burst even with lower amounts of BcPG1. Furthermore, it remains questionable whether an oxidative burst in cell suspensions reflects physiological responses in *B. cinerea* challenged green tissue.

We favour the explanation that the main (if not exclusive) role of endopolygalacturonases is in tissue colonisation and maceration. Firstly, all *Botrytis* species studied thus far seem to possess a gene family encoding multiple endopolygalacturonases (Wubben et al., 1999). This seems redundant for *Botrytis* spp. specialised on a single host species if their function was to act as an elicitor. Secondly, if the function of endopolygalacturonases was to act as an avirulence protein, one would predict that the genes evolve in such a way that enzyme activity of the gene product is lost. Yet, all *B. cinerea* endopolygalacturonase genes studied encode active enzymes (G. Krooshof et al., unpubl.). Thirdly, the presence in plants of an R gene that recognises BcPG1, and thereby confers susceptibility to *B. cinerea*, would put strong selection pressure on the host for loss of R gene function.

4.1.3. Exopolygalacturonase

Exopolygalacturonases cleave monomeric or dimeric glycosyl groups from the pectic polysaccharide, thereby providing the fungus with potential nutrients. *B. cinerea* and *B. allii* produced exopolygalacturonases in necrotic leaf sections of onion, while *B. squamosa* did not (Hancock et al., 1964a, b). Exopolygalacturonase activity was also detected in *B. cinerea*-inoculated tomato petiole and fruit (Verhoeff and Warren, 1972). The total exopolygalacturonase activity in *B. cinerea*-infected

bean plants remained at a similar level for 6 days whereas the symptom development progressed (Kapat et al., 1998). Johnston and Williamson (1992a) were the first to purify and characterise two *B. cinerea* exopolygalacturonases with molecular weights of 65 and 70 kDa, respectively. The same two isozymes were detected in *B. cinerea* cultures containing as carbon sources either citrus pectin, polygalacturonic acid or its monomer, galacturonic acid (Lee et al., 1997; Rha et al., 2001). Tobias et al. (1993), however, detected four exopolygalacturonase isozymes produced by *B. cinerea* grown on apple pectin. Secretion of exopolygalacturonases was detected in cucumber leaves from 9 h after inoculation with *B. cinerea*, using polyclonal antibodies (Rha et al., 2001), suggesting that these enzymes play a role in early stages of infection and in subsequent tissue maceration.

A *B. cinerea* exopolygalacturonase gene sequence was deposited in a database (J.W. Kim and E.G. Rha, Gyeongsang National University, Korea, unpubl.), but transcripts of this gene in *B. cinerea*-infected tomato leaves were only detected at low levels in late stages of infection (I. Kars et al., unpubl.).

4.1.4. Pectin lyase and pectate lyase

Pectin lyase is a pectin-degrading enzyme that cleaves homogalacturonan with a high degree of methyl esterification. Pectin lyase is inactive at acidic pH. Several groups investigated *B. cinerea* pectin lyase activity *in vitro* and *in planta* (Movahedi and Heale, 1990b; Chilosi and Magro, 1997; Doss, 1999). Pectin lyase isozymes were detected in extracts of ungerminated conidia and in the extracellular matrix of *B. cinerea* germlings. Pectin lyase was produced early after inoculation of soybean hypocotyls and zucchini fruits, but not in infected apple tissue (Chilosi and Magro, 1997). Tissue-specific enzyme production may be correlated to the ambient pH in uninfected tissue. Apple tissue is very acidic (pH 3-4), while zucchini fruit and soybean hypocotyls have a more neutral pH. Since *B. cinerea* acidifies its environment (Section 3.2) prior to pectin degradation, pectin lyases are in any case unlikely to contribute significantly to pectin degradation in the early stages of infection by *Botrytis*. A pectin lyase gene from *B. cinerea* was cloned (J. van Kan et al., unpubl.) that is expressed at low levels in *B. cinerea*-infected tomato leaves (I. Kars et al., unpubl.). The generation of mutants has been unsuccessful so far.

Pectate lyases catalyse the cleavage of pectate, i.e. unmethylated homogalacturonan. Pectate lyases strictly require Ca^{2+} ions for catalysis and are inactive at acidic pH. This enzyme is therefore also unlikely to contribute significantly to cell wall degradation in early stages of infection by *Botrytis*. *B. cinerea* strain B16 produced pectate lyase in liquid cultures and on French bean leaves (Zimand et al., 1996; Kapat et al., 1998). One pectate lyase gene was cloned from *B. cinerea*, but mutants in this gene have not yet been made.

4.1.5. Rhamnogalacturonan hydrolase

Rhamnogalacturonan hydrolase (RGase) specifically hydrolyses non-esterified galacturonosyl-rhamnosyl linkages in the modified hairy regions of pectin. RGase

activity was first detected in *B. cinerea* culture filtrates by Gross et al. (1995). Chen et al. (1997) cloned a rhamnogalacturonan hydrolase gene from *B. cinerea*, present in a single copy in the genome. The biochemical characteristics of the RGase gene product, including substrate specificity were determined (Fu et al., 2001), but its role in pathogenesis remains to be investigated.

4.2. Non-pectinolytic cell wall-degrading enzymes

Although *B. cinerea* is considered to be a pectinolytic fungus (Ten Have et al., 2002), the degradation of plant cell walls may also require a number of non-pectinolytic CWDEs such as cellulases, xylanases and arabinases (Hancock et al., 1964a, b; Verhoeff and Warren, 1972; Drawert and Krefft, 1978; Urbanek and Zalewska-Sobczak, 1984; Cole et al., 1998; Ten Have et al. 2002). *B. alli*, *B. squamosa* (Hancock et al., 1964a, b) and *B. fabae* (Cole et al., 1998) also produce non-pectinolytic CWDEs.

4.2.1. Cellulases

The cellulolytic complex comprises, among others endoglucanase, cellobiohydrolase and β -glucosidase and it degrades cellulose into cellobiose and glucose. Cellulase activity was neither detected in ungerminated nor in germinating conidia of *B. cinerea* (Verhoeff and Warren, 1972). Cellobiohydrolase activity was not detected in *B. fabae*-infected bean leaves (Cole et al., 1998). Transcripts of a *B. cinerea* cellobiohydrolase gene were not detected in infected tomato leaves (I. Kars et al., unpubl.). These observations indicate that cellobiohydrolase does not play an important role in infection. *B. cinerea* was found to produce three intracellular β -glucosidases (Gueguen et al., 1995), and one extracellular β -glucosidase (Sasaki and Nagayama, 1994) in liquid cultures. The extracellular β -glucosidase and one intracellular β -glucosidase were purified and characterised, but only the extracellular β -glucosidase was suggested to be involved in plant cell wall degradation.

4.2.2. Xylanase and arabinase

Xylan and arabinan are constituents of hemicellulose in the primary cell wall. The hemicellulolytic complex degrades these polymers by a range of enzymes including xylanases and arabinases. Urbanek and Zalewska-Sobczak (1984) reported that *B. cinerea* secreted one arabinase and three xylanases during apple cell wall degradation. No further studies were published on xylanases and arabinases in any *Botrytis* spp. Neither of the genes has been cloned nor were the enzymes purified. Involvement of these enzymes in pathogenesis remains to be resolved.

5. Other enzymes potentially involved in pathogenesis

5.1. Aspartic proteases

Already over a decade ago, Movahedi and Heale (1990a) demonstrated that *B. cinerea* produces aspartic protease (AP) both in liquid culture and during early stages of the infection process. AP activity was detected prior to the appearance of pectinases. When the inoculum was supplemented with the specific AP inhibitor pepstatin, AP activity was blocked and infection was strongly reduced. This led Movahedi and Heale (1990a) to propose that a secreted fungal AP is important for pathogenesis. This conclusion, however, did not consider the possible role of plant (aspartic) proteases in the infection. Expression of a tomato AP is induced by wound responses (Schaller and Ryan, 1996) and the infection of tomato leaves by *B. cinerea* was shown to induce the expression of wound responsive genes in the host (Diaz et al., 2002). The effect of pepstatin that Movahedi and Heale (1990a) observed may therefore have been caused by its inhibition of a plant rather than a fungal AP. Recent studies have identified important roles for various types of plant proteases in R-gene mediated defence responses (Tör et al., 2003).

In filtrates of liquid cultures of *B. cinerea*, the total protease activity was highest when the assay was performed at low pH and it was fully inhibited by pepstatin, indicating that all enzyme activity was due to an AP (Manteau et al., 2003; Ten Have et al., 2004). Five genes encoding an AP were cloned. All were expressed *in vitro*, as well as *in planta*. Expression of these genes may be modulated by the *Bcg1* gene, because deletion of this gene resulted in reduced secreted proteinase production (Schulze Gronover et al., 2001). The AP isozyme sequences cluster in four distinct phylogenetic groups. One of the *B. cinerea* AP isozymes is presumably secreted, while a second one is undoubtedly a vacuolar protease. Two isozymes show characteristics of membrane-bound APs, although this remains to be verified (Ten Have et al., 2004). Single and double mutants were generated for all five *Bcap* genes (J. van Kan et al., unpubl.). Analysis of the mutants is in progress.

5.2. Laccases

Extensive studies on laccase activity have been performed over at least two decades by Mayer and co-workers. The production of gallic acid-inducible laccases in a *B. cinerea* culture was suppressed by secondary metabolites from Cucurbitaceae called cucurbitacins, while the activity of other fungal enzymes was not affected (Viterbo et al., 1993a). Cucurbitacins protected cucumber fruit and cabbage leaves from infection by *B. cinerea* (Bar-Nun and Mayer, 1990), leading to the hypothesis that laccase plays an important role in pathogenesis (Viterbo et al., 1993a; Staples and Mayer, 1995). Some cucurbitacin forms were more effective than others in reducing secreted laccase activity (Viterbo et al., 1993b). Radiolabeled cucurbitacin was taken up by *B. cinerea* mycelium suggesting that it acts intracellularly. Cucurbitacin induced a lower production of laccase activity (Viterbo et al., 1994), supposedly as a consequence of repression at the mRNA level (Gonen et al., 1996). These mRNA expression studies were however performed using a probe corresponding to the gene

Bclcc1, whereas the gallic acid-inducible laccase gene is in fact *Bclcc2* (Schouten et al., 2002b). These two genes show poor cross-hybridisation (A. Schouten, unpubl.).

Three laccase genes were cloned, of which one (*Bclcc2*) was expressed in several host species and the second (*Bclcc3*) was expressed only in ageing mycelium, both *in vitro* and *in planta*. The third gene, denominated *Bclcc1*, was never expressed at any detectable level. Deletion of either the *Bclcc1* gene or the *Bclcc2* gene did not result in detectable reduction of virulence on a range of host species tested (Schouten et al., 2002b). It can thus be concluded that at least the laccases BcLCC1 and BcLCC2 are not important virulence factors. Deletion of the *Bclcc3* gene has not (yet) been performed, but its expression pattern suggests that it is unlikely to play an important role in early steps of pathogenesis.

The biochemical data of A. Mayer and co-workers (Hebrew Univ. Jerusalem, Israel) seem to point to an important role for laccases in the infection process, while the molecular-genetic data exclude such a role. How can one reconcile these two seemingly contradictory conclusions? There are differences in the experimental procedures and the fungal strains used. In most of the biochemical work, *B. cinerea* was grown in pectin-containing medium in which inducers were present throughout the culture. Growth was generally over a great length of time, up to well over 14 days. Schouten et al. (2002b) grew pre-cultures in defined synthetic medium and showed that *Bclcc2* mRNA could be induced within 1 h after addition of tannic acid or resveratrol. Laccase activity was detected after overnight incubation in the presence of an inducer. It is possible that the enzyme activity detected by Mayer and co-workers throughout their work is encoded by the *Bclcc3* gene, or by a novel laccase gene that has not yet been identified.

5.3. Counteracting host defence responses

Throughout the course of an interaction between *B. cinerea* and its host, the plant (often vigorously) attempts to defend itself against pathogen invasion. Largely the same spectrum of defence responses is activated during an infection by *B. cinerea* as during a hypersensitive response (HR) to avirulent races of a biotrophic pathogen: lignification (Maule and Ride, 1976; Heale and Sharman, 1977), biosynthesis of phytoalexins (e.g. Bennett et al., 1994) and accumulation of PR proteins (e.g. Benito et al., 1998; Diaz et al., 2002). These defence responses are presumably local, being restricted to the infection site(s). Although *B. cinerea* is a necrotising pathogen it does not trigger Systemic Acquired Resistance (Govrin and Levine, 2002). The total spectrum of defence responses results in a primary necrotic lesion in which the fungus is effectively restricted. Depending on the type of host tissue and yet unidentified physiological aspects of the host, fungal growth enters a lag phase in which the lesions do not expand. A proportion of the primary lesions eventually develop into aggressive, expanding lesions (Van den Heuvel, 1981; De Meyer and Höfte, 1997; Benito et al., 1998). The pathogen is not killed in the non-expanding lesions since viable fungal mycelium could be recovered from all lesions (E.P. Benito and J. van Kan, unpubl.). Thus, an active defence contributes to (temporarily) restricting the fungus within the primary lesions, giving rise to a period of

quiescence. It is as yet unknown which factors determine the transition from quiescence to the aggressive, expanding infection phase. Chapter 9 will deal in more detail with the defence compounds (metabolites and enzymes) produced in plants in response to *Botrytis* infection and their effectiveness in restricting the outgrowth of the pathogen. In order to be a successful pathogen on their respective host species, *Botrytis* spp. obviously have to cope with these defence compounds, either by active suppression of their synthesis or by counteracting their growth inhibiting effect. Chapter 9 discusses mechanisms that *Botrytis* spp. have evolved to overcome the deleterious effects of pre-formed or induced host defence compounds.

The oxidative burst at the host-pathogen interface (Chapter 8) imposes stress on the host as well as the pathogen. *B. cinerea* is able to cope with external oxidative stress in order to survive in the necrotic tissue. Successful detoxification of H_2O_2 is mediated by an extracellular catalase BcCAT2 with glutathione S-transferase presumably functioning as intracellular back-up (Prins et al., 2000a; Schouten et al., 2002a). Targeted mutagenesis of the catalase gene *Bccat2* did not affect the survival within the oxidative environment of a necrotic lesion. Virulence of *Bccat2* deficient mutants was indistinguishable from wild type (Schouten et al., 2002a).

6. Conclusions

This chapter illustrates that *Botrytis* is equipped with a large toolbox of enzymes and metabolites that enable the pathogen to infect a spectrum of host plants. There can be functional overlap between different types of enzymes attacking the same substrate and there is apparent redundancy within a family of isozymes. Some enzymes supposedly act in concert, while others may be irrelevant for a particular host. Altogether these tools are needed to fulfil their job: killing plant cells and facilitating the conversion of plant tissue into fungal biomass.

Research on *B. cinerea* pathogenicity factors has focussed for many decades on the production of secreted enzymes and their correlation with pathogenesis. Such studies are still pursued by certain researchers up until today. With our current knowledge of the occurrence of gene families encoding multiple isozymes, often with quite distinct biochemical properties, we recommend not to initiate further studies on total enzyme activity. It will be much more informative to study individual isozymes, their biochemical characteristics and expression during pathogenesis. Information on their temporal and spatial accumulation, substrate preference, end-product release and other isozyme-specific characteristics will substantially contribute to understanding enzymatic processes during pathogenesis.

Targeted mutagenesis provides an excellent tool to study the role of specific gene products in pathogenesis. The generation of single, double or even triple gene-specific mutants is feasible (Chapter 4) and will provide a better understanding of the complex role of the different enzymes secreted during *Botrytis*-host plant interactions. We look forward to witnessing the design of rational control strategies, based on such knowledge, in the upcoming decade.

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CHAPTER 8

BOTRYTIS CINEREA PERTURBS REDOX PROCESSES AS AN ATTACK STRATEGY IN PLANTS

Gary D. Lyon¹, Bernard A. Goodman² and Brian Williamson¹

¹Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK; ²ARC Seibersdorf research GmbH, A-2444 Seibersdorf, Austria

Abstract. Electron transport processes play vital roles in the functioning of biological systems, and oxygen is the driving force for many of these reactions. A consequence of this is the production of several oxygen-derived molecules, known as active oxygen species (AOS), whose reactivity is greater than that of oxygen itself. There is increasing evidence that *Botrytis cinerea* exploits the production of AOS in colonising plant tissues, and this is reviewed in the present chapter. Specific considerations are given to the interactions between hydrogen peroxide and other AOS that are produced by the fungus and the plant-based antioxidant systems in determining the outcome of the infection process. In addition, biochemical processes that appear to be of importance for lesion development are discussed and the evidence to support them critically evaluated. These are considered in separate sections dealing with the perturbation of the free radical chemistry and transition metal redox processes (particularly those involving iron), the regulation of enzymes (of both plant and fungal origin), the production of toxic metabolites in the host, and host signalling and programmed cell death. Attention is also drawn to the need for the scientific community to adopt standard procedures (both chemical and biological) to facilitate comparison between results from different groups. Finally, consideration is given to strategies that could be used to resolve some of the outstanding questions relating to our understanding of the *Botrytis* infection process.

1. Introduction

Electron transport processes are fundamental to the functioning of biological systems, although the range of reactions differs between different types of organisms. At the molecular level, many of these reactions function through a change in oxidation state of a transition metal ion, e.g. in metalloenzymes, but in other cases reactions may be based purely on organic compounds, often involving the generation of free radical species (an expression used to describe non-transition metal chemical species with unpaired electrons). Reversibility is a common feature of biological electron transport processes, and the chemical species involved are stable in both reduced and oxidized forms, with the act of cycling between the states being given the name “redox”. Thus redox reactions involve the transfer of electrons

between two chemical species; compounds that lose an electron are described as oxidised whilst compounds that gain an electron are reduced.

Molecular oxygen (or more specifically $3O_2$) is often the driving force for oxidation reactions, leading to the generation of molecules, known as active oxygen species (AOS), sometimes referred to as reactive oxygen species (ROS), whose reactivity is greater than that of oxygen itself. AOS include the superoxide radical anion (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical ($\cdot OH$), singlet oxygen (1O_2) and lipid-derived species such as LOOH and the LOO \cdot and LO \cdot radicals. AOS are thus normal products of primary metabolic processes in cells, and are produced through a wide range of biochemical processes, including the enzymes NADP dehydrogenase, NADPH-cytochrome c reductase, cytochrome P450, and NADPH oxidase. Other chemical processes involved in their production include autoxidation, photochemical reactions, and the reaction of metalloenzymes with oxygen, unsaturated fatty acids (e.g. β oxidation), and other compounds in several subcellular compartments, including chloroplasts, mitochondria and microbodies such as peroxisomes and glyoxysomes.

In healthy cells, the production of the various AOS is controlled through a number of fundamentally different processes, including compartmentalisation of precursors, the speciation of metal complexes which behave as catalysts, and the activity of a variety of enzymes. Enzymes are also involved in the scavenging of AOS reaction products, along with antioxidant molecules, which include the reduced forms of ascorbic acid (AA) and glutathione (GSH) as two of the best known examples. Also, because of their widely different chemical reactivities, a variety of approaches are necessary to control the behaviour of the AOS themselves. Some, such as $\cdot OH$ and 1O_2 , are very reactive and organisms have to possess a number of processes to minimise any damage they might cause under normal conditions. Such processes include control of AOS production, scavenging of the secondary products of their reactions, and repair of any resulting DNA damage. However, because of their high, and largely indiscriminate reactivity, the production of specific scavengers for these chemical species is not a realistic option for an organism (although there are several, largely erroneous, reports in the literature of specific hydroxyl radical scavengers). In contrast, the production of specific scavengers seems to be the favoured approach for controlling the levels of the more stable AOS, such as O_2^- and H_2O_2 , where the roles of the enzymes superoxide dismutase (SOD) and catalase are well known.

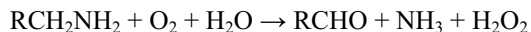
A frequent consequence of the existence of stress processes (of both abiotic and biotic origin) is a perturbation in the production of AOS and changes in redox potential in the organism. The chemical processes involved are complex, particularly during infection by pathogens; many interconnected biochemical pathways are affected and with diverse consequences, including exacerbation of damage to either the plant or pathogen, or signal transduction to regulate transcription. In plant-pathogen interactions, both the plant and the pathogen are involved in AOS production (Mayer et al., 2001), and both possess extensive antioxidative machinery to moderate their damaging effects. It has been widely reported that an early plant

response to infection by an incompatible pathogen is the so-called 'oxidative burst', which involves a rapid, transient and localised production of AOS (Lamb and Dixon, 1997; Wojtaszek, 1997). This is generally presented as a specific reaction that is initiated by the pathogen, since the oxidative burst is not seen during infection by a compatible pathogen. However, a virtually instantaneous burst of oxidative activity occurs in plant tissues during maceration (Goodman et al., 2002), which is not a pathogen-derived reaction, but a manifestation of physical damage to the host.

There is now evidence (e.g. Edlich et al., 1989; Urbanek et al., 1996; Von Tiedemann, 1997; Govrin and Levine, 2000) that the generation of AOS assists the colonisation of plant tissues by necrotrophic organisms, such as *B. cinerea* (Chapter 2). Thus the reactions that are utilised in defence against attack by biotrophic pathogens appear to increase vulnerability to attack by necrotrophs. The factors which determine whether the plant or the pathogen is ultimately successful are complex, but their relative ability to deal with oxidative reactions is a major factor. The evidence for the exploitation of oxidation processes by *B. cinerea* will be presented in this Chapter.

2. Hydrogen peroxide and other AOS

The main source of H_2O_2 in plants is usually considered to be the Mehler (1951) reaction, where atmospheric oxygen is reduced to H_2O_2 in photosystem I. A similar reaction also occurs through the action of numerous enzymatic oxidases. Examples include glycolate oxidase, which catalyses the conversion of glycolate to glyoxylate in peroxisomes, NADPH oxidase, which is normally associated with the plasmalemma, and various copper-containing amine oxidases that catalyse the oxidation of a wide range of amines, including mono-, di- and poly-amines:



Amine oxidases are located predominantly in the extracellular matrix and H_2O_2 formed by oxidation of amines may be directly utilized by wall-associated peroxidases during lignification (Bolwell and Wojtaszek, 1997). H_2O_2 has numerous effects, many of which are related to the ease with which it can generate the highly reactive $\cdot OH$ radical. Thus H_2O_2 accumulated in the apoplast may be directly toxic to invading pathogens by initiating DNA damage via $\cdot OH$ attack. Also it can oxidise and inactivate thiol-containing enzymes, such as the thioredoxin modulated enzymes in chloroplasts, and it may also be involved in intracellular signalling. The half-life of $O_2^{\cdot -}$ in plants is relatively short compared to H_2O_2 , and it is converted to H_2O_2 in the presence of the enzyme superoxide dismutase (SOD). In contrast to H_2O_2 , superoxide cannot readily cross cell membranes because of its anionic charge, and it is therefore relatively immobile. Superoxide can reduce transition metal ions, which in turn can reduce H_2O_2 , generating $\cdot OH$ in the process. In addition to DNA damage mentioned in the preceding paragraph, this radical can cause lipid peroxidation, and other degradative reactions in cells.

B. cinerea has been shown to produce H_2O_2 when grown on autoclaved flax stems (Bratt et al., 1988), possibly as a result of oxidase activity, since sugar oxidases are commonly produced by many fungi; glucose-, xylose-, galactose- and ascorbate-oxidases have been shown to be produced by *B. cinerea* (Edlich et al., 1989; Liu et al., 1998). However, the glucose oxidase isolated by Liu et al. (1998) appeared to be an intracellular enzyme that differed in molecular structure from the typical secreted sugar oxidases of other pathogens and it was repressed by glucose. This localisation and the repressing effect of glucose makes it very unlikely that this particular enzyme was involved in the interaction postulated by Edlich et al. (1989). Recently, a knock-out *Botrytis* mutant for a putative secreted glucose oxidase gene, *bogod1*, when inoculated on to leaves of *Phaseolus vulgaris* displayed normal virulence (Rolke et al., 2004).

Antioxidants applied to the infection drop or sprayed on plants suppressed grey mould of several crops (Elad, 1992). Similarly, infiltrating barley leaves with antioxidants prior to inoculation with the fungal necrotrophs *Rhynchosporium secalis* and *Pyrenophora teres* also reduced the rates of growth of the fungi in compatible interactions (Able, 2003). Further evidence for the involvement of AOS in the infection process has been provided by Von Tiedemann (1997), who showed that aggressive isolates of *B. cinerea* induced the formation of H_2O_2 and 'OH radicals during early stages of infection of *P. vulgaris* leaves, though he did not show whether the H_2O_2 was produced by the fungus or the plant. AOS (as hydrogen peroxide) and the antioxidant enzymes peroxidase, superoxide dismutase and catalase and lipid peroxidation were measured in *Phaseolus vulgaris* infected by *B. cinerea*. *B. cinerea* infection was found to be associated with increased AOS in the infection site and at a distance around the lesion, and antioxidants were elevated (Lapsker and Elad, 2001).

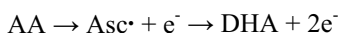
Increases in the levels of the enzymes glutathione peroxidase (GPX) and glutathione S-transferase have been observed as a result of the application of H_2O_2 to plants (Levine et al., 1994), suggesting that these enzymes have a role in AOS scavenging. In mammals, glutathione peroxidase is an important peroxide scavenging enzyme that is inactivated by nitric oxide (NO), but there is still debate about the importance of NO in plant responses to *Botrytis* spp. Exogenous application of H_2O_2 to plants can induce transcription of a number of genes including glutathione S-transferase, lipoxygenase, and peroxidase (Desikan et al., 2001).

3. Low molecular mass antioxidant molecules

As mentioned in the previous section, organisms possess multiple systems for containing, regulating, or eliminating AOS. Scavenging of some of the more stable AOS is performed by a range of compounds that are collectively referred to as antioxidants (Nagata et al., 2003). There is a wide range of such compounds in plants, including water-soluble phenolics, GSH, ubiquinone, cysteine, α -tocopherol,

and enzymes such as the SODs (Mn-SOD, Cu/Zn-SOD, Fe-SOD), monodehydroascorbate reductase (MDHAR), glutathione peroxidase and catalase that can act as antioxidants and scavengers of AOS (Sroka and Cisowski, 2003). However, AOS also function as signalling molecules and can activate the synthesis of antioxidants in host cells (*e.g.* AA and anthocyanin in *Arabidopsis*, Nagata et al., 2003). Thus the oxidative and antioxidative processes are closely interlinked, and often it is difficult to differentiate between cause and effect when investigating these processes.

The chemistry of the action of biological antioxidants is complex and as illustrated below for ascorbate and glutathione, they are cycled through reduced and oxidised states as a result of redox processes that occur within cells. The redox cycling of ascorbate involves the production of a free radical intermediate (Asc.) between the reduced (AA) and oxidised (DHA) forms



whereas with glutathione, the reduced form (GSH) is a monomer and the oxidised (GSSG) form is a dimer



There are small differences in the biochemistry of antioxidant systems between fungi and plants. For instance fungi synthesise erythroascorbic acid and not ascorbic acid (Loewus et al., 1995). Both the fungus and the plant need antioxidant systems to survive free radical damage that arises from normal life processes. An important issue is the relevant tolerance of each partner to the AOS being released, *e.g.* both produce H_2O_2 , but is one partner more resistant to the effects of that H_2O_2 .

AA has a number of potential roles related to photosynthesis including scavenging of AOS generated by oxygen photoreduction and photorespiration, regeneration of α -tocopherol from α -tocopheryl radicals, acting as a cofactor for violaxanthin de-epoxidase and donation of electrons to photosystem II (Smirnoff, 2000). High light intensity has been reported to increase both the production of AOS and several AOS scavengers (Karpinski et al., 1997), and AA can reach very high concentrations in chloroplasts (20-300 mM). The concomitant production of both AOS and AOS scavengers may be the explanation for apparently conflicting observations concerning diurnal variations in AA levels. For example, Smirnoff (2000) has reported that the AA pool size increases with high light intensity, whereas Muckenschnabel et al. (2003) showed a decrease in AA concentrations in leaves of *P. vulgaris* between morning and afternoon. It seems likely, therefore, that there is a delicate balance between oxidation and reduction processes and that this can be tipped by relatively subtle environmental changes to result in either increases or decreases in the levels of antioxidants such as AA. However, variations in light regulated antioxidants may be a contributory factor influencing the time of infection, although, in unpublished work, I. Muckenschnabel (pers. comm.) found that

decreasing tissue AA levels through abiotic stress did not influence the rate of lesion development in inoculated *Phaseolus* leaves.

In tissue rotted by *B. cinerea* a massive depletion of AA pools is generally observed (Vanacker et al., 1998; Loewus, 1999; Muckenschnabel et al., 2002, 2003). One consequence of this AA depletion is likely to be changes in gene expression, since low AA levels in cells have been reported to induce PR proteins, whereas high AA levels suppressed their expression (Pastori et al., 2003). Glutathione is directly involved in reactions with AOS, and in addition it binds to electrophiles such as 4-hydroxy-2-nonenal (4-HNE) and dopaquinone (Sect. 9). These reactions may cause some 'loss' of total glutathione in infected tissues, though the amounts of free 4-HNE are small (micromolar) compared with much larger (millimolar) amounts of glutathione. It seems unlikely, therefore, that a significant reduction in free glutathione could be accounted for by conjugation to 4-HNE alone though there are many other related molecules and the sum of their reactions could be appreciable.

Although direct evidence for free radical mediated changes ahead of infection has been observed in electron paramagnetic resonance (EPR) spectroscopy experiments (Sect. 4), there are varied reports of the effects of *B. cinerea* on antioxidant levels in plant tissue remote from soft rot lesions. For example, Muckenschnabel et al. (2002, 2003) observed a massive depletion in AA levels in uninfected regions of infected leaves of *A. thaliana* and *P. vulgaris*, whereas Kuźniak and Skłodowska (1999, 2001) found little change in total AA levels in chloroplasts from tomato leaves infected by *B. cinerea*. The latter authors did, however, observe an increase in the proportion present as DHA, indicating a significant pro-oxidative shift in the ascorbate redox status. Moreover, Kuźniak and Skłodowska (1999, 2001) found that *Botrytis* infection resulted in a decrease in GSH and total glutathione content, but Muckenschnabel et al. (2001b) found that glutathione levels in *Phaseolus* leaves were little affected by *Botrytis* infection. Therefore, although these various reports all show that *B. cinerea* infection results in a shift in overall redox status to more oxidized forms, there are major differences in the behaviour of the individual antioxidant molecules. This suggests that a great deal of caution needs to be exercised in interpreting the results of analyses based on a small number of chemical species.

4. Perturbation of free radical chemistry as a result of *Botrytis* infection

As mentioned previously, free radical production, particularly via photosynthesis, is a normal feature of cell metabolism and healthy cells have a variety of mechanisms for containing them and preventing unwanted reactions. However, during infection, when many biochemical processes are perturbed, changes in the amounts and chemical forms of free radicals may occur. Direct evidence for such changes can be obtained from EPR spectroscopy and some examples will be briefly summarised in this paragraph.

One of the most obvious and consistent effects of *B. cinerea* infection is a large increase in the EPR free radical signal in rotted tissue compared to controls; similar results have been obtained with leaves and fruit (e.g. Deighton et al., 1999;

Muckenschnabel et al., 2001a, b, 2002). Also with leaves, the spectroscopic properties of rotted tissue are distinctly different from those of uninfected photosynthetic tissue, although it is not immediately obvious whether the new free radical species is derived from processes occurring within the plant or the fungus or both. However, in a careful study of the free radical signal in different parts of leaves of *A. thaliana* that contained *B. cinerea* lesions, Muckenschnabel et al. (2002) were able to deconvolute the spectra from uninfected regions of infected leaves into the components seen within lesions and control tissues. Thus a free radical with spectral properties identical to those from the lesions can be produced in tissue that does not contain the fungus.

In experiments of the type described in the preceding paragraph, it is not possible to distinguish between stable free radicals and steady-state concentrations of free radicals that are turning over rapidly (as is the case with photosynthesis). An approach to identifying the existence of unstable free radical species in a sample is to use a molecule, known as a spin trap, that can react selectively with the radical to form a (relatively) stable adduct radical. Such spin traps are often nitrones, which yield nitroxide radicals on reaction. An example is the molecule α -(4-pyridyl-1-oxide)-N-*t*-butylnitron (POBN).

Muckenschnabel et al. (2001a) have reported the detection of free radical adducts of POBN in tissue from *B. cinerea* infected fruits of *Capsicum annuum* that were infiltrated with the spin trap. In contrast, only the ascorbate radical (Asc \cdot) could be detected in the EPR spectra in healthy control fruits. With infected fruits the EPR spectra showed a progressive change from the free radical adduct to Asc \cdot . With increasing distance from the surface of the lesion. Although the radical adduct could be observed in tissue up to 50 mm beyond the edge of the lesion, the change to Asc \cdot was more rapid in tissue that did not contain a major vascular bundle. It appears, therefore, that vascular transport processes play an important role in bringing about chemical changes in the host tissue ahead of colonisation by *B. cinerea*.

Although it is tempting to interpret these EPR results as showing direct evidence for *Botrytis*-induced production of free radicals in uninfected tissue, there remains the possibility that these spectra are produced as a consequence of the sample preparation procedure, which involved excision of sections of tissue, infiltration with the spin trap under partial vacuum, then centrifugation to extract the resulting solution. Physical damage to plant tissues is known to generate free radicals and the radicals detected in these experiments may have been derived through the excision process. However, even if this were the case, the different responses between the different tissues indicates *Botrytis*-induced alteration of the radical reaction pathways, and demonstrates a direct effect of *B. cinerea* on the free radical scavenging machinery in the plant.

Similar results to those described for *Capsicum* fruits have been observed with leaves of *P. vulgaris*, where time dependent differences in the free radical chemistry occurred at positions remote from the edge of the lesions (Muckenschnabel et al., 2003). In that work, there appeared to be a fundamental change at around 48 hours

post infection (hpi). Prior to that time, spectra from both Asc. And the radical adduct of POBN could be observed in tissue infiltrated with the spin trap. The Asc. signal was absent from samples recorded at longer hpi, although it was present in all samples which did not have added spin trap. Furthermore, there was an increase in the Asc/ AA ratio at this time, indicating a perturbation of the ascorbic acid redox chemistry.

5. Production of oxalic acid

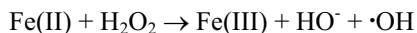
Many fungi, including *B. cinerea*, produce oxalate in culture (Dutton and Evans, 1996) and are presumed to also produce it *in planta*, though it is important to remember that plants can also synthesise oxalic acid (Loewus, 1999). D-erythroascorbic acid has been suggested to be a precursor of oxalate in fungi (Loewus et al., 1995), though several other biosynthetic pathways have been proposed (Dutton and Evans, 1996). Under alkaline conditions, H₂O₂ can cleave D-erythroascorbic acid to yield oxalic acid, i.e. peroxygenation of D-erythroascorbic acid (Keates et al., 1998), but it is not clear to what extent this might occur *in vivo*.

Oxalate production by fungi is thought to be advantageous to their development, and may contribute to pathogenesis through sequestration of calcium from cell walls and acidification of host tissues to a pH that is more optimal for degradation of plant cell walls by fungal polygalacturonases (Chapter 7). Binding of oxalate to calcium in cells may also interrupt Ca²⁺ signalling. A role for oxalate in preventing up-regulation of the plant antioxidant defences is supported by the work of Jiang and Zhang (2003), who showed pre-treatment with Ca²⁺ chelators and Ca²⁺ channel blockers suppressed the up-regulation of antioxidant defences in plants exposed to paraquat (which generates O₂⁻). Oxalic acid can also bind directly to copper and may, therefore, affect the functioning of copper-containing proteins, though evidence for this is still sparse. However, oxalic acid has been shown to inhibit the activity of phenolase, which has a copper ion at the active centre (Satô, 1980).

Genes for oxalate oxidase, which is potentially capable of converting oxalic acid into CO₂ and H₂O₂, has been isolated from a number of monocotyledonous plants (Bolwell and Wojtaszek, 1997). This enzyme has been described as having an important role in the resistance to fungi, such as *Blumeria* (syn. *Erysiphe*) *graminis* f. sp. *hordei*. It is interesting that *B. cinerea* is not a pathogen of monocots, suggesting that this may be a consequence of the presence in monocots of the oxalate oxidase. Although it appears that dicots do not possess oxalate oxidase *per se*, they do possess related genes (identified on the basis of sequence similarity to oxalate oxidases from monocots), but their enzymes do not have the ability to degrade oxalate (Membre et al., 2000; Patnaik and Khurana, 2001). Dicots transformed with wheat oxalate oxidase genes have increased resistance to infection by fungal pathogens (Liang et al., 2001; Lane, 2002), and it seems, therefore, that there is a correlation between host range for *B. cinerea* and absence of oxalate-degrading oxalate oxidase in the plant.

6. Dynamics of iron redox chemistry

Hydrogen peroxide (produced by either the plant or the pathogen) can oxidise Fe(II) to Fe(III) with the formation of the highly reactive, and hence cytotoxic, $\cdot\text{OH}$ radical through the Fenton reaction (Haber and Weiss, 1934; Fenton, 1899):



In healthy plant cells this process is controlled by the rapid scavenging of H_2O_2 (Sect. 2), but in stressed cells potentially damaging amounts of $\cdot\text{OH}$ can be formed as a result of increased generation of H_2O_2 . Subsequent reduction of the Fe(III) through the action of antioxidants or reductases ensures continual recycling of the Fe, and hence continued $\cdot\text{OH}$ generation. Also, although Fenton chemistry is predominantly associated with iron, $\cdot\text{OH}$ can be formed in the presence of other low oxidation state transition metal ions, such as Cu(I).

Depending on the plant species, iron is transported as either Fe(II) or Fe(III) complexes (Briat and Lobréaux, 1997), although storage as ferritin seems to be common to all types of plant. A feature of the ferritin protein is that it can accumulate up to 4,500 Fe atoms in the form of Fe(III) oxyhydroxides within the core of the organic molecule. The ability of the ferritin protein to sequester large amounts of Fe means that much of the Fe in plants is held in a fairly unreactive form. This is important because as mentioned above the presence of appreciable amounts of Fe(II) is potentially dangerous for any organism. Additionally, the speciation of iron during transport needs to be carefully controlled. In plants, nicotianamine (NA) plays an important role (Hell and Stephan, 2003) because its complexes with Fe(II) are resistant to oxidation and do not participate in the Fenton reaction (Von Wirén et al., 1999). Fe(II) speciation is, however, strongly pH dependent, and even a small reduction in pH from physiological levels can shift the Fe(II) speciation in favour of complexes with organic acids, such as citric acid, which readily participate in the Fenton reaction. Thus a role for oxalic acid in the *Botrytis* infection process, in addition to those mentioned in the previous section, is to alter the Fe(II) speciation to increase AOS ($\cdot\text{OH}$) production.

The ability of ferritin to inhibit participation of Fe in Fenton chemistry is illustrated by the work of Deák et al. (1999), where they showed that transgenic tobacco plants expressing the alfalfa ferritin protein exhibited increased resistance (seen as lesions with smaller diameter) to both viral and fungal infection, including *B. cinerea*. Deák et al. (1999) proposed that the ferritin sequestered iron that might otherwise have been available for Fenton chemistry, thereby reducing oxidative damage. However, Fenton chemistry is not completely prevented by the binding of Fe to ferritin, since solubilization of Fe from oxyhydroxides is accomplished readily by oxalate, and reaction of this Fe(III) with reductases or antioxidant molecules, such as ascorbic acid, yields the Fe(II) for the Fenton reaction. It is interesting to note this role for antioxidants in the generation of free radicals, a reaction which

illustrates the complexity of the chemical reactions for such molecules in biological systems.

Changes in the oxidation states of transition metal ions in plant tissues can be observed directly with EPR spectroscopy. Iron and manganese are the most frequently encountered in plant tissues, where they can be present as either EPR-detectable [e.g. Mn(II) and Fe(III)] or EPR-silent forms [e.g. Mn(III) and Fe(II)]. Therefore, in addition to detecting and characterising free radicals, EPR has the potential to provide direct information on the redox status of tissue samples. Indeed, using EPR, increased levels of Fe(III) have been observed in *B. cinerea* lesions on fruit of *C. annuum* (Deighton et al., 1999), and leaves of *P. vulgaris* (Muckenschnabel et al., 2001b) and *A. thaliana* (Muckenschnabel et al., 2002). However, whereas with the *C. annuum* and *P. vulgaris* tissues the Fe(III) EPR signal dropped rapidly outside of the lesion to levels comparable to those in the healthy tissues, the *A. thaliana* samples contained sizeable chlorotic areas surrounding the lesions with Fe(III) signal intensities comparable to those in the lesions. In all of these samples the changes in Fe(III) were accompanied by large decreases in ascorbic acid confirming the close relationship between antioxidant levels and the oxidation state of iron. The *Arabidopsis* samples showed a virtually complete loss of AA from the uninfected regions of infected leaves, whereas with the other tissues there was a progressive decrease in AA with decreasing distance from the lesion.

In fungi a considerable fraction of the Fe is bound to strong chelating agents known as siderophores. *B. cinerea* produces several trihydroxamate siderophores, the principal one being ferrirhodin (Konetschny-Rapp et al., 1988). Siderophore production *in vitro* is accompanied by rapid acidification of the incubation medium (possibly caused by oxalic acid production). Whilst there is a relationship between iron in its various forms and oxidative damage, there is no evidence for a direct role of siderophores in the infection process.

7. Regulation of plant enzymes

Peroxisomes are subcellular organelles that carry out a wide range of functions in eukaryotes including β -oxidation of fatty acids, glyoxylate metabolism, and generation and metabolism of H_2O_2 (Van den Bosch et al., 1992). Peroxisome biogenesis genes have been shown to be up-regulated by H_2O_2 in both animal and plant cells (Lopez-Huertas et al., 2000), suggesting that peroxisomes play a key role in regulating AOS/redox in cells.

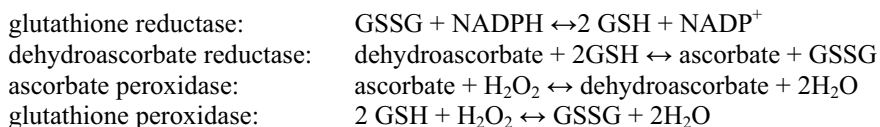
Superoxide dismutases (SOD; EC 1.15.1.1) are a family of metalloenzymes that catalyse the conversion of $O_2^{\cdot-}$ to O_2 and H_2O_2 . Several isozymes of SOD have been reported including Cu/Zn-SOD located in the cytosol, chloroplasts and peroxisome; Fe-SOD in chloroplasts, mitochondria and peroxisomes; Mn-SOD in mitochondria (Bowler et al., 1994) and a SOD in the plasma membrane (Vuletić et al., 2003). The H_2O_2 generated by SOD activity is removed by catalases and peroxidases.

Catalases, which occur in peroxisomes and glyoxysomes, are generally considered to be responsible for removing the majority of the H_2O_2 by catalysing its conversion to O_2 and H_2O ; ascorbate and glutathione peroxidases (APX and GPX,

respectively) remove most of the remainder. APX occurs in both the cytosol and chloroplasts (in the ascorbate-glutathione pathway), and has a higher affinity for H_2O_2 than catalases. Also its production may be regulated by H_2O_2 , since its activity in tobacco leaves has been observed to increase on treatment with H_2O_2 (Gechev et al., 2002).

Catalase deficient mutants of *Nicotiana tabacum* have been used to dissect some of the processes associated with AOS production leading to modification of redox status. Catalase-deficient plants were more sensitive than wild type plants to external application of H_2O_2 (Dat et al., 2001), and exposure of these plants to moderate or high light intensity resulted in cell death as leaves were unable to scavenge the H_2O_2 released during photorespiration. In addition, transgenic plants with reduced levels of catalase and APX are hyper-responsive to pathogens and activate a programmed cell death (PCD) at lower pathogen levels than control plants (Mittler et al., 1999). These results demonstrate that suppression of AOS-scavenging enzymes can occur during infection and plays a role in enhancing pathogen-induced PCD.

APX and glutathione reductase (GR) are enzymes specifically associated with the ascorbate-glutathione cycle and increases in their activities have been associated with counteracting the production of AOS caused by oxidative metabolism (Tommasi et al., 2001). GR is involved in maintaining the supply, or pool, of GSH, which is utilised for the reduction of DHA. In addition, both GSH and AA can directly scavenge AOS. Examples of some of the enzymes involved in common pathways are:



Down regulation of enzymes may occur in the presence of some pathogens. For example, transcription of catalase genes has been reported to be down-regulated in rice treated with H_2O_2 (Agrawal et al., 2001), tomato inoculated with incompatible *Pseudomonas syringae* pv. *tomato* (Mysore et al., 2002), and tobacco inoculated with TMV (Rizhsky et al., 2002). In contrast, Morita et al. (1999) found that APX increased in cell suspension cultures of rice treated with H_2O_2 . Thus the mechanisms governing gene regulation are complex, just as those involved in antioxidant production, and the factors which determine whether a particular gene is up- or down-regulated are not well understood.

Several workers have reported that *B. cinerea* causes the down-regulation of enzymes, such as APX and catalase, which are involved in the control of AOS levels. Differential display of mRNA has been used to isolate a gene from tomato infected with *B. cinerea* that shows sequence similarity to the amino terminal region of CCH, a copper chaperone from *A. thaliana* (Company and Gonzalez-Bosch, 2003). The gene in tomato is down-regulated after infection suggesting a relationship between copper homeostasis and plant defence responses. Copper

chaperones are involved in controlling intracellular concentrations of copper and facilitating its transfer to specific copper-requiring proteins. Interestingly, Hsp33, a molecular chaperone from *E. coli*, is redox regulated (Jakob et al., 1999) and oxidizing agents such as H₂O₂ activate its chaperone function. This may explain how redox changes can affect transcription in plants and fungi. In plants, copper is a cofactor for a number of enzymes including superoxidases, mono- and di-oxygenases, and enzymes involved in the elimination of superoxide radicals such as Cu/Zn SOD and ascorbate oxidase (Wintz and Vulpe, 2002). Knock-outs of the *bcsod1* gene in *B. cinerea* profoundly reduces the pathogenicity of this fungus on *P. vulgaris* (Rolke et al., 2004).

The large number of plant genes requiring copper suggest that changes in redox potential of the cell could have multiple and perhaps profound effects. However, EPR measurements have failed to positively identify the formation of mononuclear Cu(II) complexes as a result of the infection of plant tissues by *B. cinerea*, although such species can be seen readily in tissues that have been exposed to severe abiotic stresses (Goodman and Newton, 2004).

8. *Botrytis*-derived enzymes

Botrytis cinerea possesses an array of enzymes for protection against AOS-mediated reactions during the infection process. For example, a number of intracellular and extracellular enzymes that are associated with the degradation of H₂O₂ are formed in liquid culture. These include guaiacol peroxidase, ascorbic peroxidase, glutathione peroxidase, laccase, and catalase (Gil-ad et al., 2000), although their levels vary with the composition of the growth media.

Schouten et al. (2002) have reported that the mRNA for the gene (*Bccat2*) encoding an extracellular catalase in *B. cinerea* was rapidly up-regulated when the fungus was exposed to H₂O₂ *in vitro*. Mutants containing a disrupted *Bccat2* gene were more sensitive than the wild type strain to H₂O₂, and showed higher levels of mRNA for the stress-responsive genes *Bcgst1*, encoding glutathione S-transferase, and *Bcubi4*, encoding for polyubiquitin, suggesting that the *Bccat2*-deficient mutant was more stressed than the wild type. There was no consistent reduction in virulence of the *Bccat2*-deficient mutant when grown on bean and tomato leaves indicating that there is no simple relationship between catalase and virulence. This further confirms that *B. cinerea* is a pathogen that is well adapted to growing in an oxidizing environment and possesses robust defences against it.

Although *B. cinerea* produces both catalase and Cu/Zn-SOD (Choi et al., 1997), Van der Vlugt-Bergmans et al. (1997) could not detect *catA* from *B. cinerea* expression *in planta* but *cat1* from tomato was detected. The fungus has been shown to rapidly break down H₂O₂ though it is unclear which enzymes are involved (Gil-ad and Mayer, 1999). Gil-ad et al. (2001) suggested that the glucan sheath surrounding the mycelium of *B. cinerea* may play a role in protecting the fungus from host responses though the evidence for this is still circumstantial. A SOD gene has been cloned (Rolke et al., 2004) and the knock-out has reduced virulence on bean leaves.

Whilst enzymes associated with the degradation of AOS are important to *B. cinerea*, such enzymes are not unique to necrotrophs; enzymes such as SOD, GR, catalase/peroxidase and microsomal glutathione S-transferase have also been isolated from the obligate pathogen *Blumeria graminis* f. sp. *hordei* (Thomas et al., 2001). An important consideration, therefore, is whether these microbial enzymes are extracellular or intracellular; the latter would suggest that they may only serve to protect the fungus from its own AOS whilst the former would indicate that they may be involved in protecting the fungus from the plant's AOS.

In addition to the production of enzymes for protection against AOS, *B. cinerea* also possesses enzymes that are capable of oxidising plant compounds associated with the resistance response (Mayer et al., 2001). *Botrytis*-derived enzymes are also able to oxidise a number of natural products, such as alcohols, *in vitro* (Fukuda and Brannon, 1971; Farooq and Tahara, 2000a, b).

Based on comparisons of the aggressiveness of six isolates of *B. cinerea* during infection of leaves of *P. vulgaris* by *B. cinerea*, Von Tiedemann (1997) suggested a primary role for AOS in the induction of plant cell death. Interestingly, all isolates suppressed plant peroxidase activity compared to non-inoculated leaf tissue.

A gene encoding a glyoxal oxidase homologue (Bcglyox1) has been cloned from *B. cinerea* (Stefanato et al., 2003). The gene product is presumed to be secreted and mutants in which this gene was replaced continued to produce oxalic acid, but were non-pathogenic and were unable to grow on minimal media containing a range of simple sugars. The existence of non-pathogenic mutants that produced oxalic acid confirms that pathogens like *B. cinerea* contain a number of so-called 'pathogenicity factors' and that pathogenicity is not associated with a single factor.

Other relevant genes isolated and characterised from *Botrytis* include *gst1* (Prins et al., 2000) and a mitochondrial alternative oxidase (AOX) (Joseph-Horne et al., 2000). These alternative oxidases in fungi have possible iron-binding sites that are conserved in AOX genes from different fungi. The AOX is an example of the 'same' enzyme occurring in both plant and pathogen, but each with different molecular organisation and regulation of activity (Joseph-Horne et al., 2000).

9. Generation of lipid peroxidation products

The toxicity of molecules such as superoxide and H₂O₂ produced during infection of plants by *B. cinerea* is thought at least in part to be related to their abilities to generate hydroxyl radicals, which in turn can produce lipid-derived peroxides and aldehydes as a result of degradation of cell membranes (Esterbauer et al., 1991). Cell membranes are dynamic structures that are continually degraded and rebuilt. However, the breakdown products of the polyunsaturated lipids, linoleic and linolenic acids, that are major components of plant cell membranes are extremely labile, cytotoxic and genotoxic (Feng et al., 2003). There is now evidence that some of these toxic products of lipid peroxidation are present in lesions and at the leading edge of expanding soft rots caused by *B. cinerea*.

Lipid peroxidation in plants can be initiated by either lipoxygenase enzymes, or reactive oxygen species, reactions which lead to the formation of either oxylipins or

phytoprostanes (Griffiths et al., 2000). For example, jasmonates are generated as a result of oxidation of linolenic acid by lipoxygenase followed by hydroperoxide dehydratase, hydroperoxide cyclase, 12-oxo-phytodienoic acid reductase, and β -oxidation. Alternatively, free radical catalysed autoxidation of linolenic acid leads to the formation of phytoprostanes, increased levels of which have been seen in tomato plants infected by *B. cinerea* (Thoma et al., 2003).

Although 4-HNE is a normal breakdown product of cell membranes, such breakdown can be increased dramatically during infection, and 4-HNE conjugated to glutathione can be transported via ATP-binding cassette (ABC) transporters that are known to be up-regulated after infection (Xiong et al., 2001). *B. cinerea* itself also possesses ABC transporters that are involved in limiting the sensitivity of *B. cinerea* to phytoalexins and fungicides (Schoonbeek et al., 2001) (Chapter 9, 12).

In *C. annuum* fruits infected with *B. cinerea*, massively elevated levels of 4-HNE and 4-hydroxy-2E-hexenal (4-HHE) were detected within lesions and at lesion margins, compared with the levels seen at positions remote from lesions (Deighton et al., 1999). Similar large increases in 4-HNE and malondialdehyde (MDA) were found adjacent to rotted tissue in leaves of four genotypes of *P. vulgaris* inoculated with *B. cinerea* (Muckenschnabel et al., 2001b), but not in leaves of *A. thaliana* inoculated under similar conditions (Muckenschnabel et al., 2002), where there was an apparent decrease in the levels of these aldehydes as a result of infection. Thus, as was the case with the Fe(III) EPR signals, the results obtained with *A. thaliana* showed fundamental differences from the other plant tissues.

Interpretation of analytical results for lipid peroxidation products is complicated by a number of factors, one of the most important being the ability of *B. cinerea* to utilise/deactivate these molecules. Additional problems in interpreting analytical results stem from the methodology used and the reactivity of the products. For example, many determinations of MDA levels are based on the TBARS method, which has been extensively criticised for its non-specificity (Janero, 1990).

In animals, glutathione S-transferases conjugate 4-HNE with reduced glutathione (GSH) to prevent cell damage (Boon et al., 1999) and a similar process involving glutathione S-transferases may also occur in plant cells. If this occurs on a large scale, presumably it could affect the production of ascorbic acid downstream in the cycle. Muckenschnabel et al. (2001b) found consistently large decreases in the amounts of ascorbic acid in infected leaf tissue of different genotypes of *P. vulgaris*, but GSH levels varied to such an extent between cultivars that no simple conclusion could be made.

10. Host signalling and programmed cell death

There is strong evidence that AOS are associated with several aspects of the plant's response to infection, including the initial oxidative burst and redox-related signalling events leading to up- or down-regulation of sets of genes whose products help to defend the plant from pathogen attack. The initial oxidative burst is not pathogen specific, since it can also be generated abiotically by physical damage (Goodman et al., 2002).

Dat et al. (2001) suggest that enhanced levels of H_2O_2 by themselves are not the direct cause of cell death, but that they trigger a signal transduction cascade that ultimately leads to an active cell death process. In view of the discussion in Section 2 of this Chapter, it seems likely that it is $\cdot OH$ radicals that are the killing agent.

A number of important processes, particularly associated with chloroplasts, can be modulated by redox changes in plants. These include gene transcription (Escoubas et al., 1995; Baginsky et al., 1999), RNA processing (Liere and Link, 1997), translation (Danon and Mayfield, 1994), and protein degradation (Garcia-Ferris and Moreno, 1993). Baginsky et al. (1999) showed that the regulation of plastid gene expression is controlled by phosphorylation and redox in *Sinapis alba*. In *Chlamydomonas reinhardtii*, redox has been shown to regulate RNA degradation in the chloroplast (Salvador and Klein, 1999). The AO (a quinol oxidase) of the respiratory chain in plants and some fungi is regulated by redox-sensitive disulphide bond formation (Djajanegara et al., 1999). Further evidence of gene activation by redox changes is provided by Enyedi (1999) who showed that rose bengal, a photodynamic AOS generator, increased salicylic acid levels in tobacco, induced the expression of the gene *PR-1a* and activated systemic acquired resistance (SAR) throughout the plant. In addition, application of AOS scavengers such as N-acetyl-L-cysteine and pyrrolidine dithiocarbamate prevented accumulation of salicylic acid and diminished *PR-1a* gene expression by rose bengal. Additional evidence that AOS may be involved in signalling comes from the use of free radical scavengers such as salicylhydroxamic acid and propyl gallate that, when applied to carrot slices at the time of inoculation with *B. cinerea*, largely prevented the accumulation of the phytoalexin 6-methoxymellein (Hoffman and Heale, 1989).

Induction of *PR-1* gene expression in tobacco by benzothiadiazole (BTH) or salicylic acid is suppressed by antioxidants (Wendehenne et al., 1998), suggesting that changes in H_2O_2 levels or the redox status of the cell may be involved in the activation of certain defence responses. Indeed, Mou et al. (2003) showed that SAR is regulated by redox changes, and reduction of the SAR-associated protein NPR1 converts it to a monomeric form that is then translocated to the nucleus where it activates gene expression. Changes in redox potential could occur very quickly in plant cells. Thus, the oxidising conditions associated with *B. cinerea* infection could prevent dissociation of NPR1 to the monomeric form thereby preventing induction of an SAR response. Govrin and Levine (2002) showed that *B. cinerea* did not induce SAR in *A. thaliana*, and induction of SAR with chemical treatments, or avirulent *Pseudomonas syringae*, failed to inhibit growth of *B. cinerea*.

Local resistance to *B. cinerea* requires ethylene-, jasmonate-, and SA-mediated signalling (Ferrari et al., 2003). Gene expression studies on *gst1* and *pall* in the mutants *nahG*, *coil* and *etr1* in *Arabidopsis* plants inoculated with *P. syringae* pv. *tomato* suggest that the oxidative burst and signalling via subsequent redox changes are independent of salicylates, methyl jasmonates and ethylene, but are related to a mitogen-activated protein (MAP) kinase (Grant et al., 2000). The MAP kinase AtMPK6 has been shown to be strongly up-regulated in *Arabidopsis* cells by H_2O_2 and potassium superoxide (Yuasa et al., 2001). In addition, the AtMPK6 was also up-regulated by a catalase inhibitor, 3-amino-1,2,4-triazole, suggesting that

AtMPK6 is potentially involved with signal transduction in response to AOS in *Arabidopsis*.

There is increasing evidence that nitric oxide (NO) plays an important role in signalling in response to infection of plants by plant pathogens (Delledonne et al., 1998; Bolwell, 1999). Indeed, a burst of NO has been reported to coincide with an increase in AOS in tobacco treated with cryptogoin from *Phytophthora cryptogea* (Foissner et al., 2000), suggesting that AOS and NO production may be cooperative phenomena. There is some evidence that NO may be capable of acting as an antioxidant in certain situations by binding to and removing AOS (Beligni and Lamattina, 1999), though NO has not been detected in plants as a *Botrytis* infection response.

Damage to cell membranes by the pathogen directly, or as a result of induced free radical generation, also triggers the production of several membrane-bound kinases that are components of signalling pathways that ultimately lead to programmed cell death. This host response is important for defence against biotrophs, but would presumably increase its vulnerability to necrotrophs such as *B. cinerea*.

Some pathogen-derived signals are able to elicit or trigger AOS-associated changes in plants. For example, polygalacturonide elicitors, derived via the action of extracellular pectic enzymes on plant cell walls, induced the accumulation of H₂O₂ in cultured soybean cells (Legendre et al., 1993). This was preceded by a rapid increase in inositol 1,4,5-trisphosphate (IP₃) production, suggesting that phospholipase C activation might constitute one pathway by which elicitors trigger the soybean oxidative burst. The ability of different non-specific elicitors to induce an AOS response indicates that more than one initial receptor may be involved or that some of these elicitors (e.g. arachidonic acid) may be triggering the AOS through different routes (e.g. via a cell death pathway?). Ethylene can be produced *in vitro* by *B. cinerea*, probably via L-methionine as a substrate (Cristescu et al., 2002; Chagué et al., 2002), though the role of ethylene produced by *B. cinerea* during the infection process remains unclear (Chapter 10).

A purified glycoprotein from *B. cinerea*, identified as endopolygalacturonase 1, has been shown to induce a number of resistance responses, including production of AOS (Poinssot et al., 2003). Poinssot et al. concluded that elicitor activity of the endoPG was not due to its enzymatic activity and release of oligogalacturonides, raising the possibility that the induction may be due to the carbohydrate or protein moiety.

Using an assay based upon ability to generate H₂O₂ in cell suspension cultures, ergosterol, and a (partially purified) proteinaceous elicitor from *B. cinerea*, have been shown to rapidly induce the accumulation of non-specific lipid-transfer protein (nsLTP) mRNA in cell suspension cultures of grape (Gomès et al., 2003). Though the function of nsLTP is still unclear, the P4 isoform of the nsLTP (but not the other isoforms tested) reduced the growth of *B. cinerea* mycelium *in vitro*, but only in calcium-free medium. LTPs have been classified as pathogenesis-related proteins, specifically PR-14 (Van Loon and Van Strien, 1999). Gomès et al. (2003) suggested that as high calcium concentrations are found in the apoplast, where the fungus is present, grape nsLTPs probably lack antifungal activity *in planta* and that the

function and mode of action of nsLTPs is still unclear. Nevertheless, this work clearly shows a relationship between a fungal product, H₂O₂ and transcriptional responses by the plant.

11. Fungus-derived metabolites

B. cinerea is known to produce a number of phytotoxic metabolites *in vitro*, with a botryane skeleton including dihydrobotrydial and botrydial that are considered to be pathogenicity factors though not a primary determinant of pathogenicity (Deighton et al., 2001; Colmenares et al., 2002a). Interestingly, botrydial was only found to be phytotoxic when plants were incubated in the light, suggesting a possible link with oxidative processes associated with photosynthesis. Four new lactones have recently been isolated from *B. cinerea* by Colmenares et al. (2002b); one of these was phytotoxic at high concentration (250 mg/litre), but the other three were not phytotoxic. Homobotcinolide, a polyhydroxylated nonalactone esterified with 4-hydroxy-2-decenoic acid, has also been isolated from *B. cinerea* (Cutler et al., 1996) and caused severe chlorosis and necrosis when applied exogenously to corn. The relationship between these phytotoxins, which reproduce symptoms of the disease when applied to plants, and AOS has not been clarified, but knowledge of their location *in planta* would help in understanding their role in disease processes.

12. Conclusions

The production of AOS during infection of plants by *B. cinerea* has been well documented and there is growing evidence that the fungus exploits this situation to aid its development *in planta*. However, understanding the details of the infection process is an extremely complex exercise, which is confounded by a considerable number of apparently conflicting observations in the literature.

A major problem that has restricted the development of our knowledge of this area of science is a lack of conformity in both the chemical and biological techniques used by different groups. To some extent these problems are imposed by the facilities available to individual scientists, but the net result is that it is often difficult to make sensible comparisons between the results. Also, because of the cross-disciplinary nature of this science, it is generally difficult to assemble groups with a sufficiently wide range of expertise, with the result that there is often an uncritical acceptance of the results from some areas of the work. This is particularly true of the approaches used for chemical analyses, where many of the methods used in publications have not been fully validated for biological systems, where the potential for interference can be high. It is important, therefore, in any evaluation of the literature to consider carefully the methodology that has been used and to evaluate critically the validity of conclusions that may be derived from the results of any particular experiment. However, in spite of our reservations discussed above, a number of observations on the *Botrytis* infection process are becoming clear.

One important observation that is coupled with *B. cinerea* infection is the production of oxalic acid. This has the direct effect of acidifying the environment,

and also influences the chemistry of metal ions, either through direct complexation, as with copper or calcium, or by pH-induced speciation changes, as with iron. There seems to be an important correlation between host range for *B. cinerea* and absence of oxalate-degrading oxalate oxidase in the plant. *B. cinerea* also produces siderophores, which are able to form strong chelates with metals such as iron, and thereby directly affect their relative availability to the plant and fungal systems.

Changes in redox potential in infected plant cells may have multiple effects on many enzymes, particularly those containing transition metal ions within their structure. The strongly oxidising conditions coupled with oxalate production may block potential responses such as SAR that require reducing conditions. In addition, the suppression of AOS-scavenging enzymes, which can occur during infection by necrotrophic pathogens, could aid their development in plant tissues.

Short-term fluctuations in the redox status of plant tissues could also influence the susceptibility of the plant to infection, and such processes could be the explanation for the observation of 'optimum times for infection' that occur naturally. Redox changes may also be responsible for the observed variations in resistance of intact tissues to *B. cinerea* as they become older and begin to senesce.

Although some components of the AOS 'system' have been identified, we still do not understand fully how they are regulated. Both fungal and plant transgenics and knockouts are going to be used along with increasingly more sophisticated chemical analytical techniques to provide answers for many of the remaining questions. With increased knowledge it may become feasible to manipulate the plant systems to increase their natural resistance to *B. cinerea*. However, because of the fundamental differences in the colonisation strategies used by necrotrophs and biotrophs, increasing the resistance to one class of pathogen may result in increased susceptibility to the other. We believe, therefore, that although it may be possible to win battles against individual pathogens, the war against the microbial kingdom in general is one that will inevitably have to continue indefinitely.

Finally, whilst *Arabidopsis* is an excellent model plant to study aspects of gene function and transcriptional regulation, there are limitations to the extent that information derived from *Arabidopsis* can automatically be related to plants in other families.

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14. References

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CHAPTER 9

PLANT DEFENCE COMPOUNDS AGAINST *BOTRYTIS* INFECTION

Peter van Baarlen¹, Laurent Legendre² and Jan A.L. van Kan¹

¹ Laboratory of Phytopathology, Wageningen University Plant Sciences, Binnenhaven 5, 6709 PD Wageningen, The Netherlands; ² University of Western Sydney, Centre for Horticulture and Plant Sciences, Locked bag 1797, Penrith South DC, NSW 1797, Australia

Abstract. Plants possess a range of tools for combating a *Botrytis* infection. This chapter will describe three types of pre-formed and induced plant defence compounds and discuss their effectiveness in restricting *Botrytis* infection. Case studies are presented on several types of secondary metabolites: stilbenes including resveratrol, saponins including α -tomatin, cucurbitacins, proanthocyanidins and tulipalin A. Evidence is presented suggesting that *Botrytis* species have evolved mechanisms to counteract some of these defence responses. Secondly, we discuss the role of structural barriers and cell wall modification in preventing penetration. Finally the contribution of PR proteins to resistance is discussed.

1. Introduction

In response to pathogens, plants are generally able to mount a spectrum of defence responses, often coinciding with an oxidative burst involving active oxygen species (AOS) that commonly confers resistance to a wide range of (biotrophic) pathogens. The oxidative burst is however not completely effective against *Botrytis* and there is evidence that the pathogen actually benefits from it (Chapter 8). Nevertheless plants possess a range of tools for combating a *Botrytis* infection. This chapter will describe three types of pre-formed and induced plant defence compounds, namely secondary metabolites, structural barriers and antifungal pathogenesis-related (PR) proteins, and discuss their role and effectiveness in restricting *Botrytis* infection. In addition, mechanisms are discussed that suggest that *Botrytis* species have developed strategies to counteract some of these defence responses. Most of the information comes from research on *B. cinerea* but we will also discuss research on other, host-specialised *Botrytis* species where available and appropriate. The terms ‘compatible interaction’ and ‘incompatible interaction’ will be used for interactions

with a host and a non-host plant species, respectively. This is especially relevant for *Botrytis* species with a narrow host range, but in some cases also for *B. cinerea*.

2. Antimicrobial secondary metabolites

There are two types of antimicrobial metabolites: phytoalexins and phytoanticipins (VanEtten et al., 1994). Phytoanticipins are preformed, while phytoalexins are induced by pathogen infection. Excellent reviews on the phytoalexins identified in plants that are hosts for *Botrytis* spp. have been published by Mansfield (1980) and Daniel and Purkayastha (1995). These reviews also discuss the evidence for specific enzymatic degradation of some of these plant defence molecules, for example the metabolism of wyerone derivatives by *B. cinerea* and *B. fabae* in *Vicia faba*. The following section will discuss case studies of secondary metabolites that display inhibitory activity to *Botrytis*. Their chemical structures are very diverse and their modes of action presumably distinct.

2.1. Resveratrol and other stilbenes

Trans-resveratrol (3,5,4'-trihydroxystilbene) is one of the simplest stilbenes. It is a product of the plant secondary phenolic metabolism by the action of resveratrol synthase on p-coumaroyl-CoA and malonyl-CoA. It occurs in unrelated groups of angiosperms (Morales et al., 2000) and has been studied mainly in grapevine where it is the most abundant stilbene (Creasy and Creasy, 1998). In this species, it constitutes one of the major components of wood (Langcake and Pryce, 1976) and acts as a phytoalexin in leaves (Langcake and Pryce, 1977a; Langcake, 1981; Jeandet et al., 1995a). It is also found in the fruit skin (Jeandet et al., 1991).

Besides *trans*-resveratrol, numerous other stilbenes have been characterized in grapevine. These include a 3-O- β -glucoside of resveratrol called piceid that is formed by the action of a glycosyl transferase on resveratrol (Waterhouse and Lamuela Raventos, 1994; Romero-Perez et al., 1999) and a dimethylated derivative of resveratrol (3,5-dimethoxy-4'-hydroxystilbene) named pterostilbene of which the biosynthetic pathway remains to be resolved (Langcake et al., 1979; Pezet and Pont, 1988a). Piceids are a water-soluble form of resveratrol that can be reconverted into resveratrol by plant glycosidases (Ayran et al., 1987). Stressed or *Botrytis*-infected leaves also accumulate oligomers of resveratrol termed viniferins, the most abundant of which being *trans*- ϵ -viniferin, a resveratrol dehydrodimer (Langcake and Pryce, 1977b; Langcake, 1981) believed to result from the oxidative dimerisation of resveratrol by a plant peroxidase (Langcake and Pryce, 1977c; Langcake, 1981; Morales et al., 1997, 2000) or fungal laccase (Pezet, 1998; Schouten et al., 2002). *Cis* isomers of resveratrol, piceids and viniferins have also been detected in mature fruit and wine (Romero-Perez et al., 1999). They result from the isomerisation of *trans*-resveratrol by UV irradiation (Roggero and Garcia-Parrilla, 1995).

An early study on the antifungal activity of stilbenes revealed that they rapidly inhibit the respiration of fungal cells, probably by acting as uncoupling agents and by forming protein-phenol complexes (Hart, 1981). Based on the structural similarity of hydroxystilbenes and aromatic hydrocarbons, it was inferred that their

mode of action may involve lipid peroxidation by blocking cytochrome c reductase and monooxygenases (Pezet and Pont, 1995). A study on structure-activity relationships of natural stilbenes and synthetic derivatives demonstrated a positive correlation between the biological activity of stilbenes and their hydrophobicity, their ability to form complexes with proteins and the electron-attractivity of their substituents (Pont and Pezet, 1990; Pezet and Pont, 1995). The hydroxystilbenes most active on fungal respiration were pterostilbene and ϵ -viniferin with respective EC₅₀ values of 20 $\mu\text{g/ml}$ (Pezet and Pont, 1988a) and 37 $\mu\text{g/ml}$ (Langcake and Pryce, 1977a).

Resveratrol displayed no immediate toxicity towards *B. cinerea* because of its hydrophilic character and inability to reach its targets in fungal cells (Pezet and Pont, 1995). Long-term incubation of *B. cinerea* with resveratrol, however, led to the inhibition of germination of conidia, as well as the elongation of germ tubes and hyphae (Adrian et al., 1997). Functional analysis of a resveratrol-inducible laccase gene, *Bclcc2*, recently clarified this discrepancy (Schouten et al., 2002). *Bclcc2* gene replacement mutants had an impaired ability to metabolise resveratrol and displayed improved growth characteristics on resveratrol containing media, suggesting that resveratrol is not toxic in itself, but the BcLCC2 protein is responsible for transforming resveratrol into a fungitoxic substance (member of the viniferins). Such a novel mechanism of activation of a phytoalexin presents obvious advantages for the plant. Plant secondary metabolites may be toxic to the plant itself and often have limited solubility. As resveratrol is one of the most soluble and least toxic stilbenes, it can be stored safely at high concentrations in vacuoles and cell walls (Morales et al., 2000) only to be activated upon contact with a microorganism. The activation of resveratrol into viniferins does not necessarily require the conversion by the pathogen. Increased ϵ -viniferin production also occurs in grapevine in the absence of a microbe, after UV irradiation (Langcake and Pryce, 1977b). The content ratios of resveratrol and ϵ -viniferin remained unchanged in several grapevine cultivars after UV irradiation or *B. cinerea* infection, even when their respective concentration increased (Douillet-Breuil et al., 1999; Adrian et al., 2000). Plant enzymes potentially involved in oxidation of resveratrol include peroxidases (Langcake and Pryce, 1977a, b). One constitutive basic grapevine peroxidase exhibits a high affinity for resveratrol (Morales et al., 1997). The peroxidase-mediated oxidation of resveratrol does not yield viniferins *in vitro*. However, the natural association of the benzene rings of resveratrol with cellulose fibres affects the stability of radical reaction intermediates such that peroxidase-mediated oxidation of resveratrol may generate viniferins *in planta* (Morales et al., 2000). Interestingly H₂O₂, a co-substrate of peroxidases, is generated during *B. cinerea* infection (Chapter 8).

Pterostilbene has the highest antifungal activity, but its concentration is less than 5 $\mu\text{g/g}$ in leaves (Douillet-Breuil et al., 1999) and fruit (Adrian et al., 2000) of various grapevine cultivars and is not enhanced by UV irradiation or *B. cinerea* infection. This raised doubts about the capacity of pterostilbene to protect plants against *B. cinerea* (Langcake, 1981; Douillet-Breuil et al., 1999; Adrian et al., 2000). The potency of pterostilbene increases in the presence of glycolic acid, an organic acid that accumulates to high concentrations in immature grape berries.

Pterostilbenes may thus act as a constitutive defence component in berries (Pezet and Pont, 1988b). In contrast, resveratrol acts in an inducible defence reaction against *B. cinerea*. A positive correlation was found between the resveratrol level in grapevine cultivars and their resistance to *B. cinerea* (Langcake and McCarthy, 1979). UV irradiation or *B. cinerea* challenge of leaves and fruit of resistant grapevine cultivars led to resveratrol concentrations up to 750 µg/g, exceeding levels needed for toxicity to *B. cinerea in vitro* (Jeandet et al., 1995a; Douillet-Breuil et al., 1999; Adrian et al., 2000). Local resveratrol concentrations may be more important than those found after grinding whole tissue. The interaction of grapevine leaves with an incompatible *B. cinerea* isolate led to a hypersensitive-like response during which stilbenes preferentially accumulated in the direct vicinity of the fungus (Derckel et al., 1999).

Raising resveratrol levels in crop plants is an attractive option because of the potential of this substance to protect plants from the attack of pathogens and to improve human health (Chiou, 2002) by preventing and curing cancers (Savouret and Quesne, 2002) and guarding against vascular diseases (Hung et al., 2000). In plant species that do not naturally produce stilbenes, the production of resveratrol was achieved by genetic engineering. Introduction of the grapevine stilbene synthase gene *Vst1* into tobacco (Hain et al., 1993) and barley (Leckband and Lorz, 1998) resulted in resveratrol accumulation and enhanced protection against grey mould. In transgenic kiwifruit, however, the presence of high endogenous levels of glycosyl transferase activity led to the preferential accumulation of piceid over resveratrol and no protection to *B. cinerea* infection was observed (Kobayashi et al., 2000). In plants that naturally have the genetic potential to produce resveratrol, the expression of stilbene synthase is low in unstressed leaves. It can be induced in this organ by a variety of biotic and abiotic elicitors such as AlCl₃ (Adrian et al., 1996; Jeandet et al., 1998), UV, paraquat, wounding, H₂O₂, salicylic acid, jasmonic acid, ethylene (Grimmig et al., 1997; Adrian et al., 2000; Lippmann et al., 2000; Chung et al., 2003), methyl jasmonate (Larronde et al., 2003), systemic acquired resistance elicitors (Busam et al., 1997), abscisic acid (Ban et al., 2000) and ozone (Grimmig et al., 1997). The exogenous application of *B. cinerea* cell wall fragments (Liswidowati et al., 1991) or a live soil-borne biocontrol *Bacillus* species (Paul et al., 1998) have also been reported to lead to increased accumulation of resveratrol in grapevine leaves.

Fruit, such as grape berries, behave differently from leaves by expressing significant levels of resveratrol constitutively. One concern is that resveratrol levels decrease during fruit ripening due to lower expression of stilbene synthase (Bais et al., 2000) and increased substrate competition for this enzyme with chalcone synthase, whose activity is stimulated during fruit ripening to provide precursors for anthocyanin accumulation (Jeandet et al., 1995b). Nevertheless, grapes keep their capacity to accumulate higher levels of resveratrol after UV irradiation at any stage of ripening (Adrian et al., 2000) and even after harvest (Cantos et al., 2001). Another concern with resveratrol accumulation in grape is that it only occurs in the skin, implying that resveratrol can only participate in protection of the berry from outside intruders when the fruit skin is intact. Cultivation practices have also been shown to influence resveratrol accumulation in grapevine leaves and fruit. A

negative correlation was found between the resveratrol level and the extent of nitrogen fertilization (Fregoni et al., 2000; Bavaresco et al., 2001) or fungicide spray application (Magee et al., 2002) while increased potassium fertilization correlated with higher levels of resveratrol (Fregoni et al., 2000). The potential of grapevine cultivars to accumulate resveratrol was positively correlated with their tolerance to *Botrytis* (Douillet-Breuil et al., 1999), leading to the suggestion that resveratrol production capacity could be used as a criterion in the selection of resistant varieties (Pool et al., 1981; Creasy and Coffee, 1988; Sbaghi et al., 1995).

The ability of *B. cinerea* to cope with stilbenes should not be underestimated. Several studies did not corroborate a relationship between resveratrol accumulation and disease incidence in grapevine (Magee et al., 2002; Keller et al., 2003). Increasing conidia concentration in synthetic media containing resveratrol led to increased resveratrol catabolism and survival of conidia (Hoos and Blaich, 1990) and a correlation between resveratrol detoxification capacity of *B. cinerea* isolates and their virulence on grapevine leaves has been proposed (Sbaghi et al., 1996). Detoxification of resveratrol and the related pterostilbene has been attributed to their oxidative dimerisation by *B. cinerea* laccases (Hoos and Blaich, 1990; Pezet et al., 1991; Sbaghi et al., 1996; Adrian et al., 1998; Breuil et al., 1999). Application of cucurbitacins led to reduced production of laccases (Sect. 2.3) and correlated with increased resistance of several hosts, even those that do not accumulate stilbenes, such as carrot and cucumber (Bar-Nun and Mayer, 1990; Viterbo et al., 1993a). This suggested that *B. cinerea* laccases are part of a more general 'attack' machinery designed to detoxify phenolic defences from many host plants (Staples and Mayer, 1995). However, the product of one specific laccase gene, BcLCC2, has been implicated in the opposite effect by activating resveratrol into a fungitoxic substance (Schouten et al., 2002). Laccase production by *B. cinerea* increases during late stages of infection or development (Roudet et al., 1992; Manteau et al., 2003) in parallel with a decrease in resveratrol concentration in its host (Adrian et al., 2000; Montero et al., 2003) and can be stimulated *in vitro* by host-derived substances such as phenolics, pectins (Viterbo et al., 1993a) and ambient pH (Manteau et al., 2003). Proanthocyanidins in fruit act as competitive laccase inhibitors (Pezet et al., 1992).

Three laccase genes were described so far (Schouten et al., 2002) and limited information is available on their distribution among *B. cinerea* populations and their physiological role during pathogenesis. This role may prove to be complex since the incubation of *B. cinerea* with resveratrol has been shown to lead to the production of an array of oxidised derivatives, including the fungitoxic ϵ -viniferin (Langcake and Pryce, 1977a; Keller et al., 2003) and six other dimerisation products: δ -viniferin (Breuil et al., 1998; Pezet et al., 2003a), leachinol F, pallidol and restrytisols A-C (Cichewicz et al., 2000). Some information is available on the toxicity of these individual, pure metabolites towards *B. cinerea*, but one should consider that such *in vitro* tests may not reflect the chemical environment that the pathogen encounters during infection. Laccase activity on resveratrol yields radical quinone intermediates that may react with very distinct (phenolic and other) compounds, giving rise to heterogeneous products. The metabolism of resveratrol *in planta* therefore depends on the temporal and spatial distribution both of the fungal laccase(s), the plant peroxidase(s), their substrate(s), as well as on the enzyme kinetics for each substrate.

2.2. α -Tomatine and saponins

α -Tomatine is a secondary metabolite produced in tomato leaves and unripe fruit (Friedman, 2002). It is a potent antifungal (e.g. Sandrock and VanEtten, 1998) and insecticidal (e.g. Kowalski et al., 2000) compound that interacts with sterols in membranes (Keukens et al., 1995). Already in the 1970s it was reported that α -tomatine inhibits mycelial growth of *B. cinerea* while not affecting germination of conidia (Verhoeff and Liem, 1975). It was proposed that its fungistatic action was responsible for maintaining *B. cinerea* infections in a quiescent state. The reduction in α -tomatine content in ripening fruit (Friedman, 2002) supposedly relieves fungistasis and permits fungal outgrowth. Furthermore, Verhoeff and Liem (1975) reported that *B. cinerea* was able to convert α -tomatine to tomatidine by sugar hydrolysis, thereby actively detoxifying the compound and facilitating fungal outgrowth into tomato tissue prior to the natural drop in α -tomatine levels.

Supporting evidence for the important role of α -tomatine in conferring resistance towards *B. cinerea* came from work of Quidde et al. (1998). Among a set of 13 isolates, one field isolate was identified that lacked α -tomatine degrading ("tomatinase") activity. This isolate, designated M3 and originating from grape, was able to form primary lesions on tomato but these lesions never expanded. Virulence of isolate M3 on *Phaseolus vulgaris* was not affected (Quidde et al., 1998). Contrary to the report of Verhoeff and Liem (1975), the α -tomatine degradation product was identified as β -tomatine (Quidde et al., 1998). On the basis of this conversion it was proposed that the tomatinase should possess xylosidase activity. Quidde et al. (1999) cloned a *B. cinerea* gene homologous to the tomatinase gene *tom1* from *Septoria lycopersici*. The gene, designated *sap1*, encodes a β -glucosidase active on saponins. Gene replacement mutants deficient in *sap1* lost the ability to degrade avenacin (a phytoanticipin from roots of oat, a non-host species for *B. cinerea*), yet they remained able to detoxify tomatin, digitonin and avenacoside (a phytoanticipin from leaves of oat). *sap1*-deficient mutants remained able to infect tomato, suggesting that the *sap1* gene does not encode the true tomatinase. Thus *B. cinerea* can produce at least three distinct saponin-specific glycosidases (Quidde et al., 1999).

2.3. Cucurbitacins

Cucurbitacins are bitter-tasting triterpenoids, produced by the Cucurbitaceae, that are toxic to insects and mammals. There are at least six forms of cucurbitacins, one of which antagonises insect steroid responses (Dinan et al., 1997) while another form disrupts the cytoskeleton in carcinoma cells (Duncan et al., 1996). Application of cucurbitacin I to host tissue prior to inoculation prevented infection by *B. cinerea* (Bar-Nun and Mayer, 1990). When cucurbitacin was added to a *B. cinerea* liquid culture, laccase secretion into the medium was reduced (Bar-Nun and Mayer, 1989) while other enzymes were not affected (Viterbo et al., 1993a). Some cucurbitacin forms were more effective than others (Viterbo et al., 1993b). Radio-labelled cucurbitacin was taken up by *B. cinerea* mycelium suggesting that it acted intracellularly, reducing laccase activity (Viterbo et al., 1994), supposedly as a consequence of repression at the mRNA level (Gonen et al., 1996). These gene

expression studies were however performed with a probe corresponding to the *Bclcc1* gene, whereas the laccase gene that is induced by gallic acid is in fact *Bclcc2* (Schouten et al., 2002). The two genes show poor cross-hybridisation (A. Schouten, unpubl.). Neither of these laccase genes is important for virulence on a range of hosts (Schouten et al., 2002). It remains to be resolved whether the reduction of *B. cinerea* infection by application of cucurbitacins (Bar-Nun and Mayer, 1990) is caused indirectly by activation of defence or directly by inhibiting virulence factors.

2.4. Proanthocyanidins

Proanthocyanidins (condensed tannins) are polymeric flavonoids that result from the condensation of two or more derivatives of flavan-3,4-diol. The term was first coined by Weinges et al. (1969) to designate these colourless tannins that form intensely coloured anthocyanidins upon heating with acid. Proanthocyanidins are widely distributed in the plant kingdom and are constitutive components in a number of discrete tissues in most plant organs. In leaves, they are mostly present in vascular tissue and in fruit they preferentially accumulate in the epidermis and seeds (Porter and Schwartz, 1962; Bachmann and Blaich, 1979; Hills et al., 1981; Jersch et al., 1989; Prieur et al., 1994; Prusky, 1996; Souquet et al., 1996). The chemical structure and composition of proanthocyanidins vary among plant species, organs and also with the stage of organ development. In grape berries, the mean degree of polymerisation of the proanthocyanidins, the proportion of epigallocatechin extension subunits and the level of anthocyanin association increase during ripening (Hills et al., 1981; Kennedy et al., 2001).

Because of the potential association of proanthocyanidins with sugars (including pectins), flavonoids and proteins (Kennedy et al., 2001), reports on their toxicity towards *B. cinerea* (conidia germination and germ tube elongation) have yielded opposite results depending on the state of purity of the proanthocyanidins (Hills et al., 1981; Jersch et al., 1989; Hebert et al., 2002). Nevertheless, these studies agree in suggesting that plant proanthocyanidins maintain *B. cinerea* in a quiescent stage, leading to delayed development of symptoms. The transition from quiescence into expansion is triggered during host senescence or ripening and occurs at a less senescent or ripe stage in susceptible varieties (reviewed by Elad, 1997). For example, grapes develop latent infections as early as the blooming stage (McClellan and Hewitt, 1973) while lesions are increasingly likely to start spreading only when the fruit ripens (Hills et al., 1981). The development of quiescent *B. cinerea* infections on grape did not correlate with conidia density (Coertze and Holz, 1997), the stage of ripening (Hills et al., 1981) or the level of resistance of the cultivar to grey mould (Pezet et al., 2003b). Even though the production of phytoalexins after pathogen entry is one way to keep *B. cinerea* quiescent (Sect. 2.1), some fruit, such as strawberries, do not mount a phytoalexin response (Jersch et al., 1989), implying that fungal quiescence can be maintained by additional mechanisms. These may involve the inhibition of fungal pathogenicity factors by proanthocyanidins (Prusky, 1996). *B. cinerea* virulence factors include a range of secreted enzymes (Chapter 7) and proanthocyanidins can complex with proteins (Haslam, 1966, 1974) resulting in non-specific inhibition of the activity of fungal polygalacturonases and

cellulases (Porter and Schwartz, 1962; Hills et al., 1981; Jersch et al., 1989). Proanthocyanidins additionally act as competitive inhibitors of *B. cinerea* laccase, thereby preventing detoxification of the phytoalexin pterostilbene. Pezet et al. (1992) reported an EC₅₀ value of 12 µg/ml for the inhibition of *B. cinerea* laccase by grape skin proanthocyanidins. Their levels in grape skin vary from 50 to 250 µg/g dry weight (Hills et al., 1981; Pezet and Pont, 1992) and 14-50 µg/g fresh weight in strawberry (Jersch et al., 1989) depending on the cultivar or the stage of ripening.

Despite the fact that proanthocyanidins do not accumulate in all plant tissues, it is suggested that endogenous proanthocyanidins contribute to maintaining *B. cinerea* in a quiescent state. More tolerant grape and strawberry cultivars accumulate larger quantities of proanthocyanidins (Hebert et al., 2002; Pezet et al., 2003b). Proanthocyanidin content decreases in grape during fruit ripening (Hills et al., 1981) while they remain constant in strawberries (Jersch et al., 1989). However, the ripening-related modifications of proanthocyanidins led to a decrease in their capacity to inhibit *B. cinerea* macerating enzymes (Hills et al., 1981; Jersch et al., 1989; Pezet et al., 1992; Pezet and Pont, 1992). This phenomenon parallels the loss of resistance of these fruit during ripening (Hills et al., 1981; Jersch et al., 1989) and occurs to a larger extent in susceptible cultivars (Pezet et al., 2003b). Finally, the addition of proanthocyanidin at 0.1% at the point of inoculation blocked the development of spreading lesions of *B. cinerea* in susceptible grapevine varieties (Hills et al., 1981).

Prolonging the quiescence of *B. cinerea* infections by increasing the proanthocyanidin content would reduce losses to grey mould, especially after harvest. However, proanthocyanidin levels are constitutive and are not known to be subject to modulation by external elicitors. Moreover, knowledge is lacking on the genes and enzymes involved in the subtle modifications of proanthocyanidin structure that affect their biological activity. The use of proanthocyanidin content as an indicator of grey mould resistance for the selection of cultivars with improved shelf-life has been suggested for grape (Pezet et al., 2003b) and strawberry (Jersch et al., 1989; Hebert et al., 2002). However, proanthocyanidins negatively affect the taste and colour (via anthocyanin binding) of fresh produce and plant-derived food products (Noble, 1990; Gawel, 1998). Attempts to modify their composition may result in a modification of the perception of these products by consumers.

2.5. Non-host resistance

2.5.1. Phytoanticipins of tulip as mediators of *Botrytis* non-host resistance

Tulip bulbs and pistils contain high concentrations of fungitoxic compounds, identified as lactones and termed tulipalins (Bergman et al., 1967; Beijersbergen, 1969). Tulipalin A is found in bulbs, whereas aerial plant parts contain mainly tulipalin B. Tulipalins A and B are stored in a glycosylated form, named tuliposides A and B. Tuliposides are less toxic to fungi than the corresponding aglycosylated lactones and are lactonised into tulipalins when the pH exceeds 5. Plant enzymes may enhance the conversion rate of tuliposides into tulipalin A (Beijersbergen and Lemmers, 1972). The lactone itself is relatively stable *in vitro* at pH values between 5.4 and 6.5 (Beijersbergen, 1969). It was suggested that precursor tuliposides, stored

in acidic vacuoles, are released upon disruption of the vacuolar membranes during cell collapse mediated by pathogens (Schönbeck and Schlösser, 1976). The lactones themselves spontaneously hydrolyse at pH values above 7.5 into the corresponding non-toxic butyric acids (Beijersbergen, 1969).

All *Botrytis* species tested so far are sensitive to pure tulipalin A, except for the tulip-specific pathogen *B. tulipae* (Schönbeck and Schroeder, 1972). Partial or complete fungistasis occurs *in vitro* at 3-5 μM . Higher doses (7-10 μM , more than 30 μM for *B. tulipae*) are lethal to *Botrytis* spp. Mycelium exposed to lethal tulipalin A concentrations does not resume growth when transferred to fresh agar without tulipalin. Microscopic observation of tulipalin A-treated mycelium showed a disappearance of hyphal cell contents and altered autofluorescence of hyphal walls as compared to vital cultures. Conidia are at least three times more sensitive to tulipalin A than mycelium (P. van Baarlen and M. Staats, unpubl.). *B. tulipae* is more tolerant to tulipalin A than other *Botrytis* species. Infection of tulips by *B. tulipae* is associated with a conversion of tuliposides into the corresponding, inactive hydroxylic acids. In contrast, infection by *B. cinerea* leads to a conversion into the active lactones (Schönbeck and Schroeder, 1972). This suggests that *B. tulipae* contains a factor that can hydrolyse the tuliposides or the lactones into the corresponding hydroxylic acids. Indeed, a total protein extract from *B. tulipae* mycelium was able to neutralise tulipalin A; incubation of tulipalin A with this protein extract resulted in loss of toxicity towards sensitive *B. cinerea* isolates (P. van Baarlen and M. Staats, unpubl.). Lactone detoxification was also reported in *Fusarium oxysporum* isolates that produce a lactonohydrolase (Shimizu et al., 1992). It is tempting to speculate that *B. tulipae* possesses an enzyme with similar activity. Such an enzyme might act as a host specificity determinant for *B. tulipae*.

2.5.2. Other monocot secondary metabolites involved in non-host resistance

The genus *Allium* contains various compounds associated with resistance to fungal disease. Some are constitutive inhibitors, such as the phenolic compound catechol that is present in the outer bulb layers of pigmented *Allium cepa* (onion) cultivars where it confers resistance to *Colletotrichum* (Link and Walker, 1933). Onion also produces a class of cyclopentane phytoalexins upon pathogen infection, designated tsibulins (Dmitriev et al., 1990), which accumulate in bulb scales at infection sites during incompatible interactions with *B. cinerea*. Tsibulins inhibited spore germination and germ tube elongation of *B. cinerea* *in vitro*. The ED_{50} values were lower than the actual phytoalexin content in bulb scale spots, where *B. cinerea* lesion formation was restricted (Dmitriev et al., 1990). Only little accumulation of cyclopentane phytoalexins was observed in onion bulb scales during infection by the specialised pathogen *B. allii* (Dmitriev et al., 1990). *B. allii* seems to either suppress tsibulin accumulation, analogous to the interaction of *B. narcissicola* and its host narcissus (discussed below) or actively degrade tsibulins, as discussed for other antifungal compounds above. It would be of interest to investigate whether *B. allii* is able to break down tsibulin cyclopentanes.

3. Tolerance of *Botrytis* to antifungal metabolites

In many cases *Botrytis* species have adapted to antifungal compounds produced by their host plants by developing mechanisms to counteract their toxicity. In fact the ability to counteract toxicity of phytoalexins and phytoanticipins is often crucial for successful host colonization. As a first line of defence to toxic compounds, fungi possess ATP-binding cassette transporters (ATR) and Major Facilitator (MFS) proteins that are able to excrete a spectrum of chemically unrelated toxic metabolites from the cytoplasm. ATR and MFS proteins serve as membrane pumps with a broad substrate range that expel chemically heterogeneous antifungal compounds at the expense of ATP or proton extrusion (reviewed by de Waard, 1997). These proteins may also be involved in fungicide resistance (Chapter 12). *B. cinerea* possesses a large family of functional transporter genes, some of which confer protection to antibiotics (Schoonbeek et al., 2002) and plant defence metabolites including resveratrol (Schoonbeek et al., 2001). The degree of tolerance that ATR and MFS transporters confer towards these toxicants is in several cases small, only rarely do they provide full resistance. This low degree of tolerance may however be biologically significant as it allows the pathogen time to activate true enzymatic detoxification mechanisms. Such true detoxification mechanisms are specific for each individual antifungal compound, as discussed in Sect. 2.

4. Structural barriers and cell wall modifications

Structural barriers can be mounted during resistance responses and non-specific wound responses (Heath, 2000, 2002) by means of incorporating and cross-linking phenolic compounds at penetration sites. Cell wall modifications may directly pose physical barriers for fungi and they may interfere with degradation of wall components that function as nutrient sources for *Botrytis* species. One form of plant cell wall modification associated with disease resistance is their lignification. The phenylpropanoid pathway generates coumaryl Co-A and cinnamyl Co-A esters that serve as precursors for diverse compounds, including lignin (Dixon and Paiva, 1995). Wall modification occurs in the incompatible interaction of *B. cinerea* with a non-host, wheat. Challenging wheat leaves with *B. cinerea* conidia results in a localised, sharp increase of sinapyl alcohol dehydrogenase, phenylalanine ammonia lyase and peroxidase expression and progressive lignification (Mitchell et al., 1994). The phenylpropanoid pathway also generates phytoalexins (discussed above). The simultaneous formation of structural barriers and phytoalexin production blocks the infection and lesion spread of *B. cinerea* during incompatible interactions with carrot, narcissus and wheat (Garrod et al., 1982; O'Neill and Mansfield, 1982; Mitchell et al., 1994). The relative contribution of wall modifications and phytoalexin production to the effective inhibition of infection is often unclear.

Other wall modifications are established by a class of phenolic amides, mainly hydroxycinnamic acid derivatives and tyramine (McLusky et al., 1999). The synthesis and incorporation in cell walls of amides is associated with the inhibition of outgrowth of *B. allii* germ tubes within modified infection sites (Stewart and Mansfield, 1985). The amides that are produced in onion cells are not fungitoxic to

B. allii and *B. cinerea* conidia and germ tubes up to concentrations of 1 mM (McLusky et al., 1999). The fact that amide synthesis and their incorporation into cell walls were associated with attempted penetrations of *B. allii*, a compatible pathogen of onions, suggests that this defence mechanism provides a general protection against *Botrytis*. The efficiency of the resistance mechanism may depend on spore concentration, H₂O₂ availability and occurrence of other stress factors.

The cell wall modification response (Heath, 2000) has been studied in lily and tulip cultivars in compatible and incompatible interactions, with *B. cinerea*, *B. elliptica* or *B. tulipae*. Cell wall modification was observed during incompatible interactions but not during compatible interactions (P. van Baarlen and J. van Kan, unpubl.). Autofluorescence of cell walls at penetration sites occurred within 18 h upon inoculation of tulip and lily leaves with dry conidia. Fluorescent vital staining of plant cells showed that in an incompatible interaction, the non-host plant cells were not killed upon expression of this resistance response. Germ tubes did not penetrate the modified walls any further. Primary lesion formation was not observed in incompatible interactions, suggesting that wall modifications can effectively restrict *Botrytis* infection. A more extensive study of wall modifications during compatible and incompatible plant-*Botrytis* interactions seems worthwhile, although it will remain difficult to distinguish the contribution to resistance of cell wall modification from that of the concomitant phytoalexin production.

Several methods by which *B. cinerea* could interfere with cell wall resistance responses may be envisaged. The pathogen may actively suppress resistance responses in a compatible interaction. Preliminary microscopy studies have shown that lily cells, at the sites of penetration by *B. elliptica*, did not show the characteristic local yellow cell wall autofluorescence observed in incompatible interactions. Also in the compatible interaction of narcissus and *B. narcissicola*, cell wall modifications were mostly absent (O'Neill and Mansfield, 1982). Both phytoalexin production and wall modification depend upon the phenylpropanoid phenolic acid pathway. It is conceivable that in a compatible plant-*Botrytis* interaction, the cell wall modifications and phytoalexin production are suppressed by (a) secreted fungal factor(s). One study has shown that during a compatible interaction of the cowpea rust fungus (*Uromyces vignae*) with its host, cell wall-associated defence responses are suppressed (Heath, 2002). In this pathosystem, suppression of defence is established through interference with wall-membrane communication via secreted fungal peptides that interfere with integrin proteins that mediate wall-membrane adhesion (Mellersh and Heath, 2001). We are, however, not aware of literature reporting active suppression of defence by *Botrytis*.

5. Pathogenesis-related proteins

Pathogenesis-related proteins (PR proteins) represent a large array of proteins coded by the host plant that are co-ordinately expressed under pathological or related situations. They have been characterised in over 70 plant species and 13 plant families including mono- and di-cotyledonous plants. They are extremely diverse in terms of enzymatic and biological activity and have been grouped into 13 protein families based on primary structure and serological relationships (Van Loon, 1999).

All share several physicochemical properties such as solubility in acidic buffers, resistance to proteolysis, a molecular mass less than 50 kDa and a lack of quaternary structure and glycosylation (Stintzi et al., 1993). They primarily accumulate in plant cell walls and vacuoles. *B. cinerea* infection leads to PR protein induction in many plants (Van Loon, 1985). Fruit tissues differ significantly from other plant organs by accumulating unusually high concentrations of a limited set of PR-like proteins that share sequence similarities with known PR proteins but accumulate with fruit development (most often during fruit ripening) and not after stimulation with pathogenesis-derived signals such as salicylic acid or wounding (Derckel et al., 1998). Grapes, for example, mainly accumulate one thaumatin-like protein and one chitinase which make up 80% of the total soluble protein content of the fruit at harvest (Derckel et al., 1998; Salzman et al., 1998; Waters et al., 1998).

Members of several PR protein families display some toxicity towards *B. cinerea in vitro*. For some of them, this may be caused by their potential to degrade chitin and β -glucan fragments of *B. cinerea* cell walls (Gomez-Miranda et al., 1981; Punja and Zhang, 1993; Simmons, 1994). However, fungitoxicity varies greatly among members of one PR protein family, just like their specific enzyme activity which may differ by up to 250-fold towards a given substrate (Kauffmann et al., 1987; Stintzi et al., 1993). A grape PR-like protein (chitinase) has one of the highest botryticidal activities. It inhibits germination of conidia with an EC_{50} value of 7.5 μ g/ml (Derckel et al., 1998) and it restricts the elongation of hyphae (Salzman et al., 1998). The chitinase is present at levels up to 26 μ g/g in fruit of resistant cultivars (Derckel et al., 1998; Salzman et al., 1998). The grape thaumatin-like protein, on the other hand, exhibited no toxicity towards *B. cinerea in vitro* (Salzman et al., 1998). In the presence of 1M glucose (a physiological concentration for a ripe grape), however, the toxicity of the grape chitinase was potentiated by as much as 70% and the grape thaumatin-like protein became equally toxic as the chitinase at similar concentrations (Salzman et al., 1998).

Despite their anti-microbial activity *in vitro*, there is little evidence to support a potential role of PR proteins in effective plant disease resistance to *B. cinerea*. The homologous or heterologous expression of PR proteins in transgenic plants, or the infiltration of single PR proteins in leaves, has rarely led to any significant level of protection against *B. cinerea* (Lawton et al., 1993; Datta et al., 1999; Neuhaus, 1999; Van Loon, 1997). Only the coordinated expression of members of different PR protein families yielded some (though limited) degree of tolerance due to synergistic effects (Zhu et al., 1994; Jach et al., 1995; Datta et al., 1999). Additionally, treatments that stimulate the coordinate expression of a large array of PR proteins in tomato leaves such as ethylene treatment (Diaz et al., 2002) or *B. cinerea* primary lesion formation (Benito et al., 1998) did not prevent the development of *B. cinerea* spreading lesions. To the contrary, a higher degree of susceptibility (higher fungal growth as estimated by the level of fungal actin mRNA) correlated with higher levels of PR proteins mRNAs (Diaz et al., 2002). In any case, the commercial potential of plants exhibiting higher levels of PR proteins will be hampered by the fact that PR proteins are associated with several undesirable effects such as the formation of haze in grape juices (Waters et al., 1996) and allergenic reactions (Breiteneder and Ebner, 2000; Hoffmann-Sommergruber, 2000).

6. Conclusions

There are interesting perspectives for altering secondary metabolism in plants to optimise the contents of antifungal compounds, either by transgenes or by classical breeding. The example of enhanced *Botrytis* resistance in transgenic tobacco producing resveratrol (Hain et al., 1993) already provides evidence for its feasibility. Now that the field of plant pathology and breeding has entered the genomics era, it will be increasingly feasible to modulate the levels of secondary metabolites, such as phenylpropanoids, to enhance defence (Dixon et al., 2002). The information on the *Botrytis* genome will be a useful tool. When plants can be equipped with antifungal metabolites for which *Botrytis* has no detoxifying enzymes, it is likely that these metabolites will be more effective. Conversely, when *Botrytis* possesses enzymes that may convert non-toxic metabolites into antifungal compounds (Schouten et al., 2002) one can perhaps make use of these enzymes. When considering the options for enhancing plant resistance to *Botrytis* by modulation of secondary metabolites, it should be taken into serious account that it is often not required to achieve complete resistance as conferred by the classical R-genes. Especially in the case of post-harvest problems, it will be sufficient to delay the disease outbreak and attenuate disease out-growth. We recommend the design of strategies that extend the latent infection phase. Chapter 20 deals in more detail with novel approaches to reduce the damage inflicted by *Botrytis* diseases.

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CHAPTER 10

PHYTOHORMONES IN *BOTRYTIS*-PLANT INTERACTIONS

Amir Sharon¹, Yigal Elad², Radwan Barakat³ and Paul Tudzynski⁴

¹Department of Plant Sciences, Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel;

²Department of Plant Pathology, The Volcani Center, Bet Dagan 50250, Israel; ³Department of Plant Production and Protection, College of Agriculture, Hebron University, P.O. Box 40, Hebron, Palestinian Authority; ⁴Institut für Botanik und Botanischer Garten, Westfälische Wilhelms-Universität 3, D-48149 Münster, Germany

Abstract. Several lines of evidence suggest that plant hormones are involved in mediating *Botrytis* interaction with plants. External treatments with some plant hormones such as auxins and gibberellins can suppress disease development, while ethylene and abscisic acid seem to enhance the disease. Increased ethylene levels by *Botrytis* infection are well documented. Not only the plant, but also the fungus is capable of producing different hormones and fungal development may be influenced by these hormones. Little direct evidence is available on the involvement of plant hormones in vegetative and pathogenic *Botrytis* development. Most of the data come from studies on the production of ethylene in infected plants, on its possible effect on the disease and on ethylene production by *Botrytis*. Production of other plant hormones by *Botrytis* and their possible role in disease and fungal development have hardly been studied. The production of various plant hormones in *Botrytis*, and the effect that they may have on disease and fungal development are reported.

1. Introduction

Plant hormones (phytohormones) are naturally occurring substances that at low concentration control various stages of plant growth and development. The important plant hormones are auxins, gibberellins, cytokinins, ethylene and abscisic acid. All classes of plant hormone have also been found in microorganisms (Tudzynski, 1997; Tudzynski and Sharon, 2002). The physiological condition of plant tissue affects susceptibility to infection and disease development. Plant hormones are involved in mediating a plant's susceptibility to pathogens. Hormone biosynthesis, transport, metabolism and action, as well as host tissue sensitivity to hormones, all contribute to the hormonal homeostatic balance of the tissue. This balance affects plant susceptibility to pathogen development and infection, and may change hormonal levels in the host tissue. Furthermore, pathogen susceptibility to plant hormones may affect its behaviour before and after infection of the plant (Elad

and Evensen, 1995; Elad, 1997). This chapter will describe the involvement of plant hormones in the interaction of *Botrytis cinerea* with plants. Ethylene is described in detail compared with the other hormones because of the much larger extent of published data that is available on its biosynthesis, the effect on pathogen development, and association with *Botrytis*-incited diseases. We will summarize the current knowledge on the biosynthesis and influence of the three major plant hormones: the auxin indole-3-acetic acid (IAA), gibberellic acid (GA₃), abscisic acid (ABA) and cytokinins in *Botrytis*.

2. Biosynthesis of plant hormones by *B. cinerea*

2.1. Ethylene

Qadir et al. (1997) have shown that *B. cinerea* produces ethylene in shake cultures. They also found that ethylene production was methionine-dependent, but were unable to determine which enzymes were involved in ethylene biosynthesis. In higher plants ethylene is produced from methionine through the intermediate S-adenosyl methionine (AdoMet or SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) (Adams and Yang, 1981; Johnson and Ecker, 1998). This pathway is uncommon in microorganisms and has been described in only a few fungal species (Amagai and Maeda, 1992; Jia et al., 1999). Two other ethylene biosynthetic pathways are known in bacteria and fungi. In the first pathway, 2-oxoglutarate is converted to ethylene by a multi-function enzyme called ethylene-forming enzyme (EFE). In the other pathway methionine is deaminated to produce α -keto γ -methylthiobutyric acid (KMBA) and ethylene is produced by spontaneous or enzymatic oxidation of KMBA (Yang, 1969). Chagué et al. (2002) and Cristescu et al. (2002) showed that in *B. cinerea* ethylene was produced from methionine through KMBA, but not through the ACC pathway. Ethylene production was light dependent: when the fungus was grown in the dark no ethylene was produced, but when the dark-grown cultures or culture filtrates were exposed to light they released large amounts of ethylene (Chagué et al., 2002). These and other results showed in *B. cinerea* that ethylene is released by photo-oxidation of KMBA produced from methionine and then secreted to the medium.

2.2. Auxins

Auxins are probably the most important group of plant growth regulators. Biosynthesis and function of the major auxin in plants, IAA, has been intensively studied for almost a century. In most plant species IAA is produced from tryptophan through the intermediate indole-3-pyruvic acid (IPA) but IAA can also be synthesized through additional pathways including tryptophan-independent pathways. Despite intensive efforts, none of the suggested pathways of IAA biosynthesis has been unequivocally established in plants. One reason for that is the complexity by which IAA levels are regulated in plants, which involves multiple pathways of IAA biosynthesis, conjugation of IAA to larger molecules, hydrolysis

of IAA conjugates by a variety of specific enzymes, and IAA oxidation (Bartel, 1997; Normanly and Bartel, 1999).

Four IAA biosynthesis pathways have been characterized in bacteria (Patten and Glick, 1996). Activities of the enzymes that compose these pathways were confirmed and the corresponding genes cloned from several bacterial species. The two primary IAA pathways identified in bacteria are the indole-3-pyruvic acid (IPA) pathway and the indole-3-acetamide (IAM) pathway, which is rare in plants. Most fungal species have the capacity to produce IAA, but relatively little information is available on the metabolic pathways of IAA biosynthesis in fungi. The IPA pathway has been identified in a few species, but only two IAA biosynthesis genes from *Ustilago maydis* have been analysed so far (Basse et al., 1996). Furukawa et al. (1996) found that fungi belonging to the genus *Rhizoctonia* converted tryptophan to IAA. Feeding experiments using indole precursors confirmed the activity of the IPA pathway in *Rhizoctonia*. No other IAA biosynthesis pathways were identified in this fungus. The bacterial pathway (through IAM) has been reported so far only in *Colletotrichum* (Robinson et al., 1998).

Tapani et al. (1993) reported that the mycelium of *B. cinerea* contained 128 ng/g IAA while less than 1 ng/ml was detected in the medium. However, we analysed IAA production by 30 *B. cinerea* isolates and found that most of the IAA was secreted to the medium (S. Haskin and A. Sharon, unpubl.). As was found in other species, IAA biosynthesis in *B. cinerea* requires tryptophan as a precursor. It has been suggested that IAA is produced via the IPA pathway, but the precise biosynthetic pathway is still unclear.

IAA biosynthesis in *B. cinerea* was studied by feeding with various IAA intermediates *in vitro* (S. Haskin and A. Sharon, unpubl.). To test the activity of the IPA pathway enzymes we used tryptophan, IPA, or indole-acetaldehyde (IAL) as precursors and then measured IAA production. Only low levels of IAA were produced in tryptophan-amended medium. Addition of IPA to the medium resulted in high levels of IAA, but similar levels also accumulated in the control treatment that included only medium without the fungus. Further analyses showed that IPA is spontaneously converted to IAA in the growth medium. Similar results were obtained by *in vitro* enzymatic assays using *B. cinerea* protein extracts under conditions specific for the activity of the enzyme indole pyruvic acid decarboxylase. These results indicated that IPA was not used as a precursor for IAA synthesis under these experimental conditions. The addition of indole-3-acetaldehyde (IAD), on the other hand, resulted in production of high levels of IAA an order of magnitude higher than the amounts of IAA that were produced with tryptophan. No IAA was detected in the control flasks that contained only culture medium without the fungus. These results suggested that the IPA pathway might not be active in *B. cinerea* under the experimental conditions, or that it is not a main pathway of IAA biosynthesis in the fungus. It also showed that *B. cinerea* was capable of producing high levels of IAA, but under the experimental conditions it could not use tryptophan efficiently and required an intermediate precursor such as IAD. These results suggested that pathways other than the IPA that lead to IAD and then to IAA production might be used for IAA synthesis. To test this possibility we added to the medium tryptamine, an intermediate of another microbial pathway in which tryptophan is

first decarboxylated to tryptamine, which is then deaminated to produce IAD. The addition of tryptamine to the medium resulted in high levels of IAA that was completely dependent on the presence of the fungus. These results showed that in *B. cinerea* the major pathway of IAA biosynthesis is probably the tryptamine pathway, and not the IPA as was previously suggested. Once the intermediate precursors tryptamine or IAD are provided to the fungus, it can produce high amounts of IAA, but it is still unknown why only low levels are produced from tryptophan.

2.3. Gibberellic acid

The best investigated fungal phytohormone system, and the only one in which all the biosynthetic genes involved have been identified, is the production of GA by *Gibberella fujikuroi*: members of the mating population C (causing the so-called Bakanae disease in rice, characterized by an enormous stem elongation) produce high titres of GA₃; high producing strains are used for efficient biotechnological production of GA₃ (Tudzynski, 1999; Tudzynski and Sharon, 2002). It has been shown recently that the genes involved in GA biosynthesis in *G. fujikuroi* are arranged in a cluster (Tudzynski and Höltter, 1998), and that the biosynthesis in major aspects is different from the higher plant pathway (Hedden et al., 2002).

So far GA production in *Botrytis* has not been proved unequivocally. In extracts of a model strain (B05.10) not even kaurenes could be detected (V. Siewers and P. Tudzynski, unpubl.). A gene (*bccps/ks1*) showing significant homology to the first gene of the GA pathway in *G. fujikuroi* (encoding the bifunctional enzyme ent-copalyl diphosphate synthase/ent-kaurene synthase) was identified in *B. cinerea*; a knock-out of this gene had no effect on growth or morphology of the mutant in axenic culture nor on virulence. Since expression of the gene could not be detected under any growth conditions (by RT-PCR) and a neighbourhood sequence analysis showed absence of any possible GA cluster genes like in *G. fujikuroi*, the analyses were not carried on (V. Siewers, S. Giesbert and P. Tudzynski, unpubl.).

2.4. Abscisic acid

Strains of *B. cinerea* have been shown to synthesize ABA (Marumo et al., 1982; Hirai et al., 1986); overproducing strains are used for biotechnological production of ABA (Toray Industries, Japan). Biosynthesis of ABA in fungi also seems to be distinct from the pathway used by higher plants. Biochemical analyses in various *Cercospora* species have presented evidence that these fungi synthesize ABA directly from farnesylpyrophosphate (FPP) via different oxidative steps, with either 1',4'-dihydroxy- γ -ionylidone acetate, 1'-desoxy-ABA, or 1',4'-*trans*-diol-ABA as intermediates, and not via the carotenoid pathway as in higher plants (Assante et al., 1977; Neill et al., 1987; Okamoto et al., 1988a, b). In *B. cinerea*, biosynthesis of ABA seems to follow the same pathway as *Cercospora densiflorae*, i.e. via 1',4'-*trans*-diol-ABA (Hirai et al., 1986). The fact that ABA is produced mainly by pathogenic fungi, and given the effects of ABA on higher plants (induction of senescence, etc.), it has been speculated that ABA might be involved in pathogenesis, i.e. represents a virulence factor (Kettner and Dorffling, 1995). This idea was supported by Shaul et al. (1996) who showed that external application of

ABA enhanced disease development caused by *B. cinerea*. The unequivocal proof for a role of fungal ABA in the host-pathogen interaction would require defined mutants which are absolutely unable to produce ABA *in vivo*. (Strains not producing detectable amounts of ABA in axenic culture have been described, but it is open to question whether they still have the capability to produce ABA *in planta*.) Identification of genes involved in the ABA biosynthesis pathway in *B. cinerea* would be interesting for several reasons: they could be used for evolutionary research for comparison of the pathways in higher plants and fungi, for biotechnological purposes for the generation of overproducing strains, and for phytopathological analyses as outlined above.

The approach to clone ABA pathway genes (Siewers et al., 2004) is based on the first "genomic" tools available for *B. cinerea* (Chapter 4). The proposed direct biosynthetic pathway from FPP suggested the involvement of P450 monooxygenases, since several oxidation/hydroxylation steps would be involved. For a first step a P450-oxidoreductase gene (*bccpr1*) was cloned using a PCR approach; deletion of this gene resulted in a drastic reduction of ABA biosynthesis in an ABA overproducing strain (ATCC 58025), strongly supporting the concept that P450 monooxygenases are involved. Therefore, all P450 monooxygenase genes contained in the available EST libraries (28) were checked for induction under ABA biosynthesis conditions. Two genes up-regulated under ABA biosynthesis conditions were deleted by a gene-replacement approach. A mutant of one of the genes (*bcp450-16*) did not produce any ABA, but an ABA intermediate, proving that this gene encodes the first identified enzyme of the ABA biosynthetic pathway. Deletion of the homologous gene in strain B05.10 (strain ATCC 58025 is almost non-pathogenic) is under way; this will allow the first unequivocal test for the role of fungal ABA biosynthesis in pathogenicity of *Botrytis*.

The above mentioned examples suggest that the phytohormone biosynthesis pathways in *Botrytis* are unrelated to plant pathways and are in contrast to the hypothesis that phytohormones represent a good example for an horizontal gene transfer between plants and fungi. Ethylene is synthesized via KMBA (Chagué et al., 2002), as in prokaryotic systems, and not via the ACC pathway used in higher plants, and ABA is synthesized directly from FPP and not through the plant carotenoid pathways (Siewers et al., 2004). The details of IAA biosynthesis are not yet fully uncovered, but they include at least one non-plant pathway in which tryptophan is first decarboxylated to tryptamine which is then deaminated to form indole-3-acetaldehyde. These examples suggest that in *B. cinerea* phytohormone biosynthesis pathways are more similar to prokaryotes than to plants.

3. Effect of plant hormones on *B. cinerea* and on disease development

3.1. Ethylene

3.1.1. Ethylene and fungal development

Stimulation by ethylene of conidial germination, germ tube elongation and appressorium formation were reported in several fungi (Kepczynski and Kepczynska, 1977; Kepczynska, 1989, 1994; Kolattukudy et al., 1995). These effects, however, are not general and may be significant in some, but not all, fungi. A considerable amount of work was dedicated to study the effects of ethylene in *B. cinerea*. Brown (1922) reported high rates of *B. cinerea* conidial germination in the presence of ripe apples, and suggested that the atmosphere of ripening fruits may have a stimulatory effect on conidial germination, and later studies supported these early observations. Kepczynski and Kepczynska (1977) reported that ethylene enhanced conidial germination of *B. cinerea*, and Kepczynska (1989) showed that 2,5-norbornadiene (NBD) (a specific inhibitor of ethylene action in plants) reversed this effect. Germination was effectively inhibited by NBD and was relieved by transfer of the conidia to fresh air suggesting an indispensable role for ethylene in the germination process. Ethylene was also reported to stimulate mycelial growth (Kepczynska, 1993, 1994). Treatment with ethylene up to 10^3 $\mu\text{l/l}$ air increased the total dry weight of *B. cinerea* grown both *in vitro* and *in vivo* on strawberries as determined by glucosamine content (El Kazzaz, 1983). It should be noted that ethylene inhibitors such as amino-ethoxy-vinyl-glycine (AVG) and NBD, and the ethylene donor ethephon (ethylene releasing agent) were used in these studies. While these results strongly suggest that ethylene affects *B. cinerea* development by enhancing conidial germination and hyphal growth, they do not present unequivocal proof for the direct effect of ethylene. Both AVG and ethephon have effects other than that of inhibition of the ACC pathway and release of gaseous ethylene, respectively. AVG was found to reduce mycelium growth and sporulation of the fungus (V. Chagué and A. Sharon, unpubl.), while ethephon is known to release phosphonic acid and is pH sensitive. To better assess the direct effect of ethylene on *B. cinerea* we conducted similar experiments to test the effect of pure ethylene on fungal development and found that it inhibited mycelium growth in culture (V. Chagué and A. Sharon, unpubl.). The rate of conidial germination and germ tube elongation on glass, or on tomato and bean leaf surfaces, were enhanced (Elad, 2002; Elad et al., 2002). Thus, ethylene may have different effects on the fungus at different developmental stages and in different systems.

Two lines of evidence appear to be significant in evaluating the possible role of ethylene in *B. cinerea*: 1) all strains tested so far produced significant amounts of ethylene (e.g. Qadir et al., 1997; Chagué et al., 2002) suggesting that this ability may be an inherent characteristic of the species; and 2) the accumulated data strongly support an effect of ethylene on fungal development, including conidial germination and mycelial growth. Taken together, these data suggest that changes in ethylene levels are sensed by the fungus and might affect its growth and development.

The mechanisms of ethylene perception and action in plants have been elucidated in a great detail. It has been shown that ethylene specifically binds to a number of "ethylene receptors" which are proteins with homology to two-component histidine kinase regulators (Kieber, 1997; Theologis, 1998). The binding of ethylene to these receptors triggers a kinase cascade resulting in transcriptional activation of nuclear genes (Kende and Zeevaart, 1997). It is rather plausible to assume that a similar cascade might mediate the effect of ethylene in fungi, including *B. cinerea*. However, in contrast to the wealth of physiological studies describing the effect of ethylene on fungal development, there are no molecular data in support of this hypothesis. Molecular studies are therefore needed to verify whether ethylene indeed affects development through transcriptional gene activation. Using differential expression techniques we were able to show differences in the transcription level of an array of genes caused by ethylene treatment (V. Chagué and A. Sharon, unpubl.). These preliminary results support the hypothesis that ethylene directly affects *B. cinerea*. More extensive research is required to determine how this effect is obtained.

3.1.2. Ethylene and disease

Enhanced ethylene production has been considered to be an early response of plants to pathogen attack. Although increase in ethylene levels has been associated both with resistance and susceptibility to disease, the working model has been that enhanced ethylene production is an early, active response of plants to pathogen attack.

Plants infected by *B. cinerea* certainly produce high levels of ethylene. Williamson (1950) noted that infection of chrysanthemum tissue by *B. cinerea* resulted in the release of ethylene. Smith et al. (1964) found that carnation infected by *B. cinerea* also produced more ethylene than non-infected plants; the ethylene predisposed the flowers to further attack by the pathogen. Leaves of pelargonium and ruscus, flowers of carnation and leaves and flowers of rose infected by *B. cinerea* produced much higher levels of ethylene (up to 12 nl/g/h) compared with wounded or healthy tissues, and ethylene production was correlated with the severity of grey mould. However, when the host became completely macerated, ethylene production diminished. Methionine sprays, incubation with exogenous ethylene, or pre-cooling of flowers at 4°C increased disease incidence considerably. On the other hand, sprays of the ethylene activity inhibitor silver thiosulphate (STS) and the ethylene biosynthesis inhibitors aminooxyacetic acid (AOA) and AVG decreased disease severity. The latter two compounds inhibited ethylene production in infected plants (Elad, 1988; Elad and Volpin, 1988). Leaves of tomato, sweet pepper, yellow bean, and cucumber behaved similarly. In addition, when *B. cinerea* was grown on autoclaved leaves supplemented with methionine it produced 0.14 nl/g/h ethylene (Elad, 1990).

Calcium can be added to cut flowers in the vase and in the fertilizing solution in the greenhouse. Ethylene production in flowers with high calcium content was decreased by 50-95% (Volpin and Elad, 1991). Calcium ions inhibited disease

development, whereas the ion chelator ethylene glycol-bis-(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA) enhanced it. Disease suppression by an excess of Ca^{2+} was correlated with repression of ethylene production by the flowers (Elad and Volpin, 1988). It should be noted that calcium can also affect the susceptibility of plants to *B. cinerea* by affecting pectin resistance to *B. cinerea* enzymes or by directly inhibiting pectolytic enzymes (Chapter 7).

There are additional reports on production of ethylene by *B. cinerea*-infected plant tissues. A 3.3-fold increase in ethylene production by cell suspension cultures of *Papaver somniferum* was observed 7 h after elicitation with a *Botrytis* fungal homogenate (Songstad et al., 1989). Kiwifruit stored at 0-10°C produced significant amounts of ethylene 20-30 days after inoculation with *B. cinerea*, while only trace amounts were detected in healthy controls (Niklis et al., 1992). Symptomless, newly abscised blackcurrant flowers of many genotypes were found by fluorescence microscopy to contain infected ovules. Inoculated flowers produced higher ethylene levels than un-inoculated controls (McNicol et al., 1989). The use of NBD then confirmed that ethylene produced in response to infection was a major factor in premature flower abscission, and the sensitivity of blackcurrant genotypes to ethylene corresponded with their known susceptibility to fruit drop.

As mentioned above, external ethylene (applied as ethephon) may enhance grey mould, whereas ethylene inhibitors may suppress the disease. *In vitro* tests showed that the plant growth regulator 4-chlorophenoxyacetic acid (4-CPA) at 10 µg/ml inhibited mycelial growth and conidial germination of three *B. cinerea* isolates. Ethephon had a slight effect on mycelium growth. Tomato plants sprayed with 4-CPA and ethephon showed 50 and 80% infection by *B. cinerea*, respectively, compared with 60% infection on an untreated control (Benliogulu and Yilmaz, 1992). On the contrary, ethylene exogenously applied as ethephon stimulated grey mould disease severity on both tomato and bean plants at 100-400 µg/ml (M.I. Al-Masri, Y. Elad, A. Sharon, and R. Barakat, unpubl.). Grapevines were sprayed with ethephon, harvested and the grapes were stored. *B. cinerea* infection and the percentage of soft berries were increased in these grapes (Hartmann, 1988).

Several compounds were tested for their ability to reduce development of *B. cinerea* on rose, tomato, sweet pepper, aubergine, French bean and *Senecio* sp. Use of potassium permanganate to absorb ethylene from the atmosphere surrounding rose flowers, or leaves of tomato and pepper, resulted in slower fungal development (Elad, 1993). Inhibition of ethylene activity by NBD controlled the disease on all crops other than tomato, and carbon dioxide controlled the fungus on roses. Inhibitors of ethylene biosynthesis such as AOA, Co^{2+} , the uncoupler 2,4-dinitrophenol and the radical scavenger salicylic acid varied in effectiveness in controlling the disease on the various hosts. The cytokinin benzyl adenine, which reduces host responsiveness to ethylene, resulted in 39-99% disease reduction in rose flowers and in leaves of tomato and *Senecio* sp. but was not effective on aubergine or pepper (Elad, 1993). Most, if not all, of the compounds affect other processes in addition to ethylene and therefore the observed effects may not be ethylene-specific. Interestingly, the opposite was reported by Hoffman et al. (1988) who worked with a below ground plant organ. Carrot slices originally resistant

became susceptible to a normally non-invasive level of *B. cinerea* conidia after treatment with AVG; ACC partially reversed the susceptibility induced by AVG.

Antioxidants restrain grey mould on various plants (Elad, 1992; Chapter 8). Ethylene production was inhibited in tomato leaves treated with the antioxidants propyl gallate, ascorbic acid and benzoic acid, but not in pepper leaves. Ethephon or H₂O₂ increased the severity of grey mould on leaves of *Senecio* sp. This effect was controlled by the antioxidants butylated hydroxytoluene (BHT) and benzoic acid, or by BHT alone, respectively (Elad, 1992). Ethylene stimulated germ-tube elongation of *B. cinerea* conidia incubated within normal and non-ripening *nor* tomato fruits, but had little influence on the total percentage of germination. Exposure of the normal and the mutant fruits to ethylene immediately after inoculation increased sporulation. When tomato fruits were exposed to ethylene for 3 days before inoculation, rot was stimulated on the mature-green normal fruits, but not on the *nor* mutant fruits. It was suggested that exogenous ethylene might directly stimulate germ tube growth of *B. cinerea* in both normal and mutant fruit, but that it may affect subsequent fungal growth indirectly, via stimulation of the ripening process, only in pre-climacteric normal tomato fruit (Barkai Golan et al., 1989).

The effect of ethylene on *B. cinerea*-host interaction was further described using French beans, tomato and *Arabidopsis thaliana* plants (Elad, 2002; Elad et al., 2002; M.I. Al-Masri, Y. Elad, A. Sharon and R. Barakat, unpubl.). Infected resistant *Arabidopsis* plants produced less ethylene than sensitive plants. Interestingly, not only did ethylene enhance *B. cinerea* germination (Sect. 1.2), but it also increased the number of infection structures per germ tube and subsequent penetration of the host tissue (Elad, 2002; Y. Elad, unpubl.).

It is possible to study the impact of phytohormones produced by plants on the host-parasite interaction using plant mutants with altered hormone susceptibility or production (Korolev and Elad, 2004). Ethylene-related mutants include ethylene-insensitive, ethylene-overproducers and ethylene-reduced mutants. Ethylene-insensitive mutants *ein2-1*, *ein-6*, *etr1-1*, *etr1-3*, ethylene-overproducers *eto1-1*, *eto2* and ethylene-reduced production mutant *hls1-1* were more susceptible than the wild type (WT) *Arabidopsis*, whereas other mutants did not differ from their WT background. AVG significantly inhibited disease on both *ein2-1* and *hls1-1* mutants, whereas ethephon did not change the level of disease on *ein2-1* and slightly stimulated disease on *hls1-1* (Korolev and Elad, 2004). It is possible that the pathway that leads to susceptibility is independent of other ethylene signalling transduction pathways.

In summary, *Botrytis* infection induces increases in both active oxygen species (AOS) and ethylene production. Ethylene induces auto-catalytic production of that same hormone in exposed tissue. Ethylene promotes deterioration of the infected tissue and the AOS development and *vice versa*. Consequently, antioxidant levels are changed in the rot area or in asymptomatic tissues surrounding the rot. The reaction can be prevented by inhibition of ethylene or external application of antioxidants (Elad, 2002). Govrin and Levin (2000) suggested that the oxidative burst during plant infection enhances infection by *Botrytis*. They found an ethylene burst from the infected tissue that coincided with the peak of oxidative burst. *B. cinerea* perturbs redox processes involvement in pathogenesis (Chapters 8 and 9).

Cristescu et al. (2002) compared *in vitro* ethylene production by *Botrytis* with the ethylene produced during plant infection and found that the levels of emission during plant infection were 100-fold higher. The time of evolution of enhanced ethylene production by infected tomatoes and the cytological observations indicated that ethylene emission was not triggered by *B. cinerea*-produced ethylene, although it was strongly synchronized with the growth rate of the fungus inside the plant. Chagué et al. (2002) showed that peroxidase is capable of catalysing *in vitro* KMBA oxidation and release of ethylene. Taken together these results suggest that *Botrytis* has the potential to produce ethylene during plant infection. Further research is necessary to determine whether ethylene is indeed produced *in planta* by *B. cinerea* and to better assess the influence of ethylene on disease development by specifically determining the effect on both the plant and the fungus.

3.2. Auxins

High auxin levels were found in infected plants, and symptoms resembling the effects of high auxin, such as epinasty and plant organ deformations, are associated with many fungal diseases (Tudzynski and Sharon, 2002). Auxin may affect both the fungus and the plant. Addition of IAA and gibberellic acid affected sporulation and cell elongation in yeast (Yanagishima, 1965; Kamisaka et al., 1967), and enhanced germination of *Neurospora crassa* conidia (Nakamura et al., 1978, 1982). Elevated IAA levels are associated with tumours and growth abnormalities caused by several pathogens. Wolf (1952) reported that only those species of *Ustilago maydis* that produced auxin in culture caused gall formation on their hosts. Naphthalene acetic acid (NAA) reduced the mycelial growth rate of *Sclerotinia sclerotiorum* *in vitro* and white mould disease severity on detached leaves and whole bean and cucumber plants at concentrations of 200-600 µg/ml (Al-Masri et al., 2002).

Various auxins such as IAA, naphthalene acetic acid ethyl ester (NAAEE) and N-*meta*-tolylphthalamic acid (NMT) reduced botrytis blight of cut rose flowers (Elad, 1995). Application of auxin to enhance fruit setting of aubergine reduced susceptibility to the disease (Elad et al., 1992). IAA and NAA reduced disease on tomato leaves at concentrations of 10^{-4} - 10^{-3} M and also reduced *B. cinerea* germination *in vitro* or on leaves, with lower efficacy of the lower concentrations of NAA combined with GA₃ being inhibitory on tomato and bean leaves. The inhibitory effect on disease was sometimes additive (Y. Elad, unpubl.). The auxins, NAA and 2,3,5-triiodobenzoic acid (TIBA) reduced the mycelial growth rate of *B. cinerea* *in vitro*, and grey mould disease severity on tomato plants at various concentrations (R. Barakat, unpubl.). Delen and Özbek (1989) observed increased grey mould severity in tomato greenhouses treated with auxins. 2,4-dichlorophenoxy-acetic acid (2,4-D) increased *B. cinerea* growth and sporulation at concentrations of 0.01–10.0 and IAA increased the mycelial growth and sporulation at 0.01–1.0 µg/ml, but decreased it at 50.0 – 500.0 µg/ml. On tomato plants 2,4-D stimulated grey mould when applied at 0.5 – 1.0 µg/ml (Delen and Özbek, 1989).

Korolev and Elad (2004) infected auxin-resistant *Arabidopsis* plant mutants. Most auxin-resistant mutants developed rot similar to the WT background, whereas

the mutants *axr1-3* and *aux1-7* were more susceptible than the WT; external application of NAA stimulated disease on *axr1-3*.

3.3. Gibberellic acid

There was less fungal decay due to *B. cinerea*, *Monilia (Monilinia) fructigena* and *Penicillium expansum* in treated nectarine fruits after gibberellic acid sprays were applied to trees in an orchard, which also delayed ripening and increased firmness (Lurie et al., 1998). GA₃ increased the mycelial growth and sporulation of *B. cinerea in vitro*, at concentrations of 1.0–50.0 µg/ml, respectively. On tomato plants it promoted disease at concentrations of 100–300 µg/ml (Delen and Özbek, 1989). When celery was treated with GA₃ 1 month prior to storage at 2°C, decay was decreased and the concentration of psoralens increased because GA₃ slowed down the conversion of (+)marmesin to psoralens, thereby increasing the resistance to *B. cinerea* and other pathogens (Afek et al., 1995). Gibberellin treatment reduces stalk rot in grapevine due to *B. cinerea* (Brechbuhler, 1982). GA₃ suppressed grey mould on tomato and bean plants (Elad, 1995, 1997). Botrytis blight of cut rose flowers has been controlled by GA₃ applications to detached petals or to whole cut flowers (Shaul et al., 1992). At the concentrations used in this work the germination, growth and development of the fungus were not affected, but in later work it was found that higher concentrations of GA₃ inhibited the fungus (Y. Elad, unpubl.). In the case of rose flowers the effect of blight suppression resulted from GA₃-imposed inhibition of senescence processes in the petals (Shaul et al., 1995a, b). GA₃ inhibits the senescence-related increase in the permeability of the cell membranes, reduced leakage of nutrients from the tissue and increased production of *Botrytis*-inhibiting phenolic compounds. The possibility that GA₃-stimulated formation of phenolic glycosides and other phenolic saccharides reduces the availability of nutrients to the pathogen and introduces inhibitory properties has been proposed (Zieslin et al., 1996). Other GA effects that may lead to reduced plant susceptibility to *Botrytis*, such as effects on pectin solubility, reduction of polygalacturonase activity and ethylene evolution, were reviewed by Elad (1997).

Korolev and Elad (2004) inoculated *Arabidopsis* mutants that affected GA metabolism and all were strongly affected by *B. cinerea*. GA-deficient mutants developed more severe rot than GA-resistant or GA-insensitive ones. External application of GA₃ or the inhibitor 2'-isopropyl-4'-(trimethylammonium chloride)-5'-methylphenyl piperidine-1-carboxylate (AMO-1618) did not change the level of disease on two GA-deficient mutants, *gal-4* and *ga2-1*.

3.4. Abscisic acid

ABA is associated with negative effects on infected plants. Kettner and Dorffling (1995) studied tomato ABA mutants and concluded that at least four processes control the level of ABA in WT tomato leaves infected with *B. cinerea*: stimulation of fungal ABA biosynthesis by the host; release of ABA or its precursor by the fungus; stimulation of biosynthesis of plant ABA by the fungus; and inhibition of its metabolism by the fungus. Application of ABA together with fungal conidia to tomato leaves caused a faster development of necrotic leaf area than conidial

inoculation only (Kettner and Dorffling, 1995). ABA is formed during plant tissue aging and may antagonize the function of gibberellins in the plant tissue and increase the susceptibility to *Botrytis* (Elad, 1997). Indeed, botrytis blight of roses is promoted by ABA and ABA antagonizes GA₃-suppression of the disease on rose flowers (Shaul et al., 1996). Similarly, tomato and bean leaf infection is promoted by ABA. Additionally, ABA and ethylene act synergistically in promoting infection by *B. cinerea*. Mevalonic acid lactone, a precursor of ABA biosynthesis, promoted disease at 1 μ M concentrations, similar to the ABA effect. It promoted germination of the conidia on glass and on leaves and even had an additive effect on germ tube elongation when combined with ABA (Y. Elad, unpubl.).

According to Audenaert et al. (2002), ABA plays a major role in the susceptibility of tomato to *B. cinerea*. Tomato mutants with reduced ABA levels (sitiens plants) are more resistant to *B. cinerea* than WT plants. Exogenous application of ABA restored susceptibility to *B. cinerea* in sitiens plants and increased susceptibility in WT plants. ABA appeared to interact with a functional plant defence response against *B. cinerea*. Thus, ABA appears to negatively modulate the salicylic acid-dependent defence pathway in tomato, which may be one of the mechanisms by which ABA levels determine susceptibility to *B. cinerea* (Audenaert et al., 2002).

Korolev and Elad (2004) infected *Arabidopsis* mutants expressing both deficient and insensitive responses to ABA that were significantly more susceptible to *B. cinerea* than corresponding background lines. Application of ABA and mevalonic acid lactone external applications did not change the level of disease on the ABA-insensitive mutant *abi2-1*, but significantly reduced disease on the ABA-deficient mutant *aba1-3*.

When *B. cinerea* infects leaves of raspberry (*Rubus idaeus*) primocanes, it causes dwarfing of the axillary buds in the growing season; axillary buds at infected nodes on overwintered canes then usually fail to develop into lateral shoots in the following spring, thus causing yield loss (Williamson and Hargreaves, 1981). This suppression of axillary buds was postulated to be due to a fungal toxin, but in view of new research on the synthesis of ABA by *B. cinerea* it seems more likely that hormone inhibition is a cause of the retardation of bud development in the first season, followed by strong correlative inhibition from larger distal buds at healthy nodes above.

3.5. Cytokinins

There are only a few reports on the role of cytokinins in *B. cinerea* infection. Increased concentration of kinetin or 6-benzyladenine decreased mycelial growth of the onion pathogens *B. allii* and *Colletotrichum dematium*. Sporulation of *B. allii* increased by 60% as amendment concentration increased from 0-10⁻⁵ M and then decreased 25% at 10⁻³ M (Russo and Pappelis, 1993). Benzyladenine and kinetin reduced botrytis blight of rose flowers (Elad, 1995) and other plants (Elad et al., 1993).

4. Conclusions

B. cinerea is capable of producing several plant hormones in axenic cultures. All tested strains produce large quantities of ethylene and low levels of IAA, while ABA is produced only by some, but not all, strains in culture. None of the tested isolates produced gibberellins. External supply of a precursor is required for (ethylene and IAA) or significantly enhances (ABA) production of the phytohormones. No ethylene or IAA are produced in media without methionine or tryptophan respectively, and mevalonic acid is necessary for production of substantial levels of ABA. Since external supply of substrates is required for phytohormone production by the fungus, *B. cinerea* must utilize plant metabolites in order to produce phytohormones during plant colonization. Production of the phytohormones by *Botrytis in planta* has not been demonstrated, and it remains uncertain whether the fungus indeed utilizes plant substrates to produce plant hormones, and whether fungal-produced phytohormones affect disease development. *B. cinerea* employs biosynthetic pathways that are different from the plant pathways for synthesis of ethylene, IAA and ABA. This fact might be utilized to obtain information on phytohormone production *in planta* by the fungus through feeding experiments and measurement of pathway-specific intermediate compounds or by using pathway-specific inhibitors.

A *B. cinerea* biosynthetic gene was recently cloned for the ABA pathway. This will contribute to our ability to study the evolution and function of this phytohormone in fungi, e.g. by sequence and expression pattern comparisons with bacteria and plants, and by testing pathogenicity and development of null mutants. Isolation of ethylene biosynthesis genes might be more difficult since only one non-specific aminoacid transferase seems to be involved in ethylene production. Other methods including isolation of ethylene-regulated *Botrytis* genes may contribute to understanding ethylene function in *Botrytis*.

Phytohormones produced by the infected plant, or external supply of plant hormones, clearly affect disease development. External supply of ABA and ethylene seem to enhance disease, while IAA and GA₃ reduce it. As might be expected, the effect is highly sensitive to phytohormone concentrations and time of application. Nevertheless, the cumulative data suggest that when administered at the right time and concentrations, plant hormones might be very useful in preventing *Botrytis* diseases.

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CHAPTER 11

DETECTION, QUANTIFICATION AND IMMUNOLOCALISATION OF *BOTRYTIS* SPECIES

Frances M. Dewey (Molly)¹ and David Yohalem²

¹Department of Viticulture and Enology, University of California at Davis, Davis CA95616, USA;

²Horsekildevvej 38. 1 tv, Valby DK-2500, Denmark

Abstract. Classical methods of detection of *Botrytis* species include plating-out of surface sterilized infected plant tissues, soils and airborne conidia on selective media and the identification, by microscopy, of the sclerotia, conidia and conidiophores, based on their characteristic shape, size and colour. Other methods are now available such as nucleic acid-based methods that can be used to track individual isolates or specific species. The determination of biomass levels in samples using these methods, however, is problematic because of the multinucleate nature of *Botrytis* conidia and thallus. Immunological methods employing genus-specific monoclonal antibodies, particularly quantitative laboratory-based plate-trapped antigen ELISAs, allow large numbers of samples to be processed easily within a few hours. These methods, combined with the modified plate spore trap, the Micro-Titre Immuno Spore Trap (MTIST), enable the quantification of conidia in microtitre wells. A rapid semi-quantitative immuno-chromatographic lateral flow device designed for use in the field or office promises to be a useful screening device for *Botrytis*. Development of species-specific monoclonal antibodies remains a challenge. The usefulness of Fourier transform infrared spectroscopy, nuclear magnetic resonance, liquid chromatography-mass spectroscopy and enzymic methods to detect and quantify specific secondary metabolites produced by *Botrytis* remains to be fully demonstrated.

1. Introduction

Detection and quantification of *Botrytis* infections in plants, seeds, air-borne conidia and sclerotia in soils has, until recently, depended on the plating out of infected material and the microscopic identification of sclerotia, conidia and conidiophores on the basis of their size, shape and colour. Although these methods yield valuable information, they are limited. Plating out is a time-consuming process in which surface sterilization is a general pre-requisite. Other methods are now becoming available that either yield more specific information, as is the case with molecular methods, or are faster and more easily replicated, as with immunological methods. These various methods will be addressed separately along with other quantitative methods.

Sampling is a problem inherent to any detection assay. There are no sampling techniques unique to *Botrytis* spp., which has a generally rare (Poisson) spatial distribution and/or clumped (negative binomial) and sampling should be performed with the biology and epidemiology of the system being examined in mind (e.g. Marois et al., 1993). In the case of epidemiological studies, some stratification is often necessary to make meaningful inferences and different techniques are needed for different environments to address specific questions.

Assessing the extent of infection within a sample is also problematic. Care has to be taken when making comparisons that employ different methods. For example, the level of infection in a sample of grape berries assessed on a weight for weight basis will be different from that assessed on a number basis because rotted berries generally weigh less (Dewey et al., 2000). Estimates of fungal biomass of foliar infections are commonly determined by measuring lesion area (Elad et al., 1994), but these estimates are clearly different from levels based on percentage of leaf area that is sporulating (Köhl et al., 1995), the numbers of conidia derived from sporulating tissues by shaking at high speed in tap water with a detergent (Gerlagh et al., 2001) or CFU/cm² from macerated leaf samples plated out by limiting dilution (Lennox et al., 2003). The use of immunological methods to determine fungal biomass in individual and massed infections is promising (Sect. 3).

2. Classical plating out method

Common methods of surface sterilizing plant material prior to plating out include immersing excised tissues/seeds or fruits sequentially in sodium hypochlorite, ethanol and sterile distilled water (Meyer et al., 2000; Coertze and Holz, 2001). Where infected material is already sporulating identification can be confirmed by plating out single conidia picked up with a sterile needle. Special methods are employed for the detection of latent infections. Dipping berries in alcohol, freezing them at -20°C for a short time and then incubating in a moist chamber at room temperature for 7-10 days until the fungus has sporulated has proved to be an effective simple method (Mundy and Beresford, 2003). Others have induced sporulation by treatment of surface sterilized tissues or fruits with paraquat to reveal latent infections in grape berries (Gindrat and Pezet, 1994; Pezet et al., 2003), strawberries (Sutton et al., 1997), sweet cherry fruit (Adaskaveg et al., 2000) and fallen rose petals (Morandi et al., 2000).

Semi-selective and differential media are based on the selective inhibition of competing microbes, the encouragement of the target organism's growth and/or the expression of a characteristic property of *Botrytis*. Viable conidia will germinate on many media, in the presence of free water, nitrogen and phosphate, and form colonies, but do not always sporulate. Confirmation of growth of any species of *Botrytis* requires microscopic examination. A medium based on Martin's rose bengal agar amended with several fungicides and high concentrations of antibiotics was found satisfactory for assaying organic soils (Lorbeer and Tichelaar, 1970). Kritzman and Netzler (1978) developed a medium for isolation of *Botrytis* species from soil and onion seed based on the development of dark pigments in the medium

due to degradation of tannic acid and resistance to PCNB and maneb. This medium was also used to monitor *Botrytis* populations in vineyards and green houses, and as a medium amended with fungicides to detect resistant populations in Israel (Elad et al., 1992). For the detection of conidia of *B. cinerea* caught in spore traps, Kerssies (1990) used a very similar medium to which fenarimol was added to inhibit growth from airborne conidia of *Penicillium* spp. However, this medium also shows the same browning with *B. aclada* and *B. allii* as with *B. cinerea* (D. Yohalem, unpubl.). A new *Botrytis*-selective medium and *Botrytis* spore trap medium have been developed by Edwards and Seddon (2001). Several basal media amended with a suite of fungicides have been used to monitor fungicide resistance within populations of *Botryotinia fuckeliana* (Baroffo et al., 2003).

3. Immunological methods

Development of immunological methods for the detection of *Botrytis* species has had a relatively long and chequered history with many unpublished reports. This is because antisera (polyclonal antibodies) raised to mycelial fragments or crude extracts lack the necessary specificity and commonly cross-react with related and unrelated fungi as well as with extracts from plant tissues (Dewey, 1996). However, Linfield et al. (1995) and Cousins et al. (1990) did raise antisera to *B. allii* and *B. tulipae* respectively that cross-reacted only weakly with unrelated species of fungi and extracts from host tissues. In testing antiserum from rabbits immunized with *B. cinerea*, Ricker et al. (1991) found that while antiserum from early bleeds was relatively specific, that from later bleeds cross-reacted strongly with *Aspergillus niger* and other fungi. Using purified, deglycosylated invertase from *B. cinerea* as the immunogen in chickens, Ruiz and Ruffner (2002) found that antibodies from the egg yolks did not cross-react with unrelated fungi or with extracts from uninfected grape berries. However, their antibodies did not recognize native invertase; they only recognized invertase in samples that had first been partially denatured by heat treatment.

The advent of hybridoma technology has made possible the production and selection of antibodies that are near-genus- or genus-specific to *Botrytis* (Bossi and Dewey, 1992; Meyer and Dewey, 2000). Attempts to raise species-specific monoclonal antibodies have not been successful but Salinas and Schots (1994) did produce three antibodies to *B. cinerea* each of which recognized a different array of *Botrytis* species. Despite comparative studies, it is still not clear what is the best source of antigens for raising taxonomically specific antibodies for detection purposes (Meyer and Dewey, 2000).

A number of different types of immunoassays have been used to detect, and to a lesser extent quantify, levels of *Botrytis* infections in plants. By far the most common are enzyme-linked immunosorbent assays, particularly plate trapped antigen-immunosorbent assays (PTA-ELISAs). Using antisera raised in rabbits, Ricker et al. (1991) demonstrated that PTA-ELISAs could be used to determine levels of *Botrytis* antigens in juice from infected grapes. More recently, the same protocol, with the genus-specific monoclonal antibody BC-12.CA4, was used to

detect and quantify *B. cinerea* in grape juice (Dewey et al., 2000), wines (Dewey, 2002), pear stems (Meyer et al., 2000), strawberries (L. Mehli, Institutt for Biologi, NTNU, Trondheim, Norway, pers. comm.), grape berries (K. Williamson, F.O. Obanor and M. Walters, HortResearch, Lincoln, NZ, pers. comm.), raspberries (Dewey, 2000), tomato fruit (Lurie et al., 2003) and latent *B. aclada* infections in onion leaves (Yohalem et al., 2004). The antigen detected by this antibody is produced constitutively, is highly stable, is not degraded by heat or freezing and is not metabolised during fermentation (Dewey, 2002). As standards for quantitative assays, extracts from freeze-dried mycelium of the fungus grown in liquid culture on grape juice have been used. The assay gives a linear correlation between absorbance values and mycelial extracts in the range of 10 ng/ml to 20 µg/ml (Dewey et al., 2000). This antibody has also been used to develop a 20-min tube assay for on-site quantification of *Botrytis* antigens in grape juice at wineries at harvest time (Dewey and Meyer, 2004), and to develop a semi-quantitative 4-minute immunochromatographic assay or lateral flow device (B-LFD) (M. Dewey and C. Danks, UC Davis, CA, USA and Central Scientific Laboratory York, UK, unpubl.). The latter, which is technically similar to LFDs developed for the detection of viruses in potatoes (Danks and Barker, 2000), is a very simple “user friendly” device that can be used in the field or office without any electrical power; the time taken for the appearance of the positive test band is related to the level of the *Botrytis*-antigen in the sample. The device is highly sensitive and has been used to detect early symptomless infections in artificially inoculated grape vine leaves (C. Aguero and M. Dewey, UC Davis, USA, unpubl.), pine seedlings (K. Capieau and E. Stenstrom, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden, pers. comm.) and tomato fruits (Lurie et al., 2003). Development of a “stand-alone” scanner that measures the level of reflectance or intensity of the test band is under way (K.G. Wilson, KGW Enterprises, Indiana, USA).

Immunofluorescence techniques have been used to immunolocalize and follow infection paths of *B. cinerea* and *B. fabae* in *Vicia faba* (Cole et al., 1998a, b). In an elegant study, Kessel et al. (1999), using the same antibody (BC-KH4), determined effectively the biomass of *B. elliptica* in lily leaves co-inoculated with the biocontrol agent *Ulocladium atrum* by digital image analysis. *Botrytis* antibodies that have worked well by immunofluorescence have also proved useful in studies at the ultra-structural level. For example, Cook et al. (2000) by use of BC-KH4 which recognizes antigens present in the extracellular matrix of *Botrytis* species (Cole et al., 1998a, b) were able to show the active attachment of a potential biocontrol bacterium, *Enterobacter aerogenes*, to the extracellular matrix of geminating hyphae of *B. cinerea* and E. Zellinger and M. Dewey (Oxford Brookes University and University of Oxford, UK, unpubl.) have shown that the *Botrytis* antibody BC-12.CA4 strongly immunolabels the walls of *B. cinerea* hyphae, but not the conidia (Figure 1).

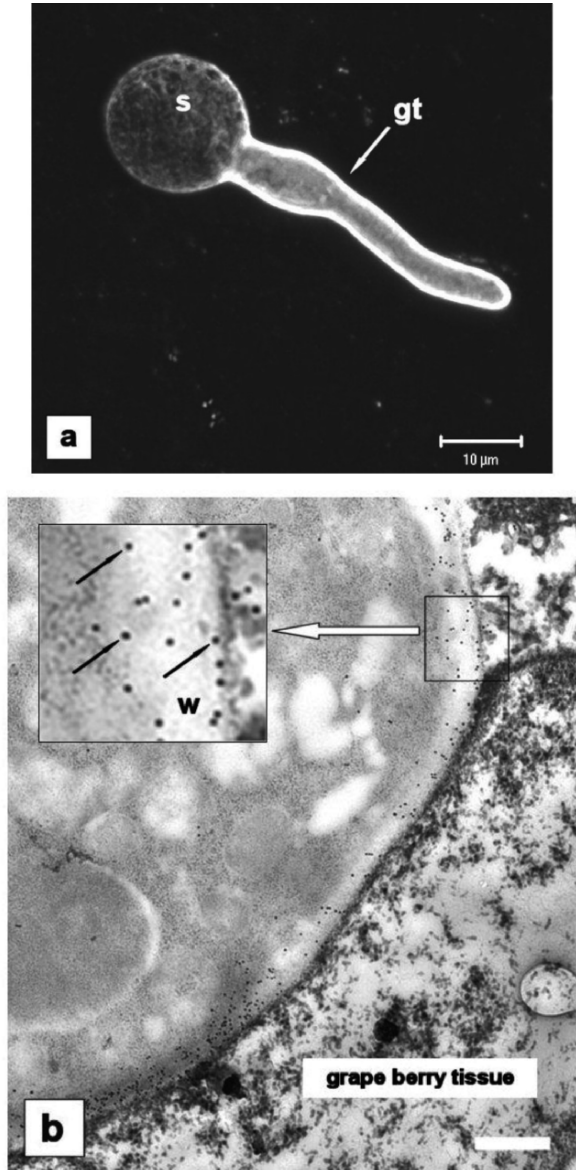


Figure 1. *Botrytis cinerea* immunolabelled in plant material. a. Confocal microscopy of *Botrytis cinerea* conidium germinated on outer surface of detached grape berry skin and immunolabelled with the monoclonal antibody BC-12.CA4 and anti-mouse FITC; note poor labelling of conidial wall but intense labelling of germ tube wall (gt); b. TEM of hypha of *B. cinerea* growing within detached grape berry tissue immunolabelled with BC-12.CA4 and anti-mouse 10 nm gold conjugate; note labelling within the wall of the hypha (w)

Immunofluorescent labelling techniques have also been used to detect, differentiate and enumerate air-borne conidia of *B. cinerea* trapped on tape in a Burkard 7-day volumetric suction spore trap (Dewey, 1996). Other methods of trapping and quantifying air-borne conidia have also been tried such as use of a modified Burkard portable air sampler for agar plates, the Micro-Titre Immuno Spore Trap (MTIST) (Kennedy et al., 2000). This device uses a suction system to directly trap air-particulates and conidia by impaction into micro-titre wells. The conidia are then allowed to germinate overnight in an appropriate buffer and are later quantified by PTA-ELISA.

4. Nucleic acid-based methods

With the development of nucleic acid-based techniques, modern epidemiology has moved closer to population genetics and population biology. Methods based on nucleic acids have been developed for the specific detection and diagnosis of subsets of the form genus (Nielsen et al., 2002), species of *Botrytis* (Mathur and Utkhede, 2002; Nielsen et al., 2002; Rigotti et al., 2002), sub-species (Giraud et al., 1999; Fournier et al., 2003; Martinez et al., 2003), populations (Luck and Gillings, 1995; Moyano et al., 2003) and individual isolates (Kerssies et al., 1997). Several of the methods are critical for proper identification of the causal organism (e.g. *B. cinerea* subspp. *vacuma* and *transposa* (Giraud et al., 1999) and *B. aclada* and *B. allii* (Yohalem et al., 2003), while others hasten diagnosis. In general, the broader groupings are detected with primer sets designed from random-sequence characterized fragments, that is, modifications of various sequence characterized amplified regions (SCAR) or from sequencing with arbitrary primer pairs (SWAPP). The finer groupings rely on either known genetic differences (e.g. Giraud et al., 1999) or randomly amplified polymorphic DNA (RAPD) or related fingerprint methods. Specific RNA transcripts have been detected, which can be used to reveal differential expression of *Botrytis* genes (Choquer et al., 2003).

4.1. Different types of molecular detection assays

Several methods have been used to detect and diagnose *B. cinerea*. Both Mathur and Utkhede (2002) and Rigotti et al. (2002) report direct detection of *B. cinerea* with specific primers sets. Mathur and Utkhede (2002) designed their primers based on sequence data obtained from the internally transcribed spacer (ITS) regions of ribosomal DNA (rDNA). They screened their primers against isolates of other common fungi associated with greenhouse tomato production and found little or no cross-reactivity. In contrast, Nielsen et al. (1998) did not find sufficient variation among ITS sequences within the genus to design primers for the detection of neck rot-associated *Botrytis* spp. Mathur and Utkhede (2002) used their primer set in a dot-blot assay which allows for the simultaneous detection of the pathogen in many samples, but also recommended it as part of an array in a reverse dot-blot test, which allows for the detection of many pathogens simultaneously in a single sample. Rigotti et al. (2002) designed 20-mer primers based on the sequence of a randomly amplified fragment common to all *B. cinerea* isolates screened. A single 0.7-kb band

was produced from all *B. cinerea* isolates tested while a 0.6-kb band was amplified from the DNA of *B. fabae*. They were able to detect 2 pg fungal DNA when mixed with 1 µg plant DNA.

The onion neck rot-associated species of *Botrytis* can be detected, as a group, by PCR amplification of a 413-bp sequence (Nielsen et al., 2002). Digestion of the amplification product with the restriction enzyme *Apo1* clearly distinguishes *B. aclada*, *B. cinerea* and *B. squamosa* (Nielsen et al., 2002). A further amplification with rDNA ITS primers and digestion with *Sph1* serves to separate *B. byssoidea* from the hybrid species, *B. allii* (Yohalem et al., 2003). Hence, the method can be used for detection of a suite of pathogens associated with the syndrome, or for diagnosis of the specific causal agent. Nielsen et al. (2002) report a detection limit of 1 pg DNA from pure cultures.

Population structure within *B. cinerea sensu lato* has been studied with a variety of genetic markers. A method was reported for differentiating benomyl resistant from susceptible isolates using PCR (Chapter 12; Luck and Gillings, 1995). A single base substitution was discovered that correlated with the mutation that conferred resistance, which was found to be detectable by cleavage into two fragments of a restriction digest of a 381-bp amplification product, while leaving the susceptible strains' product undigested. They also designed primers that were specific to the substitution site. The method proved useful for both pure cultures and for direct assay from infected plant tissues. Kerssies et al. (1997) used RAPD markers to distinguish isolates, but found no correlation between their markers and pathogenicity, time, nor sampling source. They reported a species-specific 45-kb band generated by primer D6 (Operon Technologies Inc., Alameda, CA, USA) from which, presumably, a SWAPP set could be designed. RAPD and amplified-fragment length polymorphism (AFLP) techniques have been directly compared and found equally satisfactory for revealing the genetic structure of populations of *B. cinerea* (Moyano et al., 2003). The *vacuma* and *transposa* groups of the *B. cinerea* complex can be separated by the absence or presence of two transposable elements called *Flipper* and *Boty*, respectively (Giraud et al., 1999; Chapters 3, 4 and 12). Multiple isolates can be screened using dot-blot hybridisation. The *Boty* transposon has subsequently been found in several *vacuma* strains (Martinez et al., 2003). However, these isolates can be distinguished using a PCR-RFLP method developed by Fournier et al. (2003). Muñoz et al. (2002) developed a duplex PCR scheme to test for the presence or absence of the transposons and, combined with four known genetic markers and RAPD-RFLP, report host differentiation between *transposa* and *vacuma* populations in Chile. Baraldi et al. (2002) have used RFLP with amplification products of four genes combined with two other genetic markers to examine diversity within and among populations of *B. cinerea* found on kiwifruits. Their data indicate recombination among populations and an association between cold-temperature adapted isolates and carbendazim resistance.

Northern blot hybridizations have been used to detect gene expression related to growth or infection by *Botrytis* and are proving to be a useful tool in elucidating infection processes. Benito et al. (1998) tracked transcripts of *B. cinerea* actin and β -tubulin genes during infection of tomato leaves, while Mengiste et al. (2003) used

the β -tubulin transcript in an *Arabidopsis* model system to make inferences about the effects of a host *Botrytis* susceptibility factor also required for stress response. Choquer et al. (2003) used real time-PCR (RT-PCR) to compare expression levels of several transcripts *in vitro* and *in planta*.

4.2. Dealing with problems related to molecular detection

Problems associated with PCR-inhibitory components of the fungal milieu (particularly soil and seed associated phenolics and tannins) can be addressed through more or less elaborate extraction and purification protocols. Nielsen et al. (2002) report reduced foaming from plant tissues extracted with a potassium ethyl xanthogenate (PEX) buffer as opposed to a cetyl trimethylammonium bromide (CTAB) buffer, which was adequate for working with pure culture material. The extraction efficiency decreases with increasing sample size in a non-linear fashion, i.e. 100- μ g samples produce proportionally lower yields of DNA than do 50- μ g samples, increasing the probability of obtaining a false negative result. These problems can be addressed partly by enhancement protocols. Magnetic capture hybridization (MCH) uses single-stranded DNA bonded to magnetic beads as a template for capturing and concentrating target DNA. DNA is lyzed from the beads and the lyzate subjected to PCR. The sensitivity of this method is reported to enhance the detection threshold for *B. aclada* using the primers of Nielsen et al. (2002) by a factor of ten (Walcott, 2003). The primary disadvantage has to do with expense and additional handling of the samples. In any case, the relation between a positive result and the amount of fungus in tissue is unclear. Because *Botrytis* spp. have multinucleate conidia and hyphae the relationship between amplification of a specific band and the amount of fungal biomass is difficult to assess. ITS sequences are found in variable numbers, often several hundred copies are found in a genome. This makes ITS-based detection very sensitive to the presence of fungal DNA, with reports of 50-100 fg detection (Moricca et al., 1998), c. 100 times more sensitive than the detection limit for a single copy amplicon (Nielsen et al., 2002).

In addition to primer design, various strategies have been developed to reduce sample handling, to automate the extraction, amplification and detection steps and to increase sample through-put (Lévesque, 2001). Sample handling is a major source of contamination, while extraction is the major bottleneck and source of system-specific procedural variation. The localization of the putative pathogen is critical to sampling from tissues (Lévesque, 2001; Taylor et al., 2001).

Reverse dot-blot hybridisation allows the simultaneous detection of many genomes from a single sample. Several probes are immobilized on a membrane and sample extract is presented to each probe. Detection of hybridisation reveals the identity of the pathogen. Mathur and Utkhede (2002) have suggested their *B. cinerea*-specific probe could be used in this manner. Multiplex PCR, in which distinct primer sets that produce amplicons with either unique electrophoretic characteristics or ligated to different fluorescent moieties, similarly allows detection of multiple pathogens simultaneously. Real-time PCR (RT-PCR), in which amplification products release fluorophores that are detected as they approach a threshold concentration, obviating the necessity for electrophoresis, offers the

advantages of reduced sample handling and risk of contamination, a semi-quantitative detection of product and a faster turn-around time. Multiplexing is also possible, as well, with RT-PCR. Combined with automated melting point analysis of the PCR products, RT-PCR provides a check that specific amplification has occurred (Taylor et al., 2001). However, both apparatus and reagents are considerably more expensive than standard thermo-cyclers. RT-PCR following reverse transcription of mRNA was used by Choquer et al. (2003) to detect the levels of chitin synthase transcripts in *B. cinerea*-infected tissues. They calibrated their amplification products against levels of plant-associated RNAs and were able to make inferences about several pathogenicity-related systems. Electronic and mass spectrophotometric detection of hybridization products improve the sensitivity of detection, remove the need for time-consuming electrophoresis and are used for detection in bio-chips in microarray procedures. *Botrytis* is among the candidate genera being included in several on-going bio-chip projects. All DNA-based methods for detection of *Botrytis* infections in plant materials are susceptible to sampling errors, in part because of the small amount of tissue from which extractions and inferences can be made. Further, sampling is, of necessity, destructive.

5. Other detection methods

Several techniques based on the detection of characteristic metabolites (e.g. Fourier transform infra-red (FT-IR) spectroscopy (Dubernet et al., 2000; U. Fischer, SLFA, Neustadt, Germany, pers. comm.) nuclear magnetic resonance spectroscopy, and liquid chromatography-mass spectroscopy (LC-MS) equipped with electro-spray injection, have been developed that do not require destructive sampling and are capable of evaluating both large numbers and sizes of samples. These methods, however, require specific calibrations for *Botrytis* products, which have not been investigated so far. Botrydial is an infection-associated sesquiterpene produced during grey mould infection. LC-MS has been used to detect its presence in various host tissues (Deighton et al., 2001). Similarly the presence of β -1,3-glucanase in grape berries and grape vine leaves has been taken as an indicator of *Botrytis* infections (Renault et al., 2000), as have laccase (Grassin and Dubourdie, 1989), gluconic acid and glycerol in grape juice (Perez et al., 1991), but great caution should be exercised before using these methods for detection of a pathogen species because other organisms may produce the same or similar compounds in tissues. Characteristic lipid profiles have been derived from pure cultures of *B. cinerea* (Cooper et al., 2000), but these have not been used for detection of the pathogen in plant tissues.

Indirect effects of *Botrytis* infection are observed in several hosts. Increases in ethylene and ethanol with concomitant decreases in acetaldehyde have been detected in stored tomatoes and correlate with grey mould infection (Polevaya et al., 2002). The protein profiles of must from grape cultivars change as a consequence of infection with *Botrytis* and can be detected by SDS-polyacrylamide gel electrophoresis (Marchal et al., 1998).

6. Conclusions

6.1. Comparative utility of the different methods

Classical methods are useful where time and space are not limited and in laboratories where a good microscope and a certain level of expertise are available. However, rapid methods are needed where decisions have to be made about applications of fungicides and the sale of fruits and flowers on the home market versus export. Where genus-specificity is sufficient, immunological methods, such as the *Botrytis* lateral flow device (B-LFD) which is semi-quantitative and takes minutes rather than hours, promise to be useful. The lack of antibodies that are species-specific means that immunological methods cannot be used for the detection of one target *Botrytis* species in the presence of others, as occurs in onion crops. Nucleic acid-based methods employing species- and isolate-specific probes are the only methods currently available that can be used to track an individual species or isolate of *Botrytis* in unlabelled wild populations. Much valuable information can be gained from such methods, but they are demanding in space, time, expertise and cost. The genus-specific antibody BC-12.CA4 has proved useful for determining the biomass of *B. cinerea* and *B. aclada*.

For quantitative assays it is important that the optimum level of dilution be determined for each type of tissue or juice and that a set of standards is run with each test. Similarly, semi-quantitative nucleic acid based-methods such as RT-PCR, which have proved useful with other fungi, promise to be useful for quantification of *Botrytis*. However, correlation between quantity of amplification product and biomass is unclear for several reasons primarily associated with DNA extraction efficiency and nuclear number, which is variable within a thallus.

6.2. Problems and recommendations

Detection and quantification of latent or quiescent infections remains the most challenging and important problem because, in most cases, classical methods are too slow. The B-LFD has proved to be highly sensitive and preliminary studies have shown that, if the devices are allowed to continue developing over a 20-min period, they can detect the presence of very low levels of *Botrytis* antigens in grape juice and tissues before any visible symptoms of infection are apparent.

Determination of biomass in fungal infections is important in many fields of research, particularly in determining levels of resistance in transgenic crops and the efficacy of biocontrol agents and new fungicides. For these tests, which often involve large numbers of samples, plate immunoassays are recommended (PTA-ELISAs). Plate immunoassays could also be used for detection of seed borne infections in crops with small seeds such as onions (cf. Detection of fungi in individual rice grains by immunoassay by Dewey et al., 1992). Localization and quantification in tissues using GUS transformants is also promising for research purposes where studies with the one isolate are sufficient as are epidemiological studies using Nit and selenium mutants (Barnes and Shaw, 2003; Weeds et al., 1998, respectively, and Chapter 3).

Assessments of the levels of air-borne *Botrytis* conidia in greenhouses and polyethylene-covered tunnels, as well as in the field by direct capture into microtitre wells, raises the possibility of using nucleic acid-based methods to quantify specific isolates for epidemiological studies. Identification of *Botrytis* sclerotia in soils, which currently depends on plating out methods involving very long incubation periods, is laborious. It is possible that immunological methods employing antibodies that recognize washings from sclerotia soaked overnight in buffer in micro-titre wells could be used to study the life cycle of the pathogen in the field. Whatever method is used however, care has to be taken at every step from sampling to interpretation of results.

Deployment of the above methods devised during the past decade to detect, diagnose and quantify species of *Botrytis* should help greatly in furthering our understanding of the biology and epidemiology of *Botrytis* infections. The usefulness of metabolomic methods, such as FT-IR, still remains to be demonstrated.

7. References

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CHAPTER 12

CHEMICAL CONTROL OF *BOTRYTIS* AND ITS RESISTANCE TO CHEMICAL FUNGICIDES

Pierre Leroux

INRA, Unité de Phytopharmacie et Médiateurs Chimiques, 78026 Versailles cedex – France

Abstract. The chemical control of *Botrytis* spp., and especially *B. cinerea* the causal agent of grey mould on many crops, can be achieved by several families of fungicides. Among those affecting fungal respiration, the oldest ones are multi-site toxicants (e.g. dichlofluanid, thiram); newer ones are uncouplers (e.g. fluazinam), inhibitors of mitochondrial complex II (e.g. boscalid) or complex III (e.g. strobilurins). Within anti-microtubule botryticides, negative-cross resistance can occur between benzimidazoles (e.g. carbendazim) and phenylcarbamates (e.g. diethofencarb), a phenomenon determined by a mutation in the gene encoding β -tubulin. Aromatic hydrocarbon fungicides (e.g. dicloran), dicarboximides (e.g. iprodione, procymidone, vinclozolin) and phenylpyrroles (e.g. fludioxonil) affect the fungal content of polyols and resistance to these various compounds can be associated with mutations in a protein histidine kinase, probably involved in osmoregulation. However, dicarboximide-resistant field strains of *B. cinerea* are sensitive to phenylpyrroles. Anilinopyrimidines (e.g. cyprodinil, mepanipyrim, pyrimethanil) inhibit methionine biosynthesis but their primary target site remains unknown. In few situations, resistance of commercial significance has been recorded. Among sterol biosynthesis inhibitors those inhibiting 14 α -demethylase (DMIs) which are widely used against many fungal diseases are of limited interest against *Botrytis* spp., whereas the hydroxylanilide fenhexamid, which inhibits the 3-keto reductase involved in sterol C4-demethylations, is a powerful botryticide. Monitoring conducted in French vineyards revealed the presence of multi-drug resistant (MDR) strains, a phenomenon probably determined by overproduction of ATP-binding cassette transporters. Resistance towards fungicides of the different groups is described throughout the chapter.

1. Introduction

Botrytis cinerea, the anamorph of *Botryotinia fuckeliana*, is an ubiquitous fungus which causes grey mould on many economically important crops including vegetables (e.g. tomato, cucumber, lettuce), ornamentals (e.g. rose, gerbera), bulbs (e.g. onion) and fruits (e.g. grapevine, strawberry, kiwifruit). It can attack many organs including leaves, stems and fruits as a necrotroph, often with heavy losses after harvest. It is also a saprophyte on senescent and dead plant material. It had long been thought that *B. cinerea* was unspecialized, thus differing from the other species of this genus. For example, *B. tulipae* is found

only on tulips, *B. squamosa* on onions, *B. allii* on *Allium* spp. and *B. fabae* on Leguminosae (Chapter 3).

Chemical control remains the main way to reduce the incidence of grey mould and other *Botrytis* diseases on major crops. The most common interventions consist of spraying aerial parts of plants with fungicides. The applied doses (Table 1) vary from 2000-3000 g/ha (e.g. maneb, thiram, dichlofluanid) to 400-500 g/ha (e.g. carbendazim, fludioxonil, pyrimethanil). The number of treatments during a season ranges from one or two, to more than twenty. Treatments of seeds or bulbs, as well as fungicide applications after the harvest of fruits, are also used (Chapter 19). The chemical control of *Botrytis* diseases is impeded by the development of resistance to many botryticides and the negative public perception regarding the safety of pesticides. As a consequence, in many countries, the regulatory authorities have restricted the use of new and established pesticides (Gullino and Kuijpers, 1994). For instance in French vineyards, the numbers of applications per season of each family of botryticide is limited to one to avoid exceeding of MRL (Maximum Residue Level) values. Some active ingredients cannot be used near harvest (e.g. fludioxonil, diethofencarb) (Couteux and Lejeune, 2003). Several families of synthetic botryticides are available (Rosslenbroich and Stuebler, 2000) and they can be classified according to their biochemical modes of action. Five categories have been distinguished: 1) fungicides affecting fungal respiration; 2) anti-microtubule toxicants; 3) compounds affecting osmoregulation; 4) fungicides whose toxicity is reversed by amino acids; and 5) sterol biosynthesis inhibitors. The properties of the various active ingredients are presented and resistance phenomena described. Data are given on the molecular basis of botryticide resistances and also on phenotypical characteristics including resistance levels expressed as ratios EC 50 resistant/EC 50 sensitive strains (Anonymous, 1988).

2. Fungicides affecting respiration

The breakdown of organic molecules (i.e. sugars, fats, proteins) provides energy for the survival of living systems. In fungi, as in other eukaryotes, the final steps of this catabolic process take place in mitochondria and lead to the synthesis of the high energy intermediate ATP. Several groups of fungicides disturb the energy supply in *B. cinerea* and all such compounds are powerful inhibitors of conidia germination (Table 1). As the effect is associated with inhibition of conidia adhesion to polystyrene microtiter plates, a rapid high-throughput alternative to traditional conidia germination assays has been developed by Slawecki et al. (2002).

2.1 Multi-site toxicants

The multi-site fungicides that have been used for a long time against *Botrytis* diseases can be classified into three main chemical classes. There are dithiocarbamates, such as thiram (dialkyldithiocarbamate), which can control *B.*

cinerea on many crops, and mancozeb or maneb (alkylenebisdithiocarbamates), which have been commonly applied against *Botrytis* leaf blight of onion. The alkylenebisdithiocarbamates are polymeric complexes whose hydrolytic metabolite DIDT (5,6-dihydro-3H-imidazo[2,1]-1,2,4-dithiazole-3-thione) is considered to be the major fungitoxicant (Roberts et al., 1999). The second class correspond to N-trihalomethylthio fungicides; captan and folpet have unsaturated rings that are derivatives of phthalimide whereas dichlofluanid and tolylfluanid are arylsulphamides. The reaction of these compounds with thiols produces thiophosgene or its monofluoroanalogue that are both highly reactive chemicals, especially with thiols (Corbett et al., 1984). These N-trihalomethylthio fungicides are widely used against *B. cinerea* on various crops including fruits, ornamentals and vegetables. Chlorothalonil, which is a chloronitrile (or phthalonitrile) derivative, constitutes the last group of multi-site botryticides; it can be applied for example on peas to control *B. cinerea* or onions to control *B. squamosa*. All these multi-site fungicides exhibit protective action and need frequent applications of high doses, generally between 1000 and 2000 g/ha. In laboratory tests, most of them are highly toxic to conidial germination in *Botrytis*, but show low activity to mycelial growth. Chlorothalonil generally appears to be the most toxic compound at the conidial germination stage (Table 1; Pollastro et al., 1996; Slawewski et al., 2002; Tremblay et al., 2003). It has been shown that these multi-site fungicides prevent conidial germination by inhibiting several thiol-containing enzymes involved in respiration (Corbett et al., 1984).

The intrinsic risk for resistance to multi-site fungicides is considered to be low. However there are several reports of failure to control *Botrytis*-incited diseases, for instance, resistance of *B. squamosa* towards alkylenebisdithiocarbamates, but chlorothalonil remaining effective (Lorbeer and Vincelli, 1990). In laboratory tests, resistance levels varying four-fold have been recorded between strains (Tremblay et al., 2003); similar observations were done in *B. cinerea* (Delen et al., 1984). Due to the fact that this class of dithiocarbamates are not toxic by themselves, the relevance of *in vitro* tests is questionable. In several European countries resistance towards N-trihalomethylthio derivatives (especially dichlofluanid) was reported in *B. cinerea* and according to Malathrakis (1989), this phenomenon also applies to chlorothalonil. The *in vitro* response to dichlofluanid in mycelial growth or conidia germination tests provided low to moderate resistance levels (between 2 and 20) (Malathrakis, 1989; Rewal et al., 1991; Pollastro et al., 1996). A recent study indicated that dichlofluanid resistance was determined by two major genes named *Dic1* and *Dic2* (Pollastro et al., 1996). Moreover, cross-resistance to various dithiocarbamates was identified among captan-resistant isolates. Biochemical studies revealed that levels of glutathione were higher than normal in these field isolates resistant to captan in response of this fungicide. Such a phenomenon could make more non-vital thiol-containing compounds available for detoxification of captan and therefore prevent damage that the fungicides could cause to vital protein thiols of fungal cells (Barak and Edgington, 1984). Glutathione S-transferases (GST) are a family of multi-functional detoxification enzymes that catalyse the conjugation of a wide variety of xenobiotics to

glutathione in mammals and plants. Few data are available in fungi, but the recent targeted inactivation of a GST from *B. cinerea* failed to show that it had a role in chemical stress tolerance (Prins et al., 2000). However, more GSTs exist and need to be studied (P. Tudzynski, Münster University, Germany, pers. comm.).

Table 1. Intrinsic toxicity of fungicides towards *Botrytis cinerea* and application rates

Fungicide		EC ₅₀ value (mg/l)			Typical Application rate (g/ha)
Common name	Selected trade names	Conidia germination	Germtube elongation	Mycelium growth	
Dichlofluanid	Elvaron, Euparen	0.20	0.05	3.00	2000
Folpet	Folpan	3.00	0.40	10.00	1500
Thiram	Pomarsol, Thiram	~ 1.00	0.10	10.00	2000
Fluazinam	Frownicide, Shirlan	0.10	0.04	0.08	750
Azoxystrobin	Amistar, Heritage	> 10.00	2.50	10.00	200
Boscalid	Endura	0.50	0.10	0.40	600
Carbendazim	Bavistin, Derosal	> 10.00	0.04	0.03	500
Diethofencarb ^a	Sumico ^b	> 10.00	0.08	0.04	500
Iprodione	Kidan, Rovral	2.00	0.80	0.15	750
Procymidone	Sumiclex, Sumilex	2.50	0.80	0.15	750
Fludioxonil	Geoxe, Switch ^c	0.06	0.015	0.004	500
Cyprodinil	Switch ^c , Unix	~ 0.10	0.008	0.01	375
Pyrimethanil	Scala	~ 0.30	0.05	0.08	800
Prochloraz	Octave, Sportak	> 10.00	0.03	0.10	250
Tebuconazole	Folicur, Horizon	> 10.00	0.20	0.30	250
Fenhexamid	Teldor	> 10.00	0.05	0.01	750

^aThe EC₅₀ values were recorded by testing benzimidazole-resistant strains (BenR1).

^bMixture of diethofencarb and carbendazim.

^cMixture of fludioxonil and cyprodinil.

2.2. Uncouplers of oxidative phosphorylation

Fluazinam is a phenylpyridinamine derivative exhibiting a broad antifungal spectrum. For instance, it shows excellent activity against potato blight. Against grey mould, fluazinam has been used in Japan since 1990, on various crops, including beans. It was introduced at the end of 1990s in European vineyards at the registered dose in France of 750 g/ha. No cross-resistance was observed between fluazinam and the other botryticides (Leroux et al., 1997; Kalamarakis et al., 2000). In laboratory tests, this phenylpyridinamine is highly toxic to conidia and mycelia with EC₅₀ values below 0.1 mg/l (Table 1, Kalamarakis et al., 2000; Slawewski et al., 2002). This high level of fungitoxicity is probably related to uncoupling of mitochondrial oxidative phosphorylation that is governed by a protonophoric cycle involving protonation/deprotonation of the secondary amino group (see Leroux, 1996).

Concerning resistance, a recent report from Japan indicated the emergence of fluazinam-resistant populations in bean crops from Hokkaido. A reduction of

efficacy has been recorded since 1996 (about 6 years after the introduction of fluazinam). Based on the inhibition of mycelial growth, sensitive strains collected mainly in fields which had never been treated by fluazinam, exhibited EC_{50} values of about 0.003 mg/l. In similar tests in treated crops, two types of strain exhibiting low (about 10) or high (about 10,000) resistance levels were detected. Under field conditions, fluazinam-resistant strains, especially in a programme involving three treatments of this botryticide, were ineffective (Tamura, 2000). Monitoring done in vineyards in Champagne and based on a test on conidial germination (discriminatory concentration of 0.5 mg/l) has failed to show any shift in sensitivity of *B. cinerea* towards fluazinam (Leroux et al., 2002a). The biochemical background of fluazinam resistance in *B. cinerea* is not known, but a mechanism involving detoxification can be suggested. This assumption is based on the fact that in mammalian mitochondria, fluazinam is metabolically detoxified probably by a glutathione conjugation mechanism (see Leroux et al., 2002a).

2.3. Inhibitors of mitochondrial complex III

Fungicides inhibiting mitochondrial respiration by binding to cytochrome b, a part of the cytochrome bcl complex (syn. complex III), were first introduced to the market in 1996. Most of them are synthetic analogues of natural strobilurins (e.g. strobilurin A) produced by a range of Basidiomycete wood-rotting fungi. Strobilurin fungicides are members of QoIs because of their binding at the Qo site (an outer, quinone oxidizing pocket) of cytochrome b (Bartlett et al., 2002). Some of them such as azoxystrobin (Figure 1) or metaminostrobin control *B. cinerea* on various crops, including vegetables, ornamentals and strawberries. For instance with azoxystrobin, good efficacies were obtained from a 7 to 10-day schedule at rates of about 200 to 250 g/ha (Dacol et al., 1998).

In some situations, especially on grapevine, the control of grey mould was not satisfactory. This might be related to the fact that the terminal alternative oxidase (AOX), constitutively present in *B. cinerea* mitochondria, allows electron flux to by-pass the blockage of the cytochrome bcl pathway caused by strobilurins (Tamura et al., 1999; Wood and Hollomon, 2003). Such a phenomenon occurs *in vitro* because increased susceptibility to strobilurins has been found either in the presence of SHAM, an inhibitor of AOX (Tamura et al., 1999) or after disruption of the gene encoding for AOX (C. Levis, unpubl.). It also explains why in *in vitro* tests, *B. cinerea* tolerates high concentrations of QoIs (Table 1). On the other hand, the participation of AOX during the interaction of *B. cinerea* with plants remains questionable (Wood and Hollomon, 2003). Tamura et al. (1999) postulated that practical control of *B. cinerea* with strobilurins is dependent on the amount of natural inhibitors (e.g. flavonoids) of AOX in plant tissues. Regarding acquired fungicide resistance in plant pathogens, it is generally caused by a point mutation in the mitochondrial cytochrome b gene leading to an amino acid change from glycine to alanine at

position 143 (Gisi et al., 2002). It remains to be determined if this mechanism is involved in natural or acquired resistance to QoIs in *B. cinerea*.

2.4. Inhibitors of mitochondrial complex II

Carboxin and related anilides or carboxamides are systemic fungicides controlling mainly Basidiomycetes. They were introduced at the end of the 1960s as seed or foliar treatments (Kulka and von Schmeling, 1995). In laboratory tests some Ascomycetes and Adelomycetes such as *Aspergillus* spp., *Fusarium* spp., *Helminthosporium* spp. and *B. cinerea*, were also inhibited by some carboxamides. These fungicides inhibit the succinate dehydrogenase complex (syn. complex II) and for instance with carboxin the I_{50} values were about 0.1 mg/l in enzyme preparations from Basidiomycetes and from some other fungi including *B. cinerea* (White and Georgopoulos, 1992; Fritz et al., 1993). In mammals, plants and the yeast *Saccharomyces cerevisiae* inhibition of complex II was observed at higher concentrations (White and Georgopoulos, 1992). From these data, it appeared that selectivity of carboxin and related carboxamides was related to differences in sensitivity among complex II and/or to accessibility of the toxicant to its target site.

Boscalid (syn. nicobifen) is a novel broad spectrum carboxamide (Figure 1) effective at 600 g/ha against grey mould to be released for use council RETURN on grapevine in 2004. In laboratory tests conducted on conidia or mycelium, the fungitoxicity of this nicotinamide depended upon the composition of the nutrient medium, especially the carbon source. For instance, in the presence of glucose, the activity of boscalid was low (the absence of any synergism by SHAM indicated that AOX was not involved) and highest toxicities were recorded with succinate or acetate instead of glucose. A survey of *B. cinerea* populations from Champagne vineyards did not detect any strains moderately or highly resistant to boscalid and showed the absence of cross-resistance with benzimidazoles, phenylcarbamates and anilinopyrimidines (Leroux et al., 2003).

Succinate dehydrogenase is composed of two main sub-units: a flavoprotein (Fp or CII 1) and an iron-sulphur protein (Ip or CII 2), which together with two membrane-anchoring proteins (CII 3 and CII 4) make up complex II. Carboxamides prevent the transfer of electrons from succinate to ubiquinone, probably by intercalating between sub-units Ip and CII 3 (White and Georgopoulos, 1992). The gene encoding the Ip sub-unit from carboxin-sensitive strains and laboratory mutants has been sequenced from a few fungal species. The deduced amino acid sequences showed a high degree of homology, particularly within the three cystein-rich clusters. Furthermore, resistance was related to a change within the third cluster, where histidine was replaced either by leucine or tyrosine (Broomfield and Hargreaves, 1992). According to the sequence of the gene encoding sub-unit Ip in *B. cinerea*, this highly conserved histidine residue is also present (A.S. Walker, INRA, Versailles, France, unpubl.).

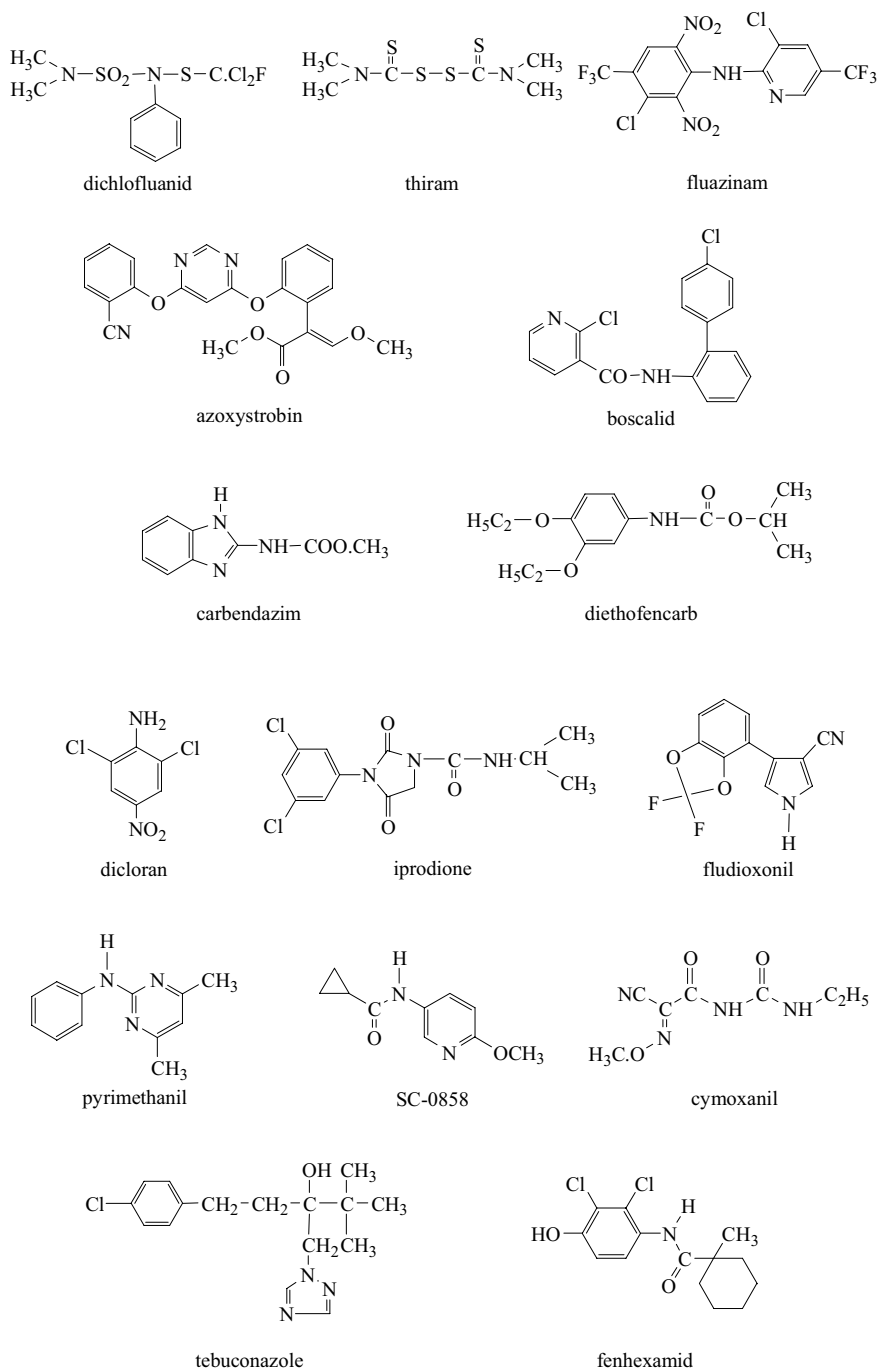


Figure 1 : Structure of fungicides with anti-Botrytis activities

3. Anti-microtubule fungicides

The benzimidazole fungicides, thiabendazole, carbendazim (Figure 1) and the related benomyl and thiophanate-methyl were developed in the late 1960s. They were the first systemic fungicides that exhibited a broad spectrum of activity. Most Ascomycetes and Adelomycetes are sensitive, especially *Botrytis* spp. (Delp, 1995). In *B. cinerea*, these fungicides do not prevent conidial germination, but at low concentrations, they inhibit hyphal growth and cause distortion of germ tubes (Table 1; Leroux et al., 1999). Such effects, probably the consequence of perturbations in the functioning of cellular microtubules, result from the binding of benzimidazoles to the β -tubulin sub-unit of fungal tubulin (Davidse and Ishii, 1995).

Shortly after the introduction of benzimidazoles, several cases of resistance were recorded and in *B. cinerea* the first report was on cyclamen in Dutch greenhouses after 2 years of use (Bollen and Scholten, 1971). A similar situation occurred in European vineyards and after only three to four seasons, use of benzimidazoles in the grape *Botrytis* market in northern Europe was severely curtailed (Leroux and Clerjeau, 1985; Smith, 1988). Many other crops have been affected and in some countries, benzimidazoles did not even reach registration for *B. cinerea* control because of the perceived resistance risk (Y. Elad, pers comm.). Most of the benzimidazole-resistant strains exhibit resistance levels greater than 1000, and are simultaneously very susceptible to N-phenylcarbamates, such as diethofencarb (phenotype Ben R1; Leroux et al., 1999). This property which was initially observed with N-chlorophenylcarbamates used as herbicides (e.g. barban, chloroprotham) also occurred with the scald inhibitor diphenylamine and with experimental N-phenyl-formamidoximes (Davidse and Ishii, 1995). The discovery of this negative cross-resistance led to the introduction of a mixture of carbendazim and diethofencarb at the end of the 1980s. In Israel, populations of *B. cinerea* resistant to the mixture were found immediately at the beginning of its commercial use in cucumber greenhouses (Katan et al., 1989). Similarly, in Champagne, after only 2 years of use of this mixture, strains of *B. cinerea* simultaneously resistant to both fungicides were detected (phenotype Ben R2; Leroux et al., 1999). Such a phenotype, which has been detected in other countries and on various crops, was moderately resistant to benzimidazoles (resistance levels from 30 to 100) (Yarden and Katan, 1993; Nakazawa and Yamada, 1997). In both phenotypes, benzimidazole resistance was conferred by alleles of the same gene named *Mbc1* encoding the structural gene for β -tubulin (Faretra and Pollastro, 1991). The changes with regard to wild type (WT) were in Ben R1, at position 198 an alanine replaced a glutamate, whereas in Ben R2, at position 200 a tyrosine replaced a phenylalanine. Two additional mutations located in codon 198 have been detected in benzimidazole-resistant strains isolated from vegetables. The first mutation resulted in a lysine replacing a glutamate and gave high resistance to benzimidazoles and diethofencarb (Yarden and Katan, 1993). The other mutation which substituted valine, conferred a weak resistance to diethofencarb (Nakazawa and Yamada, 1997). In several fungi,

including *B. cinerea*, binding of carbendazim to β -tubulin of benzimidazole-resistant strains was low compared to WT strains (Davidse and Ishii, 1995).

Surveys of benzimidazole-resistance in *B. cinerea* can be achieved in assays with mycelium or conidia using discriminatory concentrations. For instance, in our laboratory, based on the inhibition of germ-tube elongation, benzimidazole-resistant strains can be detected by 1 mg/ml carbendazim. At 10 mg/l carbendazim highly resistant-strains Ben R1 are detected and they are the only ones which are inhibited by 10 mg/l diethofencarb. The mixture diethofencarb (10 mg/l) + carbendazim (1 mg/l) allowed the detection of the phenotype Ben R2. Moreover, according to the molecular basis of benzimidazole-resistance, PCR (Polymerase Chain Reaction)-based methods can be developed (Koenraadt and Jones, 1992). In *B. cinerea*, the successful analysis of samples from fungal mycelium or from infected plant tissues was achieved by AS-PCR (Allele Specific PCR) or by PCR-RFLP (PCR-Restriction Fragment Length Polymorphism) using *Tha*I as restriction enzyme. This method allowed discrimination between WT strains and Ben R1 (Luck and Gillings, 1995).

Under laboratory conditions, many mutations in the β -tubulin gene can confer benzimidazole-resistance. However, under field conditions, in most plant pathogenic fungi, they are limited to positions 198 and 200 (Davidse and Ishii, 1995). A possible reason is that mutation in other codons might impose a selective disadvantage under field conditions. Results from greenhouse and field studies indicate that for several plant pathogens benzimidazole-resistance is generally persistent in the absence of treatment. The survey conducted in Champagne vineyards illustrates this phenomenon in *B. cinerea* for the Ben R1 phenotype (Leroux, 1995; Leroux et al., 2002b). This observation indicates that the substitution of an alanine by a glutamate in position 198 does not reduce the fitness of *B. cinerea*. On the contrary, the appearance of field mutants with a substitution of phenylalanine by tyrosine in position 200 was less surprising. Indeed, in various organisms, naturally resistant to benzimidazole fungicides, methionine or tyrosine are found, suggesting a reduced constraint in this position (Davidse and Ishii, 1995). On the other hand, the fact that the frequencies of Ben R2 strains decrease in the absence of treatments by the mixture carbendazim + diethofencarb suggests that the presence of tyrosine in position 200 interferes with the fitness of such mutants. Moreover, the generalization of programmes involving one annual application of carbendazim + diethofencarb maintain high proportions of Ben R2 strains. Consequently, discontinuous use of this mixture is advised in French vineyards. An additional constraint for these anti-microtubule compounds is their long persistence and tendency to accumulate excessive residues, and consequently only a single spray at flowering is advised for the mixture carbendazim + diethofencarb (Leroux, 1995; Leroux et al., 2002b).

4. Fungicides affecting osmoregulation

One of the common features of the pesticides mentioned in this paragraph is their cross resistance in numerous laboratory mutants from various fungal species

Moreover this phenomenon is connected with an increased osmotic sensitivity, revealed when resistant mutants are inoculated to nutrient medium containing high concentrations of mineral salts (e.g. NaCl) or sugars (e.g. glucose) (Leroux and Fritz, 1984; Fujimura et al., 2000b). The three following chemical families are recognized: “dicarboximides”, “phenylpyrroles” and “aromatic hydrocarbons”. Dicarboximides or DCOFS are cyclic imides (e.g. chlozolinate, iprodione, procymidone, vinclozolin), also characterized by the presence of a 3,5-dichlorophenyl group (Figure 1). They superseded the benzimidazoles in the late 1970s for the control of grey mould. The phenylpyrrole antibiotic pyrrolnitrin is an antibiotic compound produced by a number of *Pseudomonas* spp. and is thought to play a role in biocontrol by these bacteria. Several synthetic analogues of pyrrolnitrin have been tested and two of them, fenpiclonil and fludioxonil have been developed as seed-dressing agents against numerous fungal species. Owing to its good light stability, fludioxonil was introduced in the mid 1990s as a foliar fungicide, especially against *B. cinerea* (Rosslenbroich and Stuebler, 2000). Aromatic hydrocarbon fungicides (AHFs) represent an old and heterogeneous group of fungitoxicants. Among them the most active compound against *Botrytis* spp. was dicloran (Figure 1); other compounds such as quintozene and *o*-phenylphenol can also partially control *B. cinerea*, respectively in soils and during fruit storage. Moreover, the *in vitro* antifungal effects of the organophosphorus tolclofos-methyl (mainly used against *Rhizoctonia* spp.) and of the herbicide dichlobenil allowed them to be classified within the AHF group.

Dicarboximides, phenylpyrroles and the most toxic AHFs (e.g. dicloran) inhibit both conidial germination and mycelium growth, but the latter process is more sensitive (Table 1). Furthermore, all these fungicides produce similar morphological alterations of germ-tubes (i.e. swelling, bursting, branching). Fludioxonil appears 30-40 times more toxic than dicarboximides in *in vitro* effects on hyphal growth, but under field conditions the registered doses for both families are similar (i.e. 500 g fludioxonil/ha versus 750 g dicarboximide/ha in French vineyards).

In *B. cinerea*, numerous studies have been done on fungicide resistant strains induced in the laboratory or collected in the fields. Laboratory mutants showing high resistance levels towards dicarboximides, phenylpyrroles and AHFs that are simultaneously sensitive to osmotic stress can be easily produced when cultivating WT strains in the presence of a fungicide belonging to these classes. Such a phenotype is unusual in the field, but some authors have been successful (Gouot, 1988). Monitoring on different crops revealed the presence of strains moderately resistant to dicarboximides (resistance levels about 10) with cross-resistance to AHFs, but not with phenylpyrroles; this resistance is coupled with either slight or no osmotic sensitivity. In some field strains resistance is restricted to dicarboximides (Leroux et al., 1999), or to phenylpyrroles (Vignutelli et al., 2002). Genetic studies conducted by Faretra and Pollastro (1991) with field strains and laboratory mutants showed that dicarboximide resistance was determined by a single polymorphic gene named *Daf1*. Furthermore in some strains, showing specific resistances to dicarboximides, phenylpyrroles or AHFs,

other genes are probably involved (Vignutelli et al., 2002). Such a multigenic resistance to the previous fungicides was also described in several fungi including *Neurospora crassa* and *Ustilago maydis* (see Leroux et al., 2002a).

4.1. Lipid peroxidation and oxidative damage

The mechanism of action of AHFs, dicarboximides and phenylpyrroles is a source of debate and their primary target site is not yet known. Many effects on various processes have been recorded: cell wall synthesis (this effect is considered to be the primary mode of action of dichlobenil on plants); metabolism of lipids; synthesis of nucleic acids; cellular and nuclear division (including mitotic instability); respiration; and metabolism of sugars (Leroux, 1996). According to Edlich and Lyr (1992) most of the effects can be attributed to the production of active oxygen species (AOS) and these authors showed that the level of lipid peroxides are in good correlation with the fungicide concentrations. Support to the involvement of oxygen free radicals in the primary action of these fungicides includes the observation that the addition of antioxidants, especially α -tocopherol, reverses their fungitoxicity. Furthermore, the enhanced levels of catalase and superoxide dismutase recorded in some resistant strains could be responsible for the detoxification of peroxi-radicals. Edlich and Lyr (1992) proposed that these fungicides act by blocking flavine enzymes, like NADPH-cytochrome c reductase, and that this leads to abnormal electron transport that produces AOS. However, in several recent investigations, the involvement of AOS in the primary mode of action of fungicides is disputed. For instance, the production of lipid peroxides in growing fungal cells was unaffected by low fungicide concentrations (Cabral and Cabral, 2000). In primary cultured fish hepatocytes, iprodione produced oxidative damage that is not due to interaction with NADPH-cytochrome c reductase (Radice et al., 2001). Another common feature of these fungicides is the fact that they are antagonized by inhibitors of cytochrome P-450 monooxygenases (P-450s) such as piperonylbutoxide or DMI fungicides (Sect. 6; Leroux et al., 1992). This effect could suggest that fungitoxicity of dicarboximides, AHFs and phenylpyrroles may depend upon an activation of these compounds by P-450s. Another hypothesis suggested by Edlich and Lyr (1992) is that inhibitors of P-450 could prevent the binding of the previous fungicides to their membrane receptor and consequently would suppress lipid peroxidation. A third possibility could be related to the induction of P-450s with deleterious effects on fungal metabolism; such a phenomenon has been observed in animals (Radice et al., 2001). In a recent study conducted with iprodione it has been shown that this dicarboximide exhibited a selective effect on lipid metabolism in *B. cinerea*. A decrease of phosphoglyceride content suggested that choline (ethanolamine) phosphotransferase would be worth investigating as a primary mode of action (Griffiths et al., 2003).

4.2. Fungal osmoregulation

Effects towards osmoregulation processes were studied in filamentous fungi, including *N. crassa*. In WT strains, stimulation of glycerol biosynthesis was recorded with dicarboximides, phenylpyrroles and AHFs (Fujimura et al., 2000a, b). Besides, most osmotic sensitive strains with *os1*, *os2*, *os-4* and *os-5* mutations showed cross-resistance to the previous fungicides (Fujimura et al., 2000a, b). The *os1* locus of *N. crassa* encodes a putative histidine kinase (os-1p). *N. crassa* os-1p shows similarities with the *S. cerevisiae* sln1p sensor, but it does not possess potential trans-membrane-spanning domains, suggesting that it has an intracellular distribution. In addition, os-1p contains a large N-terminal domain with six tandem repeats of 90 amino acids, which is predicted to form a coiled coil structure. These cytoplasmic helical linker domains, also named 'HAMP domains', because of their presence in histidine kinase adenyl cyclases, methyl accepting proteins and phosphatases, are common to signalling proteins. In fungal histidine kinase, the association of these HAMP domains might form a regular superstructure that is necessary for mediating interactions of these proteins with target molecules (Aravind and Ponting, 1999). Among *os-1* mutant strains, those carrying either a stop codon (position 308) or a frame shift (at position 294) in the second HAMP domain appear highly resistant to fungicides, but are moderately sensitive to osmotic stress; they are probably null mutants. In another phenotype characterized by high osmo-sensitivity and moderate fungicide resistance, single base-pair mutations resulting in single amino acid changes have been recorded within the fifth HAMP domain (Ochiai et al., 2001). Several other mutants combining resistance to fungicides and sensitivity to osmotic stress show single amino acid changes within the third, the fourth or the sixth HAMP domains; in one case a deletion of a single codon at an amino acid of the sixth HAMP domain was observed. The disruption of the *os1* gene is responsible for similar alterations in response to fungicides and osmotic sensitivity (Miller et al., 2002). Recently the *os-2*, *os-4* and *os-5* genes of *N. crassa* have been characterized. They encode putative protein kinases respectively homologous to the *S. cerevisiae* Hog1p, Ssk22p and Pbs2p (Zhang et al., 2002; Fujimura et al., 2003); suggesting that the hyper-osmotic stress response pathway of *N. crassa* can be the primary mode of action of dicarboximides, phenylpyrroles and AHFs. A direct interaction with a protein kinase involved in the MAPK cascade cannot be excluded because Pillonel and Meyer (1997) have shown that, in *N. crassa*, an intracellular c-AMP- and Ca-independent protein kinase is inhibited by phenylpyrroles. On the other hand, in *U. maydis*, a c-AMP-dependent protein kinase implicated in dicarboximide resistance and AHFs is not inhibited by these fungicides (Ramesh et al., 2001).

Very recently, a gene designated *BcOS1* (Leroux et al., 2002a) or *Bos1* (Cui et al., 2002) homologous to *os1* of *N. crassa* has been cloned and sequenced from various strains of *B. cinerea*. Translation of the gene *BcOS1* predicted a 1315 amino acid protein (BcOS1p) with an organization similar to that of os-1p from *N. crassa*, including the presumed phosphoryl acceptors histidine and aspartate at positions 735 and 1158 respectively, an ATP-binding motif and a

linker region with six HAMP domains. According to Cui et al. (2002), *Daf1* and *Bos1* correspond to the same gene. Regarding laboratory mutants highly resistant to fungicides and sensitive to osmotic stress, single base-pair mutations determine single amino acid changes within the second HAMP domain (at position 325 or 346), the third one (at position 386), the fourth one (at position 538) or the fifth one (at position 585). A stop codon at position 684 (sixth HAMP domain) leads to a similar phenotype. Concerning laboratory mutants osmotically sensitive but showing low resistance levels to dicarboximides, they carry single amino acid changes either in the second (at position 345) or in the fourth (at position 531) HAMP domain (Cui et al., 2002; Leroux et al., 2002a). In most dicarboximide-resistant strains of *B. cinerea* from the field, a point mutation at codon 365 of the second HAMP domain which encodes an isoleucine in WT strains, is substituted by a serine; in isolates from French vineyards arginine or asparagine were also found instead of serine (Oshima et al., 2002; Leroux et al., 2002a). In Japanese populations of *B. cinerea*, two additional groups of genotypes were characterized either by four amino acid substitutions at positions 225, 368, 369 and 447, or by two substitutions at positions 369 and 373 (see Sierotzki and Gisi, 2003). These observations as well as those obtained in *N. crassa* suggest that the linker region of the *os1*-type histidine kinase of filamentous fungi could be the target site of dicarboximides, phenylpyrroles and AHF fungicides, but direct binding studies are needed to confirm this hypothesis.

4.3. Acquired resistance in the field

In *Botrytis* spp., the only case of practical resistance concerned dicarboximides and, as mentioned previously, this phenomenon is mainly related to moderately resistant strains. The scarceness of strains highly resistant to dicarboximides, phenylpyrroles and AHFs is probably related to their osmo-sensitivity that induces a reduced fitness. Surveys in *B. cinerea* can be done using assays on mycelium or conidia using discriminatory concentrations. For instance in the presence of procymidone (10 mg/l) or vinclozolin (5 mg/l), conidia of WT strains do not germinate whereas those of dicarboximide-resistant ones produce germ-tubes; using iprodione at 25-40 mg/l, highly resistant strains can be specifically detected (Leroux and Clerjeau, 1985). During *in vitro* tests involving mycelial growth there is the risk of inducing highly resistant sectors during the time of the experiment. Recently a PCR-RFLP method using *Taq1* as restriction enzyme was proposed for detection of the most abundant mutation (I 365 S) in the *BcOs1* gene (see Sierotzki and Gisi, 2003).

The first dicarboximide-resistant field isolates of *B. cinerea* were found in a German vineyard (Mosel growing area) at the end of 1978, 3 years after the first registration of dicarboximides. In 1980 many European vineyards were concerned, but due to the lack of good alternative fungicides, dicarboximides use continued and the number of resistant strains increased considerably in these regions in the early 1980s. Further reports of dicarboximide-resistant *Botrytis*

strains are mainly concerned with strawberries, vegetables and greenhouse crops of a wide variety (Lorenz, 1988). Failures of control have been reported for instance on protected crops (Katan, 1982) or in vineyards (Leroux and Clerjeau, 1985), but sometimes only decreased efficacy was observed (Lorenz, 1988).

Monitoring done on various crops generally shows a decline in frequency of dicarboximide-resistant isolates following discontinuation of fungicide applications (Gouot, 1988; Pak et al., 1990; Leroux, 1995; Pommer and Lorenz, 1995). This could represent a reduced fitness of dicarboximide-resistant strains. According to Raposo et al. (2000), such a phenomenon occurs during the saprophytic phase rather than the parasitic phase in the life-cycle of *B. cinerea*. Some authors also suggest that in *B. cinerea*, phenotype instability and heterokaryosis lead to a decrease in resistance after cessation of dicarboximide treatments (Faretra and Pollastro, 1993; Yourman et al., 2001).

As observed for *B. cinerea* in vineyards, the selection pressure for resistance is related to the number of dicarboximide sprays (Leroux and Clerjeau, 1985). As a guide, FRAC (Fungicide Resistance Action Committee) recommends that the number of dicarboximide-based treatments should be restricted to two or three per crop and per season. In the case of French vineyards, the recommendation was to apply them only once a season. In addition to limiting the number of applications of dicarboximides, another anti-resistance strategy consists of mixing these botryticides with a fungicide with multi-site mode of action, such as chlorothalonil or thiram. In most cases the performance of the combination was better and more stable than dicarboximides alone (Gullino and Garibaldi, 1982; Katan and Ovadia, 1985; Lorbeer and Vincelli, 1990; Lorenz et al., 1994). With respect to the selection pressure, full dosages of dicarboximides alone or in mixtures caused in general the same increase in the resistant population. On the other hand, mixtures with reduced dosages of dicarboximides often delayed the selection of resistant strains (Vali and Moorman, 1992; Lorenz et al., 1994; Leroux, 1995). In the case of fludioxonil that was first used in 1995 in French vineyards, it is recommended for use (alone or in mixture with cyprodinil) only once a season. To our knowledge after several years of monitoring, no strains moderately to highly resistant towards phenylpyrroles have been isolated so far.

5. Fungicides whose activity is reversed by methionine

The anilinopyrimidine fungicides (syn. APs) cyprodinil, mepanipyrim and pyrimethanil are chemicals closely related to each other (Figure 1). They are effective against a wide range of Ascomycetes and Adelomycetes and are mainly used as foliar sprays at 200-1000 g/ha. They were introduced as botryticides in various European countries in the mid-1990s. *In vitro* studies with *B. cinerea* revealed that anilinopyrimidines strongly inhibit germ tube elongation but effects on mycelial growth varied according to the composition of nutrient media. Fungitoxicity was generally low with complex media, for instance those containing yeast extract. This phenomenon seems to be related to the ability of

the fungus to obtain nutrients by methods that circumvent the mode of action of anilinopyrimidines. Several amino acids, particularly methionine, have been shown to antagonize the fungitoxicity of anilinopyrimidines and a similar effect was recorded with the experimental anilide SC-0858 (Leroux, 1994; Masner et al., 1994). Because cystathionine, the precursor of methionine, only antagonizes the fungitoxicity of SC-0858, it has been suggested that the primary target of anilinopyrimidine could be cystathionine β -lyase and the target of SC-0858 is cystathionine γ -synthase. In *N. crassa*, resistant mutants to this anilide are allelic to *met7*, the gene encoding cystathionine γ -synthase. However, enzymatic and biochemical studies have never shown any effect of SC-0858 on this enzyme and methionine biosynthesis (see Leroux, 1996).

Biochemical studies in *B. cinerea* with $\text{Na}_2^{35}\text{SO}_4$ indicated that the levels of radio-labelled methionine and homocysteine from mycelia were lower after a treatment by pyrimethanil; at the same time a slight increase in radio-labelled cystathionine was recorded. This result constitutes an indirect proof of a primary effect of anilinopyrimidines on cystathionine β -lyase (Fritz et al., 1997). However, enzymatic studies revealed only weak inhibition of cystathionine β -lyase by anilinopyrimidines (Sierotzki et al., 2002; Fritz et al., 2003). Besides, several cyanooxime derivatives, including the Oomycete fungicide cymoxanil, can inhibit the mycelial growth of some *B. cinerea* strains at low concentrations. This *in vitro* effect is reversed by several amino acids including methionine, cystathionine, and also serine or glycine. However, biochemical studies did not reveal major changes in the composition of sulphur-containing amino acids. Moreover, the most sensitive strains quickly metabolized cymoxanil, suggesting that cyanooximes are probably activated to a fungitoxic compound (Leroux et al., 1987; Tellier et al., 2002).

The anilinopyrimidines also have the ability to prevent secretion of hydrolytic enzymes (e.g. proteases, cellulases, lipases, cutinases) involved in the infection process (Miura et al., 1994). In *B. cinerea*, the same phenomenon concerns laccases and could explain the reduced laccase activity in wines produced on grapevines treated with anilinopyrimidines (Milling and Richardson, 1995). The exact mechanism of action in the protein secretion pathways is not yet understood; it has been hypothesized that the target of anilinopyrimidines could be a step involving the Golgi complex or a later stage. A similar phenomenon seems to occur in rat hepatocytes treated by mepanipyrim, because this anilinopyrimidine inhibits the transport of very low density lipoproteins (VLDL) from the Golgi apparatus to the cell surface. This effect could result from abnormalities in the final stage of lipoprotein assembly (Terada et al., 1998). However it remains to be determined if in fungi the inhibition of secretion of proteins is really the primary mode of action of anilinopyrimidines.

Monitoring in vineyards of various countries including France, Switzerland, Croatia and Chile, has detected anilinopyrimidine-resistant strains. In most cases they were moderately to highly resistant to anilinopyrimidines in which ever *in vitro* assay was used. Resistance was also confirmed by *in vivo* methods

(phenotype AniR1; Birchmore and Forster, 1996; Leroux et al., 1999). In field uses reduced performances were recorded when these botryticides were used intensively. Such a situation was observed for the first time, in a Swiss trial (Forster and Staub, 1996) and has also been reported recently in commercial table grapevines in Chile (Latorre et al., 2002). According to Petsikos-Panayotarou et al. (2003), strains of *B. cinerea* with low resistance levels (below 10) can also lead to decreased efficacy of pyrimethanil in greenhouse-grown tomatoes. In French vineyards, similar phenotypes were also detected, but they did not seem to lead to resistance of practical importance; in fact, they have been characterized as multi-drug resistant phenotypes (Sect. 7). Some moderately to highly resistant strains of *B. cinerea* appeared unstable and that could explain why they were not selected as rapidly as those resistant to benzimidazoles or dicarboximides. However, crosses conducted with stable strains revealed that resistance was monogenic and consequently selection of the “disruptive” or “qualitative” type might be expected (Hilber and Hilber-Bodmer, 1998; Chapeland et al., 1999).

Uptake and metabolic profiles of pyrimethanil were similar in both WT strains and anilinoypyrimidine-resistant strains suggesting that specific resistance to anilinoypyrimidines might be related to a change at the target site. The cystathionine β -lyase gene was cloned and sequenced from sensitive and resistant field strains of *B. cinerea*. It encodes a 459-amino acid protein and the sequence polymorphism in the coding region of this gene revealed 13 single nucleotide substitutions within strains collected in French vineyards. Among these substitutions only one found in two anilinoypyrimidine-resistant strains led to a change from a threonine (in WT strains and most anilinoypyrimidine-resistant ones) to a valine at position 302. From this result, it can be concluded that cystathionine β -lyase does not contribute to the mode of resistance towards anilinoypyrimidines (Fritz et al., 2003). Another study conducted by Sierotzki et al. (2002) on the gene encoding cystathionine γ -synthase (the potential target of SC-0858) revealed some differences between field isolates of *B. cinerea* sensitive and resistant to anilinoypyrimidines. Among the three detected point mutations, only two led to amino acid changes. The WT strains contain a serine at position 24 and an isoleucine at position 64; the respective amino acids were phenylalanine and valine in resistant ones. These mutations were located in the regulatory part of the gene suggesting a by-pass reaction in methionine biosynthesis as a mechanism of resistance. However this hypothesis remains questionable because these point mutations were found in a few sensitive strains; besides there was no cross-resistance between anilinoypyrimidines and SC-0858. Further studies are needed to characterize the primary target site of anilinoypyrimidines, as well as the product of the gene (named *AniI*) responsible for specific resistance to this family of botryticides.

To date the detection of anilinoypyrimidine-resistance within field populations of *B. cinerea* is based on either *in vitro* or *in vivo* methods (Birchmore and Forster, 1996). The fact that on complex media, there may be variability in response to anilinoypyrimidines, it is essential to use well-defined synthetic

nutrient media, especially in tests involving mycelial growth. Another alternative consists of testing the effects of anilinopyrimidines on germ tube elongation, for instance with pyrimethanil discriminatory concentrations between 1.0 and 2.5 mg/l allowing the detection of resistant isolates. As mentioned previously, the intensive use of anilinopyrimidines can lead to resistance (Forster and Staub, 1996; Latorre et al., 2002; Petsikos-Panayotarou et al., 2003). Consequently, the first approach to resistance management consists of restricting the number of treatments involving anilinopyrimidines. For instance, the recommendations of FRAC, which depend upon the total number of botryticide applications per season, were as follows: 1) more than seven treatments, maximum of three anilinopyrimidines; 2) up to six treatments, maximum of two anilinopyrimidines; two treatments, only one anilinopyrimidine. In French vineyards, since the introduction of anilinopyrimidines in 1994, the strategy based on only one annual application, over a total of two or three treatments, remains satisfactory because very few resistant strains have been detected (Leroux et al., 2002b). Among anilinopyrimidines, solo products are available for pyrimethanil and mepanipyrim whereas cyprodinil is proposed in a mixture with fludioxonil. On grapevine, in a trial where anilinopyrimidine-resistance was well established in Switzerland, it was shown that in programmes involving four treatments, those with solo products were ineffective whereas a mixture fludioxonil + cyprodinil gave effective control. However, in both situations, at the end of the season, most isolates of *B. cinerea* were resistant to anilinopyrimidines (Forster and Staub, 1996). According to this experience, the same basic management strategy consisting of a limitation of anilinopyrimidine-containing fungicides must be applied to solo products and mixtures.

6. Sterol biosynthesis inhibitors

Four main groups of sterol biosynthesis inhibiting fungicides (SBI) can be distinguished according to their target sites: i. inhibitors of squalene epoxidation including allylamines (e.g. terbinafine) and thiocarbamates (e.g. tolnaftate) are mainly used in medicine; ii. DMIs or inhibitors of 14 α -demethylation are widely used in agriculture on many crops against numerous Adelomycetes, Ascomycetes and Basidiomycetes; iii. several amines such as fenpropimorph, fenpropidin, tridemorph and spiroxamine which act either on Δ 14-reduction or Δ 8 \rightarrow Δ 7 isomerisation are powerful powdery mildew fungicides; iv. Hydroxyanilides, such as fenhexamid recently introduced into the *Botrytis* market blocks the C4-demethylations (Rosslenbroich and Stuebler, 2000).

In *B. cinerea*, these various fungicides do not prevent conidial germination but at low concentrations they inhibit the elongation of germ tubes and mycelial growth (Table 1). Additionally, the germ tubes produced on fungicide-supplemented media are distorted, bulge and their cytosol has a granular appearance (Leroux et al., 1999). However, few of them effectively control *B. cinerea* under field conditions. Among the DMIs, prochloraz and some triazoles, such as tebuconazole, are used against grey mould on various crops. The main

reason for the limited field performance of DMIs towards *B. cinerea* seems to be related to the low recommended rates compared to other botryticides. Applications of higher rates of triazoles, which would allow better control of grey mould, may be limited by phytotoxicity. This problem can be overcome by using mixtures with multi-site fungicides (e.g. tebuconazole + dichlofluanid) (Yunis et al., 1991; Stehmann, 1995). Additionally, strains with reduced sensitivity towards several DMIs including tebuconazole have been associated with poor grey mould control on vegetable crops in greenhouses (Elad, 1992). According to Stehmann (1995), reduced accumulation of tebuconazole in fungal mycelium did not account for resistance to DMIs in field isolates of *B. cinerea*.

The most effective SBI fungicide against grey mould currently is fenhexamid. When *B. cinerea* is grown in the presence of this hydroxyanilide derivative, the ergosterol content is reduced and three 3-keto compounds (4 α -methylfecosterone, fecosterone and episterone) accumulate, suggesting an inhibition of the 3-keto reductase involved in C-4 demethylations (Debieu et al., 2001). In cell free systems, this enzyme is inhibited by 1-2 mg/l (I_{50} values) of fenhexamid (D. Debieu and J. Bach, INRA, Versailles, France, unpubl.). Surprisingly, this novel SBI exhibits a narrow antifungal spectrum compared to DMIs. It shows high preventive activity against *B. cinerea* in various crops and also controls related fungi such as *Monilinia* spp. and *Sclerotinia* spp. (Rosslenbroich and Stuebler, 2000).

Before the release of fenhexamid on the market, strains highly resistant to this promising botryticide were easily detected in field populations of *B. cinerea*. This phenomenon was mainly expressed in mycelial growth tests, whereas the germ tubes produced by these strains (Hyd R1) appeared sensitive to this hydroxyanilide (Leroux et al., 1999). Since under field conditions fenhexamid was found to act preventively against *B. cinerea*, this could explain why Hyd R1 strains do not apparently lead to resistance of practical importance (Suty et al., 1999; Baroffio et al., 2003). Hyd R1 strains seem to be a genetic entity (group I or *B. 'pseudocinerea'*), naturally resistant to fenhexamid (Leroux et al., 2002b). According to Giraud et al (1999), *B. cinerea* consists of at least two sympatric sibling species *vacuma* and *transposa*. The sibling species *transposa* is characterized by the presence of two transposable elements, *Boty* and *Flipper*, whereas *vacuma* strains have neither. In recent studies, the polymorphism observed in several genes reveals a new structuring of *B. cinerea* natural populations into two groups. Group I contains only *vacuma* strains exhibiting laboratory resistance to fenhexamid, whereas group II includes all *transposa* strains and the fenhexamid-sensitive ones in *vacuma* (Albertini et al., 2003; Fournier et al., 2003).

Hyd R1 strains quickly metabolized fenhexamid and the main route corresponded probably to hydroxylation in the cyclic ring. Synergism of fenhexamid activity in Hyd R1 strains (and not in WT strains) by cytochrome P-450 inhibitors such as DMIs provided indirect evidence of the involvement of a P-450 monooxygenase in fenhexamid detoxification. The negative-cross resistance to edifenphos in Hyd R1 strains suggested that the same P-450 was

responsible for activation of this phosphorothiolate used against rice blast and for detoxification of fenhexamid (Suty et al., 1999; Leroux et al., 2002a).

Recently, a *B. cinerea* putative 3-keto reductase gene (*ERG27*) homologous to mammalian 17 β -hydroxysteroid dehydrogenase type 7 gene was cloned and sequenced. The inferred 535-amino acid protein encoded by the putative *ERG27* gene possesses the two common features of most reductases concerning the catalytic site and the N-terminal NADP(H) binding site (Albertini and Leroux, 2003). A dozen amino acid differences were recorded between Hyd R1 and non-Hyd R1 strains. This polymorphism, that constitutes an additional proof of the genetic differentiation between these two groups of strains, also suggests that laboratory resistance to fenhexamid in Hyd R1 strains could partially result from reduced sensitivity of the target site. The increased susceptibility of Hyd R1 strains towards DMIs and inhibitors of sterol Δ 14-reductase did not seem to be related to activation of these SBIs. Sequence comparison of the *CYP51* gene encoding eburicol 14 α -demethylase (this putative protein contains 522 amino acids), revealed a high polymorphism at this locus. The existence of five dimorphic regions within the two introns provided a way of distinguishing Hyd R1 (group I) from non-Hyd R1 strains (group II). Moreover, two expressed mutations were present in all Hyd R1 strains, namely phenylalanine to leucine at position 15, and serine to asparagine at position 105. These differences could explain why Hyd R1 strains respond differently to DMIs in *in vitro* tests (Albertini et al., 2003).

In a few strains of *B. cinerea* (group II) from the field, fenhexamid resistance (revealed in *in vitro* tests with mycelia) was not associated with increased susceptibility to SBIs and edifenphos. Among them, one phenotype (Hyd R2) was weakly resistant to fenhexamid at the germ tube elongation stage and, as in Hyd R1 strains, DMIs exhibited synergistic effects towards this hydroxyanilide (Leroux et al., 2002a). Moreover, the sequence of the putative *ERG27* gene was similar in Hyd R2 strains and WT strains. These data suggest that in Hyd R2 strains resistance to fenhexamid is mediated by P-450. The second phenotype (Hyd R3) was highly resistant to fenhexamid whatever the fungal stage. According to the sequence data of the *ERG27* gene, two expressed mutations were specifically found in Hyd R3 strains. They determine changes from phenylalanine to isoleucine at position 412 and from arginine to threonine at position 496. The amino acids found in the putative 3-keto reductase from Hyd R3 strains are usually found in mammalian 17 β -hydroxysteroid dehydrogenase (Albertini and Leroux, 2004). This result and the fact that in cell free systems from the Hyd R3 strain, the 3-keto reduction is not affected by 30 mg/l fenhexamid (D. Debieu and J. Bach, unpubl.) suggest that resistance in this phenotype results from a reduced sensitivity of the target site. According to their phenotype characteristics, Hyd R3 strains are a threat for the future of fenhexamid and their evolution should be followed within field populations of *B. cinerea*. The only reliable biological test involves checking the effect of a high concentration of fenhexamid (above 4 mg/l) on germ tube elongation. Till now, Hyd R3 strains have never been detected in French vineyards and they were only

found in one location in Germany. To avoid or slow down the selection of this phenotype, a reduction in spray number per season is advised. For instance, in French vineyards, the number of applications is limited to one, whereas in other crops such as tomato or strawberry, a maximum of two applications is recommended (Couteux and Lejeune, 2003).

7. Multi-drug resistance in *Botrytis cinerea* and fungal transporters

Multi-drug resistance (MDR) is the simultaneous resistance of organisms to a variety of unrelated toxic compounds. MDR was first described in mammalian cells resistant to anti-tumour drugs. It is generally caused by over-production of multi-drug transporters named P-glycoproteins that results in a decrease of intracellular concentrations of drugs. MDR has been described also in various other classes of organisms and among fungi this phenomenon was extensively studied in *S. cerevisiae* under the name of pleiotropic drug resistance (PDR). Several genes are associated with PDR and they appear to encode membrane proteins belonging either to the ABC (ATP-Binding Cassette) or to the MFS (Major Facilitators Super-family) transport families and transcription regulation factors (Balzi and Goffeau, 1994). See also chapter 9 for information on MFS.

ABC transporters are able to bind and hydrolyze nucleotide tri-phosphates (mainly ATP) and to use the energy generated to transport a wide variety of compounds across cell membranes. The structural unit of an ABC transporters is composed of two homologous halves, each containing six trans-membrane domains (TMDs) and a conserved nucleotide binding fold (NBF). The majority of ABC transporters have a [TMD₆-NBF]₂ or [NBF-TMD₆]₂ topology and are composed of 1300-1600 amino acid residues. Half-sized transporters with TMD₆-NBF or NBF-TMD₆ structures and multi-drug resistance related proteins (MRP) with a TMD₆ [TMD₆-NBF]₂ topology have also been described in various organisms, including *B. cinerea* (Stergiopoulos et al., 2002). MFS transporters do not hydrolyze ATP and they facilitate the transport of various compounds using the energy from electrochemical gradients across membranes. They are composed of 400-800 amino acid residues and share a common topology consisting of two-times six TMDs separated by a large cytoplasmic loop (12 TDMs); some of them exhibit two additional TMDs at the C-terminal domain of the protein (14 TDMs) (Stergiopoulos et al., 2002). In filamentous fungi these membrane proteins are able to transport a wide variety of natural and synthetic compounds of either endogenous or exogenous origin (Del Sorbo et al., 2000; Stergiopoulos et al., 2002).

7.1. Characteristics of transporters from *Botrytis cinerea*

Complete sequencing of the *B. cinerea* genome revealed the existence of 46 putative ABC proteins and 13 of them have been cloned (*BcatrA-N*) (Yoder and Turgeon, 2001; Vermeulen et al., 2001). The basal level of transcripts of these genes vary from undetectable (*BcatrC*, *BcatrI*, *BcatrN*) to low (*BcatrA*, *BcatrB*,

BcatrC, *BcatrG*, *BcatrK*) or high (*BcatrF*, *BcatrH*, *BcatrI*). Treatments with fungicides can increase transcript levels of several of these genes, especially *BcatrB*, *BcatrD*, *BcatrG* and *BcatrK* (Stergiopoulos et al., 2002). See also Chapter 9 regarding ABC transporters.

BcatrB encodes a protein with a [NBF-TMD₆]₂ topology and increased transcript levels of this gene in WT strains are observed in the presence of phenylpyrrole fungicides. Surprisingly this effect was not recorded with pyrrolnitrin, dicarboximides, anilinopyrimidines and DMIs. Functional analysis, by means of gene disruption, showed that $\Delta BcatrB$ mutants display increased susceptibility to phenylpyrrole fungicides and also to resveratrol, suggesting a role of *BcatrB* in virulence of *B. cinerea*. In addition, accumulation of fludioxonil by *BcatrB* replacement mutants was higher than by WT isolates, whereas for laboratory mutants over-expressing *BcatrB* the reverse was observed (Schoonbeek et al., 2001; Vermeulen et al., 2001).

BcatrD encodes a protein with a [NBF-TMD₆]₂ topology and its transcript level is up-regulated by various fungicides including DMIs, dicarboximides and anilinopyrimidines, as well as the antibiotic cycloheximide. A positive correlation between increased transcript levels of *BcatrD* and resistance to DMIs was recorded. Replacement mutants of *BcatrD* exhibit increased sensitivity to this class of SBIs and accumulate relatively high amounts of DMIs. In laboratory mutants over-expressing *BcatrD*, the reverse situation occurs; i.e. decreased sensitivity to DMIs and lower accumulation (Hayashi et al., 2001, 2002a).

BcatrK (syn *BMRI*) encodes a protein with a [NBF-TMD₆]₂ topology and increased transcript levels of this gene in WT strains are observed after treatments with phenylpyrroles and DMIs. However, disruption of *BcatrK* did not result in a hypersensitive phenotype to these fungicides, suggesting that *BcatrK* is probably not responsible for decreased accumulation of phenylpyrroles and DMIs. On the other hand, the increased susceptibility of $\Delta BcatrK$ mutants towards the antibiotic polyoxin and the phosphorothiolate fungicide iprobenfos, which respectively inhibit the biosynthesis of chitins and phospholipids, implies that *BcatrK* is an additional MDR transporter in *B. cinerea* (Nakajima et al., 2001).

Up to now, three MFS genes (*Bcmfs1*, 2 and 4) have been characterized in *B. cinerea* (Stergiopoulos et al., 2002). Treatments with anilinopyrimidine and DMI fungicides increased transcript levels of *Bcmfs1* (this gene encodes a putative MFS transporter with 14 TMDs) whereas a reverse effect was recorded with phenylpyrrole fungicides. $\Delta Bcmfs1$ mutants display an increased sensitivity to natural toxic compounds such as camptothecin and cercosporin, but their response to fungicides was unmodified. On the other hand, mutants over-expressing *Bcmfs1* showed an increased tolerance to the above natural compounds, as well as to several fungicides including DMIs, anilinopyrimidines, dicarboximides and fenhexamid. Moreover deletion of *Bcmfs1* in a $\Delta BcatrD$ mutant increased the sensitivity of this mutant to DMIs. These results demonstrated that *Bcmfs1* is a MFS transporter with a function of MDR, capable

of transporting both natural toxic compounds and fungicides (Hayashi et al., 2002b).

7.2. MDR in field strains of *Botrytis cinerea*

Monitoring in French vineyards since 1994 detected two anilinopyrimidine-resistant phenotypes (AniR2, AniR3) also showing resistance to other fungicides. In both cases this phenomenon was mainly recorded at the germ tube elongation stage and generally the resistance levels were below 10 (Leroux et al., 1999). Genetic analysis showed that fungicide resistance was determined in each phenotype by a major gene. Both phenotypes were weakly resistant to anilinopyrimidines, SC-0858 and dicarboximides. Moreover, AniR2 strains were resistant to phenylpyrroles while AniR3 strains displayed decreased sensitivity to DMIs, fenhexamid, boscalid and cycloheximide. Within inhibitors of squalene epoxidase, both phenotypes were sensitive to the allylamine and terbinafine, and resistant to the thiocarbamate, tolnaftate (Chapeland et al., 1999). The phenotype of AniR2 strains collected in the field is close to that of laboratory generated mutants selected on fludioxonil and showing reduced sensitivity to phenylpyrroles, dicarboximides and anilinopyrimidines. In these mutants accumulation of fludioxonil was lower than in WT strains and correlated with the expression level of *BcatrB* (Vermeulen et al., 2001). It remains to be determined whether this ABC transporter is also involved in AniR2 strains.

The resistance of AniR3 strains to DMIs is associated with decreased accumulation of this type of SBI. The addition of fluazinam led to an accumulation of DMIs by fungal cells, suggesting that an efflux occurred (Chapeland et al., 1999). Similar results has been reported in laboratory mutants (Stehmann, 1995; Hayashi et al., 2001, 2002a). According to the profiles of uptake kinetics, efflux appears inducible in WT strains and seems to be constitutive in resistant isolates (field strains or laboratory mutants). In studying the putative role of ABC transporters, Hayashi et al. (2001) showed that the basal level of one of them (*BcatrD*) correlated with DMI resistance in laboratory mutants of *B. cinerea*. Similar experiments conducted with AniR3 strain indicated that *BcatrD* was not involved in these DMI-resistant field strains. This observation indicated that the transporter involved in efflux of DMIs in the AniR3 isolate is different from that in laboratory strains, since these latter mutants do not show cross resistance to anilinopyrimidines (Hayashi et al., 2001, 2002a). Precise identification of the genes responsible for MDR in field strains of *B. cinerea* will reveal if they encode ABC transporters or transcription factors as reported in *S. cerevisiae* (Balzi and Goffeau, 1994; Stergiopoulos et al., 2002).

8. Conclusions

The use of botryticides is an efficient way to protect crops against *Botrytis* spp., but control necessitates preventive applications of chemical fungicides at dose rates that are generally higher than those used against other fungal diseases such

as powdery mildews or rusts. Furthermore, especially in the case of fruit production, applications of botryticides just before or after harvest is desirable, but this practice is restricted because of the toxicological risks presented by their residues. Another serious constraint is linked to the resistance phenomena associated with several major botryticide families including benzimidazoles, phenylcarbamates and dicarboximides. However, newly introduced compounds including anilinopyrimidines, phenylpyrroles and hydroxyanilides, represent powerful tools for use in anti-resistance management strategies. For instance in French vineyards, against grey mould, the present recommendation is to alternate the various groups of fungicides with a maximum of one spray per year for each chemical family. However, due to the development of MDR in field strains of *Botrytis* spp., such a strategy can only be partially successful. In greenhouses alternation of various groups is recommended, but in this case c. 10-20 applications may be carried out and the development of resistance is common (Katan et al., 1989; Shtienberg and Elad, 1997).

For the future, there is still a need for novel highly active botryticides but in the framework of Integrated Pest Management (IPM), less efficient compounds can be used. Mineral salts (Palmer et al., 1997) or plant activators such as acibenzolar, an analogue of salicylic acid (Terry and Joyce, 2000), and biological control (Chapters 13, 18; Shtienberg and Elad, 1997) are examples of such alternatives to classical botryticides. Moreover, the increased production of plant defences (e.g. phytoalexins, PR proteins) can also be achieved with transgenic crops (Chapter 20; Wurms et al., 1999).

9. References

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CHAPTER 13

MICROBIAL CONTROL OF *BOTRYTIS* SPP.

Yigal Elad¹ and Alison Stewart²

¹Dept. of Plant Pathology and Weed Sciences, ARO, The Volcani Center, Bet Dagan 50250, Israel;

²National Centre for Advanced Bio-Protection Technologies, P.O. Box 84, Lincoln University, Canterbury, New Zealand

Abstract. Biocontrol of *Botrytis*-incited diseases has been extensively investigated over the last 50 years. This chapter reviews the literature on microbial control of *Botrytis* species with a particular emphasis on the importance of gaining a full understanding of the biological and ecological attributes of a biological control agent as a means to developing appropriate strategies for its effective use under commercial cropping conditions. The key microbial genera that have shown greatest potential for *Botrytis* disease control include the filamentous fungi *Trichoderma*, *Gliocladium* and *Ulocladium*, the bacteria *Bacillus* and *Pseudomonas* and the yeasts *Pichia* and *Candida*. Commercial success has been achieved in glasshouse and post-harvest environments where stable environmental conditions allow greater control over the application of the biocontrol agent and expression of its biological activity. Considerable progress has also been made in achieving more consistent biocontrol under field conditions, particularly in vineyards, but the complexities of the plant, microbe, environment interaction and its inherent variability will always pose a severe challenge to achieving effective and consistent field biocontrol. In recognising this, current research aims to define more clearly the biological and economic barriers that limit biocontrol efficacy and future research should focus on the strategic integration of biocontrol systems with other cultural, chemical and genetic methods to provide more sustainable disease control.

1. Introduction

Extensive knowledge has been gained on biological control of plant diseases of fruit, vegetable and flower products during cultivation or post-harvest. Biocontrol of *Botrytis*-incited diseases with filamentous fungi, bacteria and yeasts has been intensively studied over the last two decades (Blakeman, 1993; Blakeman and Fokkema, 1982; Dubos, 1992; Tronsmo, 1992; Elad and Freeman, 2002). Biocontrol offers an attractive alternative or supplement to the use of conventional methods for disease control since microbial biocontrol agents (BCAs) are perceived to be less demanding to the environment and their generally complex mode of action reduces the risk of resistance development. The world market for *Botrytis* control products is estimated at US\$15-25 million. This highlights the need for the development of cost effective biocontrol products either for conventional agricultural systems or for organic farming. Organic and minimal pesticide production systems, which are

gaining momentum in many countries, can significantly benefit from use of biocontrol preparations.

Early examples of biocontrol with common species of microorganisms are from the works of Newhook (1951) and Wood (1951) who inoculated senescent lettuce leaves with antagonistic *Fusarium* spp. and *Penicillium claviforme* that originated from the same crop and prevented primary establishment of *B. cinerea*; Wood (1951) believed that saprophytic activity on dead lettuce tissue was, to a large extent, responsible for disease control under natural conditions. Later, Newhook (1957) controlled grey mould on glasshouse tomatoes by spraying a spore suspension of *Cladosporium herbarum* and *Penicillium* sp. on the floral debris attached to the fruit. *C. herbarum* also effectively controlled strawberry grey mould by protecting the flowers under field conditions (Bhatt and Vaughan, 1962). Blakeman and Fraser (1971) and Blakeman (1972) reported the antagonistic effect of bacteria on *B. cinerea* on chrysanthemum and beetroot leaves. Subsequently, antibiotics were shown to nullify the inhibiting effect of the bacteria on germination of *B. cinerea* on beetroot leaves (Blakeman and Szejnberg, 1974). Blakeman and Brodie (1976) later described the inhibition of *B. cinerea* and other pathogens by epiphytic bacteria as a general phenomenon. Since the 1970s, *Trichoderma* spp. have been commonly used for *B. cinerea* control on snap bean blossoms (Nelson and Powelson, 1988), grape (Gullino, 1991; Dubos, 1992; O'Neill et al., 1996), strawberry (Tronsmo and Dennis, 1977) and greenhouse crops (Elad et al., 1995). *Ulocladium* species have been used to suppress *Botrytis* conidiation (Köhl and Fokkema, 1993). Yeasts (e.g. *Pichia* and *Rhodotorula*) and bacteria (e.g. *Bacillus* and *Pseudomonas*) have also been reported to be effective in controlling *B. cinerea* (Redmond et al., 1987; Edwards and Seddon, 1992; Elad et al., 1994b).

2. Biocontrol agents and their mechanisms of action

The behaviour of *Botrytis* during the pre-penetration phase of its development on the healthy plant surface and the relative importance of necrotic tissues within the crop as an inoculum source determines the biocontrol strategy that is most likely to be effective. *Botrytis* is extremely susceptible to competition because of its dependence on an exogenous supply of nutrients for germination, germ-tube growth and infection. On the plant surface, *Botrytis* conidia or germ tubes are sensitive to the effect of antibiotics and lytic enzymes produced by microorganisms that may inhibit germination and lyse germ tubes. Disease suppression may be based on reduction of pathogen saprophytic ability, spore dissemination, virulence or induced resistance in the host plant (Elad and Freeman, 2002). Induced resistance may also be effective against quiescent infection, otherwise not targeted by secreted products of biological control agents (BCAs).

2.1. Modification of plant surface properties

Microorganisms can change the wettability of plant surfaces as shown for the surface-active *Pseudomonas* spp. The surface effect can interfere with attachment and growth of the pathogen on plant surfaces (Bunster et al., 1986). *Bacillus brevis*

applied to Chinese cabbage caused water drops to spread and dry, thus decreasing the wetness periods and restricting the period suitable for *B. cinerea* germination and infection (Edwards and Seddon, 1992). It is common to observe similar effects when formulations are applied to plant surfaces, providing a secondary benefit from the formulated biocontrol product. This phenomenon has largely been overlooked in biocontrol research and warrants further investigation.

2.2. Attachment to pathogen surfaces

Biocontrol activity may, in some cases, be associated with or even require the attachment of BCAs to the surface of the host cells. Attachment mechanisms play a role in cell-cell interactions in fungi and other microorganisms (Douglas, 1987). The antagonistic yeast *Pichia guilliermondii* attaches to *B. cinerea* hyphae (Wisniewski et al., 1991). Agents that alter protein integrity (salts, proteases, etc.) and certain sugars can block the attachment. The attachment of the yeasts *Rhodotorula glutinis* and *Cryptococcus albidus* to conidia of *B. cinerea* is associated with the formation of a fibrillar material (Elad, 1996). This material is likely to be the pathogen's polysaccharide extracellular matrix (ECM) and the involvement of lectin-like compounds was suggested (Elad, 1996). The ECM can be manipulated and detected by antibodies. The monoclonal antibodies BC-8.DH2 and BC-12.CA4 (Chapter 11) detected significant reductions in antigen levels in the infection site of *B. cinerea* on bean and tomato leaves treated with *Trichoderma* conidia and the immunofluorescence pattern of the antigen was weaker and more dispersed than the control. Scanning electron microscopy showed attachments of the *Trichoderma* conidia to the ECM of *B. cinerea* (Meyer et al., 2001). Cook and Long (1995) successfully used the attachment phenomenon to screen potential BCAs among phyllosphere bacteria and yeasts. Further, yeasts that bound directly to *B. cinerea* germings were assessed for the ability to suppress spore liberation of conidia from *B. cinerea*. When α -cellulose with *Candida pulcherrima* was applied as a dry powder to sporulating *B. cinerea* colonies on kiwifruit leaf discs, the particles from the formulation attached to conidiophores and conidia and suppressed the liberation of conidia by 50% (Cook, 2002).

2.3. Competition

Bacteria, yeasts and filamentous fungi can inhibit fungal pathogens by competing for nutrients such as nitrogen, carbon, macro-elements and micro-elements. Reduction of the concentration of nutrients generally results in a reduced rate of pathogen spore germination and in slower germ tube growth, thereby reducing the number of infection courts and the extent of subsequent necrosis incited by the pathogen (Blakeman and Fokkema, 1982; Blakeman, 1993; Elad et al., 1995). For example, a *Pseudomonas* sp. removed amino acids faster than the *B. cinerea* conidia (Brodie and Blakeman, 1975).

The ability to colonize wounds rapidly is an important feature of BCAs that can be used for protection of *Botrytis* stem infection (Eden et al., 1996). A similar strategy has been used to protect post-harvest wounds (Chapter 19). Competition for nutrients or space was demonstrated with various yeasts applied to apple fruits, e.g. *Candida* spp.

(McLaughlin et al., 1990), *Cryptococcus laurentii* (Roberts, 1990), *Sporobolomyces roseus* (Janisiewicz et al., 1994) and *Candida oleophila* (Mercier and Wilson, 1994). Similarly, several bacterial and fungal isolates were able to reduce germination of conidia of *B. cinerea* through competition on detached leaves and this reduced the severity of rot symptoms (Elad et al., 1994b; Zimand et al., 1996).

2.4. Cell wall-degrading enzymes and parasitism

Parasitism of fungi by various microorganisms has been recorded in many systems (Adams, 1990; Wisniewski et al., 1991; Elad, 1995), and relies on the production of fungal cell wall degrading enzymes (CWDEs) (Elad, 1995). *Trichoderma*, *Gliocladium* and *Pythium* spp. are among the best-known mycoparasites (Elad, 1995). Dubos (1987) observed proliferation of *Trichoderma* on the margins between healthy and necrotic areas on rotting grape berries. Microscopic observations revealed coiling and penetration of the mycelium of *B. cinerea* by the antagonist. *Pythium periplocum* has been found to be an aggressive mycoparasite of *B. cinerea* in culture; infected mycelium of *B. cinerea* failed to infect grapes (Paul, 1999). Parasitism of sclerotia has also been described. Köhl and Schlösser (1989) reported parasitism of *B. cinerea* sclerotia at 5°C, and production of sclerotia on grapevine was partially hindered by *Trichoderma* spp. applied in late summer (Dubos et al., 1982).

Several CWDEs have been associated with antagonism. Labudova and Gogorova (1988) found isolates of *T. reesei* and *T. harzianum* capable of producing proteinase, mannanase, laminarinase and chitinase and postulated their role in mycoparasitism. One member of the group of hydrolytic enzymes, believed to be involved in this mechanism of biocontrol, was purified and characterized as a serine-protease (Geremia et al., 1993). An endochitinase (Chit36), isolated and characterised from a strain of *T. harzianum*, was subsequently over-expressed in the same strain. Culture filtrates containing secreted Chit36 as the sole chitinolytic enzyme completely inhibited the germination of *B. cinerea* conidia in culture (Viterbo et al., 2001). A strain of *Serratia plymuthica* reported to protect cucumber against grey mould, produced a 58kDa endochitinase as its main secreted chitinolytic enzyme. However, two mutants of the bacterial strain, one with increased chitinolytic activity and the other deficient in chitinolytic activity, did not differ appreciably from the parent strain in the production of other antifungal compounds or in suppression of *B. cinerea* (Kamensky et al., 2003). Similarly, there was no correlation between chitinase and β -1,3-glucanase production and *Botrytis* disease control achieved by five isolates of *Trichoderma* (Zimand et al., 1991) or by 74 isolates of *Trichoderma* (Elad et al., 2001). Thus, a BCA's ability to produce CWDEs does not necessarily guarantee effective biocontrol.

2.5. Inhibitory compounds

Antibiosis as a means of biocontrol has been reported for many microorganisms (Andrews, 1992; Whipps, 1997). It is sometimes difficult to predict the importance in natural ecosystems of inhibitory compounds that are produced *in vitro* by antagonists. The ultimate proof for the role of these compounds in biocontrol is the loss of activity in non-producing mutants of the antagonist. However, in many cases

the mutants still possess biocontrol activity, probably because other modes of action still operate. *Trichoderma* and *Gliocladium* spp. are common producers of antibiotics. *T. hamatum*, which produces inhibitory volatiles, reduced grey mould of snap bean pods and blossom by 77-97% (Nelson and Powelson, 1988). The peptaibol antibiotics, trichozianins A1 and B1 from *T. harzianum* and gliotoxin from *G. virens*, inhibited spore germination and hyphal elongation in *B. cinerea* (Di Pietro et al., 1993; Schirmböck et al., 1994). A pyrrolnitrin-producing isolate of *Pseudomonas cepacia* was effective against *Botrytis*, *Penicillium* and *Mucor* rots of apples and pears (Janisiewicz and Roitman, 1988) and an isolate of *Penicillium chrysogenum* produced inhibitory products, which reduced conidial germination of *B. fabae* and lesion development on faba bean (Jackson et al., 1994). *B. cinerea* has been controlled by antibiotic producers of certain *Bacillus* species in various hosts. For example, *B. brevis* secretes the antibiotic gramicidin S that is known to be very effective against *B. cinerea* (Edwards and Seddon, 1992) and control of the pathogen by *B. subtilis* and *B. pumilus* was related to antibiotic production (Leifert et al., 1995). A UV-induced antibiotic deficient mutant of *B. subtilis* was not active. However, the development of antibiotic resistance in *B. cinerea* is a potential limitation to the use of antibiotic producing BCAs (Li and Leifert, 1994).

2.6. Reducing pathogenicity of the pathogen

Synthesis of hydrolytic enzymes by *B. cinerea* during the first phase of host-pathogen interactions is crucial for the infection process (Chapter 9). Interference with pathogenicity processes was reported for *T. harzianum* T39; there was no difference in the amount of pathogen mycelium observed 24-48 h after inoculation, nevertheless, penetration of the host tissue and disease was significantly reduced (Zimand et al., 1996). The activities of exo- and endo-polygalacturonase, pectin methyl esterase and pectate lyase (Zimand et al., 1996), chitinase, β -1,3-glucanase and cutinase (but not cellulase) produced by *B. cinerea* was reduced in the presence of T39 (Kapat et al., 1998). It was shown that *T. harzianum* T39 produced protease on the leaves. The protease reduced *B. cinerea* germination, activity of pathogenicity-related enzymes and subsequent disease development (Elad and Kapat, 1999). The use of a GUS transformant of *T. harzianum* T39 illustrated the role of diffusible compounds on leaf surfaces infected with the pathogen (Freeman et al., 2002). It has been suggested that the accumulated pectic enzyme products, e.g. oligogalacturonides, act as elicitors of defence mechanisms of plants (Cervone et al., 1989; Zimand et al., 1996).

2.7. Suppression of inoculum production by the pathogen

Antagonistic interactions can also be exploited during the saprophytic stage of necrotrophs such as *B. cinerea*. Reduction in inoculum production, followed by a suppression of the pathogen's ability to infect, may create an accumulative effect over several disease cycles in pathosystems where inoculum produced within the field is the major contribution to the development of epidemics (Köhl and Fokkema, 1993). Several microorganisms have been reported to suppress sporulation of *B. cinerea* on strawberry (Peng and Sutton, 1991). The same phenomenon was later exploited in

various crops (Sutton et al., 1997; Morandi et al., 2000). *Ulocladium atrum* reduced sporulation of *B. cinerea* on dead leaves of lily and onion exposed to field conditions (Köhl et al., 1995a, b). Interestingly, *U. atrum* was effective against *B. cinerea* in commercial cyclamen crops, but was not effective against *B. elliptica* in lily. The interaction between *Botrytis* spp. and *U. atrum*, resulting in a biocontrol effect, only takes place in necrotic plant tissue. Colonisation of necrotic tissue by *U. atrum* prevents saprophytic colonisation of those leaves by *B. cinerea*. In contrast, conidia of *B. elliptica* directly infect healthy lily leaf tissue. *U. atrum* applications aimed at blocking the infection pathway from a saprophytic base are, therefore, not effective against *B. elliptica* (Kessel et al., 2001). Bacteria are also capable of suppressing *Botrytis* sporulation. Two antibiotic-producing isolates of *Serratia liquefaciens* significantly reduced sporulation on irradiated grape leaves. However, the application solution strongly influenced the degree of suppression, probably by affecting the antibiotic production by the bacteria (Whiteman and Stewart, 1998).

2.8. Induced resistance

Induced resistance (IR) is recognized as an important mode of biocontrol in vegetative tissues (Sequeira, 1983). Resistance may be induced locally or may be systemic. Induced systemic resistance (ISR) caused by various microorganisms can protect plants against soil or foliar pathogens (Paulitz and Matta, 1999). ISR has been attributed to a variety of microorganisms e.g. non-pathogenic micro-organisms such as saprophytes, plant growth-promoting microbes, and avirulent races of the pathogen and can result in control of multiple pathogens.

Dead cells of antagonistic yeasts, bacteria and *Trichoderma harzianum* were capable of inducing local resistance and, thereby, reducing grey mould, to the same extent as live cultures in some cases (Elad et al., 1994b; Elad and Kapat, 1999). Unlike locally induced resistance, ISR is demonstrated by applying a BCA at a location separated from the plant organ that is challenged by the pathogen. *T. harzianum* and *Pseudomonas aeruginosa* produced an ISR effect on bean, challenged by *B. cinerea* (De Meyer et al., 1998), *T. harzianum* also induced plant defence against *B. cinerea* in tomato, lettuce, pepper, bean and tobacco. The BCA was applied to the soil or to the lower leaves, whereas the disease was suppressed on the upper canopy parts of the plants. The effect of disease suppression declined with time and the decline was faster on old rather than on young leaves (De Meyer et al., 1998; Elad, 2000).

P. aeruginosa 7NSK2 induces resistance to *B. cinerea* infection on tomato and bean leaves. It produces three siderophores: pyoverdine, pyochelin and salicylic acid (SA), a precursor of pyochelin. SA-negative mutants of 7NSK2 are unable to induce resistance. KMPCH, a pyoverdine and pyochelin-negative mutant of 7NSK2 still produces nanogram amounts of SA on roots in non-sterile soil that are sufficient to induce phenylammonia lyase (PAL) expression in the roots and to increase free SA levels in leaves. In contrast to KMPCH, however, the wild type strain 7NSK2 does not produce SA on roots and does not induce PAL expression in the roots (Audenaert et al., 2001). These authors found that resistance induced by the wild type 7NSK2 is not caused by SA production, but by a combination of pyochelin and pyocyanin production. A pyochelin-negative mutant of 7NSK2 (7NSK2-562) and a

pyocyanin-negative mutant of 7NSK2 (Phz1) were unable to induce resistance to *B. cinerea*. However, when both mutants were inoculated together on tomato roots, the ability to induce resistance was restored. The authors suggested that active oxygen species are produced on plant roots resulting in induced resistance (Audenaert et al., 2001).

L-form bacteria, i.e. those with modified or no cell walls, have been shown to form symbiosis with plants including Chinese cabbage. The plant L-form association results in enhanced resistance to infection by *B. cinerea*. Induction of chitinases was found in Chinese cabbage plants treated with L-form bacteria of *Pseudomonas syringae* pv. *phaseolicola* during seed germination. The L-forms were systemically distributed throughout the plant, with the highest population in roots (Daulagala and Allan, 2003).

B. cinerea infection is associated with increased active oxygen species (AOS, as hydrogen peroxide and nitric acid) and elevated antioxidant enzymes such as peroxidase, superoxide dismutase and catalase (Chapters 8 and 9; Elad, 2002). *T. harzianum* T39 treatment to soil caused a decrease in AOS in uninfected leaves and in *B. cinerea*-affected leaves. Soil treatment also was associated with increased antioxidant activity in *Botrytis* free leaves and with decreased antioxidant activity in infected leaves. The latter was probably due to the reduction in AOS (Lapsker and Elad, 2001).

Arabidopsis mutants that are deficient in or insensitive to abscisic acid (ABA), resistant to or deficient in gibberellic acid (GA) and which over-produce or under-produce ethylene were more susceptible to *Botrytis* compared to the wild type plants (Chapter 10). *T. harzianum* T39 did not suppress *B. cinerea* on the ABA and GA mutant plants, but was effective on the ethylene mutants. Apparently, ethylene related factors are not associated with T39 biocontrol activity, whereas GA and ABA features may be involved in T39-disease control (Okon-Levy et al., 2003).

2.9. Combination of mechanisms

In many cases, it can be assumed that biocontrol is a result of several combined modes of action of the antagonist. *T. pseudokoningii* and *T. viride* controlled *Botrytis* rot of fruits by direct parasitism and antibiotic production (Tronsmo and Dennis, 1977). Synergism between different forms of antagonism may occur, for example, the *in vitro* inhibition of conidial germination of *B. cinerea* by antibiotics combined with the action of CWDEs (Lorito et al., 1993). CWDEs of *Trichoderma* spp. synergistically increased the toxicity of the *P. syringae* antibiotic syringomycin SP25-A against *B. cinerea* normally resistant to SP25-A alone. Purified bacterial enzymes and metabolites were also synergistic against fungal pathogens, although this mixture was less powerful than the combination with the *Trichoderma* CWDEs (Fogliano et al., 2002). A mutant of *T. harzianum* CECT which produced between two and four times more chitinase, β -1,3- and β -1,6-glucanase activities than the wild type also produced three times more extracellular proteins and secreted more yellow pigment (α -pyrone). This mutant gave better protection than the wild type against *B. cinerea* on grapes (Rey et al., 2001). The modes of action of post-harvest antagonistic yeasts involve nutrient and space competition, direct parasitism and

induced resistance (Chapter 19). The latter coincides with an increase in chitinase and β -1,3-glucanase activity in the protected fruits (El Ghaouth et al., 2001a, b, 2003).

Brevibacillus brevis uses gramicidin S and a biosurfactant as its modes of action (Edwards and Seddon, 1992; McHugh and Seddon, 2001). In tomato crops, treatments containing only the biosurfactant were as effective as *B. brevis* WT in reducing grey mould leaf symptoms. However, *B. brevis* WT gave far superior control of grey mould stem lesions as the treatments containing only one active component. In spring lettuce, treatments containing either active component performed equally well whereas in a more rapidly growing preliminary trial, spores of WT (containing only gramicidin S) had given superior control (McHugh and Seddon, 2001). They suggested that the biosurfactant is mainly responsible for disease reduction in regions of free air movement such as open leaf canopies. However, when the environment is such that high humidity renders the biosurfactant inactive, gramicidin S is responsible for disease control.

As mentioned previously, *T. harzianum* T39 exerts its biocontrol effect via a combination of competition for nutrients and interference with the production of pectolytic enzymes of the pathogen. Thus, in addition to slowing the germination of the *Botrytis* conidia, T39 prevents the penetration of the host tissue and the maceration process, and has also been shown to induce resistance (De Meyer et al., 1998; Kapat et al., 1998). A mixture of *P. guilliermondii* and *Bacillus mycoides* resulted in additive activity, compared to separate application. The combined activity was due to the summation of biocontrol mechanisms such as competition, antibiosis and induced resistance of both agents (Guetsky et al., 2002c).

3. Commercial implementation

The world market for pesticides is about US\$30 billion and, of this, less than 1% is for biopesticides - about US\$300 million. This includes all non-synthetic chemicals, such as microbial pesticides, beneficial arthropods, natural pesticides and pheromones. Most microbials are accounted for by *Bacillus thuringiensis* and most biopesticides are used in countries that export vegetables, fruit and ornamentals (Jarvis, 2001; Ravensberg and Elad, 2002).

Development of commercial BCAs involves not only screening for potential microorganisms, studying the mode of action of the microorganism, determining the ecological and biological requirements for efficacy and compatibility with commonly applied pesticides, and testing under commercial conditions but also the up-scaling of production and formulation, achieving acceptable shelf life, toxicology, marketing, patenting and distribution of the product and, especially, cost effectiveness. However, biocontrol preparations are often aimed at relatively small markets and expensive toxicological and environmental impact tests are often a limiting factor to successful commercialisation. Furthermore, at present the demands of the regulatory authorities in certain countries pose an economic constraint on the feasibility of registration (Elad, 2001; Ravensberg and Elad, 2002).

3.1. Commercial products

Although currently holding only a small percentage of the world pesticide market, biopesticide numbers are increasing and are expected to continue to increase albeit at a relatively slow rate. Products registered for use against *Botrytis* include the following:

Binab (Binab Bio-Innovation AB, Sweden) is based on *Trichoderma harzianum* and *T. polysporum*. It was first registered in France in 1976 for use on tree wounds. It is aimed at soil-borne fungal diseases in greenhouses (tomatoes, cucumbers and ornamentals) and *Botrytis* in strawberries. **Mycostop** (Kemira Gro Oy in Finland) is based on *Streptomyces griseoviridis* (K61) and is sold for control of damping-off, root and stem rot diseases in greenhouse cucumbers, tomatoes, peppers, lettuce, and ornamentals. While it mainly targets *Fusarium*, it also controls *Pythium*, *Phomopsis*, *Rhizoctonia*, *Phytophthora* and *Botrytis* (White et al., 1990). **Plantshield** (Bioworks Inc., USA) is based on *Trichoderma harzianum* (T22) and is sold as a biofungicide for control of damping-off diseases and root diseases in greenhouse and other crops for control of *Fusarium*, *Rhizoctonia*, *Pythium* and others. It has also been shown to be effective against *Botrytis* (Utkhede and Mathur, 2002). **Serenade** (Agra Quest, USA) is based on *Bacillus subtilis* (QST 713) and is sold for control of many soil-borne and foliar fungal and bacterial diseases in field and protected crops. **TRICHODEX** (Makhteshim, Israel) is based on *T. harzianum* (T39) and is sold for control of *B. cinerea* in grapes and greenhouse crops. It is also reported to be active against *Sclerotinia* white mould, *Cladosporium* leaf mould and powdery mildew (Elad, 2000; Elad et al., 1994a, c, 1995, 1998, 1999; O'Neill et al., 1996). **Botry-Zen** (Botry-Zen Ltd, New Zealand) is based on a strain of *Ulocladium oudemansii* that out-competes *Botrytis* on necrotic plant tissue and is recommended for use against *Botrytis* grape mould. **Aspire** (Ecogen, USA) contains the yeast *Candida oleophila*. "I-182" and is used for post-harvest control of decay-causing pathogens including *B. cinerea* of fruits (Droby et al., 1991). **Bio-save** (Eco Science Corp., USA) contains *Pseudomonas syringae* and is used for the post-harvest control of *B. cinerea* on fruits. A number of other products are at various stages of commercial development. Additional information about biocontrol products can be obtained at <http://www.epa.gov/pesticides/biopesticides/ingredients/index.htm> and <http://www.apsnet.org/online/feature/biocontrol/>

3.2. Delivery of biocontrol preparations

The system by which a BCA is delivered to the appropriate plant site can significantly influence its success. Biocontrol products for control of *Botrytis* diseases are usually sprayed on to target plants. In this situation, establishment of the BCA on the plant is crucial for effective biocontrol (Elad and Kirshner, 1992, 1993). Long term survival in the hostile environment is desired and well achieved by several BCAs, i.e. spore forming bacilli (McHugh and Seddon, 2001) and other BCAs. Subsequent dispersal of the BCA from treated to non-treated plants, as reported for *T. harzianum* T39 in the greenhouse, is an additional benefit that should enhance the level of biocontrol achieved (Elad et al., 1993). There are a number of

examples where insects have been used as vectors for BCAs. A conidial preparation of *Gliocladium roseum* was transferred to strawberry flowers by bees resulting in suppression of *B. cinerea* on the flowers and fruits (Peng et al., 1992). In a similar study, *T. harzianum* was delivered to strawberry flowers using bees as a vector. Although fewer flowers collected from the bee-delivered treatments had detectable levels of *T. harzianum* and those flowers generally had half the density of *T. harzianum*, this treatment reduced *Botrytis* infection by 72% compared to 40% for the sprayed treatment (Kovach et al., 2000; Bilu et al., 2003, 2004).

3.3. Barriers that limit implementation

Most of the studies reporting high efficacy of BCAs have been conducted under controlled environments. For example, a survey of 60 experiments in commercial greenhouses (mostly unheated) conducted worldwide revealed that in c. 70% of cases, *T. harzianum* T39 suppressed *B. cinerea* infections in tomatoes and cucumber proving as effective as the chemical fungicide applied for comparison (Shtienberg and Elad, 1997). In contrast, there is often inconsistent control shown by BCAs used to target diseases in the field. Environmental conditions are usually unpredictable and may influence the survival, establishment and activity of the BCA. Under field conditions, the plant surface is subjected to fluctuating temperature, vapour pressure deficit (VPD), surface wetness, gases and air movement (Burrage, 1971). These factors affect all microorganisms. For example, *T. harzianum* T39 was less effective in suppression of cucumber grey mould under wet conditions and temperatures below 20°C than under dry conditions at elevated temperatures (Elad et al., 1993). High day temperature and high night VPD were associated with reduced efficacy of *Aureobasidium pullulans*, *Cryptococcus albidus* and *T. harzianum* T39 for suppression of *B. cinerea* in cucumber and tomato (Dik and Elad, 1999).

Chemical exudates on the plant surface contain macro- and micro-elements, sugars, sugar alcohols, pectic substances, amino acids, and organic acids. The quality and quantity of leachates from plants are affected by plant age and factors such as temperature, VPD and surface moisture, light, fertilization and pollen that change constantly. These changes may affect plant surface microflora directly or have an indirect impact by modifying leaf characteristics, e.g. metabolic state, morphology and surface chemistry (Cutter, 1976). As nutrients fluctuate, there are community changes in colonization by bacteria, yeasts and filamentous fungi (Blakeman, 1985) and this may significantly affect, either beneficially or detrimentally, the colonization and biocontrol potential of the BCA. The production of rhodotorulic acid, a siderophore synthesized by *Rhodotorula* strains, was increased by up to 60% in the presence of urea as a nitrogen source and this promoted its *in vitro* antifungal activity against *B. cinerea* (Calvente et al., 2001). Thus, it is clear that in some cases the presence of naturally occurring microflora can be beneficial to biocontrol whilst in others it can be detrimental.

3.4. Combined application

3.4.1. Mixtures of biocontrol agents

Application of mixtures of BCAs can provide a number of advantages. Dual or multiple isolates may be able to target different life cycle stages of *B. cinerea*, e.g. prevention of infection and reduction of sporulation (Elad et al., 1994b). Alternatively, different organisms may be effective under complementary nutritional and/or environmental conditions. For example, isolates with different nutritional profiles had different niche requirements and could coexist in wounds of apple allowing better control of post-harvest blue mould (Janisiewicz, 1996). A mixture of two yeast isolates and one bacterium controlled the post-harvest pathogens *Penicillium expansum*, *B. cinerea* and *Pezizula malicorticis* on apple (Leibinger et al., 1997), and powdery mildew and grey mould on cucumber were controlled by a combination of AQ10 (*Ampelomyces quisqualis*) and TRICHODEX (*Trichoderma harzianum*) (Elad et al., 1998). Nunes et al. (2002) combined *Candida sake* with *Pantoea agglomerans* to improve control of post harvest *P. expansum* and *B. cinerea* on pears and apples.

Guetsky et al. (2001) suggested that application of more than one antagonist, provided that they had different ecological requirements, would increase the reliability and decrease the variability of biocontrol. Combined application of *Pichia guilliermondii* and *Bacillus mycoides* resulted in significant suppression of *B. cinerea* in strawberries, under a wide range of controlled relative humidity and temperature regimes. Application of both BCAs also reduced the variability of disease control (Guetsky et al., 2001). Under field conditions, the mixture significantly improved disease suppression compared to the BCAs applied alone (Guetsky et al., 2002a, b).

3.4.2. Mixtures with chemicals

In some circumstances, BCAs can be effective when used as a stand-alone treatment. For instance, better control of cucumber grey mould was achieved by application of either *Aureobasidium pullulans* or *T. harzianum* T39 than the broad-spectrum fungicide tolylfluanid under all tested climate regimes (Dik and Elad, 1999). However, in other cases, BCAs may be combined with other control methods in order to achieve acceptable levels of disease suppression. For example, *T. harzianum* T39 and *Ampelomyces quisqualis* AQ10 were applied with oils to ensure activity against *B. cinerea* and powdery mildew (Elad et al., 1998). Similarly, TRICHODEX mixed with iprodione was superior to either component alone for the control of grey mould (Elad et al., 1994c) and the same BCA mixed with Phyton 27 (a copper-based pesticide) was most effective (97% control) on greenhouse tomato against grey mould (Bourbos and Skoudridakis, 1994). Knowledge of fungicide-BCA compatibility is essential for their successful combination.

Post-harvest applications of BCAs in combination with other treatments has been used successfully to maximize disease control. For example, control was obtained by successful integration of *Candida saitoana* with sugar and sugar analogues such

as 2-deoxy-D-glucose, calcium and magnesium salts, chitosan and chitin derivatives, coating materials, UV-c irradiation and an antifungal lytic enzyme (Janisiewicz, 1998; El Ghaouth, 2001a, b).

3.5. Application timing

In past research, BCAs were generally applied in field trials in the same manner as chemicals, i.e. applied at pre-determined fixed intervals, either on a weekly basis (in greenhouses) or according to the developmental stage of the crop (Elad et al., 1993; O'Neill et al., 1996). However, it was soon recognised that most biological treatments do not have the same knock-down effect as chemicals and, therefore, have to be strategically applied at a time when the BCA has maximum chance of successful establishment and elicitation of its biocontrol effect. Alternation of biocontrol treatments with chemical treatments on a calendar basis was found to be more effective and more reliable for control of grey mould on grapes than biocontrol alone (Elad et al., 1994c). However, disease suppression achieved by the alternation treatment was sometimes insufficient if the BCA was applied in weather highly stimulatory to grey mould epidemics (Shtienberg and Elad, 1997). The integration of biological and chemical controls, aided by the use of a forecaster to predict disease outbreaks in non-heated greenhouses, was realized in the development of 'BOTMAN' (*Botrytis* manager) and 'GREENMAN' (greenhouse disease manager). Using these systems, timing of the biological (*T. harzianum* T39) and chemical agents was optimised and chemical use was decreased by two thirds, thus limiting development of *Botrytis*-resistant populations achieving suppression of disease equivalent to a full chemical programme (Chapter 18).

4. Conclusions

The fact that there are a number of microbial products available commercially for control of *Botrytis* diseases indicates that biocontrol can succeed and can provide a valuable additional management tool for growers. At first glance, the breadth of microbial agents forming the active ingredients is surprising. However, this just mirrors the diversity of different types of diseases caused by *Botrytis* species such as seedling and flower blights, field rots and post-harvest rots. This serves to illustrate the fact that different microorganisms have different biological attributes and, therefore, some will be better able to function in one particular ecological niche than another.

Knowledge of the biology and epidemiology of the pathogen is crucial to the development of an appropriate biocontrol strategy. It is only once the key stages of the disease cycle are defined that potential targets for biocontrol can be identified. Similarly, elucidating the biotic and abiotic factors that influence pathogen development at each of these key stages is crucial if an accurate 'blueprint' of the BCA is to be developed.

It is always difficult to decide what preferred mode of action may be the most appropriate for a particular plant disease biocontrol situation. Laboratory assays are generally biased towards antibiotic producers and clearly there is great potential for

such microbes in biocontrol. These BCAs are most closely aligned to chemical pesticides through their production of a bioactive molecule/s with a knock-down effect. Such BCAs are best utilised in a post-harvest environment where antibiotic production by the BCA can be closely controlled and the risk of resistance building up in the pathogen population is minimized. An alternative strategy for post-harvest biocontrol is the use of microbes that can preferentially occupy wound sites thereby excluding the pathogen from gaining entry into the plant tissue. Yeasts have been shown to possess such attributes and their success is reflected by the commercial products now on the market.

Organisms which operate by competitive exclusion or which exhibit several modes of action are likely to be more robust under field conditions. However, extensive knowledge of their population dynamics and interaction with the pathogen across a range of environmental extremes is essential if an appropriate formulation and application strategy is to be developed. Although there is a plethora of examples in the literature of microbes with biocontrol activity against *Botrytis* diseases, very few have given acceptable levels of reproducible control across a number of sites and seasons. Those which have stood the test of time have been ones where rigorous laboratory, glasshouse and field research has been conducted over many years. Two fungal groups, *Trichoderma* and *Ulocladium*, have given the greatest success.

Intensive research has been devoted to *Trichoderma* as a commercial BCA. The fungus survives comparably well on plant surfaces under field conditions (Elad, 1994a; Dik and Elad, 1999) and is compatible with chemical control agents commonly used in commercial crops (O'Neill et al., 1996). It can now be readily produced and formulated to give an active product with good shelf life (Elad, 2001) and its complex mode of action assures its activity against various forms of the disease (Elad, 2000). More recently, research on *Ulocladium* has shown that isolates from this genus have specific attributes that make them very effective competitive colonizers of necrotic tissue allowing them to be used to suppress *Botrytis* sporulation and subsequent disease development. Detailed *in vitro* studies have elucidated the mechanism of action and defined the key nutritional and environmental factors that influence BCA efficacy. For example, its ability to withstand repeated dry periods contributes to its effective colonisation of dead plant tissue ahead of the pathogen (Köhl et al., 1995a, b). Both BCA groups are unlikely to be used predominantly as stand-alone treatments since sometimes season-long control is not achieved. BCA applications have, therefore, been used successfully in combination or alternating with other biological (e.g. Serenade) or chemical (e.g. dicarboximides and anilinopyrimidines) treatments (Chapter 18).

It is expected that better performing BCAs will be developed in the future and that their activity will probably be based on multiple modes of action. There is still great potential for the discovery of microbes with increased competitive and survival abilities and/or which produce novel bioactive compounds. If such microbes are also capable of inducing host plant resistance then this would provide the best 'package' for *Botrytis* control. The relative risk of pathogen resistance development to antibiotic-producing BCAs will not be able to be judged until they have been in commercial use for 5-8 years.

There are now relatively few technical limitations to the selection of promising biocontrol isolates, elucidation of their mechanisms of action and understanding their population ecology under field conditions. There is also an excellent range of liquid fermentation and solid substrate technologies available and access to formulation technology is also becoming easier through standard licence agreements. Thus, as long as a satisfactory quality assurance system is put in place and the producer/supplier provides good technical back-up for growers so that the product is used according to the label recommendations, there is no reason why biological products should not provide disease control equal to that of other treatments. However, BCAs that meet all these necessary biological requirements may still never reach the market place because of certain economic limitations. These include factors such as developing cost effective mass production capability, meeting expensive regulatory requirements for registration, increasing registration costs, having a market size large enough to provide satisfactory profit margin for the company and competitive pricing of BCA products against other products. In some countries, there is also increasingly stricter regulations for the importation and field release of exotic organisms which could severely affect the commercialisation of biocontrol products on an international scale. *Botrytis*-incited diseases rank amongst the top economically important diseases worldwide, therefore the economics of a biocontrol product against *Botrytis* should be reasonable (certainly better than many other plant disease biocontrol systems). If such economic constraints can be overcome then it will be possible for biopesticide development to move from its current niche market environment to an international business arena. Such products can be used in organic and conventional farming systems that are seeking more rational means of *Botrytis* control.

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CHAPTER 14

EPIDEMIOLOGY OF *BOTRYTIS CINEREA* IN ORCHARD AND VINE CROPS

Philip A.G. Elmer¹ and Themis J. Michailides²

¹HortResearch, Ruakura Research Centre, Private Bag 3132, Hamilton, New Zealand; ²Department of Plant Pathology, University of California, Davis/Kearney Agricultural Center, 9240 South Riverbend Ave. Parlier, CA 93648, USA

Abstract. Substantial economic crop losses occur worldwide in tree fruits, nut crops, vines and small fruits as a result of infection by *Botrytis cinerea*. Fungicide-based management, once an accepted practice, is becoming increasingly restricted, a trend likely to continue in the future. Greater emphasis on alternative, non-chemical control will require improved knowledge of *B. cinerea* ecology and epidemiology in affected crops. Epidemics are often initiated in the spring from conidial inoculum produced on over-wintering structures on a very wide range of plant species. From floral infection in the spring, several infection pathways to fruit infection and crop loss at harvest are described. The majority of these pathways include a degree of symptomless latency, or quiescence, in the host tissue. In some crops (e.g. grapes) multiple pathways are described, each one dependant upon many complex host, pathogen and environmental factors. In other crops (e.g. berry fruits), a single dominant pathway is described. Latency, once poorly understood, has become the focus of research in the last decade. Several host defence mechanisms are described which may account for this period of enforced dormancy. Once pathogenic growth resumes and typical *B. cinerea* symptoms appear, many factors affect the subsequent rate of *B. cinerea* epidemics and we describe some of these in detail. The growth of organic production in the last decade has high-lighted the need for a greater understanding of the complexities of epidemic development in order to develop durable and sustainable disease control strategies.

1. Introduction

Epidemics caused by *Botrytis cinerea* can be severe and economically damaging to many agricultural and horticultural crops in conditions conducive to infection (Chapter 1). These losses continue to occur despite the availability of new botryticides (Chapter 12). However, the freedom to apply fungicides is becoming increasingly restricted and in some cases, forbidden. These restrictions are based upon public concerns about the adverse effects of synthetic pesticides on human health and the environment and zero chemical residue tolerance in many export markets. The growth of organic farming in the last decade has encouraged a re-

orientation of thinking for plant pathologists as nil and significantly reduced fungicide programmes become common. To maintain yields in these systems, disease suppression strategies based on robust epidemiological knowledge will need to be devised. In some fruit crops (e.g. raspberries), many years of research has led to a greater understanding of the epidemiological processes which contribute to *Botrytis* outbreaks, while in other crops (e.g. grapes) our understanding of epidemiological processes is continually evolving. The objective of this chapter is to review our understanding of *B. cinerea* epidemiology in selected fruit crops. We focus on the grape system since this crop is one of the most economically important fruit crops with c. 8 million ha (Vivier and Pretorius, 2002) and estimated crop losses due to *B. cinerea* of 2 billion \$US per annum.

2. Sources of primary inoculum for host infections

Conidia produced in late winter and early spring on over-wintering mycelium and/or sclerotia on host tissues and sclerotia on the surface of soil are considered the most important infective unit of *B. cinerea* for infection in the spring. Numerous alternative hosts exist in orchards and vineyards including herbicide-treated or senescing weeds and other infected crops in the vicinity (Figure 1). Over 200 host species were identified as sources of *B. cinerea* inoculum in one study (Sutton et al., 1991). Surveys from a wide range of fruit growing regions consistently rank conidia of *B. cinerea* as an important part of the aerial microflora (Bisiach et al., 1984). Insects in the spring and invertebrates may also act as casual vectors of *B. cinerea* inoculum (Sect. 7.2). Reports of apothecia in orchards and vineyards exist, but they are sporadic (Chapter 3). Although *B. cinerea* is readily detectable in the soil, it represents a small proportion of the soil fungal biomass (Dorado et al., 2001). In grapes, the most important primary inoculum source in the Hunter Valley region of New South Wales, Australia was sclerotia that initiated the disease cycle in the spring (Nair and Nadtotchei, 1987; Nair and Martin, 1987; Nair, 1990). In contrast, sclerotia were not detected at budburst in the relatively dry Marlborough wine growing region of New Zealand (NZ) (R. Balasubramaniam, HortResearch, NZ, unpubl.). A subsequent study identified the dominant sources of inoculum at 80% capfall as; old rachides on the ground, tendrils, leaf petioles and cane debris (Seyb et al., 2000b). In contrast, leaf petioles under vines were identified as the dominant source of over-wintering inoculum in Hawkes Bay, NZ (P. Wood, HortResearch, NZ, unpubl.). *B. cinerea* mycelium survived for up to 30 weeks in grape vine prunings, with weed control practices and temperature having a significant impact on mycelial longevity (Thomas et al., 1983). In California, conidial production on sclerotia and from over-wintering mycelium can be easily found on grape bunch clusters that remain on the vine and on canes, leaf petioles and laminae on the orchard floor (Figure 1). Significant positive correlations were found between over-wintered inoculum sources and flower infection in the Hunter Valley, New South Wales with carry-over inoculum accounting for 70% of the variation in flower infection in the canopy (Nair et al., 1995). In kiwifruit, *B. cinerea* sporulated profusely on infected fruit on the orchard floor, senescing leaves of weeds, necrotic

kiwifruit leaf and fruit remnants or senescent leaves from nearby crops at the end of winter and occasionally on remnants of floral tissues of male vines in California (Michailides and Elmer, 2000). The relative contribution of several inoculum sources during the kiwifruit growing season were quantified in NZ (Elmer et al., 1995). Early in the growing season as female flowers open, most of the inoculum was produced on over-wintering mycelium on prunings on the ground. Occasionally, sclerotia on prunings produced conidia. *B. cinerea* was rarely detected in leaf litter from beneath vine canopies from dormancy to harvest. As in California, profuse conidia production occurred on paraquat-treated perennial weeds (*Rumex* spp.) and senescent flowers of male vines.

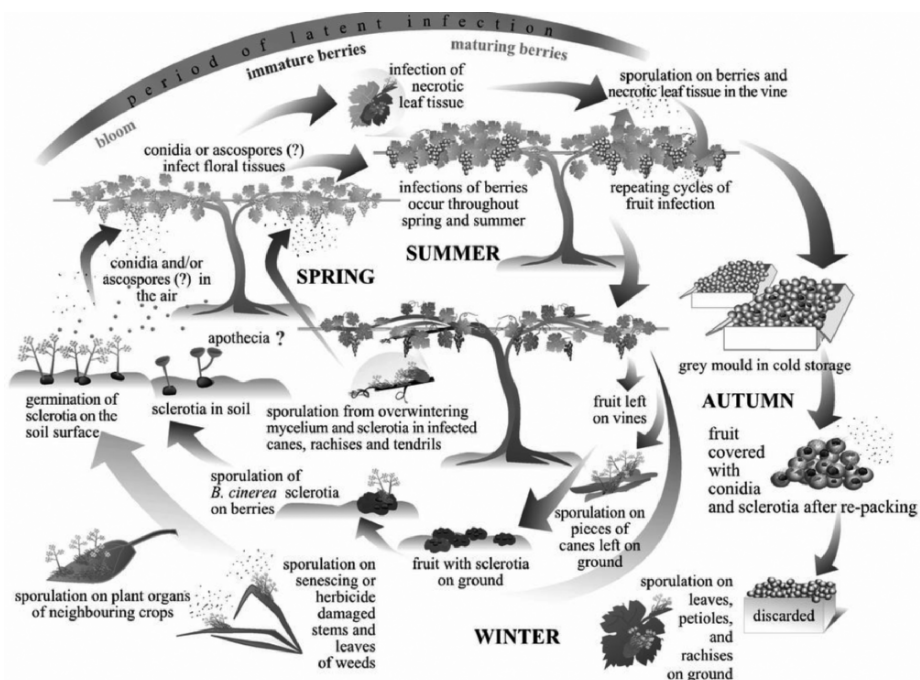


Figure 1. Proposed life cycle of *Botrytis cinerea* and disease cycle of grey mould in wine and table grape vineyards

In most temperate regions, by early spring there is ample inoculum in the environment for infection. Even newly emerged and partially expanded young green strawberry leaves were susceptible, the infection remaining in a latent stage until onset of leaf necrosis (Sutton et al., 1991). Latent infections of young green grape leaves have also been reported (Holz et al., 2003), but these early latent infections did not appear to play an important role as inoculum sources for flower infection, unless the host tissues senesced prematurely. To our knowledge latent infections on immature leaves of kiwifruit and berry fruits other than strawberries have not been reported. Apparently healthy kiwifruit sepals in California had latent infections early in the season, but did not sporulate and contribute to flower infection.

In blueberries, *B. cinerea* over-winters as dormant mycelium or sclerotia in or on plant debris. In raspberries it survives mainly as sclerotia and mycelium on over-wintering canes and on pruning debris (Williamson and McNicol, 1986). In the spring conidia produced on these tissues infect blossoms, twigs and fruit. After flower blight the fungus spreads down the peduncle to girdle the stem, killing all flowers and young green berries above the point of girdling (Bristow and Milholland, 1995). In NZ, leaf litter on the ground, herbicide desiccated primo-canes and receptacles left on the vines were sources of inoculum in boysenberry (Walter et al., 1997). The dominant source of inoculum in strawberries was over-wintering dead leaves (Braun and Sutton, 1988; Sutton et al., 1991).

Epidemiological studies show significant correlations between *B. cinerea* colonisation of senescent floral tissues in the spring with incidence and severity of grey mould at harvest and during cool storage. Flowering is therefore fundamentally important in studies of *B. cinerea* ecology and epidemiology and the current state of knowledge of infection pathways from flowering to fruit infection and crop loss are described (See also Chapter 2).

3. Flower to fruit infection pathways

Flowers of grape and many other fruit crops are highly susceptible to *B. cinerea* infection when they senesce (Jersch et al., 1989) and low resveratrol synthesis may contribute to the high susceptibility of grape flowers (Keller et al., 2003; Chapter 9). The abundance in pollen over the flowering period of most hosts increases severity of infection (Ogawa and English, 1960; Chou and Preece, 1968; Lehoczky, 1972; Michailides, 1991; Rose, 1996). In addition to stimulating conidia and pathogenicity, pollen favours many natural *B. cinerea* antagonists (e.g. *Sporobolomyces* spp.), thereby moderating *B. cinerea* epidemics (Lehoczky, 1972). Flower infections generally result in latent infections in immature fruit (Williamson, 1994), but not all fruit infections at harvest are the result of floral and latent infections at that time (e.g. grapes). Here we review current knowledge of infection pathways, with a special focus on grapes.

Pathway I - Conidial infection of the style and ovules - commences with conidia infecting styler tissues, followed by slow systemic hyphal growth into the ovule where *B. cinerea* enters a latent phase. As host defences weaken, often coinciding with berry ripening, *B. cinerea* resumes growth and rots the fruit. This sequence of events has been postulated for grapes, kiwifruit, raspberry and blackcurrants. Infection of grape stigmas early in the season was proposed as the most likely pathway to fruit infection (McClellan and Hewitt, 1973; Nair and Parker, 1985; Nair and Hill, 1992). Berry rot resulted from latent infections at the 'styler abscission' zone but in some wine growing regions, styler infections were considered less important (Pezet and Pont, 1986; Holz et al., 2003), while in other areas they played a significant role in the ecology and epidemiology of *B. cinerea* (Keller et al., 2003). In raspberries, conidia of *B. cinerea* germinate in the stigmatic fluid; hyphae grow in the transmitting tissues of the style and continue in the styler vascular tissues into the fruit carpel (McNicol et al., 1985; Williamson et al., 1987). In favourable conditions, reactivation of *B. cinerea* in the styler tissues produces conidia, thereby

providing an additional source of inoculum for infection of other senescent and necrotic styles. Saprophytic survival within aborted carpels amongst swollen drupelets can also provide an inoculum source (Williamson et al., 1987). The role of floral organs other than the styles was investigated further by Dashwood and Fox (1988). Direct infection of individual raspberry drupes by *B. cinerea* conidia contributed minimally to fruit rot (Jarvis, 1962; Williamson and McNicol, 1986). The styles are the main infection pathway to fruit rot at harvest in boysenberries (*Rubus* hybrid) and these remained susceptible to infection up to harvest (Boyd-Wilson et al., 1996; Walter et al., 1997; Walter et al., 1999b). In kiwifruit stylar infections were rare (Bisiach et al., 1984) and were not associated with post-harvest storage rot (Fermaud and Gaunt, 1995). In blackcurrants, conidia germinated in the stigmatic fluid then spread symptomlessly, via the style, into the pericarp and ovules. These latent infections were an important inoculum source and contributed to premature abscission of fruits (McNicol and Williamson, 1989). Symptomless fruit infection from inoculations at flowering were not detected in another study (Pappas and Jordan, 1997), but *Botrytis* infected blackcurrant flowers frequently aborted.

Pathway IIa - Conidial infections of the stamens and or petals - followed by systemic hyphal growth through floral tissues to the receptacle, results in latent infections which become aggressive infections as the fruit ripens (e.g. grapes, kiwifruit and strawberries). In grapes, histological studies have shown that *B. cinerea* colonises the stamens, grows basipetally to infect the receptacle and then grows systemically to the pedicel and vascular tissues in the berries (Pezet and Pont, 1986). Seyb et al. (2000a) found no evidence for stamen infections. Cultivar host defence mechanisms could account for an apparent lack of growth in the stamen tissue. Alternatively, the dry climate of this region desiccated the delicate stamen tissues before *B. cinerea* reached the pedicel/receptacle tissue. In some cultivars, stamens detach rapidly rendering this pathway redundant.

A link between floral infection of petals and anthers by *B. cinerea* and post-harvest rot was proposed in kiwifruit (Sommer et al., 1983, 1984) and confirmed as an important source of inoculum for sepal and receptacle infections (Duncan, 1991; Michailides and Morgan, 1996a). The sepals remained receptive to *B. cinerea* infection for the entire growing season. Symptomless infections of sepals and receptacles in the stem-end region were cumulative. Artificial inoculation with a conidial suspension at full bloom resulted in the highest incidence of grey mould in cool storage, suggesting that conditions for infection and establishment of latent infections were favourable over the flowering period (Michailides et al., 1999). In kiwifruit, inoculation studies demonstrated a link between colonisation of senescent carpels in the spring and stem-end rot after cool storage in NZ (Beever et al., 1984). Receptacle infections at harvest ranged from 5 to 55% (1984-1986) from flowers with conspicuous infections on their petals. In contrast, infection frequency in the receptacles ranged from 0 to 10%, when fruits were sampled randomly, indicating a link between petal infection and subsequent receptacle infection (S.R. Pennycook, DSIR, NZ, unpubl.). Despite the presence of *B. cinerea* in the receptacle at harvest, many studies found no correlation with stem-end rot in cool storage (M. Manning, HortResearch, NZ pers. comm.) and the majority of fruit infections occurred at

harvest from conidia redistributed from the hairy fruit surface to the picking wound (Pennycook and Manning, 1992; Manning and Pak, 1993; Pak and Manning, 1994). In contrast, this was not found important in Californian and Italian kiwifruit vineyards (Bisiach et al., 1984). In strawberry, early studies suggested that senescent flower parts were important sources of inoculum for subsequent spread into ripening fruits (Powelson, 1960). However, systemic infection and growth of *Botrytis* through the filaments of infected stamens into the receptacle to form a symptomless infection is considered the primary infection pathway for fruit rot (Bristow et al., 1986). Conidia were not regarded as the primary inoculum source for fruit infection (Jarvis, 1962).

Pathway IIb - Fruit infection via the fruit pedicel - A 6-year research programme in the wine growing region of the Western Cape province of South Africa (SA) has contributed significantly to our knowledge of the ecology of *B. cinerea* in grapes (Holz et al., 1997, 2000, 2003). Inoculation confirmed that the pedicel, lateral, and to a lesser extent the rachii, were susceptible to *B. cinerea* at the early stage of grape berry development, but with the exception of the pedicel, their resistance to infection increased as the season progressed (Holz, 2001). At the point where the berry is attached to the pedicel up to 30% of berries yielded *B. cinerea* following surface sterilisation and freezing. The rachises and laterals yielded c. 20% *B. cinerea* and berry cheek infections were infrequent at 5%. The stomata and lenticels were proposed as entry point for infection of the rachii and pedicel (Holz et al., 2003).

A high proportion (76 points to 90%) of fruit infections were initiated from the stem-end of table grape berries in California (Michailides et al., 2000b). In a separate study, table grape clusters of Red Globe were periodically inoculated from bloom through to harvest. Results indicated that the greatest incidence of infection post-harvest was derived from inoculations of bunches during early flowering and full bloom (Michailides et al., 2000b, c). Pedicel infections occur frequently in French vineyards (Dubos and Roudet, 2000), and fungicide timing studies in many vineyards world-wide have confirmed the importance of flowering infection. For example, a single botryticide application at the late flowering stage reduced bunch rot at harvest by 78% compared to the untreated controls (Viret and Keller, 2000). However, as described in Figure 1, multiple infection pathways occur, and conidial infections of ripening berries later in the growing season are as important as latent infections of green structural tissues earlier in the growing season (Wilcox, 2002).

Pathway III - Conidial infection and extensive colonisation of floral debris-in grapes (e.g. aborted fruitlets and calyptas) this is followed by a saprophytic over-summering phase in these tissues trapped in the developing bunch, but little or no fruit infection occurs due to preformed host barriers. At veraison, host defences weaken as the berries ripen; berry exudates reactivate saprophytic mycelium, provide sufficient inoculum potential to overcome remaining preformed barriers and direct fruit infection occurs. At bloom, the calyptra separates along a preformed abscission zone at the base of the ovule. This zone consists of a localised band of necrotic tissue ('cap scar') and is considered to be the initial site of *B. cinerea* colonisation leading to the establishment of latent infections (Keller et al., 2003). EM studies of the cap scar supported this hypothesis based upon *B. cinerea* germ tube growth and penetration into this tissue. The proportion of rapidly senescing and

necrotic tissues in grape bunches in Australian vineyards consisted of calyptras (67%) and stamens (22%), but at veraison, the calyptras were the more prevalent necrotic tissue type in the bunch (Nair and Hill, 1992). Up to 10% of the flowers did not set fruit ('aborted fruitlets') within an inflorescence and when infected were considered to be a major source of inoculum within developing bunches (Nair and Parker, 1985). Infected floral tissues potentially provide a large base of saprophytic mycelium, the extent of which varies significantly between cultivars. Collectively, the necrotic tissues within bunches in NZ are referred to as 'bunch trash' (Seyb et al., 2000a) and consist of stamens, aborted flowers, aborted berries, calyptras ('caps'), tendrils and leaf pieces. *B. cinerea* infection of bunch trash ranged from 13 to 72%, indicating that prior to bunch closure there was considerable *B. cinerea* inoculum potential within the bunch, immediately adjacent to berries. Aborted fruitlets, damaged berries and calyptras were considered the most important contributors to bunch rot at vintage, being the more numerous in terms of biomass and the most frequently infected. These components were also significantly positively correlated with the incidence of infected berries at harvest, but not in all seasons (Seyb et al., 2000a). Stamen infections were not detected for three consecutive years (1998-2000).

Pathway IV - Conidial accumulation within the developing bunch - Similar to Pathway III with extensive floral tissue colonisation *B. cinerea* enters a saprophytic over-summering phase in the bunch and during conducive conditions crops of conidia are produced resulting in contamination of the external surfaces of the ripening berry with conidia inside the bunch itself. Preformed host barriers prevent the majority of *B. cinerea* latent infections from becoming established until host defences weaken as berries ripen; leakage of exudates from the most mature berries in the bunch provide sufficient stimuli for conidial fruit infection and a rot focus commences. Inoculum is readily dispersed within bunches between flowering and veraison to pedicels, rachii and laterals (Holz et al., 2003), but also to immature fruit surfaces. Active sporulation on aborted fruitlets and calyptras in immature bunches is frequently observed (P.N. Wood and P.A.G. Elmer, unpubl.) resulting in conidial contamination of the berry surfaces within the bunch. Conidia of *B. cinerea* were detected in the receptacle/cap scar zone, and represented a resting state as opposed to a latent infection. These trapped conidia are in an ideal position at the berry base to infect the berry when host and environmental conditions favour infection (Sarig et al., 1998). Microscopic examination of the receptacle/berry attachment area and cap scar has confirmed that conidia are present (M. Cole, Monash University, Australia, pers. comm.),

Pathway V - Conidial infection of ripening fruit - As host defences weaken (e.g. in a small number of physiologically more mature berries), symptoms appear and a new crop of conidia are dispersed to new infection sites and the "classical" pre-harvest polycyclic epidemic commences. In vineyards world-wide, peaks of airborne conidia were consistently found at flowering, veraison and harvest (Vercesi and Bisiach, 1982). Therefore, when ripening commences there is ample inoculum for both canopy and fruit infections. The importance of fruit infection post veraison was well illustrated in NZ (P.N. Wood, HortResearch, NZ, unpubl.). All experimental plots were treated with repeated applications of a selected botryticide

(cyprodinil/fludioxonil) to suppress *B. cinerea* populations early in the growing season. Control plots ('unsprayed', Figure 2), received no botryticides, then at véraison, different treatments were applied. In the control plots without botryticide pre- and post-veraison, the grey mould epidemic developed as predicted and continued after bunches were incubated in high humidity chambers. In the absence of sprays applied post-veraison ('ver. & pre-harvest omitted'), the epidemic continued and was not significantly different to that in the unsprayed plots (Figure 2). Plots that received botryticides post-veraison ('industry full season', Figure 2) had low *Botrytis* infection at vintage, which did not change even after bunches were incubated for up to 10 days at high relative humidity (Figure 2). Therefore, post-veraison infections were not likely to have come from the bunch trash or latent infections of the green structural components (e.g. pedicels) since these were suppressed with botryticides over flowering and bunch closure ('ver. and pre-harvest omitted'). The importance of post-veraison conidial infections have been described in several wine growing regions (Latorre et al., 2001; Wilcox, 2002). An increase in latent infection frequency from véraison (20%) to harvest (100%) was believed to be the result of secondary infections of the ripening berries from air-borne conidia (Keller et al., 2003).

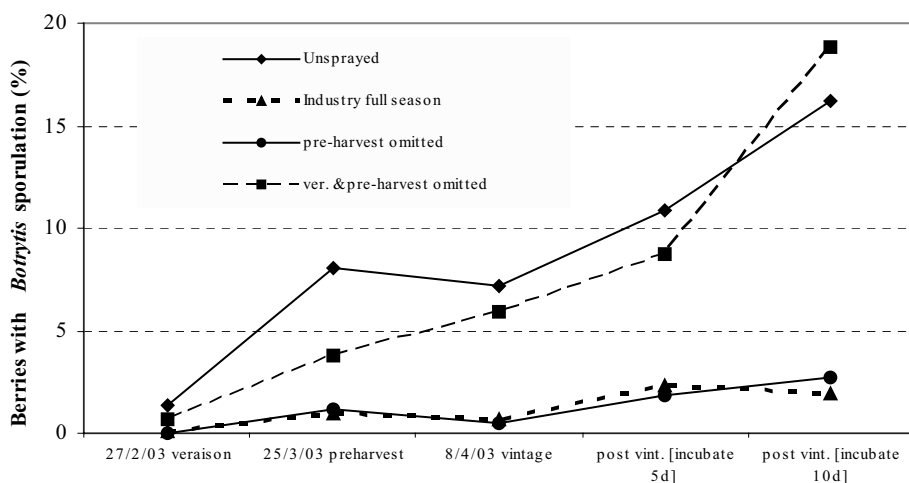


Figure 2. Effect of post-veraison berry infections on bunch rot at vintage and after bunches were incubated in *Botrytis* conducive conditions in 2002 (cv. Sauvignon Blanc, Hawke's Bay, NZ). 'Post vint.' data points refer to the number of days that harvested bunches were incubated in *Botrytis*-conductive conditions (From P.N. Wood, HortResearch, NZ, unpubl.)

In stone fruit orchards, primary inoculum was identified as fruit 'mummies' (desiccated old fruits) with *B. cinerea* sclerotia and mycelium (Hong and Michailides, 2000). *Botrytis*-infected petals of early blooming cultivars are difficult

to distinguish from those caused by *Monilinia* spp. when conditions are non-conducive for sporulation. When conditions are conducive, however, blossom blight occurs as infected by *M. fructicola*. In the Western Cape province of SA, epidemiological investigations found that flower and early fruit infections were less important compared to direct conidial penetration of the fruit during the growing season (Fourie and Holz, 1987, 1994, 1995). The importance of the late season stone fruit infection pathway was confirmed in later studies (Fourie et al., 2002) using surface sterilisation and paraquat (Gindrat and Pezet, 1994). The spatial pattern of *B. cinerea* that emerged from the fruit surface was random and not associated with specific floral tissues. Inoculum for fruit infections within the orchard included herbicide-treated weeds and conidia from early-maturing cultivars (Fourie et al., 2002). *B. cinerea* was unable to directly infect the cuticle of the fruit, but entered the sub-stomatal cavity where further growth ceased due to host defence responses. Close to harvest, conidial infections directly penetrated the fruit cuticle. In addition to stomatal infections, cuticular micro-cracks (Fogle and Faust 1975) have been suggested as possible infection pathways, as in grapes (Pucheu Plante and Mercier, 1983; Commenil et al., 1997) and sweet cherries (*Prunus avium*; Sekse, 1998). These cracks originate from microscopic fractures on the cherry surface which rapidly enlarge as the cherry swells after rainfall near harvest (Glenn and Poovaiah, 1989), thereby facilitating infection (Børve et al., 1998, 2000).

Pathway VI - Conidial accumulation on fruit and dispersal to picking wounds - If conditions are dry after flowering in kiwifruit plantations, *B. cinerea* enters a resting over-summering phase in the canopy. Wet periods during the growing season result in the production of successive crops of conidia which are then dispersed into the canopy where contamination of the external hairy surface of the fruit occurs, but not infection. These conidia are then re-dispersed to the picking wound at harvest. Post-harvest rots occur after a period of cool-storage and this pathway is believed to be specific to kiwifruit in NZ. Accumulation of *B. cinerea* on the fruit surfaces occurs early in the growing season as a result of infections of floral tissues in NZ (Elmer et al., 1995, 1997). Under conditions favourable for the pathogen, profuse sporulation was observed on attached petals. On developing fruitlets, inoculum loads were 100-fold higher on fruitlets with attached petals compared to those where petals had abscised (Elmer et al., 1995). Flowering is therefore epidemiologically important in the ecology of *B. cinerea* in NZ kiwifruit plantations and new, more efficient sampling strategies have been devised, based upon knowledge of the spatial pattern of *B. cinerea* on flowers at full bloom (Elmer et al., 1993). Spatial autocorrelation analysis (Cliff and Ord, 1981; Gaunt and Cole, 1992; Elmer et al., 1998) was used to provide information on the size and orientation of *B. cinerea* foci in kiwifruit plantations.

Kiwifruit shoots and leaves are susceptible to physical damage, providing ample opportunity for *B. cinerea* infection from air-borne conidia (Greaves et al., 2001). Wind-blown senescent shoots, green leaves with necrosis (Hoyte et al., 1994; Elmer et al., 1995, 1997) and senescent leaves in the canopy (Manning and Pak, 1993; Pak and Manning, 1994) were identified as important inoculum sources for external fruit contamination in the pre-harvest period. Methods to quantify the relative contribution of each inoculum source to 'total potential inoculum production' (TPIP)

within vine plots were devised (Hoyte et al., 1994; Elmer et al., 1995). Significant linear relationships between TPIP per plot and the level of external fruit contamination at harvest were identified (Elmer et al., 1997) and also between the level of external contamination at harvest and stem-end rot in storage (Michailides and Elmer, 2000). Conidia have been considered 'short lived propagules' when exposed to direct sunlight (Rotem and Aust, 1991) or high temperatures and dryness (Yunis and Elad, 1989), but long-term survival of *B. cinerea* conidia on fruit surfaces has been reported (Spotts, 1985; Walter et al., 1999a). *B. cinerea* applied to kiwifruit fruits early in the growing season survived exposure to field conditions for up to 16 weeks (Walter et al., 1999a). In contrast, conidia did not survive for 'extended periods' on grape berry surfaces (Coertze and Holz, 2002).

4. The phenomenon of latency in *B. cinerea* epidemiology

Latency, once described as an 'enigmatic aspect of *Botrytis* ecology' (Coley-Smith, 1980), has been the focus of many research studies in order to define its epidemiological role and relationship to crop loss. Several methods have been used to detect latent infections of fungi (Sinclair and Cerkauskas, 2000), including the paraquat induced senescence technique (Cerkauskas and Sinclair, 1980; Gindrat and Pezet, 1994) and freezing of green immature tissues, a method initially designed for *M. fructicola* (Michailides et al., 2000a). Both methods break down any preformed and constitutive host barriers allowing for rapid *B. cinerea* re-growth and sporulation. Many host and pathogen factors could account for the inhibition and apparent suspended state of *B. cinerea* and we examine several host defence mechanisms that may be responsible for latency. Three important stages are often recognised; 1) establishment, 2) containment/arrested phase and 3) resumption of active growth, sometimes referred to as 'the escape phase' (Wade and Cruickshank, 1992a; Latunde-Dada, 2001).

Containment of latent infections is achieved in grapevines by many pre-formed constitutive and inducible defence mechanisms that may account for the high relative resistance of green immature berries to *B. cinerea* (Nair and Hill, 1992; Barnavon et al., 2001; Keller et al., 2003). Preformed morphological features act as a physical barrier, while cuticular waxes are capable of preventing infection due to their antimicrobial and hydrophobic properties (Padgett and Morrison, 1990). Cuticle thickness (Prudet et al., 1992) and chemical composition change as berries ripen, often at a time coinciding with increased susceptibility to *B. cinerea* (Rosenquist and Morrison, 1989; Nair and Hill, 1992; Commenil et al., 1997). If the cuticle is breached, uninfected cells adjacent to the wounded cells commence suberisation, thereby completely isolating invading mycelium (Hill, 1985; Nair and Hill, 1992; Forbes-Smith, 1999). In the *M. fructicola*/apricot system, suberisation and periderm formation was greatest during the early stage of fruit development and was absent from infections on mature fruit. Suberisation around latent infections may also impede nutrient diffusion to the pathogen, thereby ensuring its containment (Wade and Cruickshank, 1992a, b) and blocking fruit ripening signals (Cruickshank and Wade, 1992). Other mechanisms of pathogen inhibition include

polygalacturonase inhibitor proteins and proanthocyanins (Nair and Hill, 1992; Pezet and Pont, 1992; Bezier et al., 2002), and their activity tends to decrease towards harvest (Nair and Hill, 1992; Pezet et al., 2003).

The best characterised inducible defence response of grapevines to fungal infections is the accumulation of phytoalexins and the synthesis of PR-proteins (Mauch et al., 1988; Renault et al., 1996; Chapters 9 and 20). The accumulation of resveratrol and grapevine resistance to *B. cinerea* are well correlated (Jeandet et al., 1995; Sbaghi et al., 1995; Adrian et al., 1997; Chapter 9), and decline in berries to low levels prior to harvest (Bais et al., 2000) associated with an increase in susceptibility (Jeandet et al., 1991; Nair and Hill, 1992). Some PR proteins are capable of causing death of conidia (Pezet, 1988) by breaking down chitin in the cell wall (Mauch et al., 1988; Giannakis et al., 1998), a mechanism that may account for the observed decline in latent infection frequency. Berry phenolics (e.g. catechin) may play a role in resistance of young berries to *B. cinerea* (Goetz et al, 1999), since these compounds were inhibitors of stilbene oxidase, an enzyme implicated in *B. cinerea* pathogenesis (Sbaghi et al., 1996). Unlike grape berries, leaves tend to become more resistant to *B. cinerea* as they mature, the result of cell wall-bound phenolics being released during attempted infection (Weber et al., 1995).

Only a small proportion of early season latent infections became invasive when fruits ripened. In contrast, latent infections derived from inoculations made approximately 30 days before harvest resulted in high levels of fruit rot (Wade and Cruickshank, 1992a, b). These findings suggest that the majority of early season latent infections may never become active, whereas post-veraison latent infections are more likely to become pathogenic when host defences decline.

5. Factors predisposing host tissues to *B. cinerea*

Any factor which causes damage to tissues will facilitate *B. cinerea* infection. Grape berries can be wounded by a range of edaphic, e.g. frost, hail, wind-blown sand, sun (Nair and Hill, 1992), and biotic factors, e.g. insects, snails, birds, excessive fruit swelling (usually in wet soils) and fruit split, berry compression and lesions caused by other pathogens (e.g. powdery mildew, caused by *Uncinula necator*; Jarvis, 1977). In California for instance, pistachio leaves and young green stone fruit wounded by hail were severely infected by *B. cinerea* (Michailides, 2002). Insects are known to be vectors of several plant pathogens, affect disease development (Michailides and Spotts, 1990) and create wounds by which pathogens can enter the host tissue, thereby by-passing several biophysical and biochemical host barriers (Webber and Givvs, 1989).

5.1. Cuticle integrity

The structure and thickness of the cuticle and the epidermal layers have long been regarded as major factors of resistance against *B. cinerea* infection (Commenil et al., 1997). Berry-to-berry contact, where the cuticle is absent or very thin, increases the susceptibility of grape berries to *B. cinerea* (Marois et al., 1986; Rosenquist and

Morrison, 1989) and clones within the same cultivar (e.g. Chardonnay) characterized by tight clusters also develop more severe bunch rot (Vail and Marois, 1991; Vail et al., 1998).

In general, stomata number (Bernard and Dallas, 1981) or natural openings (Pucheu Plante and Mercier, 1983) were independent of susceptibility to *B. cinerea*. However, a recent study found that the number of stomata in the berry epidermis was negatively correlated, while the number and thickness of epidermal and hypodermal cell layers and cuticle and wax contents were positively correlated with resistance to *B. cinerea* in a wide range of table grape cultivars (Mlikota Gabler et al., 2003). Vines grown under UV screens had less cuticular wax and lower lipid oxidase (an indicator of membrane damage) than those grown under ambient light, suggesting that an increase in UV light could lead to thicker wax on the fruit and leaf tissues, which may reduce susceptibility to *B. cinerea* (Steel, 2001).

Developing vegetative and floral tissues are highly susceptible to frost damage, but the role of freezing injury and early season build-up of *B. cinerea* epidemics have not been well studied in orchard crops, though profuse *B. cinerea* sporulation was visually observed on terminal grape shoots after frost injury prior to flowering in Chardonnay grapes (P.A.G. Elmer, HortResearch, NZ, unpubl.).

5.2. Association with insects, invertebrates and vectors of *B. cinerea* inoculum

Botrytis outbreaks in grapes have been shown repeatedly to be associated with the grape berry moth, *Lobesia botrana*. The first generation of the pest attacks flowers, the second feeds on immature berries promoting green berry rot (Fermaud and Giboulot, 1992) and the third generation damages ripe berries. (Bulit and Verdu, 1973; Fermaud and Le Menn, 1992). SEM studies revealed that numerous conidia contaminated the ornamentations of the cuticle segments of the larva and ingested conidia remained viable after passing through the insect's digestive system (Fermaud and Menn, 1989). Supplying viable conidia to second-generation larvae resulted in an increase in the proportion of injuries infected by *B. cinerea* in grape berries (Fermaud et al., 1992). The first instar larvae were attracted by *B. cinerea* infection on grape berries, possibly due to volatile kairomones produced by *Botrytis* (Mondy et al., 1998) and a mutualistic relationship between the two partners was proposed. The presence of *B. cinerea* infected grapes consistently increased insect fecundity and attracted females to oviposit (Mondy and Corio-Costet, 2000).

In NZ, adult thrips of *Thrips obscuratus* (Walker, 1985), often occur in large numbers on both male and female flowers of kiwifruit (Mound and Walker, 1982), feed on pistillate flower petals, act as vectors of *B. cinerea*, increase *Botrytis* inoculum on the fruit surface (Fermaud et al., 1994) and infection of berries (Fermaud and Gaunt, 1995). Use of a marker strain of *B. cinerea* deficient in nitrate reductase to track *B. cinerea* from flowering to vintage (Weeds et al., 1998; Beever and Parkes, 2003; Parkes et al., 2003) showed that the presence of thrips increased berry infection in cv. Riesling, but not in cv. Pinot Noir (Marroni et al., 2003).

Severe outbreaks of *Botrytis* in wine growing regions of Australia have been associated with infestation by light-brown apple moth, *Epiphyas postvittana* (Bailey et al., 1997). The larvae vectored conidia on their cuticles and viable propagules were recovered from larval faecal pellets. Larvae remained resident within a bunch with little bunch-to-bunch movement, increasing the severity of an existing infection by distributing *B. cinerea* to uninfected berries in a bunch. *Drosophila* spp. have long been known as disseminators of microorganisms especially in vineyards (Capy et al., 1987) and tree fruit orchards (Michailides and Spotts, 1990). Conidia of *B. cinerea* were carried externally on the cuticle of *D. melanogaster* and may be carried internally through the digestive tract. The explorative behaviour of these flies on grapes suggests that they spread conidia to ripening fruit and to surfaces where sugary exudates exist, although their precise role as potential *B. cinerea* vectors requires further study. See also Chapter 2.

To ensure adequate pollination of kiwifruit in NZ eight bee hives per hectare are recommended (Bryant, 1986). When foraging, 87% of honey bees were found to carry viable *B. cinerea* propagules (Rose, 1996). Foraging honey bees effectively transferred up to 1.4×10^4 *B. cinerea* CFU per bee from artificially inoculated flowers to control flowers, thereby effectively spreading the pathogen within the canopy (Rose, 1996). However, honey bees are cost effective pollinators and the benefits of good pollination outweighs their potential to vector *B. cinerea*. Another possible vector of *B. cinerea* is the raspberry beetle, *Byturus tomentosus* (Woodford et al., 2000; Woodford et al., 2002). Larvae of raspberry beetle feed on the receptacle and the drupelets often causing significant physical damage to the basal drupelets (Taylor and Gordon, 1975), resulting in greater post-harvest storage losses. However, the precise role of the beetle in *B. cinerea* epidemiology in red raspberries is not clear.

In California, the brown garden snail (*Helix aspersa*) consumes sepal tissue around the receptacle area of the stem-end of kiwifruits resulting in more post-harvest grey mould than in un-damaged fruits. Snail slime also enhanced the germination of *B. cinerea* conidia (Michailides and Morgan, 1996b). Wounds cause by birds ('bird pecks'), especially on the shoulder of the bunch (Chambers, 1993) caused by the birds claws provide an ideal infection site for *B. cinerea*. Birds are attracted to maturing fruit and in order to protect crops, bird control is routinely carried out to reduce berry damage. Infection by *B. cinerea* of fleshy fruits damaged by birds was also observed in apples, pears, late cultivars of stone fruits, citrus, pomegranates, persimmons, quince and kiwifruit.

6. Effect of plant nutrition on *B. cinerea* epidemics

The effects of specific nutritionally relevant ions on host susceptibility and development of *B. cinerea* epidemics has been well documented (Jarvis, 1980; Goodman et al., 1986). Nitrogen (N) and calcium (Ca^{2+}) have been the two most studied (Elad and Shtienberg, 1995), but often with conflicting results (Elad, 1997).

6.1. Nitrogen nutrition

Low N nutrition is a significant problem in viticulture, associated with 'stuck' fermentations (Tromp, 1984; Conradie and Saayman, 1989) and deterioration of wine aroma (Marangoni et al., 2001). An over-supply of N leads to excessive growth in terms of vine vigour, berry number, bunch compaction and cuticle thinning - factors all known to increase grey mould (Delas et al., 1984, 1991; Keller et al., 2001). Other studies report no adverse effect of N on wine quality (Conradie and Saayman, 1989) and no increase in grey mould (Chambers et al., 1993).

Excessive N fertilization in kiwifruit in Italy did not increase plant growth or leaf number, but *B. cinerea* incidence in cool-stored fruits was higher (Pertot and Perin, 1999). In NZ *Botrytis* incidence in cool storage was strongly linked to excessive N (Prasad et al., 1990; Prasad and Spiers, 1991), but a later study found no evidence of a link (Smith and Buwalda, 1994). *B. cinerea* populations pre-harvest were not measured in the studies described above and we suggest that the relationship between N and post-harvest *Botrytis* is indirect, perhaps leading to an increase in the susceptibility of leaves and shoots to physical damage, reducing disease resistance of leaves, as reported in related host-pathogen systems (Daane et al., 1995), thereby increasing inoculum potential in the canopy.

6.2. Calcium

Ca²⁺ plays a vital role in fruits and vines (Ferguson, 1984), increases resistance to disease (Volpin and Elad, 1991; Conway et al., 1991), reduces leakage of exudates to the host surface thus, reducing their availability to the pathogen (Volpin and Elad, 1991) and modulates various cell functions (Conway, 1982; Elad et al., 1992). In contrast, Ca²⁺ deficiency increases susceptibility to *B. cinerea* (Schwab et al., 1993). Grape cultivars differ in their response to Ca²⁺ and enzymatic degradation by *B. cinerea*, indicating that the relationship between Ca²⁺ and *B. cinerea* is complex (Chardonnet and Doneche, 1995; Chardonnet et al., 1997). When Ca²⁺ was applied before veraison to a range of grape cultivars, infection was reduced. In contrast, Ca²⁺ applied after veraison had no effect on epidemic development (Doneche and Chardonnet, 1996). When Ca²⁺ applications were made to table grapes in the field, resistance to *B. cinerea* was increased and correlated with increased levels of cellulose and of both oxalate and alkali-soluble pectins (Miceli et al., 1999). Incubating *B. cinerea* conidia in increasing concentrations of CaCl₂ decreased conidial germination and germ tube length (Chardonnet et al., 2000). However, the inhibitory effect of CaCl₂ on *B. cinerea* could be overcome by the addition of glucose to the medium (Wisniewski et al., 1995). This has important implications since sugar leakage from grape berries increases post-veraison (Kosuge and Hewitt, 1964), potentially neutralising the beneficial effects of Ca²⁺. The inhibitory activity of Ca²⁺ on *B. cinerea* was also dependant on isolate (Chardonnet et al., 2000). In strawberries, variable results with Ca²⁺ have been reported. In one study, vinclozolin and captan were replaced with CaCl₂ to protect strawberries from *B. cinerea*, but the CaCl₂ treatments did not

reduce the epidemic (Erincik et al., 1998). In contrast, increasing fruit calcium content reduced the incidence of *Botrytis* (Cheour et al., 1990; Karp and Starast, 2002; Wojcik and Lewandowski, 2003).

7. Host management factors and *B. cinerea* epidemics

7.1. Rootstocks and rooting depth

The effect of rootstocks on *Botrytis* bunch rot of grapes has been well studied (Egger et al., 1979; Delas et al., 1984; Ferreira and Marais, 1987; Cristinzio et al., 2000) and generally is indirect in its nature, primarily affecting scion vigour and bunch compactness (Ferreira and Marais, 1987). The rootstock may impart a 'resistance factor' to the scion, for example leaves of cv. Falanghina produced smaller lesions on rootstock SO4, compared to three other rootstocks (Cristinzio et al., 2000). However, inoculum production from such lesions and the nature of the resistance mechanism requires further investigation. Extensive research in France on Mèdoc and Graves soils on the impact of rooting depth and water up-take on skin splitting and grey mould have been made (Ribèreau-Gayon et al., 1980); deep rooted vines were much less susceptible to splitting and grey mould than shallow-rooted vines. Rootstocks also had a significant impact on the extent of fruit micro-cracking in sweet cherries and differences in soil moisture uptake by rootstocks were believed to be responsible (Cline et al., 1995).

7.2. Cultivars

In grapes, cultivar is one of the most important variables affecting grey mould epidemics. The morphological, anatomical and chemical characteristics of 42 cultivars with a range of resistance to *B. cinerea* were measured to establish whether resistance was linked to specific characteristics. Eleven were classed as highly resistant and the number of naturally occurring pores was negatively correlated with resistance to *B. cinerea* (Mlikota Gabler et al., 2003), thus confirming earlier studies (e.g. Eibach, 1994). Cultivar resistance was attributed to higher cuticle and wax contents and certain anatomical features, rather than induced or constitutive antifungal host defence mechanisms (Mlikota Gabler et al., 2003). In some raspberry cultivars, the stigmatic fluid was inhibitory to *B. cinerea* thereby avoiding latent infections (Williamson et al., 1987; Williamson and Jennings, 1992). These findings suggest that cultivar selection will play a major role in future *Botrytis* management strategies.

7.3. Canopy management

There have been continued global efforts to find the best combination of optimal canopy type, training and pruning systems and *Botrytis* suppressiveness to optimise fruit quality in grapes.

7.3.1. Vine training and pruning systems

Grapes grown in dense canopies are exposed to greater periods of wetness after rainfall, resulting in increased susceptibility to *B. cinerea* (Steel, 2001). A range of different vine training systems were evaluated in Italy on several grape cultivars to identify systems that were non-conducive to pathogen development. The highest incidence of *B. cinerea* was reported in the 'Pergola' system, while vines pruned to the 'Guyot' system had the lowest disease development (Cargnello et al., 1991). Vines trained in the horizontal bilateral cordon ('traditional Moser system') had improved exposure to light and lower incidence of *Botrytis* and powdery mildew (*Uncinula necator*), higher yields and better quality grapes than the 'high' cordon system supported by a one-wire trellis (Redl, 1988). Also, the practice of leaving 60 rather than 40 nodes per vine in vigorously grown Chenin Blanc grapes reduced bunch rot in spur- or cane-pruned systems, and the *Botrytis* reduction was attributable to less compact clusters (Christensen, 1981).

In Australia, the practice of 'lighter pruning' the vine canopy reduced berry-to-berry-contact within the bunch and *B. cinerea* development (Martin, 1990). Non-contact Riesling berries had 15.7 and 35% more epicuticular wax and cuticle compared to the contact samples, explaining the lower incidence of bunch rot (Percival et al., 1993). Similarly, reducing epicuticular waxes in grapes by spraying an adjuvant can increase bunch rot (Marois et al., 1987). Along with the training system itself, the bunch architecture can also affect development of the pathogen. Infection of Cabernet Sauvignon clusters after veraison by *B. cinerea* was significantly influenced mainly by cluster compactness (Vail and Marois, 1991; Fermaud et al., 2001a, b); reduced *Botrytis* was correlated with less compact clusters, associated with lower berry number and reduced cluster weight. Thus, training and pruning systems adopted to reduce the risk of *Botrytis* at vintage may be cultivar-specific and dependant upon a range of other factors.

Vine 'hedging' is the practice of pruning off the over-hanging current season growth at veraison. Vines trained on a two-wire trellis, sprayed and hedged, had a 39% reduction in bunch rot as compared to vines sprayed and not hedged. Hedging improved air circulation in the bunch zone, reduced relative humidity in the canopy and exposed more fruit bunches to light (Savage and Sall, 1982). This practice has now been widely adopted in Australasian (Clingeleffer, 1984; Sommer et al., 1995) and in North American vineyards (Reynolds and Wardle, 1993) as a cost-effective alternative to hand pruning and as a cultural operation aiming to reduce bunch rot. A better practice was proposed subsequently, based on careful selection of node number at winter pruning, providing better shoot spacing and thus creating a canopy with optimal density (Smithyman et al., 1997).

Different cultivars respond to pruning regimes quite differently. In seasons conducive to infection, the practice of removing or thinning 'distal' clusters just before veraison reduced infection in northern Italian vineyards. The level of cluster thinning depended on the particular cultivar, e.g., bunch rot incidence at harvest was 21% for no thinning, 10% for the 20% cluster thinning and 7% for the 40% cluster thinning level. In contrast, cluster thinning in Cabernet Sauvignon had no significant effect on bunch rot at vintage (Palliotti et al., 2000).

7.3.2. Leaf removal

Leaf removal from the fruiting zone of vines ('leaf plucking') has significantly reduced epidemics thereby improving *Botrytis* control in grapes in European (Zoecklein et al., 1992), Californian and Australian vineyards (Gubler et al., 1987; Percival et al., 1994). Leaf removal affects the microclimate (temperature, vapour pressure deficit, wind speed and wetness) in and around the receptive bunch, often reducing bunch rot at vintage. Increased wind speed after leaf plucking (English et al., 1989) increased the evaporative potential on the berry surface, thereby significantly reducing *B. cinerea* infection and development (Thomas et al., 1988; English et al., 1993). In addition, stimulation of phytoalexin production by increased UV light has been reported as a result of leaf removal (Langcake, 1981). Following leaf removal, exposed berries of Riesling grapes had 19 and 35% more epicuticular wax and cuticle, respectively, compared to the shaded bunches resulting in significantly less grey mould (Percival et al., 1993). Leaf removal has been adopted globally as an effective non-chemical practice to manage *B. cinerea* in vineyards.

7.3.3. Removal of potential substrates for the pathogen

A less well-adopted practice to manage *Botrytis* is the removal of potential substrates to reduce inoculum potential in the bunch early in the season. Removal of senescent floral tissues and aborted berries ('bunch trash') reduces *B. cinerea* by c. 30% in Merlot grapes (Jermini et al., 1986). The relationship between senescent floral debris retained in fruit clusters of Chardonnay and *Botrytis* bunch rot was investigated for three seasons in California. Compressed air was used to remove bunch trash at early or late fruit set. Removal of inoculum in bunch trash significantly reduced bunch rot in some, but not all vineyards, indicating that other factors besides bunch trash biomass may contribute to subsequent bunch rot at harvest (Wolf et al., 1997). In addition, in a Californian kiwifruit plantation, removal of flowers from male vines, a potent source of *B. cinerea*, reduced stem-end rot by 60% in neighboring female vines compared to fruit from vines where the male flowers were retained (Michailides and Elmer, 2000).

The impact of removal of necrotic tissue on epidemics was also demonstrated in The Netherlands (Köhl et al., 1992). In this study, removal of up to 30-50% of necrotic tissues by hand reduced the number of *Botrytis* spp. conidia in the air by 34% and subsequently delayed the *Botrytis* epidemic. This finding was used as the rationale for developing a new biocontrol strategy, based upon saprophytic colonization of necrotic tissues by selected antagonists (Köhl et al., 1995; Chapter 13). Use of compressed air to remove necrotic canopy and bunch tissue in grapes in NZ reduced the *B. cinerea* epidemic and at harvest, bunch rot incidence was reduced by c. 50% (P.N. Wood, HortResearch NZ, unpubl.). These and other studies demonstrate the importance of necrotic tissue substrates for *B. cinerea* epidemics.

7.3.4. Harvest practices to limit *B. cinerea* losses

Harvesting earlier than scheduled is the commonest cultural practice used to limit losses of mature grapes by *Botrytis* (Nair, 1985). For premium grade Chardonnay wines, grapes are harvested between 23 and 25° Brix (soluble solids content). If conditions favour *Botrytis* development, the crop will be harvested at 18° Brix to limit losses in NZ.

In kiwifruit, a different relationship between ° Brix and *B. cinerea* infection of the picking scar was established (Pennycook and Manning, 1992; Pyke et al., 1993). The majority of kiwifruits are harvested at 6.2° Brix to optimise storage keeping quality, but a common practice in organic kiwifruit plantations is to harvest at a more advanced maturity (e.g. 7.5° Brix). Field and experimental data support that more mature fruit at harvest have increased levels of resistance to *B. cinerea* than less mature fruit (Pyke et al., 1993).

8. Effect of growing system

Despite the strong increase in production of organic food, driven by consumer concern for food safety and the environment, only eight papers have been published since 1979 on *B. cinerea* in organic fruit systems, thus indicating clear needs for future research. Organic grapes are perceived to sustain more frequent and severe *Botrytis* epidemics than those under conventional production systems. However, *B. cinerea* was reported to be less in an organic system in Germany when compared to the conventional integrated system (Hoffman et al., 1997), but the factors responsible for this reduction of grey mould were not identified.

Greater uptake of organic viticulture may be achieved if naturally occurring genetic resistance could be identified that produced wines with high quality attributes. Some cultivars are regarded as highly resistant and possess a wide range of active and preformed defence mechanisms (Stein and Blaich, 1985; Stein, 1985; Nair and Hill, 1992). Efforts to combine *B. cinerea* resistance and grape quality have progressed slowly, for example, 28 years were required to produce a new grape cultivar (cv. Regent) with resistance to *B. cinerea*, downy mildew (*Plasmopara viticola*) and powdery mildew (*Uncinula necator*), suitable for use in organic wine production. The yield and sensory characteristics of the new cultivar were reported to be similar to other grape cultivars (Topfer and Eibach, 2002).

In organic Italian kiwifruits, *B. cinerea* is a significant cause of crop loss and summer pruning is the only strategy recommended to increase air flow in the canopy (Giusti and Rossi, 2002). Organic kiwifruit plantations in NZ generally have low *Botrytis* rots in cold storage, compared to conventional systems, a finding supported in one comparative study (Michailides and Elmer, 2000). The epidemiological basis for the differences is not yet fully understood since canopy density and area of necrosis were not significantly different between these two systems, whereas the incidence and severity of *B. cinerea* populations in necrotic canopy tissues were significantly different.

Necrotic strawberry leaves were the primary source of *B. cinerea* inoculum in strawberries (Braun and Sutton, 1987). An obvious strategy to reduce *B. cinerea*

epidemics is to remove these tissues, but this practice has not slowed epidemic development (Daugaard, 1999). Increasing aeration around the plants by single row planting has reduced grey mould incidence (Schmid, 1996) compared to double and triple rows (Dijkstra and Van Oosten, 1985; Strik et al., 1997). Plant age affected the severity of *B. cinerea* over growing seasons, indicating perhaps an inoculum build-up; short cropping cycles were recommended to delay the build-up of inoculum (Daugaard, 2000). A significant positive relationship between N and *B. cinerea* fruit rot has led to low N use (Kopanski and Kawecki, 1994; Wilcox et al., 1994; Cooley et al., 1996). Finally, although no specific genetic sources of resistance have been found (Hortynski et al., 1991; Maas and Galetta, 1997), differences in *B. cinerea* susceptibility in strawberry cultivars have been identified (Daugaard, 1999; Barth et al., 2002; Rhainds et al., 2002).

9. Conclusions

When environmental conditions favour *B. cinerea* in the spring, epidemics are common and associated with an abundance of senescing host tissues. Removing the dominant sources of *B. cinerea* substrates reduces inoculum loading in orchards, but does not eliminate it, since conidia are so common in the air. During the summer months it is often perceived that *B. cinerea* ‘virtually disappears’ and this period of relative inactivity is frequently referred to as ‘over-summering’. At the end of the growing season, coinciding with fruit ripening, a sudden resurgence of *B. cinerea* can occur especially in disease favourable conditions.

Considerable research has focused on the dominant infection pathways from flowering to fruit rot with a view disrupting this part of the *B. cinerea* disease cycle, thereby reducing epidemic development later in the season. There is an even greater imperative in the future for acquiring this new knowledge because it will form the foundation for sustainable disease control strategies for the ‘reduced pesticide’ and organics era. Most of the infection pathways described included a latent or inactive period. Several host mechanisms were described to account for this enforced state of inactivity (Chapter 9); however, the trigger that reactivates *B. cinerea* growth in latent infections remains a mystery. The epidemiological importance of the infection pathways described differs by location and cultivar. Saprophytic development of mycelium in floral debris, aborted fruitlets and calyptras in grapes, calyces (shucks) in stone fruit and almond, bud and bud scales in pistachio and flower sepals in kiwifruit are important in disease epidemics. Further research on the ‘within bunch conidial accumulation’ pathway to fruit rot, and the ‘conidial infection of the external fruit surfaces pathway’, proposed for grapes, kiwifruit (California), stone fruits, pome fruits, blueberries, blackcurrants, pomegranates, and figs are required.

Significant knowledge gaps exist for the host and microclimate conditions required for latent infection to occur during the host’s phenology. If these parameters were more precisely defined, then predictive models for *B. cinerea* would be more accurate. Latent infection detection is based upon surface sterilants and there are variations in the type, number, duration and sequence of these. There is a need for faster and more efficient methods of latent infection detection and the use

of real time polymerase chain reaction (RT PCR) will provide the basis for efficient, accurate and more rapid detection of *B. cinerea* inoculum.

Factors predisposing host tissue to infection by *B. cinerea* included biotic (insects, invertebrates, and humans) and abiotic (nutrition, chemical and cultural practices). Controlling insects (e.g. grape berry moth), modifying microclimate (e.g. leaf removal, special pruning) and reducing substrate availability for *B. cinerea* has significant impacts on *B. cinerea* survival and epidemic development.

Breeding for resistance has not been as successful as hoped for but significant *B. cinerea*/cultivar interactions indicate that greater emphasis should be placed on cultivars with morphological, anatomical and inherent host defence mechanisms which reduce susceptibility (Mlikota Gabler et al., 2003). Finally, organic producers frequently observe *B. cinerea* epidemics declining over time, yet the host, pathogen, environmental and human variables responsible have not been resolved and represent an exciting challenge for the future.

10. Dedication

We dedicate our review to the memory of the late Professor Roy Edward Gaunt, an internationally respected epidemiologist and a close colleague.

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CHAPTER 15

BOTRYTIS SPECIES ON BULB CROPS

James W. Lorbeer¹, Alison M. Seyb¹, Marjan de Boer² and J. Ernst van den Ende²

¹Department of Plant Pathology, Cornell University, Ithaca, New York 14853, USA; ²Applied Plant Research, Flowerbulbs, P.O. Box 85, 21650 AB Lisse, The Netherlands

Abstract. A number of *Botrytis* species are pathogens of bulb crops. *Botrytis squamosa* (teleomorph=*Botryotinia squamosa*) causal agent of botrytis leaf blight and *B. allii* the causal agent of botrytis neck rot are two of the most important fungal diseases of onion. The taxonomies of several of the neck rot pathogens of onion have been revised on the basis of recent molecular sequence analysis studies. *B. allii*, *B. aclada*, and *B. byssoidea* are now recognized as distinct species causing neck rot diseases of onion. *B. cinerea* is also pathogenic on onion, primarily causing botrytis brown stain on onion bulbs. *B. tulipae*, *B. elliptica*, and *B. gladiolorum* are important pathogens of flower bulbs and are the causal agents of leaf blight in tulip, lily, and gladiolus, respectively. Leaf blight in the major flower bulb crops is called 'fire' referring to the fire-like symptoms occurring on the leaves of flower bulb plants when epidemics occur in production fields. In both the onion and flower bulb production systems chemicals are still heavily relied upon to control the major diseases, however, alternative disease management systems also are used and undoubtedly will become increasingly important in controlling the diseases. Infected plants and colonized plant debris are considered important sources of inoculum for *B. squamosa*, *B. tulipae*, and *B. elliptica*, particularly when sclerotia are formed. Sclerotia of *B. squamosa* serve as the source of conidia, as well as apothecia producing ascospores, in onion production areas in New York. The primary inoculum sources of *B. allii* and *B. gladiolorum* are believed to be infested seed and infected corms, respectively.

1. Introduction

Botrytis spp. that cause major problems in onion growing and storage and in the flower bulb industry are discussed. *Botrytis* spp. that attack flower bulbs include *Botrytis tulipae*, *B. elliptica* and *B. gladiolorum*, the causal agents of leaf blights respectively in tulip, lily and gladiolus. *Botrytis* spp. causing diseases in other flower bulb crops are briefly discussed. The devastating leaf blight in the major bulb crops is also called 'fire' because of the rapidity of the necrosis caused by infection in a production field. Severe epidemics can cause yield losses up to 60% as a result of the early withering of the leaves due to *Botrytis* infection during the period in which a substantial increase in size and weight of new bulbs takes place (Beaumont et al., 1936). Two species of *Botrytis* pathogenic to onion which have been studied

relatively intensively in recent decades are *B. squamosa*, the causal agent of botrytis leaf blight (Lorbeer, 1992, 1997), and *B. allii* (*B. aclada*), the causal agent of botrytis neck rot (grey mould) of onion (Lacy and Lorbeer, 1995b). *B. cinerea* is also pathogenic on onion, causing botrytis brown stain (Clark and Lorbeer, 1973a) and superficial leaf flecks (tiny leaf spots), when artificially inoculated on to onion leaves under controlled environmental conditions (Hancock and Lorbeer, 1963). However, in the latter, the fleck symptoms are not followed by leaf blighting as occurs when *B. squamosa* is inoculated on to an onion leaf resulting initially in leaf spots which penetrate into the lacuna (hollow space of the leaf) of the leaf and subsequently leaf blighting occurs. *B. squamosa* also is the cause of small sclerotial neck rot of onion (Walker, 1925b; Owen et al., 1950b). Several species of *Botrytis* have been associated with a fungal neck rot disease of onion. *B. aclada* was first described by Fresenius (1850) as a pathogen of onion. *B. allii* was described as the cause of neck rot of onion by Munn (1917). Walker (1925b) described *B. byssoidea* as the cause of mycelial neck rot of onion. Hennebert (1963) synonymized *B. aclada* and *B. allii* as *B. allii* and then later accepted *B. aclada* as the valid name for the species (Hennebert, 1973). He also accepted *B. byssoidea* as a valid species. Recent molecular and taxonomic studies have indicated that *B. allii*, *B. aclada*, and *B. byssoidea* are each valid species associated with a neck rot disease of onion (Nielsen et al., 2001, 2002; Nielsen and Yohalem, 2001; Yohalem et al., 2003). The results of molecular studies conducted by Nielsen et al. (2001), which focused on *B. aclada* and *B. byssoidea*, suggest there are two genetically distinct populations within the *B. aclada* group and that the populations are distinct species. They reported that *B. aclada* and *B. allii* produce conidia which are smaller than those produced by *B. byssoidea*, and that the conidia produced by *B. aclada* are slightly smaller than those produced by *B. allii*. Yohalem et al. (2003) have proposed that the name *B. aclada* Fresenius be applied to the small-spored subgroup and that the name *B. allii* Munn be applied to the larger-spored subgroup within the formerly recognised *B. aclada* species as recognised by Hennebert (1973). In addition, since molecular sequence data indicated that two different cloned fragments from *B. allii* are identical with those of *B. aclada* and *B. byssoidea*, Yohalem et al. (2003) have suggested that during the simultaneous infection of onion by *B. aclada* and *B. byssoidea* a hybridization event involving hyphal anastomosis between the two species occurred. This event resulted in the origin of *B. allii* which has 32 mitotic chromosomes and other distinct molecular characteristics. *B. aclada* and *B. byssoidea* each have 16 mitotic chromosomes. See also Chapter 3.

2. *Botrytis* species attacking onion

Research reports over the years have referred to either *B. allii* or *B. aclada* as the cause of the botrytis neck rot (grey mould) disease. In this review the fungus reported in the literature as either *B. allii* or *B. aclada* is referred to as *B. allii*. Future studies will determine the relative occurrence and importance of *B. aclada* Fresenius and *B. allii* Munn in causing the botrytis neck rot disease and if there are major or subtle differences in their etiology and epidemiology on onion and the methods by

which they can be controlled. In this review *B. byssoidea* is accepted as the cause of mycelial neck rot of onion and not considered the cause of botrytis neck rot of onion.

2.1. *Botrytis squamosa*

Botrytis squamosa was first described as the cause of small sclerotial neck rot of onion (Walker, 1925b). Later the fungus was shown to be the cause of a leaf spot and blight disease of onion that for many years was described as 'blast', but is now referred to as botrytis leaf blight (Hancock and Lorbeer, 1963; Lorbeer, 1992). The taxonomy and classification of the fungus is described in Chapter 3.

The symptomatology of onion leaf infection by *B. squamosa* has been reviewed in recent years by Lorbeer (1992) and Lacy and Lorbeer (1995a). When *B. squamosa* infects onion leaves, the initial symptoms are distinct necrotic areas that are whitish in colour and c. 2 mm in diameter. These areas quickly develop into lesions (leaf spots) each of which usually becomes surrounded by a light green halo (1-1.5 mm in width) that appears water-soaked when first formed. Within several days the centres of the lesions become sunken, straw-coloured, and frequently develop a slit oriented lengthwise in the lesion. The halos followed by the formation of the slit in the lesion are diagnostic for the first phase of onion leaf infection by *B. squamosa*. Under field conditions the portions of onion leaves not directly exposed to sunlight (undersides of leaves when the leaves are oriented in a more or less horizontal position) are generally free of lesions, whereas lesions are common on the leaf surface exposed to the sunlight. The lesions when fully formed extend directly into the lacuna of the leaf. As they age the lesions may expand slightly and the halos generally disappear, but most remain limited in size. The blighting phase of the disease generally begins within 5-12 days after initial lesion formation. Older onion leaves are more susceptible to infection, lesion formation and blighting than younger leaves. Outbreaks and epidemics of botrytis leaf blight usually result in reduced plant growth, onion bulb size and marketable yield.

The histopathology of *B. squamosa* infection of onion leaves has been extensively studied (Hancock and Lorbeer, 1963; Hancock et al., 1964a, b; Clark and Lorbeer, 1976a; Alderman and Lacy, 1983; Sutton et al., 1984) and reviewed in depth (Clark and Lorbeer, 1976b; Lorbeer, 1992). Clark and Lorbeer (1976a) reported that when conidia of *B. squamosa* in a water suspension were sprayed on to onion leaves, germ tubes emerged laterally from that portion of each conidium facing either the region over the nearest anticlinal wall juncture of the epidermis or the nearest stoma. The fungus then grew either to the region above the wall juncture or to the nearest stoma if it was located closer than the nearest wall juncture. The germ tube apex usually functioned as an appressorium, deposited mucilage, and then penetrated the cuticle without formation of a septum. In some instances, the conidium itself functioned as an appressorium without formation of a visible germ tube. Stomatal penetration, when it occurred, was infrequent. If a conidium of *B. squamosa* was deposited immediately adjacent to a stoma, the conidium usually produced a germ tube that grew towards, and then into, the stomatal aperture without forming an appressorium. When conidia were inoculated in a nutrient

solution, the fungus formed hyphae that frequently passed over anticlinal wall junctures that were near the conidia and then formed appressoria over anticlinal wall junctures located at distances from the conidia. After appressorium formation, the fungus penetrated the cuticle. When inoculations were made with conidia in the same nutrient solution, the fungus after germination frequently penetrated stomata without forming appressoria. The typical post-penetration development that occurred when water inoculations were utilized was not altered by the incorporation of an exogenous nutrient. In both situations following cuticular penetration by the fungus, the outer periclinal wall of the epidermis was traversed in all directions by the infection hyphae. In both water and nutrient inoculations, penetration between epidermal cells occurred after the anticlinal walls collapsed and the middle lamellae of the cells became dissolved. Likewise, when stomatal penetration occurred, enlarged infection hyphae formed beneath the epidermis, ramified mostly intercellularly, and then entered the lacuna of the leaf. A cavity resulting from the collapse of epidermal and mesophyll cells then was formed in the centre of each lesion. The typical mature lesion with the characteristic longitudinal slit usually became fully formed within 2-4 days after leaf inoculation. Following lesion formation, some of the lesions continued to expand and hyphae grew through the leaf tissue as well as on the surfaces of the leaf lacuna. Blighting of the leaf occurred following substantial growth of the fungus in the leaf.

Alderman and Lacy (1983) reported that lesion expansion and subsequent blighting of leaves was controlled in part by the duration of leaf wetness. When continual leaf wetness exceeded 12 h after mature lesion formation, *B. squamosa* then grew beyond the initial borders of some lesions resulting in expanding lesions that continued to enlarge under constant leaf wetness. After 4 days of continuous leaf wetness, the expanding lesions resulted in the initiation of the leaf blight phase of the disease. The studies by Clark and Lorbeer (1976a) and Alderman and Lacy (1983) both demonstrated that the fungus sporulated on the leaf tissue it had killed when favourable environmental conditions of temperature and moisture were established. Sclerotia of *B. squamosa* sometimes develop on the leaves and outer neck regions of onion plants colonized by the fungus prior to top removal during the harvest procedure (Ellerbrock and Lorbeer, 1977b, c). If the discarded leaves and neck tissues colonized by the fungus remain somewhat succulent, or remain moist after becoming necrotic, sclerotia of the fungus may form on those plant parts. Small sclerotial neck rot (Walker, 1925b) caused by *B. squamosa* can develop on the necks and upper regions of the bulbs of the onion plants if the infected plants remain in the field for extended periods after maturation. *B. squamosa* also infects onion flower heads resulting in a flower blight and reduced levels of seed production (Ellerbrock and Lorbeer, 1977a).

The factors leading to and regulating outbreaks and epidemics of botrytis leaf blight caused by *B. squamosa* involve the production and dispersal of primary and secondary inoculum, deposition of the inoculum on the onion leaf surface, the infection process and pathogenesis and the subsequent survival of the fungus (Lorbeer, 1992). Environmental factors then interact and control these events include temperature, rainfall, relative humidity (RH), leaf wetness duration, and crop nutrition and phenology as well as other factors that presently are not well

understood. The major epidemiological considerations for the occurrence of botrytis leaf blight include the nature and source of the initial conidial inoculum, the continual production of secondary conidial inoculum, dispersal and survival of the conidia, infection and pathogenesis, and the ability of *B. squamosa* to colonize and sporulate on the leaves killed by the fungus (Ellerbrock and Lorbeer, 1977b, c; Sutton et al., 1978, 1983, 1984, 1986). The disease cycle for botrytis leaf blight under commercial onion field conditions can involve as many as four sources of primary inoculum (Lorbeer, 1992). Three of the sources involve conidia of the fungus released from blighted leaves colonized by the fungus on onion plants in nearby onion seed fields, on re-sprouted onions in nearby cull piles, and on volunteer onion plants growing in or near the production fields. The fourth source of primary inoculum involves sclerotia of the fungus. Conidia produced on sclerotia of *B. squamosa* present on the surface of the soil, either in the production fields or in nearby fields or locations, on the onion bulbs in cull piles and on the bulb portion of onion plants being grown for seed production, can infect the leaves of onion plants in production fields. In the Orange County onion-growing area of New York, apothecia of the fungus have been found arising from over-wintering sclerotia formed on those onion leaves previously invaded and killed by the fungus. Such leaves with sclerotia that produce apothecia usually are found in matted clumps of dead onion leaves located at the sides or ends of the production fields. Ascospores released from these apothecia can infect leaves of onion plants in the production fields and thereby serve as a source of primary inoculum of the fungus. Once initial infection of the leaves of onion plants in commercial fields has been achieved and leaf blighting has occurred, the fungus then can produce a number of cycles of secondary inoculum (conidia) which can lead to severe outbreaks and epidemics of leaf blight in the fields.

Under field conditions, *B. squamosa* usually produces conidia at night during wet periods when temperatures range between 8-22°C (Sutton et al., 1983; Alderman and Lacy, 1984). Formation of conidia on necrotic leaf tips usually is favoured when leaf wetness duration is greater than 12 h. When the leaf wetness period is 5.5 to 12 h, formation of conidia generally occurs only following a wet or humid day with RH equal to or greater than 70% for 6 or more hours and if the necrotic tissue remains moist (Sutton et al., 1984). Dispersal of conidia alternates in a diurnal pattern with maximum numbers of airborne conidia detected during the daylight period of increasing temperature and decreasing humidity. Sutton et al. (1978) reported that the maximum peaks of airborne conidia of *B. squamosa* occurred between 9 a.m. and 12 noon. The two primary factors that influence leaf infection by *B. squamosa* are temperature and the duration of leaf wetness (Alderman et al., 1985). Different optimum temperatures and leaf wetness durations for germination of conidia of *B. squamosa* have been reported (Shoemaker and Lorbeer, 1977; Alderman and Lacy, 1983; Sutton et al., 1984). Those studies and others (Sutton et al., 1978; Tanner and Sutton, 1981) have indicated that the greatest numbers of lesions develop at 18° to 20°C and that lesion numbers increase as the leaf wetness duration extends to 48 h. Several studies have reported a leaf wetness period of at least 6 to 8 h is required for infection at 20°C (Shoemaker and Lorbeer, 1977; Alderman and Lacy, 1983; Alderman et al., 1985).

Cultural controls for reducing the incidence and severity of botrytis leaf blight involve destruction of onion cull piles, locating onion seed fields well apart from onion production fields, and removal of volunteer onions during the early part of the growing period. Following harvest and the topping of the onion plants, all leaf debris should be removed from the field and destroyed (Lorbeer and Andaloro, 1983). Botrytis leaf blight is controlled in north-eastern North America by the application of effective fungicides applied singly or in combinations on a 7-10 day schedule beginning in mid-June and continuing until 7-14 days or more prior to harvest, depending on label requirements of the fungicides utilized (Lorbeer and Vincelli, 1990). Disease predictive systems to time fungicide applications are utilized in north-eastern North America (Lacy and Pontius, 1983; Sutton et al., 1986; Vincelli and Lorbeer, 1989; Lorbeer et al., 2002; Chapter 18).

2.2. *Botrytis allii*

Although *B. allii* and *B. aclada* are now recognised as different species (Yohalem et al., 2003), for the purpose of uniformity in this review the fungus will be referred to as *B. allii* Munn. The new taxonomy and classification of *B. allii* Munn and *B. aclada* Fresenius are described in Chapter 3. The decay of onion bulbs caused by *B. allii* is one of the most important fungal bulb diseases of onions. The disease, first described and studied in detail by Munn (1917), has been referred to in the literature as both botrytis neck rot and as grey mould. *B. allii* also causes superficial leaf flecks (Segall and Newhall, 1960; Hancock and Lorbeer, 1963), scape blight and flower blight of onions grown for seed production (Ramsey and Lorbeer, 1986a), and has been implicated as the possible cause of soil-line rot of onion plants grown under certain conditions (Crowe et al., 1995).

Botrytis neck rot is a decay manifested in the neck area of bulbs that develops slowly (Lacy and Lorbeer, 1995b), only becoming evident after 3 to 4 months in storage (Stewart and Franicevic, 1994). The tissues of the bulbs soften as the decay advances, but generally remain somewhat firm in texture and brownish in colour. The decayed tissue later loses firmness and the upper portions of the bulb appear sunken. Sclerotia frequently form between the scales in the upper regions of the bulbs and on the shoulders of the bulbs. White mycelium frequently forms between the scales and on the outer surfaces of the upper portions of the bulbs. Masses of conidia in the form of a greyish mould may form in the neck area and upper shoulders of bulbs infected by the fungus. *B. squamosa* and *B. byssoidea* have also been implicated as causal agents of neck rot disease of onion (Walker, 1925b), in the form of small sclerotial neck rot and mycelial neck rot, respectively. It is now clear that *B. squamosa* causes small sclerotial neck rot as a continuum of its pathogenicity of onion after first blighting the leaves and that *B. byssoidea* causes mycelial neck rot which decays onion bulbs in a similar manner to *B. allii*, but with much mycelial growth and few, if any, sclerotia and conidia being present.

Stewart and Mansfield (1984) reported that when *B. allii* was inoculated on to onion bulb scales the fungus could produce spreading lesions, however, infection on leaves tended to be confined to the inoculation site. With age, onion leaves were observed to become more susceptible to infection by *B. allii* (Stewart and Mansfield,

1984). Infection of leaves by *B. allii*, particularly symptomless infection, has been considered to be a pathway resulting in neck rot in storage (Tichelaar, 1967). The infection has been considered to remain symptomless with the fungus restricted to the epidermal cells until a specific physiological stage, such as senescence, when the fungus spreads through the leaves (Tichelaar, 1967).

In contrasting the etiology of *B. allii* on onion with that of *B. cinerea*, Maude (1983) suggested that the neck rot pathogen was not as ubiquitous as *B. cinerea* and was found to occur only where onion crops were planted from infected seed. *B. allii* was found to survive in seed for up to 3 years when stored at 10°C with 50% RH. The fungus has been recovered from the surface of onion seeds, as well as from under the seed coat (Maude, 1983). The proportion of seed that is infected in a seed lot can be very high; for example Maude and Presly (1977a) found up to 70% of the seeds in some commercial seed lots were infected with the pathogen. Maude and Presly (1977b) considered the progressive increase in the numbers of bulbs with neck rot symptoms in store was due to planting contaminated seed and the subsequent expression of symptomless infections. Evidence for this was provided in the form of a correlation between the incidence of disease in the field and the incidence of neck rot in storage (Maude and Presly, 1977b). Stewart and Franicevic (1994) also detected a clear relationship between the level of seed infected with *B. allii* and the level of bulb rot in storage. Stewart and Mansfield (1984) did not find evidence of a biotrophic phase during successful colonization by *B. allii* and when a biotrophic phase was observed, fungal growth was restricted, though a pathway for seedborne infection with *B. allii* of symptomless infection of leaves was described by Maude (1983). Stewart and Mansfield (1984) suggested that since the work by Maude and Presly (1977a) was based on macroscopic symptoms the fungus may still have been invading necrotrophically but the symptoms were invisible.

Several reports have suggested that the neck of the bulb, presumably after cutting for harvest, is the site of penetration for *B. allii*. Under natural conditions *B. allii* is thought to infect onion bulbs via senescent or dead tissue at the neck or through wounds on the bulb (Walker, 1926; Owen et al., 1950a; Pappelis et al., 1974). Owen et al. (1950a) considered this occurred because the phenolic compounds to which the pathogen is sensitive are present in lower quantities in leaves than in the bulb scales. A number of inoculum sources have been suggested, including contaminated soil, cull piles and overlapping autumn- and spring-grown crops (Maude, 1983; Linfield et al., 1995). In storage, conidiophores present on the dry scales on the outside of the bulbs were not found to penetrate the side or necks of bulbs (Maude and Presly, 1977a). These dry scales were found by Walker (1926) not to support the growth of the pathogen, and Maude (1983) considered the tissue to be a barrier to spread by contact.

The mode of infection of onion leaves has been debated. Segall and Newhall (1960) reported that *B. allii* hyphae did not penetrate through the cuticle or stomata, while Kritzman et al. (1981) and Tichelaar (1967) showed germ tubes growing through stomata and penetrating the epidermis directly. Stewart and Mansfield (1985) identified two types of hyphae of *B. allii* on onions: 1) short germ tubes that preceded development of appressoria, and 2) superficial mycelium composed of long germ tubes that infrequently developed appressoria. Stewart and Mansfield

(1985) also found that failure to produce distinct infection hyphae was associated with the accumulation of deposits of granular reaction material (RM) in the underlying living cells. Following infection by *B. allii*, RM at infection sites rendered the cells resistant to degradation by a mixture of cell wall-degrading enzymes. Deposits of RM in onion epidermal cells at sites of attempted penetration were identified as primarily feruloyl-3'-methoxytyramine and feruloyltyramine (McLusky et al., 1999). Other phenolics were also present. Shumway et al. (1988) also identified cellulose at the site of *B. allii* penetration, but did not suggest a role for cellulose in a resistance response. Based on histochemical and ultrastructural studies, Stewart and Mansfield (1985) suggested that in response to the penetration of the cuticle by the fungus, accumulation and/or synthesis of phenols is elicited in the endoplasmic reticulum. Despite finding no antifungal activity in three of the phenolic compounds, McLusky et al. (1999) proposed that the phenolics have a key role in preventing degradation of the plant cell wall by the fungus and thus are important for the resistance response to infection by the fungus. Stewart and Mansfield (1985) suggested that the success or failure of *B. allii* to infect onion bulb scales depends on the speed with which it can penetrate and kill the cells and thereby suppress the RM deposition and the associated cell wall alterations. *B. allii* has been reported to secrete a number of enzymes, including cutin methylesterase, pectin methylesterase, polygalacturonase, cellulase and polyphenol oxidase (Bhattacharya and Pappelis, 1982). Mankarios and Friend (1980) found the major enzyme produced by *B. allii* at all stages of growth was polygalacturonase (PG), of which there are multiple isoenzymatic forms (Magro et al., 1979). The PG produced by *B. allii* has been reported to be inhibited by high concentrations of host phenolics such as catechol and tannic acid. However, a polyphenol oxidase produced by the fungus can convert the phenols to larger non-inhibitory polymers (Kritzman and Chet, 1980).

Several environmental factors have been shown to affect the conidial germination and mycelial growth of the pathogen, as well as infection of host tissue and symptom expression. The mycelial growth of *B. allii* in onion tissue as well as sporulation on agar medium has been reported to be optimal at 20°C (Alderman and Lacy, 1984; Presly, 1985a). The cardinal temperatures for infection of onion umbels by *B. allii* were found to be near 9, 21 and 30°C (Ramsey and Lorbeer, 1986b). Tian and Bertolini (1996) found an inverse relationship between spore size and incubation temperature. The larger conidia produced from mycelia when incubated at the lower temperatures germinated much earlier and resulted in faster germ tube growth, particularly when they were produced below 0°C. Isolates of *B. allii* also sporulated most freely in darkness compared to those of *B. byssoidea*, *B. cinerea*, *B. fabae* and *B. squamosa* (Presly, 1985a; Honda and Mizumura, 1991). Stewart and Mansfield (1984) found that symptom development on onion bulb scales by *B. allii* was dependent on inoculum concentration, with a minimum of greater than 10⁴ conidia per ml required for the production of consistent spreading lesions. A number of factors related to the host also affect the development of neck rot. Ward (1979) reported that as onion bulb size increases, so does the proportion of bulbs that rot in storage. Owen et al. (1950a) noted that strongly pungent onion varieties had consistently lower neck rot indices than the mild varieties tested. High phenol

content has been associated with resistance to neck rot in coloured onions (Bhattacharya and Pappelis, 1982), likewise white onions with a low phenol content are more susceptible to infection by *B. allii* (Link and Walker, 1933; Mankarios and Friend, 1980).

One of the most common methods of control of neck rot has been the application of chemical fungicides. Seed treatments with fungicides such as benomyl and iprodione have been recommended (Stewart and Franicevic, 1994). Seed dressings using benomyl were reported to have virtually eliminated infection from seeds and produced crops that remained free of neck rot in storage (Maude and Presly, 1977b). The effectiveness of chemical control applied as a seed treatment for reducing neck rot in storage is dependent on the level of seed borne inoculum. Fungicide sprays have also been recommended to control infection in the field. Presly (1984) found that using iprodione (four sprays) gave better control than using a mixed programme of thiram and iprodione. The relationship between application volume of iprodione and retention of the fungicide on onion leaves was non-linear; the use of an oil-based formulation of iprodione was also more effective than a wettable formulation because of superior retention (Presly, 1984). Once harvested, the bulbs may then be treated with a chemical fungicide to reduce neck rot. One method is to dip the bulbs in a fungicide (Ali et al., 1979; Kritzman, 1983b). A number of disadvantages with this system have been identified, including wet bulbs in storage, the uneven deposition of the chemical because the procedure is labour intensive (Grinstein et al., 1992). Developments in fungicide application techniques have improved the efficacy of control achieved by fungicides. Grinstein et al. (1992) described a technique of reduced volume application of fungicides in which the droplets of fungicide remain discrete on the onion bulb.

The antagonistic activities of several fungi against *B. allii* have been investigated for some time, particularly with *Trichoderma* (Rod, 1984; Roulston and Lane, 1988; Köhl et al., 1992), *Ulocladium* (Köhl et al., 1997) and *Gliocladium* species (Walker and Maude, 1975; Köhl et al., 1999). Antagonists used as biological control agents (BCA) against plant pathogenic fungi have been targeted against different stages of the disease cycle. Work carried out in The Netherlands on developing a BCA for control of *B. allii* has primarily focused on suppression of sporulation. Köhl et al. (1995b) found that under continuously wet conditions sporulation of *B. allii* was not detected when certain antagonists, isolated from the necrotic tips of field-grown onion leaves, were present, while on leaves in the control group more than 2×10^5 *B. allii* conidia were detected per cm^2 . Interrupting wetness affected the efficacy of several of the antagonists. Köhl et al. (1995b) suggested that the efficacy of antagonists to suppress sporulation of *Botrytis* spp. on necrotic tissue is dependent on their ability to survive dry periods and begin growth rapidly during the subsequent favourable conditions. Köhl et al. (1997) considered *U. atrum* to be an important potential biocontrol agent because the fungus could colonize dead onion leaves quickly and extensively, and could do so under a range of environmental conditions (Köhl et al., 1995a). Application of *U. atrum* after *B. allii* had become established on a substrate was considered by Köhl et al. (1995a) to be ineffective. Köhl et al. (1995a), however, cited two important advantages for using a BCA that competes for resources. Firstly, the interaction may not be specific to a certain

pathogen, and secondly presence of the agent does not have to strictly coincide with the presence of the pathogen. See Chapter 13 for BCAs.

One recommended planting technique for neck rot control is to avoid planting leek and onion crops together as they may serve as inoculum sources not only for *B. allii*, but also for *B. byssoidea* and *B. squamosa* (Presly, 1985b). Several harvesting and drying techniques have been tested in an effort to reduce the incidence of neck rot in storage. In the initial stage of harvesting, the bulbs are undercut to sever the root system. Curing the bulbs for at least 3 weeks in the field, after undercutting and before topping, reduced the incidence of neck rot in storage (Ali et al., 1979). However, field-drying onion bulbs has been shown to increase the risk of staining of the outer bulb scales (Maude et al., 1984). Metcalf et al. (2003) found that *B. allii* spread through the senescent leaves to the neck of onion bulbs in the windrow, and that bulbs at the bottom of the windrow were at greater risk. The authors reasoned this was due to increased humidity and lack of sunlight exposure, and suggested that wider shallower windrows of bulbs may alleviate the problem. Alternatively, the bulbs may be exposed to an artificial curing process. Sel'men (1985) suggested onion bulbs destined for storage could be heated in water (46-50°C for 30 min) followed by hot-air drying (46-50°C for 2 h). The majority of research has focussed solely on forcing hot air through the onion stack, typically at temperatures above 30°C (Gunkel et al., 1972, 1976; Maude et al., 1984). The rationale for this system is to rapidly dry the neck region that is left following mechanical removal of the onion foliage (Maude et al., 1984), and so remove an infection court for *B. allii* (Walker, 1925a). The effect of the drying system on the incidence of neck rot in storage is, however, dependent on the infection pathway. Maude et al. (1984) considered 30°C sufficient to eliminate late infections, but inadequate if the infection was seed borne as it would have become well established.

In spite of the advances made in the efficacy of the aforementioned chemical, biological and cultural control methods, neck rot still occurs in stored onions. If the amount of potential neck rot could be determined, either in the seed lot or the bulbs destined for storage, growers would have the ability to assess the storage potential of their crops. Methods of detection have included selective media (Lorbeer and Tichelaar, 1970; Kritzman and Netzer, 1978), staining of potentially infected tissues (Kritzman, 1983a), ELISA using polyclonal antibodies (Linfield et al., 1995) and molecular diagnosis (Nielsen et al., 2002; Walcott et al., 2004). The advantage of using a technique such as ELISA for detecting *B. allii* is that the infection can often be detected earlier and more consistently than by using selective media (Linfield et al., 1995). This is important for a pathogen such as *B. allii* that is thought to remain latent in the bulb and usually cannot be detected in the grading lines as the symptoms of neck rot only become evident after at least 8 weeks in storage. Linfield et al. (1995) found that the ELISA test could detect the fungus in advance of the mycelium, indicating some metabolite diffusion. A serological relationship does exist between *B. allii* and *B. cinerea* (Linfield et al., 1995). Cross reactivity between *Botrytis* species has been observed by other workers (Bossi and Dewey, 1992; Salinas and Schots, 1992), limiting the precision of the technique (See Chapter 11).

Nielsen et al. (2002) developed a detection method based on universally primed polymerase chain reactions (UP-PCR) that was able to distinguish among five onion

neck rotting species. This included being able to distinguish between *B. aclada* AI and AII. The work carried out by Nielsen et al. (2002; Chapter 11) used *B. allii*-infected bulbs and mature leaves. Detection of *B. allii* in onion seeds is unfortunately hampered by compounds in the seed that inhibit the PCR reaction (Walcott, 2003; Walcott et al., 2002). To purify DNA from crude seed extracts Walcott et al. (2004) used magnetic capture hybridisation (MCH). The authors reported MCH-PCR was effective in detecting *B. allii* in seed lots, and showed a 10-fold increase in sensitivity over direct PCR.

2.3. *Botrytis cinerea*

B. cinerea is a common saprophyte on onion leaves after they have been killed by other leaf blighting pathogens attacking onions. Under the most favourable environmental conditions *B. cinerea* can cause superficial leaf flecks (ring leaf spots) on mature onion leaves (Hancock and Lorbeer, 1963). The flecks never penetrate to the intercellular spaces of the onion leaves. *B. cinerea* does not cause blighting of onion leaves and most likely the leaf flecks have little effect on plant growth, bulb formation, or yield. The fungus also causes a blight disease of the flower parts of onion plants grown for seed production (Ellerbrock and Lorbeer, 1977a). *B. cinerea* has been described by Clark and Lorbeer (1973a) as the causal agent of brown stain of onion. The typical symptoms of brown stain of onion are superficial discoloration of the outer, dry scales of onion bulbs, of which there are two distinct symptom types. The discoloration can be expressed as discrete localized spots on all regions of the bulb or as a stain that fans out from the neck to the bulb shoulder area. Both symptoms are readily observed on onion bulbs in the field at harvest (Clark and Lorbeer, 1973a).

When *B. cinerea* was inoculated on to surface sterilized onion bulbs, Clark and Lorbeer (1973b) observed typical brown stain symptoms similar to those found on onion bulbs produced in commercial fields. Onion bulbs were either wounded or non-wounded and inoculated with a conidial suspension or with leaf tissue previously infected by *B. cinerea*. Little difference was noted between the wounded and non-wounded method of inoculation, but the bulbs inoculated with the infected leaf tissue had a much greater incidence of infection than did the conidial suspension group. Clark and Lorbeer (1973b) have reported two distinct symptom patterns and postulate that different means of inoculation are responsible for the different symptoms. Discrete spots result from direct penetration by the pathogen in a localized area, whereas the discoloration that fans out from the neck region is caused by an infection of the leaf or leaf sheath with an advance of the fungus into the neck and shoulder area of the bulb. It is possible that this infection can further advance into the bulb scales.

3. *Botrytis* species attacking flower bulbs

3.1. *Botrytis tulipae*

Three sets of disease symptoms may be produced by *B. tulipae* which are called 'fire', 'spot' and 'bulb rot' (Beaumont et al., 1936; Hopkins, 1921). In a tulip bulb

production field 'tulip fire' is the name of *B. tulipae* infection officially adopted by the British Mycological Society. The typical fire lesions are grey or greenish grey, variable in size and shape, and somewhat sunken compared with the surrounding healthy tissues. There is a definite dark margin to the lesions, which in moist weather can sometimes be split up into three zones, the light green area outside with no conidia, the light grey area with a few conidia, and the dark grey or grey-brown zone with abundant conidia. The fire symptoms in the field may appear early in the season, shortly after the shoot has speared through. If the plants near to a tulip bearing fire lesions are examined, small spots will probably be found, and these will have arisen from spores that have been blown or splashed by rain from the primary fire lesion. These spots are small, dry, very slightly sunken and yellowish white or greyish white in colour. The bulk of the evidence suggests that the fungus in the spots is dead, but there is some evidence that some of the spots at least may contain viable mycelia. The third type of decay, the bulb rot, is very different in character from the other two. The whole plant is a sickly-pale or yellowish green, under-sized, with flaccid leaves and often no flower, or only a small flower which is unmarketable. The plants can be dug up easily, and with rare exceptions black sclerotia are found on the scales. Bulb rot is much less common than fire or spot and probably only occurs on the most susceptible varieties and only when bulb infection is unusually large or the bulbs have been badly bruised (Hopkins, 1921; Beaumont et al., 1936). Some infected bulbs rot completely to release sclerotia in the soil, others develop into 'primaries', but the majority of infected bulbs have healthy shoots (Doornik and Bergman, 1973; Anonymous, 1979).

B. tulipae is able to induce spotting on other flower bulb crops during the growing season (Hopkins, 1921; Sumner, 1994), but fire symptoms never develop. It is, therefore, surprising that *B. tulipae* sclerotia are found on lily debris. This saprophytic growth of *Botrytis* species on non-host plant material has important consequences for the present rotation schemes in bulb production fields (Van den Ende and Pennock-Vos, 1997a).

The over-wintering phase of *B. tulipae* covers the period between harvest and planting. The main life strategy of *Botrytis* during this phase is by quiescent sclerotia in crop debris and/or soil or by latent infections in bulbs. Latent infections in bulbs are inactive during the storage period (Doornik and Bergman, 1975). After planting the fungus spreads over the surface or the scale, or through the epidermis starting new lesions without sharp defined borders (Doornik and De Rooy, 1971). These latent infections in bulbs result in massive sporulation of *Botrytis* on emerging shoots in early spring (the so-called 'primaries') (Price, 1970a; Doornik and Bergman, 1971, 1973, 1975). Profound sporulation of these primaries results in secondary leaf and flower infection under favourable weather conditions. Therefore, the source of the disease in the field often is the grower's own stock (Anonymous, 1979).

Sclerotia of *B. tulipae* on plant debris and/or in the soil can also be an important primary source of disease (Van den Ende and Pennock, 1996b). Sclerotia formation, sporulation and survival have been studied for *B. tulipae* (Coley-Smith and Javed, 1972; Price, 1970a). Mycelial growth from sclerotia can result in direct infection of the bulb scales or emerging shoots, causing primaries. Adding sclerotia to the soil

resulted in a significant increase of primaries in the next growing season (J.E. van den Ende, PPO-Flowerbulbs, The Netherlands, unpubl.), confirming results of Doornik and Bergman (1974) and Price (1969) who showed that *B. tulipae* infections could arise from contaminated soil. These infections occur only when these structures are in close contact with the tip of the bulb, or when shoots grow through sclerotium-infested soil. *B. tulipae* apparently lacks the ability to grow from sclerotia through non-sterile soil, for relatively long distances (Coley-Smith and Javed, 1972; Doornik and Bergman, 1974). In addition, sclerotia can produce conidia directly. By producing successive batches of conidia (Coley-Smith and Javed, 1972) the sclerotia remain a source of inoculum during several weeks at the start of the growing season.

The real epidemic of fire is caused by the conidia. Conidia are formed on dead tissue both under high and low light conditions by mycelium or sclerotia. The first infections from conidia appear as small spots on green leaves. In fact, these spots are a hypersensitive response of the host. The fungus is not able to sporulate within these spots. Usually these spots are seen before flowering. However, under extreme conditions (susceptible cultivars, high inoculum pressure, prolonged wetness) the fungus may progressively invade the tissue causing large brown lesions and early die-back of the leaves. Sporulation occurs on the dead tissue. Aggressive lesions usually appear near primaries suggesting that their formation is associated with large numbers of conidia dispersed from primaries either in low concentrations during protracted periods of time or deposited in a few heavy showers (Price, 1970b). The formation of aggressive lesions usually occurs after flowering. However, in some years aggressive lesions were seen on tulip leaves 5 weeks from shoot emergence, well before flowering (Price et al., 1971). At the end of the season sclerotia are formed on plant debris forming the overwintering phase. There are some reports of survival of *B. tulipae* conidia in soil (Coley-Smith, 1980), but this overwintering method is probably unimportant.

3.2. *Botrytis elliptica*

Symptoms of fire on lily, caused by *B. elliptica*, usually include development of circular or elongated brown or reddish-brown spots that in severe attacks, coalesce to kill an entire leaf. Sometimes prominent yellow streaks appear, more frequently on older leaves of post-anthesis plants (Doss et al., 1988a). From the leaves *B. elliptica* migrates to the stem where it can block vascular transport and kill distal plant parts above the stem infection (Van Beyma thoe Kingma and Van Hell, 1931). The flower buds may be killed outright or they may produce flowers that are distorted (Moore et al., 1949). The bulbs are very rarely attacked but occasionally the fungus passes into them from the stem and causes rotting of the scales (Moore et al., 1949). The host plant range of *B. elliptica* is practically limited to lily although alternative hosts have been reported (MacLean, 1946).

Although *B. elliptica* has been the subject of detailed studies (Van Beyma thoe Kingma and Van Hell, 1931; Doss et al., 1984, 1988b), the over-wintering pathogen phase has received little attention. Recently in indoor-experiments it has been shown that latent infection in lily bulbs may occur, but bulb infection seems not to play a

role in survival of *B. elliptica* under field conditions as primaries have never been found (Van den Ende and Pennock, 1996b). Moore (1949) assumed that the renewed activity of the fungus in spring depends on the production of sclerotia on plant debris of diseased lilies. Van den Ende and Pennock (1996b) demonstrated that indeed sclerotia are the most important structures involved in the survival of *B. elliptica*. These sclerotia produce apothecia with ascospores and/or successive batches of conidia that can infect lily leaves under favourable conditions.

Sclerotia formation on lily plant debris by *B. elliptica* occurs well after harvest between October and December (Van den Ende and Pennock, 1996c). The wide temperature range (5-25°C) at which sclerotia are formed suggests that formation in the field is rarely limited by temperature in normal years. In early spring the sclerotia produce apothecia with ascospores (Van den Ende and Pennock, 1996c). Ascospores obtained from these apothecia produced typical *B. elliptica* cultures on malt agar and were pathogenic to lily plants. Crosses made by combining microconidia and sclerotia of single ascospore cultures resulted in the production of apothecia under laboratory conditions, indicating the presence of both mating types. Results showed that *B. elliptica* is heterothallic with a diallelic compatibility system. Most wild-type isolates bear both microconidia and sclerotia. The relative importance of ascospores for the initial infection in the field is yet unknown. The presence of the teleomorph is a source of genetic variability in the fungus, and might therefore affect disease management strategies (Van den Ende and Pennock, 1996c). The sclerotia also produce successive batches of conidia later during the growing season. Conidia are produced within a broad temperature range with an optimum between 13 and 20°C (Doss et al., 1984; Van den Ende and Pennock, 1997). Both ascospores and conidia can infect green leaves resulting in small spots that will enlarge under prolonged periods of leaf wetness. Severe infections result in early die-back of the crop. The fungus is unable to sporulate within spots but sporulates on dead plant tissue (Doss et al., 1984; Kessel et al., 2001). At the end of the season sclerotia are formed on plant debris, forming the overwintering phase.

3.3. *Botrytis gladiolorum*

Botrytis disease of gladiolus has two distinct phases: corm rot in storage and a leaf and flower spot and collar rot in the field (McClellan et al., 1949). Timmermans (1941) described the several symptoms of *Botrytis* disease of gladiolus. The corms can be completely rotten with white mycelium growing on the surface of the corm combined with *Botrytis* sclerotia. More often, however, the rot is only confined to the corm, or even the core of the corm, and not the outer scales. The core rot can expand to the surface of the corm through the vascular tissue or through the parenchyma. When corm infection is arrested, the lesion is surrounded by a layer of suberized cells that delimits the disease. Another form of *Botrytis* infection of the corm leads to the formation of small specks that resemble dry rot symptoms caused by *Sclerotinia gladioli*. The above ground part of the gladiolus plant can also be infected. Infection can take place directly above the soil leading to wilting of the outer leaves and formation of *Botrytis* sclerotia on the stem. This above ground infection can lead to infection of the newly formed corm. *Botrytis* infection on the

leaves leads to elongated, withered, brownish red-rimmed spots or small round rusty like spots. In these small spots *Botrytis* mycelium and conidia can be detected. Severe leaf infection leading to early die back of the plant results in smaller newly formed corms.

The precise over-wintering phase of *B. gladiolorum* is still unknown. It is highly probable that botrytis leaf blight and stem infection result from planting *Botrytis* infected corms. Sclerotia, and perhaps apothecia formed on these corms, produce conidia and ascospores that will result in botrytis leaf blight and stem infection. Timmermans (1941) stated that infection of young corms was mainly caused by contamination from infected above ground parts. The occurrence of a botrytis leaf blight epidemic is dependent on the presence of *Botrytis* conidia and the weather conditions. McClellan et al. (1949) demonstrated that in regions in the USA with conducive climatic conditions, such as temperatures between 13-18°C combined with moist conditions like rain and fog, *Botrytis* can develop quickly on the foliage and the flowers, decreasing flower quality. Severe botrytis leaf blight only leads to corm rot when favourable conditions for its development occur. The optimum temperature for infection of freshly harvested corms is 2°C. Infection did not occur on corms that had been allowed to cure for 18 days. Lack of infections of cured corms and of those inoculated and incubated at high temperatures was assumed to be due to the rapid suberization and wound periderm formation that took place at the higher temperatures (McClellan et al., 1949). Timmermans (1941) concluded that the best way to prevent corm rot was by early harvesting and rapid drying in a heated shed after harvesting.

The potentially high rate of increase of infection during an epidemic, combined with the lack of curative control options, have induced strongly risk-adverse control practices among Dutch bulb growers. Most growers spray on a weekly bases with protective fungicides from the start of the season until harvest to control the disease, amounting to an input of active ingredients of approximately 25 kg/ha per year. Such an input of and reliance on fungicides increases the risk of development of resistance in *B. elliptica* (Chastagner and Riley, 1990; Migheli et al., 1990), poses a burden on the environment, and is in conflict with public and political thrusts to develop more sustainable ways for flower bulb production. Emphasis has been placed on integrated control of *Botrytis* spp. in flower bulbs. During the past decades different measures to control botrytis leaf blight have been developed and practiced. These control measures include cultural measures and/or the use of weather-based field spray programmes. At the moment, integrated strategies have been developed in which these different measures are combined. Selection and use of healthy bulbs is especially important in gladiolus and tulip production. Bulbs with visual signs of infection should be removed before planting. Several fungicides are used in bulb dipping before planting to protect the bulbs from infection by conidia present on the bulbs or in soil. Both measures are applied in order to reduce the occurrence of heavily sporulating shoots in the next growing season.

To reduce the risk of a *Botrytis* epidemic, at a minimum growers should use crop rotation schemes of 1:3. In different studies it was shown that the survival of sclerotia of *B. tulipae*, *B. elliptica* and *B. gladiolorum* is unlikely to exceed more

than 2 years (Coley-Smith, 1980; J.E. Van den Ende, unpubl.). Some Dutch tulip growers use flooding of their fields after the growing period to reduce the inoculum potential of the soil, since sclerotia of both *B. tulipae* and *B. elliptica* do not survive the flooding treatments (A.W. Doornik, PPO Flowerbulbs, The Netherlands, unpubl.). In tulip production the removal of primaries (infected plants at the beginning of the growing season) remains the most important cultural measure to reduce the primary inoculum and thereby limiting secondary spread of tulip fire (Price and Briggs, 1974; Doornik and Bergman, 1975). As bulbs increase in size it is common practice to decrease the plant density, which may result in shorter leaf wetness periods and hence, less favourable conditions for *Botrytis* infection, as shown for other *Botrytis* species (Maude, 1980). Since the flower tissue is extremely susceptible to infection it is important to remove the flowers during the growing season, to lower the potential of *Botrytis* epidemics in tulip, gladiolus and lily. In the case of narcissus, removal of flowers is a very effective strategy, as the flowers are the main infection pathway for *B. polyblastis*, the causal agent of Narcissus 'fire'.

Although resistance against *Botrytis* spp. in different bulb crops exists, it is not an important factor in crop management for most Dutch growers. Growers are advised to design a new planting system such that sensitive cultivars are grouped together and less sensitive cultivars are similarly grouped. This way the growers can adjust their spray programme to the sensitivity of the cultivars. In practice, however, planting designs are based more on logistics (optimising mechanical harvesting) than on the possibility of a selective spray programme.

In order to reduce fungicide use, a warning system has been developed for the control of botrytis leaf blight (BoWaS; *Botrytis* Warning System) in flower bulbs (Bastiaansen et al., 1997; Van den Ende et al., 1999; Chapter 18). Infection of flower bulbs (lily, tulip, gladiolus) by *Botrytis* spp. was studied under different temperature and RH conditions in climate chambers. BoWaS uses data from these studies and the regional forecast of the weather variables 'leaf wetness duration' and 'leaf temperature' to predict critical periods for *Botrytis* infection in the specific crops for the next 5-day period. Critical periods and hence action thresholds depend on cultivar susceptibility. Between 1994 and 1998 the warning system was tested in several lily, tulip and gladiolus cultivars grown at different research and on-farm locations. Application of BoWaS resulted in good control of fire and a 30-80% reduction of input of active ingredients could be achieved depending on cultivar susceptibility and year. The success of BoWaS has resulted in the introduction of several warning systems in Dutch flower bulb culture. At present around 20% of the bulb growers use a warning system for *Botrytis* control. At the end of the growing season proper ploughing after harvest limits the sporulation potential and the viability (Coley-Smith, 1980) of the sclerotia in the spring time of the next year. Leaving plant debris in the production fields or in organic waste piles results in inoculum sources in spring. Leaving plant debris of lily in the field may not only affect lily growing in the following season, but also cause problems in tulip culture as sclerotia of *B. tulipae* may occur on the dead lily stems (Van den Ende et al., 1997a).

New control strategies such as the use of antagonists like yeasts and bacteria, or pesticides of natural origin like plant extracts and essential oils, are under investigation (De Boer et al., 2003) and show promising results under field

conditions (M. De Boer, Applied Plant Research, Lisse, The Netherlands, unpubl.). It was shown that application of antagonists during the over-wintering phase of the pathogen resulted in lower inoculum pressure in spring (Van den Ende and Pennock, 1996a). Attempts to control *Botrytis* spp. during the growing season with antagonists have given variable results. Kessel et al. (2001) showed that although *Ulocladium atrum* proved to be a good control agent against *B. cinerea* in cyclamen, control of *B. elliptica* was not possible. This was due to *B. elliptica* being more virulent than *B. cinerea*, with conidia and mycelium both capable of directly infecting healthy lily leaf tissue.

4. Conclusions

The primary *Botrytis* species attacking onion and flower bulb crops are mostly pathogenic to the specific crops which they attack in contrast to the wide host range of *B. cinerea*. Sclerotia, conidia, and in specific situations ascospores are the primary inoculum involved in the etiology of onion and flower bulb crop diseases. The associations between these propagules and over-wintered crop debris, volunteer plants, and plants grown for seed production have resulted in cultural disease control practices recommending the removal of those sources of inoculum. The importance of seed-borne inoculum for initiating the occurrence of botrytis neck rot of onion in storage has now been well established, and as a result chemical fungicides are applied to onion seeds as one of the several procedures to control botrytis neck rot. The primary strategy for control of leaf diseases in both types of crops is currently by chemical methods. Extended periods of rain, leaf wetness, and high humidity along with critical temperature regimes for each *Botrytis* species are the primary factors involved in the epidemiology of *Botrytis* leaf diseases of onion and flower bulb crops. In recent years predictive and warning systems for indicating the potential for the development of disease outbreaks (and epidemics) have been developed to ensure timely application of control procedures. Biocontrol systems for the control of the diseases have been studied and it is anticipated that effective systems involving cultural and biological control will be forthcoming.

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CHAPTER 16

BIOLOGY AND MANAGEMENT OF *BOTRYTIS* SPP. IN LEGUME CROPS

Jenny A. Davidson¹, Suresh Pande², Trevor W. Bretag³, Kurt D. Lindbeck³ and Gali Krishna-Kishore²

¹South Australian Research and Development Institute, GPO Box 397, Adelaide, 5001, South Australia;

²International Crop Research Institute for the Semi-Arid Tropics, Patancheru 502 324, Andhra Pradesh, India; ³Victorian Department of Primary Industries, Private Bag 260, Natimuk Road, Horsham, Victoria,

3401, Australia

Abstract. Grey mould and chocolate spot, caused by *Botrytis cinerea* and *Botrytis fabae* respectively, are serious diseases of pulse crops worldwide. The severity of epidemics is dependent upon the prevailing environmental conditions, particularly high humidity and temperatures in the range of 15-25°C. The fungi attack all aerial parts of the plants, and flowers and growing tips are particularly susceptible. Yield loss occurs through the death of plants, reduced growth, or shrivelled seeds in diseased plants. In severe epidemics 100% yield loss can occur. Seed-borne infection can result in seedling rot, resulting in seedling death and stunted plants. The pathogens can survive on infected debris and seed, either as conidia, mycelium or sclerotia, or directly in the soil as sclerotia. Desirable levels of host-plant resistance against *Botrytis* spp. infection are scarce and chemicals are expensive. There is also the potential threat of the development of fungicide tolerant strains. Hence integrated disease management involving a combination of foliar fungicides and seed dressings, resistance breeding, and cultural methods (reducing crop density, wider row spaces, erect plant habit) to alter the microclimate is the most viable option for effective management of *Botrytis* spp.

1. Introduction

Legume crops are important as a source of protein for millions of people, especially providing essential amino-acids for vegetarian populations. They are also important because of their ability to fix nitrogen and to build soil fertility. *Botrytis* spp.-incited diseases occur on a wide range of crop species including chickpeas (*Cicer arietinum*), lentils (*Lens culinaris*), faba beans (*Vicia faba*), field peas (*Pisum sativum*) and vetch species (*Vicia* spp.). *B. cinerea* is ubiquitously distributed and results in significant production losses. Grey mould, generally caused by *B. cinerea*, occurs worldwide and is considered a serious problem in most chickpea growing areas, but particularly in south Asia (Ellis and Waller, 1974a; Nene and Reddy, 1987; Nene et al., 1991; Kaiser et al., 2000). This pathogen is also a severe problem on lentils in

India, Syria, Morocco (Beniwal et al., 1993), and Australia (Bretag et al., 1996) and on field peas in Europe, North America, Japan and New Zealand (Biddle, 2001). It is less important on soybeans (*Glycine max*), groundnut or peanut (*Arachis hypogea*), lima bean (*Phaseolus lunatus*), common bean (*P. vulgaris*), cowpea (*Vigna spp.*), pigeon pea (*Cajanus cajan*), lupin (*Lupinus spp.*), clover (*Trifolium spp.*) and tropical pasture legumes (*Stylosanthes spp.*) (Allen and Lenné, 1998). *B. fabae*, the causal agent of chocolate spot, has a more restricted host range than *B. cinerea*, occurring most commonly on *Vicia spp.*, especially faba beans, common vetch (*Vicia sativa*) and lentils (Lindbeck et al., 2003). *B. cinerea* also occurs on faba beans, but is less severe on this crop than *B. fabae* (Harrison, 1988). Outbreaks of grey mould or chocolate spot are highly dependent on microclimate within crops, causing most damage in humid regions. Rainfall and water-retentive soils will also influence the occurrence of outbreaks. When conditions are favourable for disease outbreaks, losses can be severe, exceeding 50% of yield. The lack of desirable levels of host-plant resistance and frequent failure of chemical control due to the widespread occurrence of fungicide resistance further contribute to the need for effective management on *Botrytis spp.* This chapter summarizes the available information on of *Botrytis spp.* in different legume crops.

2. Chickpeas

Grey mould caused by *B. cinerea* is of serious concern in India, Bangladesh, Nepal, Pakistan, Australia and Argentina (Bakr et al., 1993; Karki et al., 1993; Haware, 1998; Pande et al., 2002) where losses can reach the entire crop when conditions are conducive. The disease has also been reported in Canada, Chile, Colombo, Hungary, Mexico, Myanmar (Burma), Spain, Turkey, USA and Vietnam (Nene et al., 1984; Pande et al., 2002). The disease first reached epidemic proportions in India in the 1978/79 season, destroying 20,000 hectares of chickpeas (Grewal and Laha, 1983; Haware, 1998; Grewal et al., 1992). In Nepal it occurs almost every year with average losses of 15% in farmers fields (Joshi, 1992). The disease was first documented in chickpea crops of Bangladesh in 1981 and reached devastating proportions in 1988, damaging almost all the chickpea crop of that country (Bakr and Ahmed, 1992). It is now considered to be the most damaging foliar disease of chickpeas in Bangladesh (Bakr et al., 2002). The effects of grey mould on pod yield depend on the onset of the disease in relation to crop growth and disease severity, both of which are determined largely by weather conditions and inoculum levels.

2.1. Symptoms

B. cinerea attacks all aerial parts of the plant, with growing tips and flowers being the most vulnerable (Haware, 1998; Pande et al., 2002). Symptoms of grey mould usually become apparent following canopy closure (Knights and Siddique, 2002). It often appears first as a water-soaked lesion on the stem, near ground level. This lesion can extend along the stem, leading to infection of other stems (Knights and Siddique, 2002). Branches break off at the rotting point and the affected leaves and

flowers turn into a rotting mass (Bakr et al., 2002; Pande et al., 2002). Initially the disease is randomly distributed within a crop and is seen as scattered, yellowing or dying branches, or if the lesions are at ground level, as scattered dead plants. Drooping of the affected tender terminal branches is a common field symptom (Haware and McDonald, 1992). The fungus can form grey or brown to light brown lesions on leaflets, branches and pods, covered with conidia (Figure 1) (Haware and McDonald, 1992; Haware, 1998). Lesions on pods are water-soaked and irregular, sometimes with black sclerotia scattered in the infected areas. Grey fungal growth and profuse sporulation will occur if conditions within the canopy are humid and will rapidly spread through the canopy resulting in patches of dead plants (Knights and Siddique, 2002). Flower drop is common leading to poor pod formation and low grain yields (Haware, 1998; Knights and Siddique, 2002; Pande et al., 2002) and is often undetected unless the crop is closely monitored. Depending on the site of infection, mature seeds from diseased plants may be shrunken, darker coloured or, when the fungus has invaded the pod, covered in a white/grey fungal mat (Bakr and Ahmed, 1992; Tripathi and Rathi, 1992; Haware, 1998; Bakr et al., 2002; Knights and Siddique, 2002). Sometimes tiny black sclerotia form on dead tissue (Nene and Reddy, 1987; Nene et al., 1991; Haware and McDonald, 1992; Haware, 1998).

B. cinerea is one of the many fungi associated with seedling disorders of chickpea (Bretag and Mebalds, 1987) creating a soft rot (Burgess et al., 1997a). In most chickpea growing regions of the world foliar infection is considered most important, however, in Australia soft rot of young seedlings resulting from seed-borne infection is also important and can result in total crop failure (Burgess et al., 1997a). Symptoms include poor emergence, yellowing, wilting and death of seedlings and pale-yellow to light tan discolouration of the taproot. Plants that develop soft rot become flaccid then die within a few days, but seldom recover.

2.2. Epidemiology

B. cinerea survives on chickpea seed (Laha and Grewal, 1983; Haware et al., 1986; Meeta et al., 1986) for at least 5 years internally or externally (Grewal and Laha, 1983), though Burgess et al. (1997a) found it to be largely external. The survival period on seed is affected by the storage temperature (Tripathi and Rathi, 1992), being greatest at 5-10°C (Pande et al., 2002) and is also affected by relative humidity (RH) (Laha and Grewal, 1983). Burgess et al. (1997a) found that survival of the pathogen on chickpea seeds fell from 95% to 2% after 12-months storage at 20°C. Heating moist seed to 50°C for 5 min reduces viable seed infection (Burgess et al., 1997a). Seed from diseased plants may not have external symptoms and a laboratory seed testing procedure is required for detection of the fungus (Haware et al., 1986). Levels of seed infection from diseased crops have been recorded as high as 95% (Burgess et al., 1997a). Seed-borne infection is thought to be the most important means of transmitting botrytis seedling rot and was first reported by Cother (1977). Burgess et al. (1997a) established the importance of seed-borne inoculum as a source of primary infection for grey mould and reported the failure of several commercial chickpea crops due to seedling rot resulting from sowing infected seed.

The pathogen also survives on plant debris for up to 8 months (Meeta et al., 1986; Singh, 1989; Tripathi and Rathi, 1992; Singh and Tripathi, 1993), or in the soil as mycelium and sclerotia. Infested soil or plant debris is considered the main source of primary inoculum in India (Mahmood and Sinha, 1990), but seed-borne inoculum appears to be most important under Australian conditions. Studies at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) have shown that infected leaves of chickpea decompose within a few months, but the stems take considerably longer (Haware and McDonald, 1992). Conidia can be blown several hundred metres from their source (MacLeod and Sweetingham, 2000). Due to the wide host range of this pathogen, the role of alternative hosts is likely to play an important part in survival from one chickpea crop to another (Haware, 1998; Knights and Siddique, 2002; Pande et al., 2002). New microscopical evidence shows that dry-inoculated *B. cinerea* conidia can germinate in the fluid secreted by multicellular stalked glands on leaves and grow through the basal cell into the mesophyll (G. Senthil, G. Ramsay, G.H. Duncan and B. Williamson, unpubl.). The disease may develop rapidly over time and space, depending on environmental conditions. RH, leaf wetness and temperature are the most important factors (Tripathi and Rathi, 1992; Butler, 1993; Pande et al., 2002). Bakr and Ahmed (1992) found that disease increased when temperatures ranged from 17-28°C and RH from 70-97%. The optimum temperature for conidial germination is 20°C and extremes are 5°C and 30°C, while distinct isolates required different light intensities and RH (Rewal and Grewal, 1989). Disease severity increased with leaf wetness periods beyond 12 h/day (Singh and Kapoor, 1984). When RH is 95% or above and temperatures are approximately 25°C in a dense canopy the epidemic can spread rapidly, with the disease cycle completed in 7 days (Haware, 1998).

2.3. Disease control

Agronomic and cultural management of the disease has been demonstrated in India, Bangladesh and Nepal (Pande et al., 2002). Manipulating sowing dates, using erect cultivars and lower plant densities all assist with reducing the level of grey mould in chickpeas (Haware, 1998). It is also important to use pathogen-free seed in order to prevent seed transmission of the disease. Later sowing reduces the vegetative growth and hence lowers disease incidence. However this can also lead to reduced grain yield (Haware and McDonald, 1992; Karki et al., 1993). Wider row spacings allow for more aeration of the crop canopy and reduce periods of leaf wetness and RH. This in turn reduces the incidence of *Botrytis* in the crop (Haware and McDonald, 1992; Bakr et al., 1993; Knights and Siddique, 2000; Pande et al., 2002). Increased plant spacing in paired rows has reduced the disease and increased grain yield, as has intercropping with linseed (Reddy et al., 1990; Bakr et al., 2002) or wheat (Tripathi and Rathi, 2000). Foliage de-topping, to reduce excessive foliage, increases the duration and intensity of light to the lower canopy and makes the microclimate unfavourable for disease development (Rathi and Tripathi, 1995). Similarly, erect and compact growth habits show lower disease incidence than bushy spreading genotypes due to improved aeration (Haware and McDonald, 1992; Sethi

et al., 1993). Erect chickpea types appear to escape the disease as the open canopy allows air movement and drying of the foliage after rainfall (Haware, 1998). Crop lodging exacerbates disease through poor canopy ventilation and genotypes with different lodging susceptibilities suffer different levels of grey mould (Knights and Siddique, 2002). In addition, crop rotation, burning infected debris and deep ploughing reduce the inoculum levels.

Seed treatments with iprodione, vinclozolin, mancozeb, thiabendazole, triadimefon, triadimenol or thiram with benomyl, carbendazim, or captan are effective at eliminating *B. cinerea* from seed (Laha and Grewal, 1983; Singh and Bhan, 1986; Singh and Kaur, 1990; Bakr et al., 1993; Haware, 1998). This practice in Australia has effectively eliminated botrytis seedling rot in chickpeas (Knights and Siddique, 2002). Foliar sprays, used at regular intervals from the first appearance of the disease, can control an epidemic in the crop (Pande et al., 2002), particularly when used in conjunction with a seed dressing (Grewal and Laha, 1983). Effective fungicides include captan, carbendazim, chlorothalonil, mancozeb, thiabendazole, thiophanate-methyl, thiram, triadimefon, triadimenol or vinclozolin at 50 days after sowing or at the first sign of symptoms (Singh and Kaur, 1990; Haware and McDonald, 1992; Bakr et al., 1993; Knights and Siddique, 2002; Pande et al., 2002). Sometimes multiple sprays are recommended though generally one spray at flowering followed by a spray 10 days later on a moderately resistant chickpea cultivar provides the best protection against grey mould (Pande et al., 2002).

However, the use of fungicides cannot be widely adopted in India by resource-poor farmers and hence integrated management of grey mould is encouraged using agronomic practices, erect cultivars, biological agents and targeted spraying with fungicides (Haware and McDonald, 1992; Bakr et al., 2002; Pande et al., 2002). Fungicide application based on disease predictive models facilitates the timely application of fungicides for effective and economical disease control. Weather variables and grey mould severity data over an 18-year period have been used to develop disease prediction models in Nepal and Bangladesh. Maximum temperature and afternoon RH were identified as the key variables during the period corresponding to crop flowering to podding. An early appearance of disease preceding this period helped in initial inoculum build up and rapid disease development if the subsequent conditions were favourable. A function of these two variables is used as a basis for a predictive scheme to schedule fungicide sprays for managing grey mould (S. Pande, O.P. Jhorar and C. Johansen, ICRISAT, India, unpubl.).

Trichoderma spp. and *Gliocladium* spp. are known for their antifungal and biocontrol activities against a diverse range of phytopathogenic fungi (Chapter 13). Greenhouse experiments with *T. harzianum* (Haware, 1998) and *T. viride* (Mukherjee and Haware, 1993) have controlled grey mould and field experiments have given encouraging results. Seed treatment with *T. viride* or *G. roseum* increased % seed germination and gave disease protection equivalent to that of thiram (Burgess and Keane, 1997; Burgess et al., 1997b; Agarwal and Tripathi, 1999). Further advantages were seen in root and shoot length, and vigour of the chickpea plants with no adverse effect on nodulation (Agarwal and Tripathi, 1999). An integrated application of fungicide-tolerant *T. viride* and vinclozolin was more

effective in combating grey mould than vinclozolin alone (Mukherjee et al., 1995, 1997). Seven essential oils or their constituents were tested for antifungal activity against *B. cinerea*, using a paper disc method. Clove oil, cinnamon oil and geraniol were the most effective. These compounds at a concentration of 1 ml/litre inhibited the *in vitro* conidial germination of *B. cinerea* by over 90% and also reduced the germ tube length up to 80% (S. Pande and G.K. Kishore, ICRISAT, India, unpubl.).

Extensive screening of chickpea germplasm has found no genotypes with high levels of resistance, however, numerous chickpea lines have been identified with moderate resistance to grey mould (Haware and McDonald, 1992; Yadav, 1992; Rathi and Tripathi, 1993; Bretag and Brouwer, 1995; Tripathi and Rathi, 2000; Bakr et al, 2002; Pande et al., 2002) and other lines have been identified with tolerance (Joshi, 1992). Good resistance has been found in wild *Cicer* spp., including *C. judaicum*, *C. bijugum*, *C. echinospermum* and *C. pinnatifidum* (Singh et al., 1991; Sethi et al., 1993; Haware, 1998; Pande et al., 2002). Little information is available about the inheritance of resistance to grey mould in chickpea, but some studies have found that a major dominant gene is involved (Yadav, 1992; Singh, 1993). Several wide and intra-specific hybridizations have been carried out to transfer the identified disease resistance in wild and land lines to commonly grown chickpea cultivars. A few hybrids with moderate levels of resistance to grey mould and with desirable agronomic traits have been identified (Singh et al., 1998). A list of other chickpea lines derived from wide hybridization and their resistant parents was provided by Pande et al. (2002). Healthy resistant chickpea plants were found to have significantly lower total soluble sugars and free amino acids, but higher total phenol levels than susceptible lines (Mitter et al., 1997). A pterocarpan phytoalexin, maackiain, was found associated with grey mould resistance in *C. bijugum*, a wild relative of chickpea (Stevenson and Haware, 1999).

Since a high level of resistance to grey mould is not available in the cultivated genotypes, and fungicides are ineffective during conditions of high disease pressure, integrated disease management (IDM) using the available options as components is essential. Judicious use of fungicides as a seed treatment and/or foliar spray in an IDM system could be economical and affordable to the resource-poor farmer. An IDM technology has been devised for Nepal consisting of a grey mould tolerant genotype Avarodhi (ICC 14344), soil application of di-ammonium hydrogen orthophosphate, wider row spacing (60 cm), seed treatment with carbendazim plus thiram, and foliar application of carbendazim strategically timed based on weather data. The IDM was evaluated in farmers' participatory research in two districts of Nepal during 1998-99 crop season. Grain yields were increased by up to 400% and net income up to 300%. As a consequence, in the 2002-03 crop season, there was a 100-fold increase in the number of farmers voluntarily adopting IDM technology (Pande et al., 2003).

3. Lentils

Grey mould of lentils is caused by both *B. cinerea* and *B. fabae* (Bayaa and Erskine, 1998; Lindbeck et al., 2003). Both pathogens have been isolated from lentil seed, and have been found to be equally pathogenic on lentils in glasshouse tests (T.W.

Bretag, unpubl.; J.A. Davidson, unpubl.). This disease has been reported as a severe problem in Australia (Lindbeck et al., 2003), Bangladesh (Bahl et al., 1993), Canada (Morrall, 1997), Nepal (Karki, 1993), New Zealand (McKenzie et al., 1986), Pakistan (Iqbal et al., 1992; Bakr et al., 1993) and Colombia (Bascur, 1993) and also occurs in India, Syria, Morocco and the USA (Beniwal et al., 1993; Ilyas, 1993; Morrall et al., 1994; Bailey et al., 2000).

3.1. Symptoms

In established crops, under disease favourable conditions, infection develops on lower leaves, which are then shed. Lesions on stems are light brown or blanched and covered with grey mould (Figure 1) and rot at the crown. Whole plants eventually become infected and dry out. As with grey mould in chickpeas, the disease is initially distributed as scattered dead plants, and rapidly spreads, under favourable conditions, to produce patches of dead plants (Beniwal et al., 1993; Bayaa and Erskine, 1998). Infected pods fail to fill properly, rot, turn brown and are covered with mould; infected seeds may be discoloured and shrivelled. Seedling blight lentils occurs if heavily infected seed is sown (Bayaa and Erskine, 1998); seed-borne *Botrytis* was responsible for 20% seedling death in Canada (Morrall et al., 1994).

3.2. Epidemiology

There are several sources of inoculum for grey mould in lentils. Both causal agents, *B. cinerea* and *B. fabae*, are known to be seed-borne in lentils (Sumar and Howard, 1983; Kaiser, 1992; Bayaa and Erskine, 1998) and may severely affect seed viability, seedling emergence and stand establishment (Morrall, 1997). Further inoculum comes from diseased host plants, infected plant debris and sclerotia (Bayaa and Erskine, 1998). Sclerotia (Figure 1) are probably the main survival structure and are highly resistant to adverse conditions. They may survive for long periods if they are not buried, but decay more quickly in wet soil than dry soil. Mycelium, in plant debris, can survive for extended periods and will produce conidia under humid conditions (Bayaa and Erskine, 1998). Due to the extreme susceptibility of lentils to either *Botrytis* spp., almost any other pulse crop or legume pasture (i.e. faba beans, vetch, or lathyrus) can be a reservoir of inoculum.

Seedling blight is distinguished by the prolific grey fructifications of the fungus on the plant at the soil line. Because of the vigorous growth of *Botrytis* spp., seedling blight spreads from plant-to-plant along rows. Hence the percentage of pre-emergence death and seedling blight can exceed the percentage of seed infected with *Botrytis* (Morrall, 1997). The development of an epidemic in lentils, as in other crops, is strongly influenced by the environment (Kaiser, 1992; Bailey et al., 2000). *Botrytis* spp. will produce masses of wind-borne conidia on infected and dead tissues under humid conditions, and epidemics can develop very quickly in comparison with most other diseases (Jarvis, 1980). The microclimate within the canopy is important in determining disease incidence. Excessive vegetative growth due to irrigation or heavy rains, close plant spacing and varieties with spreading habit lead to humid conditions within the crop which favour disease. Temperatures

between 15-25°C and RH > 95% after canopy closure and at flowering or podding favour disease outbreaks (Nene et al., 1991; Haware, 1998; Lindbeck et al., 2002).

3.3. Disease control

Dense canopies encourage epidemics and several agronomic practices are possible to avoid this (Bretag and Materne, 1998a). These include adjustment of sowing dates and rates, wider row spacing, weed control and optimum fertilizer use, particularly avoiding high nitrogen levels (Bayaa and Erskine, 1998; Lindbeck et al., 2002). Farmers can also practice on-farm hygiene by only retaining seed from disease-free crops for the following year's sowing, and using seed with less than 5% seed infection. They may undertake a programme of stubble reduction through grazing, burning or burying, to reduce the carry-over of infected stubble into the following season. In addition volunteer lentils, faba beans, vetch, lathyrus or chickpeas should be controlled to reduce the early build up of inoculum (Lindbeck et al., 2002). Careful selection of the field for growing lentils can also reduce epidemics. There should be a break of more than 3 years between lentil crops, and lentils should not be grown adjacent to, or sown into, stubble of a lentil, faba bean, chickpea, vetch or lathyrus. Nor should lentils be grown adjacent to an early sown crop of faba beans, chickpea, vetch or lathyrus as these can harbour *Botrytis* inoculum (Lindbeck et al., 2002).

Seed treatments with fungicides such as benomyl, carboxin, chlorothalonil or thiabendazole minimise seed-borne inoculum and seedling blight (Morrall, 1997; Bayaa and Erskine, 1998; Bretag and Materne 1998b; Lindbeck et al., 2002). While Bayaa and Erskine (1998) state that fungicide control for grey mould in lentils is uneconomic this is a crucial part of disease control in Australian crops (Lindbeck et al., 2002, 2003). Several applications are generally required if conditions remain favourable for disease development, the first shortly prior to canopy closure and further sprays during flowering and pod fill depending on presence of the pathogen and weather conditions. Carbendazim, chlorothalonil, mancozeb and procymidone are the products used in Australia (Lindbeck et al., 2002). Iqbal et al. (1992) evaluated 14 fungicides and found that benomyl, thiabendazole and tridemorph were the most effective against *B. cinerea*. However, frequent use of fungicides can lead to the development of pathogen strains resistant to the most commonly used fungicides (Elad et al., 1992).

Four antagonists of *B. cinerea*, viz. *Pantoea agglomerans*, *Pseudomonas fluorescens*, *Penicillium griseofulvum* and *Trichoderma hamatum*, have been shown to reduce the incidence and severity of botrytis stem and blossom blight of lentil, and increase seed yield when applied as a foliar spray in greenhouse and field environments (Erickson and Huang, 2002). Essential oils have been tested for their antifungal activity; clove oil, cinnamon oil and geraniol were found to be most effective (S. Pande and G.K. Kishore, ICRISAT, India, unpubl.).

Lentil germplasm with resistance to grey mould has been identified in Australia (Bretag and Materne 1999; Lindbeck et al., 2003), Canada (Morall 1997), Nepal (Karki, 1993) and in Pakistan (Tufail et al., 1993; Erskine et al., 1994). Trial data have shown that there is variability within Australian lentil germplasm for resistance to *Botrytis* (Bretag and Materne, 1999) and crosses made with the Canadian variety

Indianhead have better resistance than current commercial cultivars (Lindbeck et al., 2003).

4. Faba beans

Chocolate spot of faba beans has been reported in many parts of the world (Deverall and Wood, 1961; Khalil and Harrison, 1981; Hanounik and Hawtin, 1982; Sundheim, 1973; Liang, 1989; Akem and Bellar, 1999), but is more prevalent in humid regions than in the arid climatic zones. Faba beans are infected by both *B. fabae* and *B. cinerea*, however, *B. fabae* is usually the major cause of chocolate spot epidemics in this crop (Sundheim, 1973; Mansfield, 1980; Saxena and Stewart, 1983). This is because *B. fabae* is able to metabolise the host's phytoalexins (particularly wyeronine acid) more rapidly than *B. cinerea* to prevent them from accumulating in the invaded tissue (Chapter 9; Mansfield, 1982). It has also been postulated that *B. fabae* suppresses the production of phytoalexins (Mansfield, 1980). Hence *B. fabae* lesions become aggressive more frequently, though it has been demonstrated experimentally that *B. cinerea* can be induced to the aggressive phase if the conditions are conducive (Hargreaves et al., 1977). In addition, severe pod rotting from *B. cinerea* infection has also been recorded (Jellis and Bond, 1980). The different *Botrytis* spp. that attack field crops are distinguishable from each other (Chapter 3). The colony characteristics of *B. fabae* are similar to *B. cinerea*, however, *B. fabae* can be distinguished from *B. cinerea* on the basis of conidial size. The conidia of *B. fabae* are much larger than *B. cinerea* and are 14-29 X 11-20 µm in size, mostly 16-25 X 13-16 µm (Ellis and Waller, 1974b; Harrison, 1983). A teleomorph of *B. fabae*, *Botryotinia fabae*, was described by Wu and Lu (1991). Yield loss from chocolate spot has been attributed to the reduced pod set and subsequent reduction in number of pods per plant from *B. fabae* infection (Williams, 1975; Griffiths and Amin, 1977). Severe epidemics can lead to total crop failure (Bretag et al., 1996). Bretag (1992) measured yield losses of over 90% in faba bean crops grown in southern Australia. Losses as high as 61% have been recorded in Ethiopia (Gorfu, 1996), 59% in the UK (Gladders et al., 1991) and over 50% in China (Liang, 1986).

4.1. Symptoms and aggressiveness

The symptoms of chocolate spot of faba beans vary from minor necrosis to extensive destruction of plant tissue. Leaves, stems, flowers and pods can all be infected (Gaunt, 1983), with flowers and pods being the most susceptible parts (Griffiths and Amin, 1977). The disease initially shows a non-aggressive phase, but as the season progresses transforms into the aggressive phase (Figure 1) (Ellis and Waller, 1974b). Non-aggressive lesions appear as small regular reddish-brown or chocolate brown spots, 1-3 mm in diameter on the leaves (Ellis and Waller, 1974b). These lesions are present in most faba bean crops every season and become quite numerous as the season progresses (Harrison, 1984), presenting a peppered appearance on the leaves (Jellis et al., 1998), but cause little damage to the plants. Continuous high RH appears to be the most important factor that encourages the

aggressive phase of the epidemic (Yu, 1945). In this phase the lesions expand to 5-10 mm in diameter and coalesce to form a black blight on the leaves and stems (Ellis and Waller, 1974a). The lesions are irregular in shape, and light and dark concentric ridges often develop during lesion expansion (Harrison, 1988). Defoliation can occur rapidly. The fungus can also grow saprophytically on fallen dead leaves, sporulate and re-infect younger, growing leaves (Jellis et al., 1998).

A wide variation in pathogenicity exists among isolates of *B. fabae*. Nine isolates of *B. fabae* from Ethiopia, with a similar conidial size, differed in their cultural characteristics, sclerotial production and infection of different genotypes (Gorfu, 1996). These variations resembled differences in cultural characteristics and virulence patterns in *B. fabae* collected from other locations (Mohammed et al., 1981). Hanounik et al. (1984) observed that in a collection of different isolates, the more virulent isolates produced fewer conidia and formed larger sclerotia than the less virulent isolates. Variation in morphology and aggressiveness between isolates has also been reported by Hutson and Mansfield (1980).

4.2. Epidemiology

Carry-over of *B. fabae* between crops occurs as mycelium in crop debris (Gaunt, 1983) or as sclerotia in the soil and in crop debris. Mycelium can survive for over a year in crop debris on the surface of the soil, but only 4 months when buried 20 cm (Gorfu, 1999), while sclerotia can survive for up to 1 year without loss of viability (Yu, 1945; Harrison, 1979). Sclerotia buried in soil are less likely to survive (Harrison, 1979). This form of survival is particularly important when there is a long summer gap between host crops as in Mediterranean climates (Jellis et al., 1998). Alternative hosts and volunteer faba bean plants may also be important for survival of the fungus between cropping seasons. Seed infection is not considered important for chocolate spot. While *B. fabae* infection has been found on faba bean seeds (Harrison, 1978; Sumar and Howard, 1983; Simay, 1992), the frequency is low and viability of the fungus lasts only 9 months (Harrison, 1978). No correlation has been observed between levels of seed infection and disease incidence, but seed infection may have a role in introducing the disease into new areas (Harrison, 1978).

Chocolate spot development is highly dependent upon environmental factors. Frequently the disease is first noticed on isolated plants that act as foci from which the disease develops rapidly in humid weather (Sundheim, 1973). Chocolate spot spreads via airborne conidia within crops where RH is above 70% and at temperatures of 15-20°C (Harrison, 1980). The conidia are released as clumps or singly, and are generally wind-borne (Fitt et al., 1985; Harrison and Lowe, 1987), but may be dispersed by rain or insect vectors, particularly thrips (Harrison, 1988). Lesions expand slowly when RH is below 66% (Harrison, 1980). The optimum temperature for lesion growth is between 15-20°C, with minimum of 4°C and maximum of 30°C. Neither light intensity nor water films on the leaves have an impact on lesion growth (Harrison, 1980) though near ultra-violet light induces sporulation (Harrison and Heilbronn, 1988) and it is common practice to use alternating cycles of 12 h darkness and 12 h of near ultra-violet light to stimulate

sporulation of *B. fabae* (Hanounik and Maliha, 1986; Dhingra and Sinclair, 1995). Plants become more susceptible to chocolate spot as they age or suffer from freezing (Creighton et al., 1986; Heilbronn and Harrison, 1989).



Figure 1. *Botrytis* spp. on legumes. Top row: sporulation (left) and lesions (centre) on infected chickpea pod and sporulation on lentil plant (right); Bottom row: sclerotia on stems of infected lentil plants (left) and non-aggressive spotting and aggressive lesions of chocolate spot on faba bean leaves (right)

4.3. Disease control

Avoidance of factors that predispose the crop to an epidemic can reduce the severity of chocolate spot epidemics. The use of crop rotation to avoid infected debris and volunteer plants, burning of infected plant debris, burying or grazing stubble and removal of volunteer plants will minimise the risk of chocolate spot in subsequent faba bean crops (Harrison, 1979; Liang, 1989). High plant densities that provide a high humidity environment should be avoided and in Europe it is recommended that early sowing be avoided to prevent spring frost damage and hence predispose the crop to the disease (Jellis et al., 1998). The association between early sowing and chocolate spot was also noted in Syria (Hanounik and Hawtin, 1982). Adequate

fertilizer, in particular potassium and nitrogen, and good soil drainage is necessary to prevent early senescence, which would provide dead leaves upon which the fungus sporulates (Liang, 1989; Jellis et al., 1998). A higher incidence of chocolate spot occurred in plants grown on acidic soils (Elliott and Whittington, 1978) and with increased nitrogen fertilizer (Hegab and Beshir, 1994).

Chemical control measures can be effective against chocolate spot. However, several applications of fungicide are generally required in situations where the environment remains favourable for disease development. In Australia it is recommended that fungicide be applied from flowering onwards, depending on conditions. Carbendazim or procymidone are recommended in high disease situations, while benomyl, chlorothalonil, mancozeb, tebuconazole or copper products are also available (El-Fiki, 1994; Marcellos et al., 1995; Panagiotopoulos et al., 2002). In Australia carbendazim was better than mancozeb or chlorothalonil for control of chocolate spot (Bretag et al., 2002) while Khaled et al. (1995) found mancozeb to be more effective than benomyl, carboxin, metalaxyl, oxycarboxin, thiram, triadimefon or dimethyl formamide. In Europe benomyl, or tank mixes of carbendazim plus chlorothalonil or iprodione or vinclozolin are used from early flowering and 3-4 weeks later (Doto and Whittington, 1980; Elliott and Whittington, 1980; Fitt et al., 1986; Giltrap, 1991; Jellis et al., 1998). Bainbridge et al. (1985) were able to reduce an early chocolate spot epidemic by using seed treated with benomyl plus thiram. In China, sprays of carbendazim or thiophanate-methyl at podding or flowering were found to be effective (Liang, 1989). Late epidemics appear not to affect yield and fungicides are not warranted in these conditions. The best indication to farmers of a potentially damaging epidemic appears to be the build-up of chocolate spot on lower leaves before or during flowering (Creighton et al., 1985). Resistance to benzimidazole fungicides, such as carbendazim, is a major problem when they are used frequently (Gladders et al., 1991; Jellis et al., 1998).

Trichoderma viride was found effective against chocolate spot in 1990 (Jellis et al., 1998). Jackson et al. (1994) reported that some isolates of *Penicillium chrysogenum* inhibited growth and development of *B. fabae* *in vitro* and showed the development of chocolate spot under glasshouse conditions was substantially reduced by this agent. Similar results were obtained with *P. brevicompactum* and *Cladosporium cladosporioides* (Jackson et al., 1997). Fourteen *Bacillus* isolates were found to be antagonistic to *B. cinerea* and *B. fabae* and reduced development of chocolate spot symptoms. However, they were not effective in the field (Sharga, 1997). Another antagonistic bacterium, *Erwinia herbicola*, which was isolated from the phylloplane of a chocolate spot-resistant genotype, was able to significantly reduce disease severity when used as a foliar spray (Abd-El-Moity et al., 1990).

Foliar application with hydroxyphaseollin, a phytoalexin obtained from soybean, induced resistance against chocolate spot. A possible disease control mechanism is the stimulation of host-specific phytoalexins, specifically wyerone acid, in sufficient quantities to inhibit the pathogen (El-Hawa, 1998). Exposure to yellow (590 nm max.) and red (650 nm max.) lights significantly inhibited the formation of infection hyphae from appressoria of *B. cinerea* on bean leaflets; inhibition is believed to enhance systemic resistance (Islam et al., 1998).

Moderate resistance to chocolate spot has been widely reported (Khalil et al., 1984; Hanounik and Maliha, 1986; Tivoli et al., 1986; Hanounik and Robertson, 1988; Liang, 1989; Robertson, 1995) and is conferred by a combination of factors such as total free amino acids and phenols, and thickness of the cuticle (Jellis et al., 1998; Kararah et al., 1991). Resistance is thought to be under additive polygenic control involving partial dominance (Elliott and Whittington, 1979; Ramsey et al., 1995), thus making it difficult to trace identifiable genes through segregating populations. The resistant lines BPL710, BPL1196 and BPL1179 which were consistently resistant in international testing (Hanounik and Maliha, 1986) originated in Ecuador and have been used in breeding programmes in Egypt and Australia (Jellis et al., 1998). A resistant variety, Icarus, derived from BPL710, is commercially grown in Australia (Ramsey et al., 1995). Four faba bean lines have been identified with high resistance to chocolate spot (Rhaïem et al., 2002). The use of cultivars with even moderate resistance can substantially reduce the levels of chocolate spot and the dependence on fungicides. Some wild accessions, *V. johannis* and *V. narbonensis*, are resistant to *B. fabae* and have the added advantage of frost tolerance (Birch et al., 1985).

5. Other legume crops

5.1. Field peas

B. cinerea can infect field peas under humid conditions and grey mould has been reported in North America, Europe, Japan and New Zealand (Basu et al., 1973; Dobson and Heath, 1991; Tivoli and Lemarchand, 1992; Sanssene and Haddad, 1995; Biddle, 2001), but in most regions it is considered a minor pathogen of peas.

The infection begins by defoliation of the lower leaves, streaking or girdling of the stem, which results in wilt of the upper plant. Under humid conditions masses of conidia are produced (Biddle, 2001). Pod damage causes the most economic loss, with up to 50% of pods destroyed (Dobson and Heath, 1991). Immature pods will shrivel and become covered with mycelium, readily infecting neighbouring pods. Infected seeds are chalky white and have poor germination (Biddle, 2001). Yield losses as high as 50% have been recorded when conditions are conducive to disease development (Basu et al., 1973). *B. cinerea* survives as mycelium, conidia or sclerotia on plant debris (Biddle, 2001), or on seeds (Sumar and Howard, 1983); conidia are splashed by irrigation, rain, or spread by wind or farm machinery to the subsequent crop (Biddle, 2001).

Adequate fertilizer will reduce predisposition to infection, particularly potassium (Biddle, 2001) and lower seeding rates may produce a more open crop, thereby increasing air circulation and reducing humidity (Bretag et al., 1996). Fungicides can be used, particularly as the first pods are developing, to protect them from infection and to prevent the falling blossoms from developing infection (Biddle, 2001). Fungicides are not necessary when weather is unfavourable to disease development (Dobson and Heath, 1991). There has been no reference to resistance to *B. cinerea* in field pea (Biddle, 2001), but purple flowering peas are reported to be less prone to grey mould than white flowering genotypes (Bretag et al., 1996).

5.2. Pigeon pea

B. cinerea grey mould is generally of minor importance in pigeon pea production, but can cause economic losses whenever it occurs. The disease has been reported from the hill regions of Garhwal, India (Kumar et al., 1991) and has completely defoliated the pigeon pea crop recently in Nepal. Pigeon pea often acts as an alternative host of *B. cinerea* causing grey mould in chickpea (Pande et al., 2002). At the time of flowering, the disease appears as dark grey fungal growth on the growing tips, flowers, and pods. Infected flowers drop, thus reducing pod set. The shed flowers and leaves on the ground are covered with sporulating mycelium. Early-maturing pigeon pea genotypes have a reduced incidence of bud damage compared to late maturing genotypes (Bajpai et al., 1995). Phytoalexin studies found that pinostrobin, chalcone and novel isoprenylated stilbene-2-carboxylic acids were induced in pigeon pea plants inoculated with *B. cinerea* (Cooksey et al., 1982).

Seed-borne inoculum of *B. cinerea* was reduced by treatment with carbendazim, captafol, captan, carboxin, or mancozeb (Kumar and Srivastava, 1985). Seed treatments with *T. viride* and *Chaetomium globosum* have been shown to reduce the number of colonies of *B. cinerea* and increase seed germination and emergence, root length, shoot length and fresh weight of seedlings (Kumar et al., 2000).

5.3. Common Bean

Grey mould (*B. cinerea*) can be a problem on common beans (*P. vulgaris*) only if conditions are conducive to disease development (Howard, 1981; Polach and Abawi, 1975; Allen and Lenné, 1998). The disease usually initiates on senescent plant parts, such as flowers or cotyledons. Infection is more prevalent if crops have been damaged by frost, hail, wind and machinery, or have been sand-blasted. Girdling of the stem can lead to plant collapse. Colonised senescent flowers remaining on the pods are the major source of pod infection, causing a watery soft rot, which can then cause marketing problems (Howard, 1981; Johnson and Powelson, 1983; Jarvis, 1991). Sclerotia may form on infected plants. These are usually black, small in size and flattened. They may not be conspicuous, but may be embedded in decayed tissue or coated with soil and other debris (Howard, 1981). Conidia can be produced from sclerotia and infected debris or host material throughout the bean-growing season. They can be dispersed via the wind, rain, and irrigation or by machinery (Johnson and Powelson, 1983; Jarvis, 1991). A film of water is necessary for conidia to germinate and infect plant tissues; they usually infect fallen petals or plant debris in contact with the stem, branches or pods and then grow into the healthy tissue (Howard, 1981). Infected seedlings bear conidia of *B. cinerea* for extended periods and serve as a local inoculum source. Dispersal studies suggest that inoculum sources are restricted to within the bean field (Johnson and Powelson, 1983).

Humidity is important in determining disease severity and so cultural practices can provide disease escape. Reduction in crop density, control of fertilizers to

prevent dense crops, and judicious watering all assist in reducing the onset of disease. Infected crop debris can be destroyed through burying, burning or grazing, and rotation with non-hosts such as cereals or corn will reduce carry-over of inoculum (Howard, 1981; Jarvis, 1991). Chemical control is rarely considered economic in green beans (Johnson and Powelson, 1983; Jarvis, 1991) unless the grey mould reaches epiphytotic proportions (Polach and Abawi, 1975). Resistance to grey mould is usually associated with plant habit that avoids the disease, i.e. resistant to lodging, open habit, non-persistent flowers and pods that do not touch the soil (Howard, 1981; Jarvis, 1991). In glasshouse and laboratory experiments *T. harzianum*, *Pseudomonas aeruginosa*, *Gliocladium roseum* and *Epicoccum nigrum* have all been shown to have some efficacy as biocontrol agents for *B. cinerea* on *P. vulgaris* (De Meyer et al., 1999; Elad and Kapat, 1999; Szandala and Backhouse, 2001; Freeman et al., 2002). Non-aggressive strains of *B. cinerea* have also been suggested as biocontrol agents (Weeds et al., 2000).

5.4. Vetch

Chocolate spot (*B. fabae*) is a common problem in vetch in Australia (Bretag et al., 1996) (described as *B. viciae* Greene by Stovold and Walker, 1980) and has been recorded as causing considerable defoliation of vetch in the USA (Henson and Schotch, 1968). Losses range from minor, to complete crop failure depending on the severity of the epidemic and environmental conditions. Yields may be reduced by 30-50% through loss of leaves and collapse of the crop (Bretag et al., 1996).

Small, dark brown spots appear on leaves and stems and may lead to complete blackening of the plant. Leaves are the main area affected, but in severe conditions the disease may also affect stems, flowers and pods. The pathogen can survive in crop debris as mycelium or sclerotia, on infected seed or on self-sown plants. Once established, the disease can spread rapidly. It is most aggressive in humid conditions particularly at flowering time (Bretag et al., 1996).

Disease risk can be reduced by destroying all vetch trash and self-sown plants before sowing, using disease-free seed and by using crop rotation. This would include avoiding stubble of other hosts of *B. fabae*, i.e. faba beans and lentils. Delayed sowing reduces crop density, which would otherwise provide a dense canopy and highly humid environment for the pathogen to thrive (Bretag et al., 1996). Fungicides can be used to control the disease when environmental conditions are likely to encourage a severe epidemic. Carbendazim, chlorothalonil, mancozeb, procymidone and copper products are recommended for use in Australian vetch crops (Bretag et al., 1996).

5.5. Peanut

In peanut or groundnut, *B. cinerea* causes botrytis blight, also known as grey mould or 'botrytis shoot disease'. The disease was first reported from Japan in 1924, and subsequently from Argentina (Marinelli et al., 1996), Hungary (Simay, 1991), Korea (Shim et al., 1996), Australia, the CIS (the former USSR), Japan, Malawi, Romania,

South Africa, Swaziland, Tanzania, USA, Venezuela, Vietnam, and Zimbabwe (Porter et al., 1984). Occasional disease outbreaks in these countries have resulted in substantial yield reductions.

B. cinerea can infect all plant parts and produce abundant conidia, but blighting of stem and leaf is the most common symptom. The first visible symptoms are sparse spots on branches that are in contact with the soil. The lesions become reddish to brown, with a distinct demarcation zone between infected and healthy tissues and covered with a profuse production of thick dark grey mycelia, conidiophores and conidia of *B. cinerea*. The infection rapidly progresses from branches to leaflets, pegs or carpophores (stalk like structures that bear the fertilized ovules at the tips), and pods. Occasionally, entire plants wilt and turn dark brown. Although apothecia have been reported, the primary source of inoculum is the mycelium originating from germinating sclerotia or omnipresent conidia. Conidia are frequently disseminated by air. Infection and disease development are favoured by cool temperatures ($\geq 20^{\circ}\text{C}$) accompanied by heavy dew or rainfall.

Peanut variety Kalina bred at Sadovo, Bulgaria, is relatively resistant to botrytis, blight, and is suitable for intense cultivation and mechanical harvesting (Georgiev, 1989). Resistant line IIRR 2069, bred for high yield and oil content, is resistant to *B. cinerea* (Popov et al., 1979). Early-maturing peanut cultivars may escape frost damage and thus reduce disease severity in some countries. Foliar sprays of fungicides such as benomyl, chlorothalonil and dichloran are partially effective in disease control.

5.6. Soybean

B. cinerea is a minor disease of soybeans that can cause a stem rot that usually occurs in wet seasons in high-density crops. A survey in Poland in 1977-80 detected the disease in 25 out of 76 fields. It has also been reported to cause damage to plants at pod formation (McGee, 1992) and can be seed borne (Sumar and Howard, 1983). No specific control measures for this disease have been recorded (McGee, 1992).

6. Conclusions

The botrytis diseases in legume crops present a range of challenges and opportunities, including understanding the biology, epidemiology and pathogenesis of *Botrytis* spp. for effective disease management. In spite of several attempts, there is a gap in our understanding of the biology and spread of *Botrytis* spp. and, in particular, the forecasting of disease epidemics (Chapter 18). Different isolates are extremely variable in many characteristics, including aggressiveness.

In the present scenario of inadequate plant resistance and expensive fungicides, alternative disease management strategies are vital. Although progress has been made in the identification of several biocontrol agents, little is known about their modes of action. Fungicide-tolerant biocontrol strains have been developed, and this approach requires further attention since it has the potential for effective disease management with reduced fungicide inputs (Chapter 13). Several plant extracts and

their components have antagonistic activity against *Botrytis* spp., but these require further investigation. Recent studies have shown that various biotic and abiotic elicitors evoke substantial levels of induced systemic resistance to fungal diseases. The use of these chemicals in combination with existing disease management practices may further enhance the disease control.

Transgenic plants with transient expression of one or more defence genes is an attractive strategy for management of *Botrytis* spp., especially because adequate levels of resistance are not available for these diseases. Engineering the host plants with defence genes (chitinases, glucanases, and thaumatin-like proteins), or other genes that trigger the signal transduction pathway leading ultimately to the induction of systemic resistance, is an emerging strategy (Chapter 20). Expression of polygalacturonase-inhibiting proteins in transgenic horticultural crops had some success for control of infection by *Botrytis* spp., and this strategy may be applicable in other crops but legumes are more difficult to transform than other plant families (Chapter 20). Nevertheless, most of the farmers in Asia and Africa, where greater opportunities exist for crop improvement, are resource-poor. Therefore, cost-effective disease management technologies that perform consistently in their fields, as single treatments or as part of an IDM package, will benefit these low-income farmers with increased grain harvested. For the present time, IDM remains the most viable option for management of *Botrytis* spp. in field legumes.

7. References

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CHAPTER 17

EPIDEMIOLOGY OF *BOTRYTIS CINEREA* DISEASES IN GREENHOUSES

Aleid J. Dik and Jos P. Wubben

Applied Plant Research, Glasshouse Unit Horticulture, P.O. Box 8, 2670 AA Naaldwijk, The Netherlands

Abstract. *Botrytis cinerea* causes problems in many greenhouse crops, such as tomato, cucumber, pepper, strawberry, sweet basil, rose, gerbera and most potted plants. In vegetables, it may infect fruits, leaves and stems. Stem infection resulting from growth through the petiole or from direct infection of wounds may cause plant death. In cut flowers, symptoms mostly occur during the post-harvest phase. In potted plants, disease is found both in greenhouses and post-harvest. In greenhouses, climatic conditions greatly influence epidemics caused by *B. cinerea*. The type of greenhouse covering influences sporulation by absorbing near-UV light, but may enhance disease by influencing the greenhouse climate. CO₂ enrichment of the greenhouse air is not sufficient to reach effective levels. Sanitation is sometimes effective, especially when infected plant parts are removed from the crop, but does not suffice as a control method. No resistant cultivars exist yet, but cultivars show highly variable susceptibility. Wide plant spacing reduces grey mould. Cropping methods, such as de-leafing in vegetable crops, can reduce *B. cinerea* infection. The effect of nitrogen in the fertilizer is not consistent, but calcium enrichment of the plant tissue generally reduces susceptibility. Timing and method of the irrigation can also be used as a control measure. A combination of measures can be used to reduce the occurrence of *B. cinerea*-incited problems in greenhouse crops. Damage in vegetable crops occurs by reducing yield by loss of infected fruits, but mainly by plant death after stem infection. In ornamental crops, plants or flowers must be discarded when affected by *Botrytis*.

1. Introduction

The epidemiology of *Botrytis cinerea* and control possibilities are very different in greenhouse crops compared to field and orchard crops. Greenhouses are used worldwide for the production of vegetables, fruits and ornamentals to protect the crop against wind, rain and cold. The structure of the greenhouse, the materials used and the cropping method have great influence on the host plants and on pests and diseases. An excellent overview of greenhouse structures, the technology used and growing systems is given by Jarvis (1992). Greenhouses can range from simple plastic tunnels and non-heated greenhouses to heated glasshouses equipped with computer-controlled technology. Plastic covered greenhouses are mostly found in warmer climates. Only a small proportion of them is provided with heating systems

and they have windows on the sides which can often be opened for ventilation. Heated glasshouses have ventilation windows in the top of the structures, which are opened automatically when the set point temperature for ventilation is reached. Obviously, these different greenhouses have very different climates. In unheated plastic tunnels, humidity is generally higher than in the heated glasshouses and the temperature fluctuations are larger. The greenhouse climate is further influenced by the height and shape of the greenhouse. Modern glasshouses are much taller than older ones because the height makes it easier to control the climate inside. The cover material itself, i.e. plastic versus glass, also influences the crop and diseases because of differential light and heat transmittance. A range of plastics is commercially available with different light absorption properties.

Compared to the open field situation, epidemiology is more predictable and less weather-dependent in greenhouses. Many other differences from field crops exist. Greenhouse crops, especially vegetables, are labour intensive, requiring tying and pruning and frequent harvesting. This causes many infection sites for *B. cinerea* and other pathogens and also stimulates transfer of spores from plant-to-plant by workers. Many greenhouse crops are grown in substrates such as rock wool, peat or in hydroponic systems. This provides the possibility to adjust the fertilizer composition and thus influence the susceptibility of the plants. For crops grown in soil this is more difficult to achieve. In unheated greenhouses crops are grown for long periods and in heated greenhouses, crops are grown year-round, providing a relatively constant environment for fungi. Plastic tunnels in warm areas may remain empty during the hot summer, forcing the fungus to survive the summer, whereas in the field over-wintering is more the issue. This chapter will describe the *Botrytis*-incited diseases in different greenhouse crops and then focus on factors that influence epidemics.

2. *Botrytis cinerea*-incited diseases in greenhouse crops

B. cinerea is a ubiquitous fungus. It causes grey mould on almost all major greenhouse crops, initially entering greenhouses probably through open windows, cracks, on people and on young cuttings and seedlings. Later in production, infected plants or infected and decaying plant parts in the greenhouse form a source of inoculum. Young plant parts are generally not very susceptible and older plants or plant parts are more usually infected. The occurrence of *B. cinerea* is expressed in different crops in different phases. Tomato, cucumber and sweet pepper are the most widely grown vegetable crops. *B. cinerea* can cause severe problems in these and in many other vegetable, flower, potted plant and herb crops.

In tomato in non-heated greenhouses, the fungus infects flowers, fruits and leaves and can grow through the petiole into the stem (Elad and Yunis, 1993; Shtienberg et al., 1998). In heated greenhouses, *Botrytis* infection is almost completely limited to stem infection. Tomato is mostly grown according to the high-wire method (Jarvis, 1992), with plants reaching lengths of 20-30 m with bundles of stems running along the floors and grown for up to 50 weeks. The leaves around the ripening fruits are removed thus causing pruning wounds. In heated greenhouses, *B.*

cinerea mainly causes stem lesions by infecting such pruning wounds. Stem lesions may girdle the plant leading to plant death, though stem infections may remain quiescent for up to 12 weeks (Jarvis, 1989).

In heated greenhouses of cucumber and zucchini (courgette), fruit infection is mainly limited to aborting fruits. Older leaves may become infected and stem infection may originate from growth of the fungus through the petioles, or from direct infection of wounds caused by pruning of side shoots. As in tomato, stem lesions may ultimately kill the part of the plant above the lesion. In pepper, grey mould infection mainly occurs on wounds caused by harvesting, or by breaking off the leaves. In lettuce, *B. cinerea* is part of the complex of foot rot fungi. In non-heated greenhouses young cucumber fruits and pepper fruits may also be infected (Yunis et al., 1994; Elad and Shtienberg, 1995).

B. cinerea is an important disease of strawberry. Flowers become infected and the fungus grows into the developing berries. Rot may occur both in the crop and after harvest (Boff et al., 2001). In Florida, epidemics were much less severe in plastic tunnels than in the field (Xiao et al., 2001).

Sweet basil is a commercial herb crop mostly grown in the Mediterranean area which can suffer severely from infection by *B. cinerea*. The plants are grown both in heated greenhouses and plastic tunnels. Within a few months after planting, the main shoots are harvested and side shoots start to develop. *B. cinerea* infects the plant through the stem wounds that are caused by harvesting and then progresses on stems, killing leaves and secondary buds. The plant may die when the lesion reaches the main stem (Sharabani et al., 1999).

In cut flowers like rose and gerbera, grey mould and botrytis blight are very destructive diseases. Lesions occur on the petals thereby strongly reducing the market value of the flowers and reducing the vase life. In heated greenhouses, flower infections are hardly ever found during the production period. However, *B. cinerea* conidia may be either present on the plants where they can survive for long periods (Salinas et al., 1989) or latent infection may exist and symptoms become visible after harvest when fluctuating temperatures during cold-chain storage and transportation promote dew deposition (Chapter 19).

Grey mould may occur in most flowering potted plants, during the production period in the greenhouse and after harvest. The prevalent symptom in most potted plants is flower infection but the fungus also infects wounds (J.P. Wubben, unpubl.). On cyclamen, naturally senescing leaves are readily infected, eventually leading to plant death (Köhl et al., 2000). Pelargonium flowers are not very susceptible immediately after opening, but susceptibility rapidly increases with age (Sirjusingh and Sutton, 1996). In some crops, such as pelargonium, stock plants are infected and cuttings taken from these plants carry the fungus. This may lead to early plant death of the rooted cuttings. *Exacum affine* (Persian violet, Gentianaceae) may suffer from *B. cinerea* cankers near the base of the stem, which may kill the entire plant within 3-5 days (Ploetz and Engelhard, 1979). Leaves and stems are also affected in some plant species.

3. Factors that influence *B. cinerea*-incited epidemics in greenhouse crops

3.1. Greenhouse climate

B. cinerea can thrive under a range of temperatures between 2 and 30°C (Elad and Yunis, 1993; Yunis et al., 1994). The optimum temperatures for the different growth phases are summarised by Jarvis (1992) and range from 12–30°C. *B. cinerea* will therefore always be a potential threat in greenhouse crops. Spore dispersal is stimulated by rising or falling humidity, and therefore atmospheres of quite constant humidity should be the objective. Temperature and relative humidity (RH) both have a direct effect on *B. cinerea* epidemics, but in greenhouses the effect of climate generally constitutes a combined effect of the two. Aeration and heating regimes greatly influence RH and leaf wetness, and thus these factors should not be considered separately. Vapour pressure deficit (VPD) is independent of temperature and is therefore a better variable to work with than RH.

There are different climates inside heated versus non-heated greenhouses. In the latter in warm areas, temperatures inside may range from quite cold during the night (especially in winter), to over 30°C during the day. Irradiative cooling during cold nights causes the plant temperature to decline below the air temperature, resulting in dew presence until late morning on colder plant parts. In tomato, for example, fruit temperature is much lower than air temperature for most of the morning during winter. *B. cinerea* causes ghost spot symptoms on fruits in these situations (Verhoeff, 1974) and can readily infect leaves. Heating may be used to prevent *B. cinerea* infection by preventing dew formation. The heating system is usually turned up before sunrise so that plant parts are already warmer than air temperature before sunrise and thus leaf wetness can be avoided; infection of fruits and leaves is thus largely prevented.

Direct stem infection in tomato and cucumber is not influenced by RH, probably because infection takes place through wounds which provide the fungus with sufficient moisture (Wilson, 1963; Eden et al., 1996; O'Neill et al., 1997). However, wounded stem pieces remained susceptible for a longer period when maintained in a low VPD environment (< 0.2 kPa) than those maintained at a high VPD; incubation at the lower VPD increased the intensity of sporulation (O'Neill et al., 1997). Increasing temperature from 15 to 20 and 25°C reduced stem infection in tomato (Eden et al., 1996). In non-heated greenhouses, however, the rate of disease progress in tomato along petioles to the nodes was increased with increase of temperature between 5 and 30°C and with high VPD (Shtienberg et al., 1998). The two important parameters associated with outbreaks of *B. cinerea*-incited epidemics in vegetables in non-heated greenhouses were duration of leaf wetness (threshold 7 h per day) and duration of night temperatures between 9 and 21°C (threshold 9.5 h per day) (Elad et al., 1992; Yunis et al., 1994).

In heated greenhouses, growers use many different heating/ventilating regimes, e.g. heat the greenhouse and simultaneously open the windows so that water vapour is released. Several regimes have been tested for their effect on grey mould epidemics in vegetable crops. Winspear et al. (1970), working with tomato, compared constant temperature of 20°C with a regime of 20°C during the day and

13°C during the night, both under uncontrolled humidity conditions and at 75 or 90% RH. Almost no fruits were infected in plants grown at constant 20°C, but infection occurred in fruits from plants grown at lower night temperatures. In the low night temperature regime, the low humidity setting reduced disease. Morgan (1984) compared three climate settings in tomato: night temperature 16°C, with and without ventilation (VPD 0.224 and 0.139 kPa, respectively) and night temperature 13°C with ventilation (VPD 0.123 kPa). The most severe disease was found at 16°C unventilated, reduced by 40-50% at 13°C with ventilation and again halved at 16°C when ventilated. This result indicates that ventilation at 16°C is a more effective control measure than low night temperatures. In similar studies, night temperatures from February until the end of cropping of 9°C resulted in more stem lesions in tomato than 13°C or than 16°C in February, 5°C in March and 13°C from April until the end of cropping (Morgan, 1985). The effect of cold nights is not clearly defined, in fact may be more complex, perhaps interacting with other factors that should be considered.

In cucumber, experiments were run in The Netherlands to establish the effect of two different heating and ventilation regimes on *B. cinerea* infection and subsequent plant death: 1) set point for heating at 22°C and ventilation at 26°C, and 2) set point for heating at 22°C and constant ventilation when ambient temperature was above 10°C. The first regime resulted in a slightly warmer and more humid (lower VPD) climate than the second regime. It was found that in the warm/humid climate infection of aborting fruits was enhanced compared to the normal/dry climate, but stem infection was similar in both regimes. Surprisingly, plant death was strongly enhanced in the normal/dry regime. Yield was not influenced by the stem lesions, only by the number of dead plants. This has led to the recommendation to retain humidity in the greenhouse when stem lesions occur, in order to prevent plant death (Dik and De Koning, 1996).

In strawberry, growing the crop in non-heated plastic tunnels reduced disease by c. 90% compared to the field crop. This was largely explained by the 60% reduction in days with leaf wetness in the tunnels (Xiao et al., 2001).

In flower crops, grey mould may be completely prevented during the production phase by controlling RH. In the canopy of gerbera crops a mean RH between 85 and 95% resulted in high incidence of airborne conidia of *B. cinerea*. Petal spots mainly occurred under these conditions when the temperature was below 20°C. Higher temperatures or lower humidity gave much lower incidence of petal spots (Salinas et al., 1989). Kerssies (1994) showed that the incidence of spots on gerbera flowers was positively correlated with mean RH preceding the harvest period and negatively correlated with external radiation. For roses it was found that RH, external radiation and spore density in glasshouses during the period before harvest are important variables in regulating the number of lesions during storage and transport (Kerssies et al., 1994). Williamson et al. (1995) showed that infection of rose petals takes place in the complete absence of water droplets when the RH is maintained at, or above, 94%. Research on greenhouse climate management has focussed on reducing disease and some work is now directed to influencing the susceptibility of the crop by climate. For the susceptibility of roses to post-harvest infection by *B. cinerea*, a linear correlation was found with air velocity during 5 weeks before harvest

(Hammer and Evensen, 1996) and a negative correlation was found with VPD during the cropping period 5 weeks before harvest (Marois et al., 1988).

In production of some potted plants temperature is often used to limit plant height and this might influence the occurrence of grey mould. However, in poinsettia, no effect was found of temperature regimes of day/night temperatures 17/17°C, 17/10°C or 10/10°C (Sammons et al., 1982). Pritchard et al. (1996) found no effect of the difference between day and night temperatures, but found an increase in disease with increase of day or night temperature. In pelargonium, experiments on temperature and wetness periods showed that wetness of > 4 h should be avoided (Sirjusingh and Sutton, 1996). Forced heated air treatment significantly reduced the incidence of stem blight and sporulation (Hausbeck et al., 1996).

The use of heating to prevent disease is effective in several crops such as tomato, rose and potted plants, but it requires high energy input which makes it costly and not sustainable. Heating regimes for tomato and flower crops are at least partly aimed at prevention of *B. cinerea* infection. The minimum set point temperature is usually 30°C in the movable heating pipe, providing constant heating at the height of the crop where pruning wounds or flower heads are present and the fungus is most likely to infect the plant. Growers have started to use horizontal energy screens at the top of the greenhouses which are closed during the night to prevent loss of heat. Growers are sometimes reluctant to use this method because it may promote grey mould, but this outcome has not been found in experiments in The Netherlands (Dik and Elad, 1999). Knowledge about the interaction between climate, host and pathogen can be used not only to develop climate regimes, but also for disease warning systems (Chapter 18). Simple measures can be taken to avoid a stimulating effect of climatic conditions on *B. cinerea* epidemics. For example, in sweet basil it is advised not to harvest during rain events (Sharabani et al., 1999).

3.2. Light

Light (intensity and wavelength) is an important factor to consider in greenhouse crop protection. Plastic and glass are quite different in their irradiation transmittance properties. Furthermore, glass is easier to clean and generally more durable than plastic. Light, especially near UV (nUV) light, increases sporulation in *B. cinerea*, both on agar plates and on different crops. About a 54-fold increase in sporulation was found when colonies were irradiated with nUV light plus white light, compared to white light only when testing 83 isolates taken from primula plants (West et al., 2000). Different types of polyethylene used for tunnels influence the sporulation of *B. cinerea* (Reuveni et al., 1989). With polyethylene films that absorbed nUV light, spore production remained < 0.05% of the control for several weeks after inoculation of tomato (Nicot et al., 1996). In a field trial in polyethylene tunnels the incidence of infection of primula and strawberries over two seasons was reduced by c. 50 and 26%, respectively, with nUV blocking film (up to 405 nm) compared to a standard film (West et al., 2000). An effect was also found using spectrally modified polyethylene films on the development of *B. cinerea* in greenhouse-grown tomato plants (Reuveni and Raviv, 1992). UV-absorbing polyvinyl chloride film

reduced grey mould incidence in cucumbers (Honda et al., 1977). However, the direct effect of light on the fungus can be counteracted by effects on the host or on the climate in the greenhouse. Grey mould in strawberry fruits was more severe under coloured polyethylene covers than under clear polyethylene or glass cloches, despite the reduced light transmittance, especially at wavelengths below 500-600 nm, probably due to the decreased VPD and more moderate temperatures found under coloured covers compared to clear plastic or glass (Jordan and Hunter, 1972). Vakalounakis (1992) compared infrared-absorbing cover to commonly used agricultural polyethylene cover and found a 40-50% reduction in tomato grey mould, due to lower mean RH and higher night temperatures. Elad (1997) also showed an effect of the far-red region of the light spectrum on the production of conidia by field isolates of *B. cinerea*. A green pigmented polyethylene film reduced spore production and infection in commercial greenhouses by 35-75%. However, some field isolates of *B. cinerea* sporulated in the dark, and clearly were not affected by light quality. Elad (1997) suggested that the effect of disease suppression under light-filtering polyethylene covers was obtained by induced resistance.

A recent development in Northern Europe is to artificially light greenhouse vegetable crops, the purpose being to start the growing season during the short-day winter period. Yields are increased in cucumber, but indications have been found that some diseases, including *B. cinerea*, are stimulated in illuminated crops. This might be due to several factors: direct effect of the light on the fungus, an effect on temperature and RH, or an effect on vigour of the host. More research on this subject is justified.

3.3. Carbon dioxide enrichment

Enrichment of greenhouse atmospheres with carbon dioxide is common practice in commercial greenhouses, usually combined with decreased ventilation to attain the target CO₂ levels. *B. cinerea* is inhibited by carbon dioxide levels of 4-5% (Svircev et al., 1984; Elad, 1993), but unfortunately these levels are not reached in normal commercial greenhouses. Decreased ventilation and CO₂ enrichment may enhance foliage density and humidity, thus stimulating *B. cinerea*, especially when CO₂ burners are used which produce additional water vapour.

3.4. Sanitation

The aim of sanitation measures is to remove *B. cinerea* inoculum from the greenhouse. Between cropping periods, it is important to remove all plant debris and thoroughly clean the greenhouse and all the materials retained within it. Growers often place young plants in between the old crop, but this is a high-risk strategy in terms of crop protection. Although grey mould is not common on young plants, spores from the old crop may land on the young plants, germinate and infect those plants later.

In all greenhouse crops, it is advised to remove *B. cinerea*-infected plants or plant debris immediately from the greenhouse within plastic bags, to prevent further progression of the fungus into the plant (for example from aborting cucumber fruits into the stem) and the build-up of inoculum. In Israel, debris left

during the warm summer was found to be a potential source of initial inoculum the following season, thus emphasizing the importance of sanitation inside and outside greenhouses (Yunis and Elad, 1989). Although sanitation measures should always be taken, in themselves they are not necessarily sufficient. The overall effect of sanitation measures is variable, as will be illustrated in several cases considered below.

In non-heated greenhouses, stem infection in tomato can largely be prevented by removing infected leaves, thus preventing growth along the petiole into the stem; in Israel, this was as effective as weekly fungicide applications (Shtienberg et al., 1998). During tomato production, pruned leaves can either be removed directly from the greenhouse or left on the floor. This latter practice is aimed at allowing natural enemies of pests, such as *Encarsia formosa* which is used against white fly, to return to the plant. However, on the picked and decaying leaves *B. cinerea* sporulates profusely and thus the infection pressure may be increased significantly. *B. cinerea* conidial counts decreased in greenhouses in which pruned leaves were removed immediately compared to greenhouses in which the leaves were left on the floor in several experiments (Figure 1), but this did not always result in lower disease incidence (A.J. Dik, unpubl.).

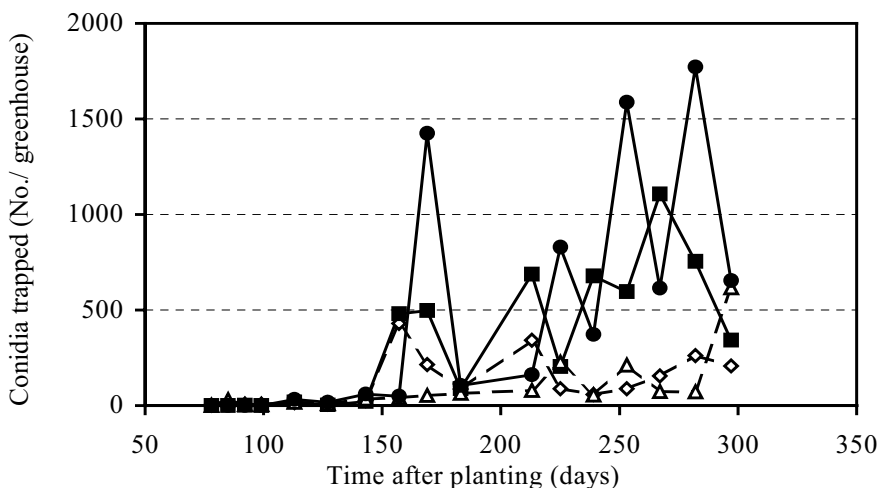


Figure 1. The number of conidia trapped during 24 h in two greenhouses in which the leaves were left on the floor after pruning (black symbols) and two greenhouses in which the leaves were removed from the greenhouse immediately after pruning (open symbols)

In cucumber, removing the dead leaves at the base of the stem once or twice per crop reduced *B. cinerea* stem infection in autumn crops (A.J. Dik, unpubl.; Table 1). Since leaves were removed in alternating rows, infection pressure does not seem to

be the cause of the decrease. More likely, the dead leaves serve as a point of entry for the fungus, which then grows through the petiole into the stem.

Plaut and Berger (1981) found that the rate of increase of *B. cinerea* blight in greenhouse-grown begonia was largest at the lowest inoculum dose, resulting in similar final disease severity as at higher inoculum doses. In strawberry, leaf sanitation reduced disease incidence, but fruit sanitation had no effect (Mertely et al., 2000). The effect of sanitation depends on whether spore density is limiting disease, or other factors are more important. In gerbera it was shown that development of petal spots on flowers was not correlated with the number of conidia in the greenhouse (Keressies, 1994). Other conditions such as RH and external radiation appear to be more important factors.

Table 1. The effect of removing dead lower leaves^a of cucumber on *B. cinerea* stem infection and plant death in autumn 1995 and 1996 in The Netherlands

Treatment	1995		1996		
	Stem Lesions (Number/plant)			Dead plants (%)	
	48 DAP ^b	54 DAP	62 DAP	62 DAP	69 DAP
Untreated	0.85 a ^c	1.50 a	3.23 a	38.3 a	80.0 a
Dead leaves removed	0.30 b	0.67 b	1.38 b	20.0 b	35.0 b

^a De-leafing was done on 7 plots of one row of 15 plants and not in 7 other plots in the same greenhouse.

^b DAP=days after planting.

^c Numbers within one column followed by a common letter are not significantly different at $P \leq 0.05$.

Hausbeck and Pennypacker (1991) showed that in pelargonium crops, the concentration of airborne *B. cinerea* conidia peaked with each grower activity. Therefore this aspect should be considered when deciding whether or not to enter the crop, though greenhouse crops are generally labour-intensive.

3.5. Cultivar

No substantial grey mould resistance has been bred into greenhouse crops so far, but there are significant differences in susceptibility to grey mould in both vegetable and ornamental crops. In strawberry, large differences were found between disease incidences in different cultivars (Legard et al., 2000; Xiao et al., 2001). In cucumber, there are indications that cultivars resistant to powdery mildew are more susceptible to *B. cinerea*, as well as to *Didymella bryoniae*. Some pelargonium genotypes are less susceptible than others (Uchneat et al., 1999). In rose and gerbera, differences have also been found, but no complete dataset has been developed. Some differences in susceptibility may be attributed to different plant architecture and canopy density.

B. cinerea resistance is often quantitative and related to different loci (genes) in the host. Breeding for resistance requires good quantitative assays to determine the susceptibility of the different cultivars. To complicate matters, it has been shown for roses, for instance, that the susceptibility of a cultivar and the differences between different cultivars can vary among sampling dates (Hammer and Evensen, 1994). In practice growers choose cultivars for their market value and end-user preference and

not for their decreased susceptibility to diseases. However, especially for flower crops, *B. cinerea* resistance is becoming more important because trading companies tend not to buy cultivars which have been shown to cause problems at the consumer level. Breeding companies are reluctant to release data on the susceptibility of their cultivars, which makes it more difficult to base a choice of cultivar on these aspects. However, it is possible that innovative molecular means may result in *B. cinerea*-resistant cultivars in the future (Chapter 20).

3.6. Plant spacing

Plant spacing influences the microclimate in the crop and the extent of direct contact between plants. In vegetables, the number of stems retained per plant influences the density of the crop. In cucumber, more grey mould occurred on fruits in a crop with two stems per plant compared with another with one stem per plant (Elad and Shtienberg, 1995). In *Exacum affine*, grey mould was increased on all floral parts in crowded plants compared to more widely spaced plants (Trolinger and Strider, 1984). In densely planted strawberries, grey mould incidence was higher than in widely planted crops, but yield was also higher in the dense crop (Legard et al., 2000). Our own results in cyclamen and saintpaulia are similar (Table 2).

Table 2. The effect of plant spacing on flower infection by *Botrytis cinerea* and rate of dead plants for saintpaulia and cyclamen

Plant spacing	Saintpaulia	Cyclamen
	Infected flowers (%)	Dead plants (%)
Normal	7.46 a	13.5 a
Narrow	16.81 b	40.6 b

Plants were assessed after a 10-week cropping period, followed by transport simulation (7 d at 15°C and 70% RH).

Numbers within one column followed by a common letter are not significantly different at $P \leq 0.05$.

3.7. Cropping methods

In vegetable crops, pruning and training methods alter the microclimate in the crop and also change the fruit:foliage ratio (i.e. the sink:source ratio). In tomato, restricting the source by shading the plants led to a higher growth rate of *B. cinerea* lesions, whereas restricting the sink by fruit reduction led to slower lesion growth rate (Shtienberg et al., 1998). Especially in cucurbits and eggplants, fruits are commonly pruned to a certain maximum in order to prevent over-burdening of the plants.

In tomato, the leaves at the height of the ripening truss are manually removed. The timing and frequency, e.g. three leaf layers every week vs. six leaves every 2 weeks, influences disease incidence. More frequent leaf removal done early in the morning reduces stem infection (A.J. Dik, unpubl.). The method of leaf removal is also important. Pruning of the leaves manually gives larger, more ragged wounds than removing leaves with a knife or scissors; the smaller wounds are much less susceptible to *B. cinerea* and heal quicker than the bigger wounds (Verhoeff, 1968).

Dutch growers now generally cut the leaves, especially in periods of high grey mould risk. Pruning makes the microclimate in the canopy less conducive to *B. cinerea*, but causes wounds which may be infected by *B. cinerea*. Pruning wounds are less likely to become infected by *B. cinerea* when they are cut close to the stem instead of leaving a small fragment of the petiole on the stem (Verhoeff, 1967, 1968).

3.8. Fertilizer

Fertilizer rates in soil-grown crops and the composition of the nutrient solution used in substrate-grown crops both influence host susceptibility to grey mould. High nitrogen rates enhance plant growth and foliage density. In general, increasing the nitrogen level is believed to result in increased susceptibility to *B. cinerea*. However, results are sometimes contradictory. Nitrogen supplied at 1.5, 3.8 and 6.0 g/m² resulted in a quadratic increase of grey mould in chrysanthemum flowers (Hobbs and Waters, 1964). In contrast, Verhoeff (1968) found that susceptibility of soil-grown tomatoes increased with decreasing nitrogen level in the soil. Hoffland et al. (1999) found a linear correlation between leaf C:N ratio and the susceptibility of tomato to *B. cinerea*. They attributed this to levels of available soluble carbohydrates. In contrast, a decrease in lesion growth rate was found in tomato in the presence of excessive assimilates (Shtienberg et al., 1998).

Calcium is generally associated with reduced susceptibility to *B. cinerea* because it plays a role in the integrity of the cell wall and increases resistance to enzymatic degradation by *B. cinerea* (Chapter 7; Volpin and Elad, 1991). In tomato, a low Ca:K ratio increases susceptibility to *B. cinerea* (Stall et al., 1965) and calcium nutrition reduced grey mould (Elad and Volpin, 1993). In roses grown in nutrient solutions to which Ca²⁺ was added, the Ca²⁺ content in the plant was raised and post-harvest botrytis blight severity was strongly reduced (Volpin and Elad, 1991). This was confirmed by Baas et al. (2000) and Bar-Tal et al. (2001) and similar results were found for miniature-potted roses (Starkey and Pedersen, 1997) in which grey mould was negatively correlated to Ca²⁺ concentration in the flowers. However, the presence of high levels of Ca²⁺ in the soil or nutrient solution does not ensure elevated Ca²⁺ levels in the plant. The passive uptake of Ca²⁺ is markedly influenced by transpiration rates of the plant, which are influenced by RH. In cucumber, the reduction of grey mould incidence by Ca²⁺ nutrition was clearer in low, rather than in high RH conditions (Yunis et al., 1990).

3.9. Irrigation regime and method

The irrigation regime in tomato has been found to influence the occurrence of grey mould stem infections. When irrigation was run from sunrise to sunset, disease incidence was much higher than in a regime with the same amount of irrigation, delivered from 1-2 h after sunrise until 1-2 h before sunset (Table 3). The effect was enhanced by high temperature (A.J. Dik and H.A.J.M. van Gulp, unpubl.).

In poinsettia flowers, a tendency was seen that *B. cinerea* infection increased when plants were overhead-irrigated compared to an ebb and flow irrigation system but differences were not significant (J.P. Wubben, unpubl.). The RH in the canopy

was higher in the crop that was irrigated from above, and especially higher RH values (more than 93%) occurred regularly in the overhead-irrigated plants and rarely in plants on ebb and flow systems. Overhead irrigation also caused more grey mould infection in *Exacum affine* compared to sub-irrigation (Trolinger and Strider, 1984).

Table 3. The effect of irrigation of tomato during a short (2 h after sunrise until 2 h before sunset) or long (sunrise until sunset) period with the same amount of water on the incidence of *Botrytis cinerea* stem lesions and the percentage dead plants, at normal and elevated temperatures (T)

Irrigation period per day	<i>B. cinerea</i> stem lesions (number/plant)		Dead plants (%)	
	Normal T	High T	Normal T	High T
Long	1.70 a	3.2 a	13.3 a	25.0 a
Short	1.15 a	1.8 b	5.0 b	7.9 b

Numbers within one column followed by a common letter are not significantly different at $P \leq 0.05$.

4. Damage relationships

As stated above, *B. cinerea* may be very destructive in most greenhouse crops when conditions are conducive for infection. In vegetables, yield loss occurs in the form of reduction of harvested product. In tomato, infected fruits may abort, but in cucumber infection of fruits mostly takes place on aborted fruits. Yield loss in vegetables is mostly correlated with plant death and not with infection itself (A.J. Dik, unpubl.), but not much data is available. In ornamental crops, yield loss can be total because infected potted plants and cut flowers are rejected at auctions. The problem with several ornamental crops is that no disease is found in the greenhouses, but symptoms become visible in the post-harvest phase due to more conducive conditions for *B. cinerea* during cold-chain marketing.

5. Conclusions

Many factors in greenhouse production systems can be used to reduce the impact of *B. cinerea* epidemics. These cultural control methods are aimed at influencing the fungus, the host and the environment. However, most measures taken by growers are based on optimum production and are generally not taken for prevention of diseases. For example, in dense crops grey mould incidence may be higher, but so is yield per unit area, and, irrigating tomato for a longer period during the day may increase *B. cinerea* stem infection, but also increases yield. Therefore, it is difficult to expect growers to preventively decide on measures that will result in sub-optimum yields. As chemical control becomes less readily available, this approach may change. In our opinion, integration of all the factors mentioned in this chapter with adequate biological and if necessary, chemical control (see also Chapter 18), should give good control of *B. cinerea* in most greenhouse crops.

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CHAPTER 18

RATIONAL MANAGEMENT OF *BOTRYTIS*-INCITED DISEASES: INTEGRATION OF CONTROL MEASURES AND USE OF WARNING SYSTEMS

Dani Shtienberg

Department of Plant Pathology and Weed Sciences, ARO, The Volcani Center, P.O. Box 6,
Bet Dagan 50250, Israel

Abstract. *Botrytis*-incited diseases are currently managed by routine application of fungicides. This strategy has become increasingly unacceptable and alternative approaches for rational disease suppression are urgently needed. This goal can be achieved either by applying fungicides only when needed, thus eliminating unnecessary sprays, or by integrating chemical and non-chemical measures so that the non-chemical measures compensate for the reduction in fungicide use. In this chapter, the methodology for rational management of *Botrytis*-incited diseases is presented, and case studies are described. The decision to omit unnecessary fungicide sprays may be guided by warning systems, several of which have been developed for *Botrytis* management over the years. All systems attempted to recognize conditions highly conducive to spore germination and host penetration, and to schedule fungicide applications accordingly. The use of biocontrol and implementation of cultural practices as stand-alone measures for *Botrytis* management are not always sufficient, but integration of these measures with chemical control has proved effective. In most cases experimental use of these approaches enabled a significant reduction in fungicide use without increasing the risks of uncontrolled *Botrytis* outbreaks. It was concluded that rational management of *Botrytis*-incited disease is feasible, and that within a few years, *Botrytis* will be primarily managed by non-chemical measures supported by a few properly timed fungicide applications.

1. Introduction

Adequate management of plant diseases is a prerequisite for stable and profitable production of food and fibers. A few *Botrytis* spp. cause severe losses in many economically important crops. It is difficult to evaluate the damage caused; however, in many crops, depending on prevailing weather, economic losses exceed 50%, and total destruction of a crop by uncontrolled epidemics is not uncommon. The control of *Botrytis* spp. is extremely difficult because of the ability of these pathogens to attack crops at almost any stage of growth, and to affect all the plant parts, including cotyledons, leaves, stems, flowers and fruits (Gullino, 1992). For the suppression of *Botrytis*-incited diseases, growers rely heavily on routine application of fungicides, and often spray weekly, whether or not it is necessary. However, such routine application of fungicides has become increasingly unacceptable, because of

environmental, public health, economic, and farm-management considerations; all dictate that fungicide use should be minimized. This can be achieved either by applying fungicides only when needed, and/or by integrating chemical and non-chemical measures so that the non-chemical measures compensate for the reduction in fungicide use. In this chapter, the methodology to be used for rational management of *Botrytis*-incited diseases is presented and case studies of its implementation are described. Finally, the possibility of implementing these concepts for large-scale management of *Botrytis*-incited diseases is discussed.

2. Reduction of fungicide use by optimal timing of spraying

Application of disease management measures when they are not needed is inefficient at best, because it results in unnecessary costs to growers, consumers, and environment (Fry, 1982). The complex interactions between the host, the pathogen and the environment force growers, extension personnel, and farming consultants to seek assistance in their decision-making procedures. One way to decide if or when to apply fungicides is to use forecasting or warning systems that predict the occurrence and/or intensity of a disease outbreak according to weather, host and/or pathogen data. Madden and Ellis (1988) distinguished between fundamental and empirical warning systems. The former are developed from experiments in the laboratory, controlled-environment chambers, greenhouses or fields and address one or more aspects of the host-parasite relationships as influenced by the environment. The latter are developed from the observation and analysis of current and historical data on disease level and biotic and abiotic factors. Newly developed warning systems should be evaluated in field experiments, by comparing their recommendations with the conventional management practice (de Kraker et al., 2000).

Fundamental understanding of the host-pathogen interactions as affected by the environment is required for development of disease warning systems. Such knowledge was accumulated over the years and the first attempts for developing warning systems for management of *Botrytis*-incited diseases were made in the 1960s-1970s (Jarvis, 1977; Coley-Smith et al., 1980). Jarvis (1964) attempted to forecast strawberry and raspberry infection by *B. cinerea* but considered that forecasting was difficult because of the complex effect of many meteorological, edaphic and biotic factors on the plant-pathogen complex. Jarvis summarized the early attempts to forecast as unsuccessful since it could not help in the correct timing of prophylactic fungicide applications (Jarvis, 1977). Nevertheless, later, warning systems have been developed for several pathosystems, mostly in the late 1990s. In this chapter, the relevant pathosystem and the common management practices used are presented first, followed by a short explanation of the concepts used in developing the relevant warning systems. Finally, descriptions of the procedures employed for their evaluation and some results are included.

2.1. The infection model: A warning system for management of *B. cinerea* in vineyards

Botrytis bunch rot, caused by *B. cinerea*, is an important disease of grapes (Chapter 14). Infection of ripe berries is the most common and destructive phase of this disease. Infected berries first appear soft and watery. Those of white cultivars become brown and shrivelled, whereas those of purple cultivars develop a reddish colour. Under high relative humidity and moisture, infected berries usually become covered with a grey growth of fungus mycelium. The rotted berries gradually shrivel and drop to the ground as hard 'mummies'. The fungus may also cause a blossom blight that can result in significant early-season crop loss. In Chile, it is common for growers of table grape to make 8-10 fungicide applications a year to control *Botrytis* bunch rot (Broome et al., 1995).

Broome et al. (1995) developed a model, which calculates the probability for berry infection by *B. cinerea* on the basis of the interaction between temperature and duration of wetness. The model was incorporated into an in-field environmental monitoring station that measures temperature, hours of free moisture, rainfall, and relative humidity every 15 min, and records an hourly average of each. The model initiates calculation of a disease index whenever leaf wetness is detected. To determine the relative risk of an infection period, logit values computed by the model are compared with the values of parameters that specify infection risk. A spray is advised as soon as either moderate or high risk is indicated. The calculation of a logit value is stopped if a dry period longer than 4 hours is recorded. If a new wet period begins after less than 4 h of dryness then the recording of accumulated hours of wetness is resumed, although the model does indicate that a split wet period occurred.

The model was evaluated in commercial table grape vineyards in Chile in 1991-1992 and 1992-1993 (Broome et al., 1995). Treatments consisted of plots maintained according to the conventional *Botrytis* management practice or according to the infection model. Over the two seasons, the fungicide programme based on model warnings proved to be as effective in controlling the disease as the conventional practice. However, vines treated as recommended by the infection model received 43 to 67% and 0 to 100% fewer applications of fungicides than those treated according to the standard practice, in the two seasons, respectively.

2.2. BoWaS: a warning system for management of *B. elliptica* in lily

Botrytis leaf blight, caused by *B. elliptica*, is an important foliar disease in lily (Chapter 15). The disease initially appears as oval shaped, reddish brown to tan spots, often with purple margins, on the leaves. Flowers and flower buds may be covered with unsightly small brown freckles. If the disease is uncontrolled the entire plants are destroyed as the spots grow together. This disease is commonly called 'fire' because the plants can become black and shrivelled as if burnt by fire. The soft tissues of flowers and damaged or decaying plant material are often covered in grey

fuzzy fungal spore masses. As the disease affects a high value crop and can be controlled only with protective fungicides, growers in The Netherlands are extremely cautious in their control strategy, which normally involves weekly fungicide sprays that deliver an average of 20–30 kg of active substance per ha per season.

A warning system called BoWaS was developed in The Netherlands for *Botrytis* leaf blight suppression (Bastiaansen et al., 1997). BoWaS is based on the data of Doss et al. (1984) on the effect of temperature and leaf wetness on infection of lily by *B. elliptica*. They determined the percentage of lily plants that become infected after inoculation with conidia of *B. elliptica* under various temperature and humidity conditions, and used linear regressions to extrapolate their data to other temperatures and durations of leaf wetness. BoWaS is operated on the basis of specific 5-day weather forecasts provided by MeteoConsult (Wageningen); the predicted leaf wetness and leaf temperature data are used to calculate the percentages of plants that are expected to be infected by *B. elliptica*. Recommendations to spray are generated when at least 14 days have passed since the previous spraying, and when the percentage of plants expected to be infected exceeds an arbitrary threshold level that depends on the susceptibility of the lily cultivar involved. Between 1994 and 1997 BoWaS was tested on several lily cultivars grown in different locations in The Netherlands. A 30 to 80% reduction in the applications of active ingredients could be achieved, depending on the susceptibility of the cultivar and the seasonal conditions (Van den Ende et al., 2000).

2.3. BLIGHT-ALERT: a warning system for management of *B. squamosa* in onion

Botrytis leaf blight, caused by *B. squamosa*, occurs in many of the onion growing areas of the world (Chapter 15). The pathogen causes leaf spots (lesions) and maceration of leaf tissue that result in leaf dieback and blighting. The lesions are whitish in colour, from 1–5 mm in length, and most are surrounded by greenish-white halos that appear water-soaked when first formed. The centres of the lesions usually become sunken and straw coloured, and may develop a characteristic longitudinal slit. Under favourable environmental conditions (high rainfall, extended periods of leaf wetness, high relative humidity and moderately warm temperatures), *Botrytis* leaf blight can result in reduced onion bulb growth and yield. Severely infected fields often take on a yellowish cast as a result of coalescing lesions and tip dieback. In New York State, USA, the disease was usually controlled through weekly applications of protectant fungicides (Vincelli and Lorbeer, 1989).

A weather-based predictive system named BLIGHT-ALERT was developed in New York for timing of applications of protectant fungicides to control *Botrytis* leaf blight of onion after the disease level had passed the threshold of 1 lesion/leaf and after the first fungicide spray had been applied. The system incorporates two components to forecast periods of likely infection by *B. squamosa*. The first is a model called the inoculum production index (*IPI*), which forecasts the production of inoculum according to temperature, relative humidity and crop development. The

IPI indicates the likelihood of sporulation occurring during the next 24-h period (Vincelli and Lorbeer, 1988a). The second component is the prediction of conditions favourable for severe infection of onion leaves by conidia of *B. squamosa*. It was found that when the forecast values of precipitation probability (*PP*) issued by the National Weather Service exceed 30%, significant development of *Botrytis* leaf blight on onion is most likely (Vincelli and Lorbeer, 1988b).

From 1987 to 1995, 45 side-by-side comparisons of BLIGHT-ALERT and commercial practices were conducted in all major onion producing areas of New York. In 1987-1993, growers averaged 5.5 fungicide applications on the basis of BLIGHT-ALERT and 7.8 fungicide applications on the basis of conventional disease control techniques, i.e., an average savings of 2.3 applications (29%). No differences in yield and quality were observed between the fields under BLIGHT-ALERT and those under conventional management. Demonstrations were continued with 11 and 12 growers during 1994 and 1995, respectively, and the results were similar to those of the earlier demonstrations, with savings averaging 1.8 fungicide applications in 1994 and 3.7 applications in 1995 (Lorbeer et al., 2001).

2.4. BOTEM: a warning system for management of *B. cinerea* in strawberry

Grey mould, caused by *B. cinerea*, is one of the most important airborne diseases of strawberry world-wide, and it can seriously reduce yield and post-harvest quality (Chapters 17 and 19). The disease begins mainly as latent infections of flower parts, which develop into rots once the fruit begins to ripen. The open flower, white bud, and senescent flower are the stages most susceptible to infection, whereas flowers at the green bud stage are relatively resistant. In the United Kingdom, grey mould is managed principally by protecting the flowers from infection by means of routine application of fungicides. This is usually effective, but under conditions particularly conducive to disease, control may be less than satisfactory (Xu et al., 2000).

A mathematical model (BOTEM) was developed in the UK to describe the influence of weather on flower infection in strawberry. The model is driven by weather variables including daytime vapour pressure deficit (VPD) and night-time temperature. During the day, when most conidia are released and dispersed, the limiting factor for conidial survival is atmospheric moisture content, measured as VPD. The higher the VPD, the less favourable are the conditions for conidial survival. In the UK atmospheric moisture content is generally very high, near saturation on spring nights but the temperature is generally low, often below 10°C. Consequently, the limiting factor for infection is likely to be temperature. The higher the temperature (up to 20°C), the higher the incidence of infection (Xu et al., 2000). The initial action threshold used by the system is reached when the predicted fruit infection rate increases to 10% or more. BOTEM was compared with a conventional management programme on strawberry crops in the UK. Use of the system enabled a reduction of fungicide inputs by up to 60%, without impairing control efficacy (Berrie et al., 2002).

3. Reduction of fungicide use by integration of chemical and non-chemical measures

Although fungicides are the primary means used for suppression of *Botrytis* spp., alternative control measures are often proposed and tested, especially in the case of protected crops (Gullino, 1992). Cultural, physiological and biocontrol measures are among these alternative methods (Elad and Shtienberg, 1995). In many instances, the efficacy of non-chemical measures, when applied alone, has been inadequate and inconsistent. However, the simultaneous implementation of non-chemical and chemical measures may enable the use of potentially environmentally harmful chemical measures to be minimized, by reducing either the amounts applied or the number of applications. Joint use of two or more control measures is advantageous for several other reasons. It may achieve acceptable disease suppression in cases where single measures are inadequate. It may increase the effective life span of a specific control measure, for example, by reducing the probability of the development of populations of fungicide-resistant pathogen. Finally, integration of control measures may reduce the inconsistency and lack of repeatability experienced with the sole application of some control measures (e.g., biocontrol, Chapter 13).

3.1. Integration of chemical and cultural measures

3.1.1. Suppression of *B. cinerea* in sweet basil

Sweet basil is an herbaceous annual plant used as a spice in the food industry and for medicinal purposes. In Israel, basil is grown in walk-in tunnels or greenhouses. Seedlings are planted in October in containers or directly in soil. When the plants are 40 to 60 cm in height, the upper part of the canopy is harvested manually with sharp knives. Harvesting breaks the apical dominance, and lateral axillary buds start to grow and develop lateral shoots that will be harvested subsequently. A crop is harvested typically every 3 to 5 weeks, depending mainly on temperature and solar radiation. Basil plants are highly susceptible to *B. cinerea* (Chapter 17), and it has been observed in commercial greenhouses that infection is initiated on the surface of stem wounds that are made during harvest. The pathogen then progresses along the stem, killing leaves and secondary buds; when it reaches the main stem, the entire plant dies. Healthy leaves that touch diseased tissues of adjacent plants may also become infected. *B. cinerea* also develops post-harvest, and one infected basil leaf may lead to the loss of an entire 1-kg package during storage, shipment and marketing. Basil growers in Israel were accustomed to spraying their crops against *B. cinerea* up to 5 to 10 times during a growing season but, nevertheless, it was not uncommon for severe grey mould epidemics to develop even in intensively managed crops (Sharabani et al., 1999).

A study of the epidemiology of the disease and of host-pathogen relationships led to the development of a management strategy based on integration of cultural and chemical measures. It was found that disease outbreaks coincided with harvests that were carried out during rain events. However, since the cut ends of stems heal within 24 h after harvest, avoidance of harvesting during rain events and application

of one fungicidal spray soon after harvest enabled the growers to protect the plants from infection (Sharabani et al., 1999). Management of basil crops according to these concepts not only improved disease management in commercial greenhouses, but also lowered the risks of contamination of the marketable products with fungicide residues.

3.1.2. Suppression of *B. cinerea* in strawberry

As indicated above (Sect. 2.4), grey mould (caused by *B. cinerea*) is an important disease of strawberries world-wide (Chapter 17). In Florida, *Botrytis* fruit rot is managed primarily by weekly applications of protectant fungicides throughout the season. Legard et al. (2002) recently examined the possibilities of planting less susceptible cultivars, using field sanitation to reduce inoculum, and manipulating the canopy microclimate to produce conditions less favourable to the pathogen. Regular applications of fungicides significantly reduced the incidence of *Botrytis* but severe losses still occurred, especially on highly susceptible cultivars. Some cultural practices improved the control of *Botrytis* fruit rot but others were relatively ineffective: the removal of senescent foliage reduced the incidence of *Botrytis* fruit rot, but not as effectively as fungicides; removal of infected fruits from the fields did not reduce *Botrytis*; the effects of cultivar resistance and within-row plant spacing were significant, and more widely spaced plants had less *Botrytis* than more crowded ones. Nevertheless, the use of non-chemical measures alone did not provide adequate suppression of *Botrytis* fruit rot. Thus, Legard et al. (2002) concluded that effective control of *Botrytis* in annual winter production systems in Florida requires a combination of chemical and non-chemical methods.

3.2. Integration of chemical and biological measures

3.2.1. Suppression of *B. cinerea* in apple

Dry eye rot on apple, also known as blossom-end rot, is caused by *B. cinerea* (Chapter 14). The fungus infects the sepals of the flower during the spring and lies latent inside the tissue until fruit maturation in the autumn. It then expands into the fruit and causes the first visible disease symptoms of intensified discoloration, and finally a brown soft rot. The expansion of the rot may be delayed into the storage season (Tronsmo, 1991). In Norway the disease was managed with fungicides, but chemical control has met obstacles. Dichlofluanid or tolylfluanid, for many years the standard treatments, have failed to control the disease, and resistance to benomyl developed after a few years of use. Vinclozolin and iprodione were then adopted, but, in view of the possibility of *B. cinerea* developing resistance to these dicarboximides, the efficacy of biological and integrated biological-chemical treatments was examined from 1983 to 1987. It was found that a fungicide-resistant isolate of *Trichoderma harzianum*, P1, either alone or together with reduced dosages of the fungicide vinclozolin, controlled dry eye rot better than the fungicide-sensitive parent strain. Although the integrated control was not significantly more effective than either biological or chemical control alone, the combination of two

different methods may provide more consistent control, and reduce the risk of fungicide resistance developing in the pathogen. Also, the use of fungicide-resistant strains of the biocontrol agent is necessary as long as fungicides are used against other pathogens (Tronsmo, 1991).

3.2.2. Suppression of *B. cinerea* in vineyards

As indicated above (Sect. 2.1), *Botrytis* bunch rot, caused by *B. cinerea*, is an important disease of grapes world-wide (Chapter 14). Suppression of grey mould is usually achieved by a programme of three to six fungicidal sprays, applied to the crop between flowering and harvest. A series of 133 experiments were carried out throughout the world, between 1988 and 1994, to compare the efficacy of a biocontrol agent, *T. harzianum* T39 (commercial preparation TRICHODEX, Chapter 13) with that of a full chemical treatment. Experiments also included an integrated treatment in which *T. harzianum* T39 and fungicides (i.e. dicarboximides, benzimidazoles, anilinopyrimidines and protectants, Chapter 12) were alternated, and a reduced chemical treatment in which chemicals were used alone, but were applied only at the times when chemicals were applied in the integrated treatment (O'Neill et al., 1996). The control efficacy achieved by *T. harzianum* T39 and by the reduced chemical treatment was inferior to that achieved by the full chemical treatment, whereas the control efficacy achieved by the integrated treatment was, in general, similar to that of the full chemical treatment. The finding that the integrated treatment was more effective than the reduced chemical treatment indicates a benefit from inclusion of the biocontrol preparation in the integrated treatment. The integrated treatment offers an opportunity to reduce the number of chemical sprays by half, with no substantial reduction in disease control efficacy. Also, the integrated treatment is advantageous, not only because it reduces the risk of chemical residues remaining in the harvested fruits, but also because it reduces the selection pressure towards development of fungicide-resistant populations of the pathogen (O'Neill et al., 1996).

4. Integration of chemical and non-chemical control measures guided by a warning system

As indicated above, fungicides are the most effective means for suppression of *Botrytis* spp. when conditions are highly conducive for the pathogen. However, when conditions are only moderately conducive, non-chemical measures may provide adequate disease suppression. Accordingly, it seems logical that integrating chemical and non-chemical measures according to the guidance of a warning system would enable the further reduction of fungicide use. A conceptual framework developed for that purpose was developed and tested in vegetable greenhouses in Israel (Shtienberg and Elad, 1997). The relevant pathosystem, the principles used for developing the warning system and the results of its evaluation are described briefly below.

B. cinerea attacks leaves, stems, flowers, and fruits of tomatoes and cucumbers grown in non-heated greenhouses in Israel and other Mediterranean countries (Chapter 17). The pathogen infects primarily leaves (in tomatoes) or fruits (in both cucumbers and tomatoes), but lesions can expand to other tissues (stems), and girdling of stems by disease can result in substantial yield losses. Grey mould epidemics are a serious problem in vegetable greenhouses and are often the reason for finishing a crop earlier than planned. In general, growers use fungicides to control grey mould and commonly apply weekly sprays. The possibility of using the biocontrol agent, *T. harzianum* T39, for management of grey mould in vegetable greenhouses was evaluated in 64 experiments carried out in non-heated vegetable greenhouses during 1988 to 1993, and it was found that *T. harzianum* T39 alone was as effective as the standard chemical treatment in 70% of the trials. In some experiments (20%), however, the disease suppression achieved by *T. harzianum* T39 was significantly inferior to that by the standard chemical treatment, and in a few experiments (10%) *T. harzianum* T39 efficacy was inadequate (Shtienberg and Elad, 1997). Inconsistency and variability in control efficacy among biocontrol agents are not uncommon; nevertheless, it precludes implementing biocontrol on a large scale. Thus, attempts were made to develop a conceptual framework for integration of the biological and chemical measures in such a way as to ensure reliable disease suppression, while reducing fungicide use.

Initially, a warning system was developed to predict the relative favourability of the environmental conditions for infection by *B. cinerea*. This tool was then used as the basis for the integration of biological and chemical measures. Decisions on whether to spray the biological agent or a fungicide were based on the ecological requirements of both the pathogen and the biocontrol agent using a 4-day weather forecast provided by the Israel Weather Forecast Service. The climate prevailing outside affects the microclimate inside the greenhouses. The integrated strategy (named BOTMAN, Botrytis Manager) was implemented as follows: when slow or no disease progress was expected, no spraying was needed; at the other extreme, when an epidemic outbreak was expected, use of a chemical fungicide was recommended; in all other cases, application of *T. harzianum* T39 was recommended (Shtienberg and Elad, 1997).

BOTMAN was compared with weekly application of fungicide in 11 experiments conducted over 3 years in greenhouse-grown tomato and cucumber. Disease reduction in plots managed according to BOTMAN recommendations did not differ significantly from that observed in plots managed according to the common practice (weekly application of fungicides), but the number of fungicide sprays in the BOTMAN plots averaged 4.2 compared with 10.5 sprays in the common practice treatment. The integrated strategy led to the addition of an average of 5.9 sprays of *T. harzianum* T39.

5. Implementation of rational approaches for management of *Botrytis*-incited diseases on a large scale

Two approaches to the rational management of *Botrytis*-incited diseases are presented in this chapter: the use of warning systems for optimal timing of fungicidal sprays, and integration of chemical and non-chemical control measures. Despite the fact that the various case studies presented above were from different countries, involved different crops and aimed at management of several different *Botrytis* species, they all share several features in common. First, they were all developed for intensive crops (that is, those for which the financial investment is high but which give a high-value yield and, therefore, high profit). Although *Botrytis* spp. may also threaten extensive crops (for example, *B. cinerea* [*Botrytis* head rot] in sunflower and *B. faba* [chocolate spot] in faba bean; Chapter 16), warning systems and integrated treatments have rarely been developed for such crops. Second, the assumption underlying all the studies was that the environment is not always conducive to the development of the pathogen. Thus, the progress of *Botrytis* epidemics is characterized by occasional outbreaks of the disease and not by a steady increase in disease intensity. In most studies attempts were made to identify these outbreaks and to schedule the application of the control measures just before, or soon after, their occurrence. Third, in light of the specific requirement of *Botrytis* spp. for free moisture during spore germination and penetration, and conversely, their broad requirements for mycelia growth and sporulation (Jarvis, 1980; Chapter 2), all the studies attempted to determine when conditions would be highly conducive for these phases in the life cycle of the fungus. Lastly, as most fungicides currently available for the control of *Botrytis* spp. inhibit spore germination, and since such fungicides must be applied soon after or, preferably, just before the infection occurrence (Chapter 12), the predictions issued by most warning systems are based on future (forecast) weather, rather than on observed (past) weather.

In view of the potential damage that may be caused by uncontrolled *Botrytis* epidemics, growers of intensive crops make all possible efforts to prevent the occurrence of disease outbreaks. In fact, growers attempt to minimize the risks of a “false negative action”, i.e., they attempt to minimize the probability of omitting a management action that eventually might prove crucial for disease control. As indicated above, the aim of most studies was to determine when disease outbreaks are likely to occur and to synchronize the management efforts with these potential outbreaks. This would enable unnecessary sprays to be eliminated (thus reducing fungicide use) without affecting the efficacy of disease suppression and crop production. Analyses of the performance of the various strategies listed above suggest that most of them fulfilled these goals. For example: in vineyards managed according to the infection model, fungicide use was reduced by an average of 36% (Broome et al., 1995); in lily fields managed according to BoWaS, fungicide use was reduced by an average of 64.5% (Van den Ende et al., 2000); in onion fields managed according to BLIGHT-ALERT, fungicide use was reduced by an average of 33% (Lorbeer et al., 2001); in strawberry field managed according to BOTEM, fungicide use was reduced by an average of 50% (Berrie et al., 2002); and in tomato and cucumber greenhouses managed according to BOTMAN, fungicide use was

reduced by 60% (Shtienberg and Elad, 1997). In all of these cases yield quantity and quality were not impaired.

As demonstrated in the cited studies, experimental implementation of rational management approaches enabled a significant reduction in fungicide use without increasing the risks of uncontrolled *Botrytis* outbreaks. However, one question remains: will growers accept and use these approaches for managing *Botrytis*-incited diseases on a large scale? Unfortunately, the answer is not obvious in all cases. There are situations in which growers would prefer to retain the conventional management strategy even if this means that unnecessary sprays are applied. This issue was explicated by Van den Ende et al. (2000), who indicated that the economic benefits of using BoWas seem to be limited, because of the high value of lilies and the low costs of fungicides. The potential saving in fungicide costs gained through the use of BoWaS is not sufficient to balance out the perceived additional risks. This situation is also common on other intensive crops. For example, MacKenzie (1981), while discussing the reasons why growers in the US would not use BLITECAST, a warning system for management of potato late blight, quoted a common response of a potato grower who said, "Why should I risk \$2,000 to save perhaps \$19 or \$38 for skipping one or two fungicide applications? I spray once a week so I can sleep at night!". The outcome is disappointing because it means that excess fungicides will continue to be applied by intensive crop growers for *Botrytis* suppression, despite the existence of reliable approaches for reduced fungicide use. The situation will not change unless the basic driving forces in the system are revised. Such a change, for example, may result from consumer demand for fruits and vegetables that are treated with fungicides only when needed ("IPM fruits and vegetables"), or from strict regulations, imposed by national authorities, to eliminate fungicide residues from the marketable products. In such cases, the risks of not being able to market the product (or to market it at much lower price) would be comparable with, or even larger than, the risk associated with omitting a few sprays.

Another problem that may hinder large-scale adoption of rational *Botrytis* management approaches lies in the risks posed by the development of other pathogens that are regularly suppressed by routine application of fungicides. If the *Botrytis* warning systems fail to predict the intensification of other potentially harmful pathogens, and if the non-chemical measures used are ineffective against these pathogens, uncontrolled epidemics of other pathogens may threaten the crop. The solution is to develop integrated systems aimed at management of all important pathogens of the relevant crops. This was done, for example, in onion fields in New York where growers use BLIGHT-ALERT for *B. squamosa* management in conjunction with DOWNCAST for downy mildew management (Lorbeer et al., 2001). Similarly, the BOTEM system provides recommendations for managing both grey mould and powdery mildew (Berrie et al., 2002) and the BOTMAN system was incorporated into a comprehensive decision support system named GREENMAN (Greenhouse Manager) used for management of all foliar pathogens that threaten greenhouse-grown tomatoes and cucumbers (Elad and Shtienberg, 1997).

6. Conclusions

A large number of research projects around the world are devoted to studying various aspects of the biology, epidemiology and control of *Botrytis* spp. Thousands of articles have been published over the years in scientific and extension journals. Consequently, it was a surprise to find that only a few published papers deal with development and implementation of rational approaches for management of *Botrytis*-incited diseases. Nevertheless, the case studies presented in this chapter demonstrate that rational suppression of *Botrytis*-incited diseases is feasible. The means to be used and the conceptual basis for their implementation do not necessarily exist at present in every country and for every species of the pathogen but, nevertheless, the knowledge and working tools have been developed and proved to be effective. The attitude of growers who still predominantly rely on fungicides for *Botrytis* suppression is changing. This has already happened for a few crops in some developed countries and this tendency is growing and continues to expand rapidly. It is possible that within a few years *Botrytis*-incited disease will be managed primarily by non-chemical measures, and fungicides will be applied as a complementary measure, only when needed, as determined by approved warning systems. As a result, not only will effective control be achieved, but also the selection pressure towards development of *Botrytis* populations resistant to chemical fungicides will be reduced.

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CHAPTER 19

POST-HARVEST *BOTRYTIS* INFECTION: ETIOLOGY, DEVELOPMENT AND MANAGEMENT

Samir Droby and Amnon Lichter

Department of Postharvest Science, ARO, the Volcani Center, P.O. Box 6, Bet Dagan, 50250, Israel

Abstract. *Botrytis* is regarded as the most important post-harvest fungal pathogen that causes significant losses in fresh fruits, vegetables and ornamentals. Its ability to attack a wide range of crops in a variety of modes of infection and its ability to develop under conditions prevailing during storage, shipment and marketing make its control a challenge. Harvested crops are particularly vulnerable to *Botrytis* infection because unlike vegetative tissue harvested commodities are senescing rather than developing. Control of *Botrytis* on harvested crops has relied mainly on pre-harvest chemical fungicides for reducing inoculum density and incipient infections before harvest. Control programmes were developed specifically for each crop and largely depend on epidemiological and etiological information. The future of many of these chemicals, however, is now doubtful and their use has come under scrutiny. This is due to severe restrictions and regulations imposed especially on post-harvest chemical treatments for the majority of freshly harvested fruits and vegetables. To develop better and more efficient methods for controlling post-harvest *Botrytis* rot it is essential to understand the relationship between infection of various plant parts in the field and incidence of grey mould in storage. This relationship has still not been fully elucidated in tomato, kiwifruit, strawberry, grapes and roses. These crops are discussed in this chapter as examples for different research strategies to tackle the problem. It is concluded that control methods based on holistic strategies which incorporate modelling and prediction systems, early detection techniques, biological and physical methods, and cultural practices, should be tailored to meet the demands of each crop.

1. Introduction

Much of post-harvest losses of fresh fruits and vegetables are due to fungal and bacterial infections. These losses are serious because the value of fresh produce significantly increases while passing from the producer to the consumer's table (Eckert and Sommer, 1967). These losses are estimated at 50% or more in developing countries due to the lack of adequate handling and refrigeration facilities; while losses may be lower in developed countries. Because of the lack of precise statistics, however, it is hard to determine the extent of real losses that may vary depending on the commodity and the producing country. Post-harvest decay can be traced to infections that occur either between flowering and fruit maturity or during harvesting and subsequent handling, storage, marketing and even after

purchase by the consumer. Intensive efforts have been made to minimize these losses through better understanding of the biology and etiology of post-harvest diseases as well as developing adequate control strategies.

A number of fungi from several genera cause post-harvest decay on agricultural produce. Among these are representatives of *Botrytis*, *Penicillium*, *Mucor*, *Alternaria*, *Colletotrichum*, *Diplodia*, *Rhizopus*, *Fusarium* and *Aspergillus*. However, *B. cinerea* is regarded as the most important since it has a wide host range (Sommer, 1985). It is an important post-harvest pathogen because environmental conditions prevailing during shipment and storage facilities are favourable to its development and infection. The losses inflicted by *B. cinerea* after harvest to fruits and vegetable crops as well as ornamentals are considerable if the disease is not properly managed. Susceptibility of vegetable, fruit and ornamental crops to grey mould infection and development increases after harvest as plant tissues senesce. Factors involved in acceleration of senescence, such as ethylene, in certain crops greatly enhance susceptibility to grey mould (Chapter 10). Delaying senescence by post-harvest treatments e.g. controlled atmosphere (CA), modified atmosphere (MA), cold storage, ethylene inhibitors and gibberellin can considerably reduce susceptibility to decay (Sommer, 1985). It was demonstrated that *B. cinerea* isolated from strawberry and kiwifruit actively produce large quantities of ethylene when grown in culture medium supplemented with methionine (Altaf et al., 1997; Chapter 10). This feature could be essential for post-harvest pathogenesis of *B. cinerea*.

This chapter will provide a general overview of post-harvest rot caused by *Botrytis* in selected crops. It will touch on the great body of research done on this subject and illustrate all aspects of the *Botrytis* problem in major crop model systems. Etiology of the infection, key current strategies and alternative approaches of control will be described.

2. Etiology of post-harvest botrytis rots

While most fungal post-harvest pathogens have a restricted host range, damage caused by *B. cinerea* is widespread on many crops causing grey mould on various plant parts, including roots (e.g. flower bulbs, sweet potato), shoots (e.g. lentils, kale), leaves (basil, lettuce), flowers (e.g. rose petals) and fruits (Jarvis, 1980; Maude, 1980). The largest damage inflicted by *Botrytis* is recorded for senescing fruit tissue after harvest since it is the most vulnerable to infection and development. Vegetables and small fruit crops that are susceptible to grey mould after harvest include artichoke, apple, asparagus, bean, beet, blackberry, black-eyed pea, blueberry, broccoli, brussels sprouts, cabbage, carrot, cauliflower, celery, chives, cucumber, currant, eggplant, endive, gooseberry, grape, kale, kiwi, kohlrabi, leek, lentil, lemons, lettuce, okra, onion, parsnip, pea, peanut, pepper, pears, peaches, plums, pumpkin, quince, raspberries, red chicory, rhubarb, rutabaga, shallot, squash, strawberry, sunflower, sweet cherry, sweet potato, tomato and turnip. *Botrytis* is a major problem for fruits and vegetables in cold storage and subsequent shipment, because the fungus is able to grow effectively at temperatures just above freezing.

The mode and time of infection vary depending on the plant species and the plant part affected. Symptoms of *Botrytis* diseases vary greatly depending on the host and plant part attacked. Symptoms include a grey to brown discoloration, water soaking, and fuzzy whitish grey to tan mould (mycelium and conidia) growing on the surface of affected tissue and restricted lesions. The following are possible modes of infection of *Botrytis* that develop after harvest:

Infection through blossoms: The pathogen can infect fruits via the flower remaining in the very early stages of development and develop only after harvest when the fruit reaches full ripeness. This mode of infection is important in strawberry and is supposed to be the main cause of decay in mature fruit after harvest. Blossom blights often precede and lead to fruit and stem end rots. Ageing flower petals of beans, carrot, celery, eggplant, onion, pepper, squash, cucumber and tomato are particularly susceptible to colonization by *Botrytis* species, and under cool, humid conditions abundant mycelium and conidia are produced. The fungus often grows from the fading petals into the rest of the inflorescence and develops on the fruit causing blossom-end rot. From there it can spread and destroy part or all of the fruit (Chapter 14).

Infection through surface injuries and cracks: Conidia can infect the fruit directly through growth cracks, cut stem scars, insect wounds or lesions made by other pathogens. Infected fruits develop water-soaked, yellowish green or greyish brown irregular lesions which can be somewhat soft and spongy in texture.

Infection of bulbs and roots: Lesions can develop on any part of the root or bulb surface, but they are more likely to form at the crown, at wounds. Lesions usually appear soft, watery and tan colour, later becoming somewhat spongy, dark brown, and light-weight. Affected tissue eventually may dry to form a greyish leathery decay. Pockets of mycelium may develop between decayed bulb scales on the surface of root lesions. Black sclerotia can also be formed on and in decayed tissue, except at low temperatures in darkness, when only a fine white mould develops. Grey mould causes considerable damage on stored carrot, parsnip, mangel, sweet potato, beet, endive, chicory, turnip and rutabaga, but usually affects only topped roots. The bulb rot phase is common on onion and garlic, but also occurs on other bulb crops (Chapter 15).

Infection through harvest cuts and trimming: Grey mould rot caused by *B. cinerea* commonly occurs after harvest on the cut surface on leafy vegetables and fresh herbs (e.g. celery, basil, shiso). The cut surface is a perfect infection site for *Botrytis* because of the presence of nutrients leaking from the damaged tissue. Once infection is established decay can proceed to entire leaf tissue. The picking wound also is known to be good site for *Botrytis* infection. In kiwifruit, picking wounds are regarded as the main site of infection pathway for the majority of infections by *B. cinerea* (Michailides and Elmer, 2000).

Insect mediated-infection: In most cases, diseases have a pre-harvest component. The activity of various types of invertebrates can play a critical role in contaminating the produce with inoculum. The most substantial evidence in this regard has been shown in relation to infection of kiwifruit through flowers with *B. cinerea* facilitated by thrips (*Thrips obscuratus*) and honey bees (Michailides and Elmer, 2000). High *Botrytis* incidence was also reported on kiwifruit with garden

snail (*Helix aspersa*) damage; slime secreted by the snail stimulated the germination of the conidia. Honey bees and other types of insects visiting flowers potentially have the capability to disseminate *Botrytis* in strawberry (Michailides and Elmer, 2000). For details see Chapter 14.

3. *Botrytis* on major crops

3.1. Table grapes

More than 2 million tonnes of table grapes are shipped from the southern to the northern hemisphere, and to a smaller extent, by other routes. Surplus yields that accumulate in the peak of production are stored for local consumption for timely distribution. The table grape season can be extended substantially by the correct composition of early and late varieties and by diversifying the local geographical production areas. Shipping and storage of table grapes requires development of commercial technologies which are focused on preserving the fresh appearance of the grapes without compromising fruit taste. The major challenge for this technology is post-harvest fruit decay caused by *B. cinerea*.

Multiple pathways of infection can lead to grape decay during storage (Coertze and Holz, 1999, 2002; Chapters 2 and 14). It is evident that *Botrytis* can infect grapes during fruit set and bloom (McClellan and Hewitt, 1973), resulting in latent infection and grape decay in the vineyard. The actual contribution of this route to decay in storage may be low since removal of external inoculum by disinfecting the surface can prevent much of the damage (Lichter et al., 2002). During ripening the susceptibility of the berries increases partly due to a decrease in the content of phytoalexins such as resveratrol (Jeandet et al., 1991). Bird, insect and mechanical damage, sun scald and rain cracking serve as vectors and pathways for infection (Broome et al., 1995; Coertze and Holz, 2002). Rain events or failure in insect control can result in grey mould epidemics in the vineyard, making post-harvest storage a challenge. However, in subtropical climates, *Botrytis* infection in the vineyard is infrequent and development of grey mould in storage remains the primary problem. Microclimate plays an important role in the epidemics of *Botrytis* in the vineyard or after storage (see Michailides and Elmer, 2000). The first symptom during storage is 'slip-skin' (separation of the skin from the flesh upon touch). This is usually accompanied by red-brown colouration easily scored on white varieties. Internal disintegration of the berries is typically followed by appearance of aerial mycelium and sporulation which serves as an inoculum source for subsequent infection. The decaying berry itself infects neighbouring berries forming 'nests' that can eventually spread over the entire bunch. Infection can also develop from the pedicel-berry interface. Less typical decay symptoms can be observed as lesions with clear boundaries and limited area.

The berries of modern table grape varieties are large with thin skin. Early season horticultural practices have a substantial effect on bunch and berry structure and quality, and hence its storage potential. For example, 'berry thinning' performed manually or by chemical induction of flower abscission with gibberellin prevents excessive fruit set (Coombe, 1973). Failure in thinning may result in 'corn'-like

appearance of the bunch with tightly compressed berries, creating a convenient microclimate for decay beyond the reach of botryticides (Marois et al., 1986; Vail and Marois, 1991). Earlier application of gibberellin results in bunch 'stretching' which loosens the berries. Gibberellin-mediated manipulation of berry size (Coombe, 1973) is another treatment with many implications for the post-harvest quality of the berry, but it may decrease fruit resistance to decay both at harvest and during storage.

Table grapes can withstand extreme cold storage and the recommended storage temperature is -0.5°C . This low temperature is of high value for prevention of fungal decay, but it is only sufficient to slow down development of *B. cinerea*. Therefore, a complementary technology of fumigation with sulphur dioxide (SO_2) was developed. SO_2 damages fungal membranes, inhibits various enzymes and if applied correctly is a very effective tool (Harvey and Uota, 1978). SO_2 is also a strong antioxidant and prevents the necrosis of the rachis. This technology is currently the only commercial practice for storage of table grapes and it is applied by different means. Gaseous application is common in California and is implemented by exposing the grapes to a large dose before storage, followed by weekly fumigations according to a formula accounting for the exposure time, volume of the room and its capacity (Combrink and Ginsburg, 1972; Smilanick et al., 1990). Most other grape producers use 'generator pads' containing sodium metabisulphite that release SO_2 in a controlled manner upon reaction with water vapours. The pad is placed over the grapes and the gas is contained in ventilated or non-ventilated liners, or without liners, wrapping the pallet with stretch-polyethylene, as practiced in Chile, South Africa and Israel, respectively. Special care is necessary to avoid high concentrations of SO_2 , expressed as bleaching, typically around the pedicel-end or around microscopic holes or cracks in the cuticle. The damage may also be expressed as an undesired taste and rare hypersensitivity in the human population. Because of the deleterious side-effects of SO_2 and the world-wide scale of the *B. cinerea* problem, several lines of alternative technologies have been suggested over the years.

Cultivar selection and horticultural practices are extremely important for integrated pest management (IPM) and have the potential to increase the quality and integrity of grapes. To date, this approach has not developed into a strategy which is sufficient for current commercial demands. Pre-harvest application of fungicides can protect grapes from post-harvest decay, but that is not always the case (Kock et al., 1991). Fungicide application during flowering, fruit set and before veraison were ineffective in reducing infection by *B. cinerea* during storage (Kock et al., 1991). However efficient fungicides may be, their acceptance by consumers is low and the potential for resistance is increased.

The practices at harvest, such as handling and removal of damaged and decaying berries, are very important for the quality of the bunches after storage. Forced-air cooling may be significant for prevention of fungal development in the initial stage of storage (Jooste, 1987), but that is not a rule (Witbooi et al., 2000). Because forced-air cooling provides benefits to the appearance of the bunch, its added potential to reduce *B. cinerea* infection is likely to be considered as part of IPM. Current technology largely ignores the microbial contamination of fruits which

are packaged with soil particles, carposphere microflora and insect debris. Dipping bunches after harvest in ethanol addresses these problems without compromising fruit quality. Interestingly, this treatment is very effective in preventing *B. cinerea* from developing decay (Lichter et al., 2002), partly due to the high sensitivity of the conidia to ethanol (Lichter et al., 2003). It is estimated that this technology can be used for short-term storage, but ethanol is unsuitable for latent and internal infection. Another treatment which can be used after harvest and before storage is vapour heat applied in the range of 50-55°C for 12 to 32 min (Lydakos and Aked, 2003). Fumigation of grapes with volatiles, such as hexanal, prevents decay after 7 days storage at 15°C (Archbold et al., 1997). UV-C (220-280 nm) is known to induce resveratrol and pre-treatment of berries with low UV-C doses followed by artificial inoculation with *B. cinerea* reduced post-harvest grey mould of table grapes (Nigro et al., 1998). Dipping bunches after harvest in resveratrol was found to inhibit development of *B. cinerea* (Urena et al., 2003). It is noteworthy that resveratrol intoxicates *B. cinerea* following laccase processing (Schouten et al., 2002).

Biocontrol is a very appealing option in IPM programmes for grapes (Chapter 13) and several reports demonstrate its efficacy against bunch rot in the vineyards and for prevention of grey mould after harvest. Among the organisms reported to be effective are *Trichoderma harzianum* (Elad, 1994), *Aureobasidium pullulans* (Lima et al., 1997), *Pythium periplocum* (Paul, 1999), *Metschnikowia fructicola* (Karabulut et al., 2003), various yeasts (Wilson et al., 1991; Zahavi et al., 2000) and *Bacillus subtilis* (Pusey, 1989). Currently, no biocontrol agent seems to be efficient enough to cope on its own merit with *B. cinerea* in the arena of table grapes and cold storage. The balance in future may, however, be shifted in favour of biocontrol with the support from IPM programmes.

Several alternative technologies have been examined for continuous protection during storage. CA of grapes with 5-10% CO₂ failed to prevent development of *B. cinerea* (Uota, 1957; Laszlo, 1985). Increasing CO₂ levels to 15% or higher suppressed *B. cinerea* (Berry et al., 1997; Crisosto et al., 2002; Retamales et al., 2003). However, a lower concentration of CO₂ was recommended for cv. Red Globe to avoid damage to the taste and appearance (Crisosto et al., 2002). Apparently, there is a narrow threshold between efficacy and damage expressed as browning of berries or rachis and undesired after-taste. Carbon monoxide at 10% was also reported to offer good decay control without loss of quality (Yahia et al., 1983). MA depends on CO₂ released from the bunch by respiration and its accumulation to the desired concentration in the liner. Probably due to its limited efficacy on its own merit, it was reported mainly in conjunction with other molecules, such as acetic acid, chlorine and volatiles (Moyle et al., 1996; Archbold et al., 1997; Zoffoli et al., 1999; Westercamp and Blargues, 2002). Fumigation with acetic acid at intervals during cold storage was also reported (Sholberg et al., 1996). Application of ozone to grapes elevated the level of resveratrol and had a significant effect on *Rhizopus stolonifer* rather than *B. cinerea* (Sarig et al., 1996). Continuous exposure to 0.3 µl/L ozone inhibited 'nesting' of *B. cinerea*, but did not reduce incidence of decay of grapes (Palou et al., 2002). Ozone applied in solution for detached berries significantly controlled grey mould, although its efficacy was

variable and dependent on grape condition (Gabler and Smilanick, 2001). This study also identified ammonium bicarbonate and ethanol as the most interesting compounds for storage of detached berries. None of the above techniques has so far reached commercial application and this situation is unlikely to change unless consumer demands reverse regulation.

3.2. Tomato

Unlike table grapes, failure to control *B. cinerea* before harvest makes it difficult to control the fungus during storage. This is partly because the optimal temperature for *B. cinerea* development on tomatoes is 15°C (Takeda and Nakamura, 1990) and close to the optimal temperature for storage of tomatoes (12°C). Another matter is the lack of an efficient method (such as SO₂ fumigation of grapes) to control the pathogen during storage. Therefore, much of the effort is dedicated to pre-harvest prevention, with a focus on IPM programmes that take into account climate and horticultural practices (Shtienberg and Elad, 1997; Chapter 18). Latent infections of tomato during flowering give rise to stem-end rot (Lavy Meir et al., 1988; Jarvis, 1994). During fruit development, wet climate in field-grown tomatoes and a humid microclimate in greenhouses play a crucial role in disease development. Hence, in a dry climate or well-ventilated indoor growth, *B. cinerea* is not a major post-harvest problem. The extent of post-harvest decay of tomato fruits is positively correlated with the amount of *B. cinerea* present at harvest (Chastagner et al., 1977). During storage, injuries such as those incurred by the calyx of adjacent fruit may be a focus for infection (Thomas et al., 1977). However, the variation in incidence and extent of infection is high between cultivars, growers, and specific growth conditions.

Fungicides have played an important role in maintaining the fruit free of decay (e.g. Chastagner et al., 1977), but fungicide resistance appeared frequently. For example, in one study more than 90% of tomato samples contained benomyl-resistant strains (Staunton et al., 1975). Such specific resistance is attributed to point mutations in the tubulin gene (Yarden and Katan, 1993; Chapter 12). New fungicides with a dual mode of action offer better protection for tomatoes (Siviero et al., 2003), but environmental concerns may limit their use. Many types of chemicals have been suggested for control of *B. cinerea* after harvest. For example, acetic acid (Sholberg and Gaunce, 1995), the anionic surfactant Nacconol (Hoy and Ogawa, 1984), the phytoalexin glucohexatose (Ning et al., 2003) and a peptide with antifungal activity (Lopez-Garcia et al., 2000). The efficacy of biocontrol agents was demonstrated with *Candida oleophila* (Saligkarias et al., 2002), *Bacillus amyloliquefaciens* (Mari et al., 1996) and *Aureobasidium pullulans* (Schena et al., 1999). The interactions between microorganisms on the fruit surface may lead to negative effects, as was observed for tissue co-inoculated with *Salmonella typhimurium* and *B. cinerea* (Wells and Butterfield, 1999). Physical treatments such as hot water dips, hot air and brief hot water rinsing and brushing (Barkai Golan et al., 1993; Lurie et al., 1998; Fallik et al., 2002) reduce the inoculum on the surface of the fruit and also decrease chilling injury, increase expression of heat shock proteins and activities of enzymes, such as peroxidase, which may limit decay development (Lurie et al., 1997). Application of UV-C light retarded ripening and decay caused

by *B. cinerea* (Liu et al., 1993) and decay was also delayed by short-term anoxia treatments (Fallik et al., 2003).

There are many reports on the contribution of MA and CA for protection of tomato from fungal diseases (Dennis et al., 1979; Marangoni and Stanley, 1991). However, this approach has never reached the practical phase, probably because the benefits did not justify the commercial adoption of the new technology. Tomato is considered a climacteric fruit that produces and requires ethylene for development and ripening. CA supplemented with reduced level of ethylene resulted in increased susceptibility to *B. cinerea*; 1-3 $\mu\text{L/L}$ of ethylene was required for optimal decay control (Geeson et al., 1986). These results correlate with the negative effect of the inhibitor of ethylene action, 1-methylcyclopropene (1-MCP), on resistance of tomato to *B. cinerea* (Diaz et al., 2002). On the other hand, ethylene induced elongation of germ-tubes of *B. cinerea* after incubation with tomato fruit and a tomato mutant with reduced responsiveness to ethylene produced less decay than the control cultivar (Barkai Golan et al., 1989; Chapter 10).

Tomato is a good model for studying molecular aspects of post-harvest *B. cinerea* problems (Botella, 2000). Functional analysis of these interactions can be carried out by testing *Botrytis* gene knock-out mutants for virulence on harvested tomato fruit, or by testing tomato mutants for susceptibility to fruit decay. Several *Botrytis* genes such as cutinase A and the polygalacturonase *Bcpg1* were evaluated for pathogenicity on tomato fruits and the *Bcpg1* mutant had reduced pathogenicity (Van Kan et al., 1997; Ten Have et al., 1998). Other *Botrytis* gene disruptants displayed reduced virulence on tomato leaves and fruits (Zheng et al., 2000; Gronover et al., 2001). Tomato fruits over-expressing pear PGIP were more tolerant to *B. cinerea* (Powell et al., 2000). Suppression of a ripening-related expansin gene which increased consistency of tomato paste did not influence fruit tolerance to *B. cinerea* (Brummell et al., 2002). In general, genes affecting cell wall metabolism have a potential to affect the interaction with *Botrytis* (Brummell and Harpster, 2001), but this issue was not addressed systematically. See also Chapters 4, 7 and 20.

3.3. Kiwifruit

Botrytis causes three types of post-harvest rots on kiwifruit (*Actinidia deliciosa*) that develop mainly during cold storage at 0°C. The major symptom is stem-end rot in which the fungus penetrates the picking wound at, or soon after, harvest. The second type is spread of the infection from fruit to fruit by contact. When fruits become over-mature *Botrytis* conidia can directly infect through cracks, wounds or through any other damaged skin area (Brook, 1992). *Botrytis*-infected fruit can also enhance premature softening of the remaining fruits in a tray or bin as a result of the enhanced ethylene production by the infected fruit. Stem-end rot begins at the picking wound of otherwise undamaged kiwifruit and progresses down the fruit; resulting in the classic 'tide mark' on the outside of the fruit. The diseased pericarp is glassy and water-soaked. Secondary rotting results in the complete disintegration of the fruit. Symptoms are not usually manifest until between 4-12 weeks of storage at 0°C, or sooner if the fruit is kept at higher temperatures. Costly repacking in the

offshore market is often necessary. In California, research showed that most grey mould developing in kiwifruit in storage was due to latent infections of *B. cinerea* in the fruit sepals and receptacles (Michailides and Elmer, 2000). The greater the level of sepal and receptacle infection prior to harvest, the greater the incidence of grey mould in cold storage. In New Zealand, however, studies demonstrated that the main site of infection is through the picking wounds. At the middle of the growing season *Botrytis* is found on senescent petals attached to fruit. By harvest, the green leaves with necrosis (dead patches) and dead leaves are the primary source of inoculum. Research has shown that the hairy kiwifruit acts as a conidia trap and thousands of *Botrytis* conidia have been found on the fruit surface. At the time of harvest, conidia on the skin surface contaminate the picking scar which leads to stem end rot in fruit under cold storage conditions. In California, fruit damaged by the brown garden snail (*Helix aspersa*) increased grey mould incidence in storage compared to undamaged fruit (Michailides and Morgan, 1996). Knowledge of the *Botrytis* life cycle after harvest is less clear, but it can be expected that infection occurs through wounds. Infection of kiwifruit at pre-harvest stages is described in Chapter 14.

Dicarboximide sprays at harvest were the common means of chemical control of *Botrytis* in kiwifruit orchards. In California, Sommer et al. (1984) showed that two sprays with vinclozolin at bloom and two more pre-harvest sprays were the most effective treatments for controlling grey mould in storage. However, *Botrytis* has developed resistance to the dicarboximides, such as vinclozolin and iprodione. Some strains of *Botrytis* are now also resistant to the benzimidazoles (benomyl). A new generation of low risk chemicals (e.g. fenhexamid; Chapter 12) for the control of grey mould have been also evaluated. The development of the BOTMON predicting system, in which the level of *B. cinerea* colonizing the sepal or receptacle is determined, has greatly improved decisions by growers regarding the timing and number of fungicide applications in the field (Michailides and Morgan, 1996). Fumigation of kiwifruit with SO₂ was also reported to reduce grey mould without fruit injury (Cheah et al., 1992).

Alternative control methods were suggested for management of the disease. An open canopy provides plenty of air movement in the plantation and subsequently creates unfavourable conditions for conidial germination. Good orchard hygiene to remove sources of *Botrytis* conidia such as dead leaves, petals etc. can reduce the risk of *Botrytis* infection of the fruit. Careful post-harvest handling of the fruit (picking, grading and packing) reduces the risk for skin injury and minimizes the prospects for infection sites. Picking bags contaminated with conidia can become a major source of inoculum; research has shown that nylon mesh bags may reduce *Botrytis* build-up compared to enclosed bags.

Incubating or 'curing' the fruit at ambient temperatures for at least 2 days before packing and cold storage significantly decreases *Botrytis* storage rots. Fruit should be checked during cold storage for presence of rots. The optimum time for checking is after 10-12 weeks of storage, by which time almost all the primary infections will have produced visible symptoms. Early condition checking (e.g. after 6 weeks storage) will identify any major problems, but fail to detect c. 80% of infections (Manning et al., 1995).

Biocontrol of *Botrytis* on kiwifruit tissues was first reported by Menzies et al., (1989) but the results were variable. In Italy, post-harvest applications of a yeast to freshly harvested fruit significantly increased the proportion of healthy kiwifruit after artificial inoculation with *Botrytis* and cool storage for 15 days (Testoni et al., 1993). Cook et al. (1999) reported that application of yeasts (e.g. *Candida sake*, *C. pulcherrima*, *Trichosporon pullulans*), or inducing host resistance by incubating kiwifruit at 10°C with high humidity were each effective in significantly reducing incidence of post-harvest stem-end rot in cool stored kiwifruit. However, when these two means were combined, the timing of the yeast application determined whether their effect was additive. There were also attempts to use *Bacillus subtilis*, *Pseudomonas syringae*, *Acremonium breve* and *Cryptococcus laurentii* as pre- and post-harvest treatments, but their efficacy was variable (Michailides and Elmer, 2000).

3.4. Roses

Botrytis blight is a widespread disease of greenhouse-grown roses (Coyier, 1985). Susceptibility to *B. cinerea* is regarded as an important factor of vase-life. Infection of petals reduces the ornamental value and can be the reason for flower collapse. Symptoms of infection are visible within 24 h at 18–25°C and > 90% relative humidity (RH) (Salinas et al., 1989) initially as restricted lesions that later become necrotic (Elad, 1988). The infection will eventually spread, leading to the necrosis of whole petals (Pie and Brouwer, 1993). The infection can spread to receptacle and then cause abscission of all the petals. The problem is aggravated by latent infections that are symptomless at harvest, but become apparent during storage and transport (Elad, 1988). Various rose cultivars show differing susceptibility to *B. cinerea* (Hammer and Evensen, 1994) and different methods have been developed for determining the relative susceptibility of the cultivars (Hazendonk et al., 1995). Cut flowers senesce after harvest during transit and marketing. Ethylene produced during senescence predisposes the flowers to infection and development of *Botrytis*. Infected flowers produce elevated amounts of ethylene that lead to premature senescence of flowers (Elad, 1988; Chapter 10).

Sanitation, cultural practices and chemical control using fungicides have been the main recommendations for reducing infection in rose production. The dicarboximide fungicides are recommended for the control of *Botrytis* in roses. However, their efficacy on greenhouse roses is limited (Elad, 1988). Flowers which may be closed at time of treatment develop and are harvested before the next spray, meanwhile later infections may have occurred. In addition, the pathogen has developed resistance to these fungicides in many areas. Cultural methods, such as reduction of RH and avoidance of free water on the rose canopy by forced aeration and heating, are currently the most effective control measures (Hammer and Evensen, 1994).

Pre-harvest sprays, fertilization or pulsing buds by fertigation with calcium compounds reduced disease severity and extended vase life of the cut flowers (Volpin and Elad, 1991; Bar-Tal et al., 2001). Microbial antagonists of *B. cinerea* are reported to reduce grey mould on rose crops. Redmond et al. (1987) identified

isolates of yeasts and bacteria that reduce the number of lesions produced by the pathogen on rose petals, and Elad et al. (1993) found that *Trichoderma harzianum* reduced symptoms on rose flowers harvested soon after treatment. Hammer and Marois (1989) used two antagonists to reduce the symptoms caused by *B. cinerea* in roses stored at 2.5°C, but control was ineffective when flowers were removed from storage and kept at room temperature.

Meir et al. (1998) have demonstrated that pulsing of cut roses with 200 µM methyl jasmonate (MJ) provides systemic protection against *Botrytis* rot by inducing resistance mechanisms in cut roses of six cultivars without impairing flower quality. Also, a direct antifungal effect of 100-400 µM MJ on conidial germination and germ tube elongation of *B. cinerea* was obtained *in vitro*, with complete inhibition at 400 µM MJ (Meir et al., 1998). These results suggest that a combined treatment of spraying and feeding with MJ may be efficient for control botrytis blight in cut roses. Indeed, application of this treatment on cvs Frisco and Red Charm resulted in excellent control. Based on these results, a practical application of MJ consisting of simultaneous pulsing and spraying was developed for use by growers. The treatment included MJ pulsing for 4 h at 20°C and MJ spraying, followed by continuation of the pulsing for additional 20 h at 6°C.

3.5. Strawberry

Botrytis fruit rot is one of the most important diseases of strawberry worldwide. This disease causes severe pre-harvest and post-harvest losses, primarily due to infection that is initiated in the field during flowering and remains quiescent until fruit ripens. Although the disease can be important pre-harvest, it is most important when developing during shipment or storage. After harvest, this disease is the major limiting factor in cold-chain marketing of strawberries.

On ripe strawberry fruits, lesions are typically found on the proximal end of the berry and are frequently associated with infected stamens, or with dead petals that stick to the fruit or become trapped under the calyx. Lesions begin as small, firm, tan spots that quickly enlarge and become covered with white fungal mycelium and grey to brown conidia. Epidemics of botrytis fruit rot in perennial strawberry production systems are initiated primarily by conidia produced in dead strawberry leaves in the field (Braun and Sutton, 1987). Conidia are either splash- or air-dispersed to infect floral parts, including stamens and petals (Bristow et al., 1986). Mycelium of *B. cinerea* then invades the adjacent receptacle as it ripens and causes fruit rot. The pathogen sporulates on diseased flowers and fruits, which become important sources of secondary inoculum in annual production systems where there are multiple flowering and harvest cycles over several months.

Effective management of fruit rot developing after harvest involves regular pre-harvest applications of botryticides during peak flowering periods. Blacharski et al. (2001) reported that weekly applications of captan and thiram were effective in reducing pre- or post-harvest incidence of grey mould. Fenhexamid (750 g a.i./ha), applied as a programme of treatments from early flowering, demonstrated good control of *B. cinerea* in the UK (Duben et al., 2002). Stensvand (1998)

has also evaluated the use of new chemicals as well as biocontrol agents for the control of grey mould of strawberry.

The removal of all diseased and unmarketable fruit from within the plant canopy is critical for effective management of botrytis fruit rot, as this fruit is an important source of inoculum that directly infects nearby flowers and fruit. The removal of senescent foliage also reduces inoculum, but provides only limited control. Use of drip- instead of overhead-irrigation reduces free moisture on plants and prevents splash-dispersal. Growing strawberries under clear plastic tunnels also reduces leaf wetness duration and botrytis fruit rot incidence (Xiao et al., 1999). Reducing plant density by use of wider plant spacing reduces the incidence of grey mould (Legard et al., 2000) but may reduce yield. The combination of cultural and chemical methods is essential for an effective control programme. Although no cultivar is fully resistant, there are significant differences in susceptibility among cultivars used commercially (Legard et al., 2000). Public concern about potential chemical pesticide residues in fruits is common. These problems high-light the need for development of alternative control methods which are effective and safe for the producer and the consumer (Wilson et al., 1998; Droby et al., 2000; Janisiewicz and Korsten, 2002).

Biocontrol of rot-causing pathogens of strawberry has been attempted in several laboratories (Peng et al., 1992; Lima et al., 1997; Helbig, 2001; Boff et al., 2002; Guetski et al., 2002). The main approaches involved either interference with pathogen infection at flowering and during fruit development, or the suppression of inoculum production (Peng and Sutton, 1991). Field application of biocontrol agents to strawberry at the flowering stage, by spraying (Tronsmo and Dennis, 1977; Peng and Sutton, 1991), or carried by bees has proved to be effective in suppressing *B. cinerea* on stamens and petals and reducing fruit rot (Peng et al., 1992; Bilu et al., 2004). *Aureobasidium pullulans* and *Candida oleophila* were more effective against *B. cinerea* storage rots on strawberries when applied in the field at bloom compared with their application immediately after harvest (Lima et al., 1997); both antagonists were shown to colonize the flower to such an extent that their activity prevented colonization of senescent floral parts (stamens) by *B. cinerea*. More recent work (Karabulut et al., 2004) showed that a pre-harvest application of the yeast *Metschnikowia fructicola* effectively reduced the development of post-harvest strawberry grey mould. The yeast significantly suppressed post-harvest incidence of fruit rot better than fenhexamid. Systemic acquired resistance (SAR) was used to suppress *B. cinerea* on strawberry fruit. Acibenzolar (S-methyl benzo[1,2,3]thiadiazole-7-carbothioate) is a chemical activator of SAR. When applied to strawberry plants at 0.25-2.0 mg/ml, acibenzolar delayed by about 2 days the development of grey mould on harvested strawberry fruit held at 5°C. This delay was equivalent to a 15-20% increase in storage life of the fruit (Terry and Joyce, 2000).

MA packaging using low density polyethylene (LDPE) is commercially used in Spain and other countries as a means to reduce botrytis rot and improve the overall quality of strawberries (Artes et al., 2001). The elevated CO₂ and reduced O₂ level inside the package seem to greatly reduce grey mould development on the fruit.

4. Conclusions and future prospects

At present, control of post-harvest decay caused by *B. cinerea* in various fruit and vegetable crops relies mainly on the pre-harvest use of chemical fungicides (Chapter 12). Control programmes were developed specifically for each crop and largely depend on epidemiological and etiological information of *Botrytis* attack (Chapter 18). The future of chemical fungicide control, however, is questioned and their use has come under scrutiny. This is because of 1) failure to effectively control grey mould in several harvested fruit and vegetable commodities due to resistance problems, 2) consumers' concerns over the possible effects of toxic residues on human health and the environment, and 3) restrictions imposed by authorities on the use of agro-chemicals. These issues that were the driving force for the development of reduced-chemical post-harvest disease control measures have now become an economic imperative, not just an option. The use of alternative non-chemical control methods as stand-alone treatments, however, does not provide the efficacy and consistency required under commercial post-harvest situations. A more realistic scenario to combat decay of harvested commodities would be the use of an integrated control approach combining biological and physical control strategies, with or without limited quantities of fungicides pre-harvest, and with efficient management and handling practices.

The availability of efficient post-harvest chemical control treatments for certain crops, such as the SO₂ technology for grapes, in some respects delayed development of alternative control means. Conversely, the lack of such dedicated control technologies in other harvested commodities has promoted the development of IPM programmes. Kiwifruit represent a crop in which horticultural and epidemiological knowledge has significantly contributed to generating control strategies for the control of botrytis rots. Strawberries are a model for a highly perishable crop in which harvesting over a relatively long season places a serious challenge as far as disease control in general and botrytis rots in particular. Roses are highly susceptible to *B. cinerea* and have relatively short shelf life, but because they are inedible, the use of chemical control strategies theoretically can be continued, subject to environmental regulation.

It is an enormous challenge to meet the demands for improved quality and safety, and reduction of post-harvest losses due to *Botrytis* infections in an affordable and environmentally compatible manner. After decades of research on *Botrytis* several questions related to the relationship between infection level occurring in the field and development of post-harvest decay remain unanswered. To develop more efficient methods for controlling post-harvest grey mould it is essential to elucidate the relationship between infection of various plant parts in the field and incidence of grey mould in storage. Development of control strategies based on a holistic approach in which modelling and prediction systems, early detection techniques, biological and physical methods and cultural practices should

be encouraged specifically to meet requirements of each crop. For example, management of grey mould on kiwifruit in New Zealand using non-chemical methods has been a success story. Adoption of summer pruning to create more open canopy, pre-harvest prediction and post-harvest curing has effectively reduced *Botrytis* losses and led to the perception by the industry that grey mould is no longer a problem. This success story may be repeated in other crop systems following the allocation of appropriate research efforts.

It is reasonable to assume that IPM approaches are unlikely to offer completely satisfactory answers for *Botrytis* control in certain commodities. However, to complement available solutions we need to systematically explore the potential of genetically manipulated plants which are more tolerant to grey mould (Chapter 20).

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CHAPTER 20

INNOVATIVE BIOLOGICAL APPROACHES TO *BOTRYTIS* SUPPRESSION

Henrik U. Stotz¹, Yigal Elad², Ann L.T. Powell³ and John M.
Labavitch⁴

¹Department of Horticulture, Oregon State Univ., Corvallis, OR 97331 USA; ²Department of Plant Pathology, The Volcani Center, Bet-Dagan, 50250, Israel; and ³Department of Vegetable Crops and ⁴Pomology Department, University of California, Davis, CA 95616

Abstract. Research into the mechanisms used by plants to protect themselves against pathogens has expanded considerably in the past few decades, fuelled, in part, by addition of genomic tools to investigators' resources. As a consequence, information about the details of many defence mechanisms and the genes that are responsible for their constitutive or induced expression is now available for utilization in crop improvement. This chapter briefly addresses many aspects of plant defences against *Botrytis* infection, including considerations of how the pathogen is detected, signalling pathways that activate and coordinate defence responses, and the expression of factors that make mechanistically different contributions to defence. Almost all of these topics are addressed more fully in earlier chapters of this book. The focus here is on use of more recent biochemical and molecular information to enhance crop plant defence against *Botrytis*. We discuss the potential for 1) introducing the *Botrytis* defence systems of wild species by cross-breeding into domesticated, genetically compatible crop species, 2) using genetic engineering to introduce into crops genes enabling the synthesis of antifungal metabolites or encoding peptides and proteins shown to have anti-*Botrytis* activity, 3) employing powerful techniques for "gene discovery" to identify sequences encoding "downstream" factors involved in defence signalling, thus offering the possibility of directly linking beneficial plant responses to the plant's perception of the pathogen while eliminating responses that might facilitate infection, and 4) specific possibilities for enhancing the ability of biocontrol microorganisms to limit damage due to *Botrytis* infection of crop plants. Broader questions that might serve as guiding principles when specific approaches to enhancing crop plant pathogen defences are under consideration are also discussed.

1. Introduction

Improvement of plant-based approaches for suppressing *Botrytis* development should consider a number of biological and economic questions. Is the goal to eliminate the development of the disease or attain an economically relevant level of reduction? Should suppression be based on molecular or conventional manipulations of the plant's recognition of, or response to, the pathogen? Do we know enough about how *Botrytis* establishes itself on hosts to devise strategies for countering

specific “attack” mechanisms? Genetic improvements in a crop plant’s defences take time; how sustainable should we expect enhanced defences to be? Are there developmental constraints that naturally limit the effectiveness of biologically based defences?

This chapter will attempt to keep these questions in mind as the authors discuss work in progress, primarily with tomato, that is aimed at reducing crop problems with *Botrytis* infections. The value of information gained from work with *Botrytis*-challenged *Arabidopsis* as a model system and from studies of necrotrophic pathogens similar to *Botrytis* will be reviewed. The authors will speculate about so far untested approaches for a plant biology-based reduction in crop losses to *Botrytis* and will examine the possible utility of transformations of both plant hosts and microbial control agents.

2. Potential use of natural genetic resources for *Botrytis* resistance breeding

Domestication and, during the past century, modern plant breeding have progressively depleted the genetic variation of crop species (Tanksley and McCouch, 1997). Limited genetic diversity is a severe threat to sustainable agriculture because it increases the vulnerability of crops to pathogen and insect epidemics. Tomato is a good example of a genetically depleted species. Fortunately, tomato is compatible with its wild relatives, all of which are diploid (Rick and Chetelat, 1995; Peralta and Spooner, 2001). Wild tomato species have been a significant asset for resistance breeding. Over half of the 42 resistance traits identified in tomato’s wild relatives have been introduced into cultivated tomato (Rick and Chetelat, 1995). Resistance to *B. cinerea* is not among those, even though it could be accessed from *Solanum lycopersicoides* (Rick, 1987; Rick and Chetelat, 1995). Stem inoculations provided the first experimental evidence for partial resistance to *B. cinerea* in F₁ hybrids of crosses between tomato cultivars and *S. lycopersicoides* accessions (Chetelat et al., 1997). Statistically significant differences in resistance to *B. cinerea* between *S. lycopersicoides* and tomato were recently recorded after spray inoculation of intact seedlings (Guimarães et al., 2004). Because individual chromosome fragments from *S. lycopersicoides* have been bred into cultivated tomato (Chetelat and Meglic, 2000; Chetelat et al., 2000), it is now feasible to map resistance genes from *S. lycopersicoides* in the cultivated tomato background. In addition, resistance to *B. cinerea* may be imported from other wild tomato relatives.

Differences in foliar susceptibility to *B. cinerea* among cultivated and wild tomato accessions were first documented by Urbasch (1986). Several accessions of *L. hirsutum* as well as an accession of *Lycopersicon esculentum* var. *columbianum* displayed strong resistance to *B. cinerea* in the laboratory and in the field. More recently, wound inoculation of tomato stems and leaves suggested elevated resistance to *B. cinerea* in accessions of *L. peruvianum*, *L. hirsutum*, and *L. pimpinellifolium* (Egashira et al., 2000). Even though the susceptibilities of stems and leaves were not correlated, all three species performed better than the commercial tomato cultivars tested in this study. However, statistical analysis

indicated that these differences were not significant (Egashira et al., 2000). Nicot et al. (2002) removed leaves from tomato plants, leaving petiole stubs that were inoculated with *B. cinerea*. Stem lesions were scored as well as secondary leaf infections. Interestingly, several *L. hirsutum* and *L. peruvianum* accessions were significantly more resistant than the tomato cv. Mospormorist when stem lesions were compared. However, when leaves were assayed, *L. chmielewskii* and *L. chilense* accessions were significantly more resistant than the tomato cultivar (Nicot et al., 2002). These lines constitute promising material for resistance breeding.

Partial resistance to *B. cinerea* is likely to be controlled by quantitative trait loci (QTL). This situation contrasts with gene-for-gene resistance relationships between races of a given biotrophic pathogen and cultivars of its corresponding host species (Flor, 1971). *B. cinerea*, like other necrotrophic pathogens, exploits a variety of mechanisms, including cell wall-degrading enzymes and toxins, to overcome host defences (Ten Have et al., 1998; Deighton et al., 2001; Colmenares et al., 2002). Genetic resources are available for mapping partial resistance to *B. cinerea*. In addition to an introgression line (IL) population of a *L. esculentum* × *S. lycopersicoides* cross (Chetelat and Meglic, 2000), mapping populations are available for the tomato species *L. cheesmanii*, *L. chmielewskii*, *L. pennellii*, *L. peruvianum*, *L. hirsutum* and *L. pimpinellifolium* (Paterson et al., 1990; Tanksley et al., 1992; Paran et al., 1995; Grandillo and Tanksley, 1996; Bernacchi and Tanksley, 1997; Fulton et al., 1997). The advantages of mapping in IL or recombinant inbred lines (RIL) are summarized in Figure 1.

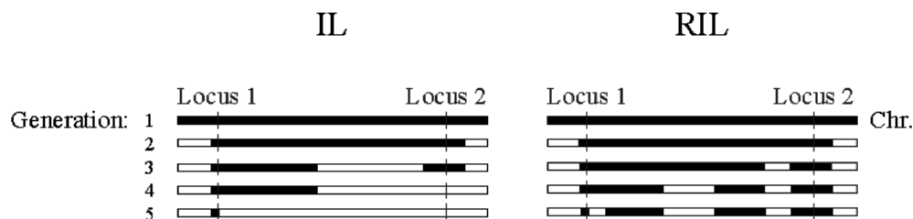


Figure 1: Quantitative trait loci (QTL) mapping strategies. Introgression lines (ILs) are generated by repeated backcrosses, which eventually result in the isolation of a chromosomal fragment that contains a locus of interest (e.g., locus 1). This process eliminates another locus that confers partial resistance (locus 2). ILs are genetically similar to the recurrent backcross parent that may facilitate detection of small genetic differences and direct application for crop improvement. Recombinant inbred lines (RILs) are selfed progenies of a cross between two species. Increased homozygosity and recombination rate in advanced generations improve mapping of loci of interest. Combinations of genetic loci may contribute to a trait if they are not separated by recombination

3. The promise of manipulating defence gene expression

The introduction of another organism’s defence mechanisms into plants to provide or enhance existing protection against infection by *B. cinerea* and other pathogens is one of the most important possibilities that molecular manipulations of crop plants

can provide. Several promising applications of this general crop improvement strategy are discussed below and in other chapters of this book. However, currently there is widespread societal reluctance to accept genetically engineered crops as an important component of agriculture. This objection to “GMOs” has little to do with whether an engineered crop will solve the agricultural problems faced by the farmer or provide the anticipated enhancement of disease resistance.

More relevant biological considerations of the gene transfer strategy demand our recognition of how a given plant defence strategy has evolved (e.g., Stotz et al., 2000) and the awareness that the strategy is now an integrated component of the biology of the plant in which it is expressed. These considerations are important because they relate to the broader “success” of the engineered crop plant, even if the introduction of a novel defence reduces pathogen susceptibility. A “spontaneous” defence mechanism may originate because a mutated DNA sequence happens to encode a protein that catalyses a reaction on a hitherto unrecognised substrate, binds to and enhances or suppresses the activity of other proteins, participates in the assembly of a cellular structure that now contains different or differently linked building blocks, etc. However, that novel defence must be compatible with the other aspects of the “life processes” of the plant where it originates if it is to be retained and, eventually, be favoured by selection. Thus, the new defence must function within the biological context (structures, pathways, integrated responses to stresses etc.) of the pre-existing plant if it is to become important to that species (e.g., Hetherington and Woodward, 2003).

As proteomic and metabolomic characterizations of plant development become more common, plant biologists will be in a better position to predict how the addition of a new cellular function, particularly a function that is over-expressed, will impact on the myriad of processes that were pre-existing in the transformed species. For now, however, we must recognize that the successful addition of a novel defence must be considered to be an early step in the process of “holistically” improving a given crop plant.

Only a few specific examples of the many possible factors that might be exploited for enhancing defences against *B. cinerea* are discussed in this Chapter. Other candidates are suggested by the more detailed discussions of specific host plant defence factors and the signalling pathways that coordinate them in Chapters 5, 6, 8, 9 and 10. The examples selected illustrate questions that must be addressed when developing strategies for enhancing crop plant defence capabilities beyond simply whether or not the factor manipulated is active against *B. cinerea*. These include: 1) Can the synthesis of a given factor be introduced in a way that is compatible with the target species’ normal distribution of potential “intermediates” so that bioactive concentrations can be reached? 2) Have selection pressures equipped target pathogen species with biochemical counter-measures that can neutralize the defence agent? and 3) Is it important to target the accumulation of anti-*Botrytis* factors to particular host tissues or cellular compartments?

3.1. Influencing pathogen intrusion into host plants

3.1.1. Polygalacturonase-Inhibiting Proteins (PGIPs)

Pectin-degrading enzymes, including the hydrolase polygalacturonase (PG), are secreted early in the interaction of pathogens and their plant hosts (Chapter 7; Collmer and Keen, 1986), thus making it reasonable to presume that plant PG inhibiting proteins (PGIPs) are components of the plant's defences. PGIPs were first described (Albersheim and Anderson, 1971) in extracts of beans (*Phaseolus vulgaris*) and proposed to be participants in the gene-for-gene defence expression. This idea was abandoned when the PGIPs of three bean cultivars all inhibited equally well each of the PGs of three races of *Colletotrichum lindemuthianum*, even though cultivar-specific patterns of resistance to *C. lindemuthianum* were evident (Anderson and Albersheim, 1972).

In 1983, PGIPs that inhibited the PGs of several fungi, including the enzyme from *B. cinerea*, were purified from pear fruits (Abu-Goukh et al., 1983; Abu-Goukh and Labavitch, 1983). The PGIP of tomato fruits was subsequently identified and purified (Stotz et al., 1994) and shown to inhibit *B. cinerea* PG, albeit less effectively than the pear PGIP. The genes for the pear and tomato fruit PGIPs were cloned (Stotz et al., 1993, 1994) and the pear PGIP gene was expressed in transgenic tomatoes to test whether PGIP over-expression would influence *B. cinerea* development. Powell et al. (2000) reported a reduction in *B. cinerea* lesion growth (20-33%) on the leaves and fruits of these transgenic tomatoes, when tests were carried out under controlled, laboratory conditions. Field trials with these pear PGIP-expressing tomato lines did not give results supporting a PGIP role in resistance, most likely indicating that PGIP is only one of the components of the tomato's resistance to *B. cinerea* and other pathogens and that the outcome of the interaction of host and pathogen is influenced by several factors. The pear fruit PGIP has subsequently been expressed in other fruit crop species i.e. grape (Aguero et al., 2002); persimmon (Tamura et al., 2004); and strawberry (D.W. Simpson and D. James, Horticulture Research International, Maidstone, Kent UK, pers. comm.) and a reduction in *B. cinerea* development has been reported for the grape and strawberry transformants. Expression of the bean PGIP in transgenic *A. thaliana* also resulted in the reduction of *B. cinerea* growth (Ferrari et al., 2003a).

Aspects of PGIP function and structure have been extensively reviewed (e.g., De Lorenzo and Ferrari, 2002) and it is generally agreed that PGIP could contribute to pathogen defence in two ways. The first is direct: PG is important to a pathogen's attack and so PGIP's inhibition of PG blunts the attack. While the logic is simple, the reality is more complicated. *B. cinerea* has several PG-encoding genes and enzyme isoforms and research has indicated that PG should be considered as a *B. cinerea* virulence factor (Chapter 7; Ten Have et al., 1998, 2001). However, while pear fruit PGIP inhibits *B. cinerea* PG in general, several of the PG isoforms the fungus produces in culture are not inhibited by pear PGIP (Sharrock and Labavitch, 1994). Furthermore, preliminary studies (K. Sharrock, HortResearch, New Zealand, pers. comm.) indicated that while much less PG accumulated in *B. cinerea* stem lesions on pear PGIP-expressing tomatoes than in lesions on controls, three or four

new, acidic PG isoforms were identified in the lesions on transgenic tissues. If *B. cinerea* and other fungal pathogens can produce PGs that are not susceptible to PGIP inhibition, perhaps in response to pathogen perception of PGIP presence, it is not surprising that over-expression of PGIPs will not always lead to a reduction in pathogen development. Work in progress that is discussed in Chapter 7 of this volume will clarify the roles of individual PGs in the infection process. This information will, in turn, help to clarify how PGIP interactions with individual pathogen PGs can contribute to defence. Understanding the role of PGIP in defence (or, perhaps, pathogen success) is further complicated by the report (Bergmann et al., 2003) that, at certain pH values, bean PGIPs can enhance the activities of some PGs that are inhibited by the same PGIPs at a more acidic pH.

The second mechanism hypothesized to explain PGIP contributions to pathogen defence is indirect. The model is that when PGIP binds to and inhibits a target PG the hydrolysis of PG's homogalacturonan substrate produces relatively large oligomeric breakdown products of the polymer (often called oligogalacturonides, OGAs) that are active in eliciting specific plant defence responses. When PGIP is not present, PG action generates smaller OGAs that are not elicitor active. This intriguing idea is based on the report of Cervone et al. (1989). The proposed mechanism of PGIP action is attractive because it would explain how PGIP could contribute to defence even if only one of a pathogen's multiple PGs were affected by PGIP. However, while the hypothesis has been widely discussed in the literature as if it were a fact, it has never been tested *in planta*. L. Yang, A.L.T. Powell, L.C. Greve and J.M. Labavitch (Univ. of California, Davis, USA, unpubl.) are now testing this idea using transgenic tomato lines with under- and over-expression of the endogenous tomato fruit PGIP. We anticipate that the over-expressing lines, like the tomatoes over-expressing the pear PGIP gene (Powell et al., 2000), will show enhanced resistance to *B. cinerea* and, after pathogen challenge, will contain different OGA populations. H-J. An, S. Lurie, L.C. Greve, C.B. Lebrilla and J.M. Labavitch (Univ. of California, Davis, USA, unpubl.) have recently begun the isolation and structural characterization of the OGAs that accumulate in *B. cinerea* lesions on tomato fruits. If the Cervone et al. hypothesis is correct, the OGAs in lesions from the PGIP-under-expressing fruits should differ from those in lesions on PGIP-over-expressing fruit and the latter, but not the former, should be elicitors of defence gene expression.

Recent studies of the PGIP from grapefruit cv. Oroblanco (D'hallewin et al., 2004) suggest an additional direct role for PGIP participation in pathogen defence. Germ tube growth in *B. cinerea* and *Penicillium italicum* was inhibited *in vitro* by the addition of grapefruit PGIP when tested in the presence of pectin. This result implies involvement of PGs in the early stages of fungal development and the possibility that PGIP can act directly to reduce that development.

Whatever its mechanism of action, the fact that PGIP has been shown to reduce the susceptibility of several crop species to *B. cinerea* infection supports its use in strategies for reducing grey mould losses. Continuing studies (e.g., Leckie et al., 1999; Stotz et al., 2000; Di Matteo et al., 2003) will more clearly define the PGIP sequence domains that are crucial for binding and inhibiting pathogen PGs. This information could guide engineering of a PGIP with broadened specificity and

inhibitory capacity and, hence, more general utility in engineering pathogen resistance. This engineering could be designed to produce high expression in crop plants or in biocontrol microorganisms (Sect. 5.2). Many plant species have PGIPs, so introduction of a PGIP from another species will not confront the transformed plant with an altogether foreign protein.

3.1.2. Cutinase

The cuticle, the waxy plant surface barrier that separates the plant from its environment, serves several functions, including protection against pathogen attack (Kerstiens, 1996). Several studies have suggested an important role for fungal cutinases in aspects of establishment of pathogen lesions on plant tissues (Kolattukudy, 1985; Deising et al., 1992; Kolattukudy et al., 1995; Francis et al., 1996). However, tests utilizing cutinase A-deficient mutants of *B. cinerea* raise some doubts about the importance of the enzyme to pathogenicity on gerbera and tomato because infection rates were no different than those resulting from inoculation with unmodified *B. cinerea* (Van Kan et al., 1997). The participation of cutinase in the *B. cinerea* infection process is discussed in Chapter 7.

More recent work using *Arabidopsis* transformants expressing the coding region of the mature cutinase from *Fusarium solani* f. sp. *pisi* (Sieber et al., 2000) has added a new perspective to studies of the role of the cuticle and associated surface barriers to interactions with *B. cinerea*. The transgenics display disturbed development, the most prominent features being multiple organ fusions involving leaves, flowers and stems. Fungal cutinase expression also correlated with a disrupted cuticle leading to increased solute permeability, although wax deposition was not impaired in cutinase-expressing plants. The waxes of cutinase-expressing plants had fatty acid and fatty alcohol compositions similar to those of untransformed lines, but a higher abundance of the hydrocarbon constituents. Interestingly, cutin monomers (the presumed products of cutinase action) did not accumulate in wax preparations from the fungal cutinase-expressing transgenic *Arabidopsis*.

Recently, C. Chassot and C. Nawrath (Department of Plant Biology, University of Fribourg, Switzerland, pers. comm.) have reported that the *Fusarium* cutinase-expressing transgenic *Arabidopsis* plants are highly resistant to *B. cinerea* development. This observation seems contrary to the idea that the cuticle serves as a penetration barrier, particularly because the earlier ultrastructural analysis (Sieber et al., 2000) showed that the cuticular layers in transgenic lines were not intact. The cutinase does not directly affect spore development and the resistance appears to be independent of defence pathways regulated by jasmonic acid/ethylene or salicylic acid. Nawrath and her colleagues speculate that the enhanced permeability of the cuticle facilitates information exchange that leads to an effective defence or that the alteration has modified a cuticle component or structure that the fungus needs to express full virulence. The compromised phenotype of the transgenic plants suggests that this manipulation will not have direct utility in crop plants. However, the

demonstrable resistance of these plants makes them excellent experimental material for the eventual identification of factors that might be specifically targeted for engineering of *Botrytis* defence.

3.2. Proteins and metabolites that influence *Botrytis cinerea* development or metabolism

3.2.1. Phytoalexins

Some information exists regarding the role of secondary metabolites in *Botrytis* resistance (reviewed in Chapter 9). The terpenoid-derived phytoalexin rishitin accumulates after UV-C treatment and/or fungal infection in tomato fruits (Charles et al., 1999). Correlative data and *in vitro* experiments suggest that rishitin is an antifungal defence compound (Glazener and Wouters, 1981; Charles et al., 1999; Fleissner et al., 2002). However, conclusive proof for an endogenous role of rishitin in grey mould resistance has to await molecular genetic data. Expression of stilbene synthase genes from grapevine results in the production of the phytoalexin resveratrol in both tobacco (Hain et al., 1993) and tomato (Thomzik et al., 1997), but resistance to *B. cinerea* is enhanced only in the former species. Whether this is a consequence of differential production of resveratrol in the two species or more efficient coordination of additional defence responses with production of the phytoalexin in transgenic tobacco is not clear. These results show that phytoalexin synthesis can be manipulated to protect against grey mould infection in some crop species (Schouten et al., 2002). Gene silencing would be required to demonstrate an endogenous role for these secondary metabolites.

3.2.2. Glycoalkaloids

Glycoalkaloids are a group of natural plant products with antifungal properties because their amphiphatic properties can disrupt biological membranes (Chapter 9; Friedman, 2002). α -Tomatine, the glycoalkaloid of tomato, is not very efficient at inhibiting *B. cinerea* growth *in vitro* (Verhoeff and Liem, 1975), perhaps because this fungus can convert this metabolite to an inactive product. Mutant studies suggest that a fungal α -xylosidase is involved in the catabolism of α -tomatine to α_1 -tomatine (Quidde et al., 1998). Because the glycoalkaloid compositions of *Solanum* and tomato species differ, their potential role in resistance could be studied in the segregating progenies of crosses between *Solanum* and *Lycopersicon* species discussed earlier. Glycoalkaloid production is not limited to *Solanum* species (Harborne, 1993). However, the number of metabolic steps required to divert potential precursors generally present in plants to the complete synthesis of an active antifungal product is rather high. Thus, genetic engineering of a pathway from one species to another is likely to be feasible only between related species with “complementary” metabolism. The manipulation of glycoalkaloid synthetic pathways from *Solanum* or other species, however, must be restricted to tissues of

crop plants that are not used as feed or forage because their toxicity would make the crops dangerous for human or animal consumption.

3.2.3. Peptides and Proteins

Antifungal peptides inhibit the *in vitro* growth of *B. cinerea* (Osborn et al., 1995; Lopez-Garcia et al., 2000; Saitoh et al., 2001; Ye and Ng, 2001). Like glycoalkaloids, antifungal peptides target microbial membranes (Thevissen et al., 1999), although there may be additional cellular targets once the membrane is compromised (Lay et al., 2003). Over-expression of the lipid transfer protein Ace-AMPI from onion increased resistance of scented geranium to *B. cinerea* (Bi et al., 1999). Unfortunately, there is limited information on the endogenous roles of plant antimicrobial peptides because definitive knock-out experiments, like those done in animal systems (e.g., Nizet et al., 2001; Blandin et al., 2002) are at present lacking. However, because these peptides are the direct products of the transcription and translation of introduced sequences, there is little concern about disruptions of normal metabolite flow when genes encoding antifungal peptides are added to crop plant genomes.

The pathogenicity related (PR) proteins chitinase and osmotin from grapevine both inhibit *in vitro* growth of *B. cinerea* (Salzman et al., 1998). Unfortunately, conclusive evidence for the role of basic chitinase in grey mould resistance is lacking because the antisense suppression approaches used to test the concept have not lowered gene expression sufficiently to give a statistically significant decrease of grey mould decay in *Arabidopsis* (Samac and Shah, 1994). Nevertheless, there are reports that the introduction of chitinase expression to some species will enhance fungal resistance (Zhu et al., 1994). Studies of endogenous β -1,3-glucanase and chitinase gene expression in bean leaves (Mauch and Staehelin, 1989) make clear that the cellular compartment (i.e., vacuole vs. apoplast) to which expression is targeted can have an important impact on the defence functions served by these enzymes and, hence, their contributions to overall defence.

Collectively, the above studies suggest involvement of a variety of genes in partial resistance to *B. cinerea*; none of them can be used as a magic bullet to prevent grey mould. Anti-*Botrytis* activity of PR proteins in non-modified situations is discussed in Chapter 9.

4. Exploitation of aspects of induced resistance for control of *Botrytis cinerea* infection: The potential for gene discovery

Manipulation of the capacity to express particular defence factors (discussed in Sect. 3) or of the induction and coordination of defence responses may be means to protect plants against *Botrytis*. Unlike other pathogens, *B. cinerea* does not trigger systemic acquired resistance (SAR) in the *Arabidopsis* ecotype Columbia (Govrin and Levine, 2002). Thus, it is not surprising that treatment with benzothiadiazole, an inducer of SAR, confers partial resistance to this ecotype (Zimmerli et al., 2001) as well as tomato (Audenaert et al., 2002). Similarly, treatment of tobacco plants with

salicylic acid (SA) inhibits the development of spreading lesions (Murphy et al., 2000). Thus, it is conceivable to use the commercial product Actigard (acibenzolar-S-methyl, Syngenta) to improve protection of crop plants against grey mould infection.

Another possibility might be the exploitation of induced systemic resistance which non-pathogenic rhizobacteria trigger (Pieterse et al., 1998). The *Arabidopsis* mutants *coil* and *ein2*, which are impaired in jasmonic acid (JA) and ethylene signalling, respectively, are more susceptible to *B. cinerea* than wild-type (WT) plants (Thomma et al., 1998, 1999). Conversely, treatment of tomato plants with ethylene enhances resistance to *B. cinerea* (Diaz et al., 2002). Even though JA did not alter disease progression in tomato (Audenaert et al., 2002), over-expression of the signal peptide prosystemin, which induces the octadecanoid (i.e., JA biosynthetic) pathway (McGurl et al., 1994), increased resistance to *B. cinerea*. Induction of wound responses may not be beneficial under all circumstances; for instance, ethylene, made in response to physical tissue damage, elevates the susceptibility of tomato fruits, apparently via its promotion of ripening (Barkai-Golan and Lavy-Meir, 1989). *Botrytis* infection induces both active oxygen species (AOS) and ethylene production in leaves of bean, tomato and other plants and the phytohormone induces additional ethylene production in exposed tissue (Elad, 1990, 2002; Chapters 8 and 10). Thus ethylene may reduce or enhance resistance, depending on the plant organ and species that perceives the signal, and on the experimental conditions.

There is considerable cross-talk between wound-, pathogen- and insect-response pathways. In addition, each of the endogenous signal molecules that participate in defence coordination (JA, SA, ethylene, abscisic acid, and AOS (Chapters 5, 6 and 8) also participate in the coordination of an array of plant developmental and abiotic environment response events. Therefore, manipulation of endogenous hormone concentrations by direct applications or genetic engineering is likely to have broad impacts on crop plant performance beyond their impacts on defence. As a result, the most effective use of information about the ways in which plants regulate their defences against pathogens will probably be to follow the pathways of signal transduction that lead to defence expression specifically. For example, an alternative form of crop protection is interference with fungal virulence mechanisms. *B. cinerea* is a necrotrophic pathogen that causes host cell death. Expression of anti-apoptotic proteins is an effective mechanism of obviating fungal pathogenesis (Dickman et al., 2001). Similarly, the *Arabidopsis dnd1* mutant which is impaired in cell death, is more resistant to *B. cinerea* than the WT (Govrin and Levine, 2000).

4.1. The promise of gene "discovery"

Some of the biochemical responses of tomato fruit and seeds to *Botrytis* spp. have been measured and compared to responses against viral and bacterial pathogens (Georgieva et al., 2000). Peroxidase activity increased in green fruit pericarp tissue 150% after grey mould infection, supporting the proposal that free radicals and AOS participate in signalling for plant responses to pathogens, even in fruit and seeds. In

infected pericarp and seed tissues, dehydrogenase activities decrease, probably due to plant cell death, although in the surrounding tissues these activities may increase so as to contribute to repairing or limiting the pathogen damage. Uniquely, in *Botrytis*-infected tomato pericarp and seeds, β -glucosidase activity increases (Georgieva et al., 2000, 2001) and this increase may be part of a specific plant response to synthesize fungitoxic compounds (Douglas, 1996). Undoubtedly, both transcriptional activation of suites and networks of genes whose products catalyse and regulate production of defence factors, as well as functional activation and synthesis of families of enzymes and proteins, are responsible for these altered metabolic functions in infection sites and the surrounding tissues. Some of these many changes may reflect the pathogen's co-opting of plant processes in order to make infection more certain; others will reflect the expression of defence factors. Unravelling the interlaced threads of the reluctant host's activated signal pathways is becoming more feasible and could provide the key to separation of desirable and undesirable crop plant responses.

One molecular mechanism to activate diverse groups of proteins for a common purpose is through simultaneous increased transcription of co-ordinately controlled genes. Initially, characterization of plant gene expression in response to *Botrytis* infection was determined by measuring changes in mRNA abundance of groups of genes suspected to be involved in plant responses. Studies of tomato leaves (Benito et al., 1998) showed that mRNAs for a group of PR proteins became more abundant in infected tissues; transcription of PR-1 and intracellular and extracellular glucanases were activated within 16 h at 20°C. At lower temperatures expression pattern differences between the glucanase and chitinase genes became clearer. Groups are beginning to evaluate pathogen-induced transcriptional activation of plant gene expression using micro-arrays of cDNAs or genomic sequences. "Global expression phenotyping" has been used to assess changes in gene expression of one-third of the *Arabidopsis* genome in response to *Pseudomonas syringae* infection (Glazebrook et al., 2003). Using global expression analysis of mutants with perturbations in salicylic acid (SA), jasmonic acid (JA) and ethylene signalling, the sequence and interdependence of changes in signalling pathways in response to *P. syringae* were deduced. Concurrently regulated genes were identified and new genes activated by the infection were identified. Previously, gene expression changes in *Arabidopsis* in response to *Alternaria brassicicola* were analysed using cDNA micro-arrays (Schenk et al., 2000), as were global transcription changes in *Arabidopsis* expressing systemic acquired resistance (Maleck et al., 2000).

Researchers are currently using micro-arrays to study *Arabidopsis* gene expression in response to *Botrytis* as well as in response to oligosaccharides that are mimics of potential plant defence signalling molecules generated during pathogenesis (Dewdney et al., 2003; Ferrari et al., 2003b). Results from these studies and others evaluating *Arabidopsis* lines with mutations in SA, JA and ethylene signalling pathways indicate that networks of gene expression are activated by pathogen contact. How and which networks are activated depends on the pathogen (reviewed in Kunkel and Brooks, 2002). The details of which pathogen functions are important for expression networks to be activated are suggested by studies looking at

gene expression changes in response to wounding (Durrant et al., 2000; Reymond et al., 2000), AOS (Desikan et al., 2001) and chitin fragments (Ramonell et al., 2002). Studies in our laboratories (L. Yang, A.L.T. Powell, S. Lurie, H-J. An, C.B. Lebrilla and J.M. Labavitch, University of California, Davis, USA, unpubl.) are examining the pectin-derived oligosaccharide signals from *B. cinerea* lesions on tomato tissues and testing their impacts on host gene expression. Correspondence between genes activated in *Arabidopsis* and in other plant species has not been demonstrated, but is expected as expression characterization using microarrays of cDNA and genomic sequences is developed further in tomato and other plant species. Additionally, it is likely that plant gene expression schemes in response to pathogens differ between tissues of the plant. Gene expression activated in leaves may not be identical to the patterns of expression seen in fruit and, indeed, during development of various plant tissues and organs, the patterns of expression are likely to change.

Changes in expression of suites of genes can be regulated by transcription factors. These proteins interact with specific sequence elements in the promoters of genes and influence the amount of transcription of genes with these sequences. The activity of the transcription factor can be regulated by the abundance of the factor, by interactions with other proteins or by modifications such as phosphorylation. The *As-1* promoter element in tobacco and *Arabidopsis* confers tissue-specific expression but is also responsive to wounding, plant hormone signalling for defence, and xenobiotic stress (Johnson et al., 2001). A family of basic leucine zipper (bZIP) transcription factors bind to this promoter element and one individual (TGA1a) has been identified as a factor activated by chemical stress. Two other bZIP transcription factors, TGA2 and TGA5 in *Arabidopsis*, interact together with the *As-1* promoter and regulate the induction of systemic acquired resistance through the NIM1/NPR1 system (Kim and Delany, 2002). Over-expression of TGA5 increases resistance to *Peronospora parasitica*. However, TGA5 probably also is involved in SA-independent pathogen resistance since over-expression in a *nim1-1* mutant was also resistant. A plant transcription factor with protein phosphatase activity, DBP1, has been identified in tobacco. It regulates the *CEVII* promoter; *CEVII* expression is defence-related in tomato (Mayda et al., 2000; Carrasco et al., 2003). It is expected that other transcription factors will be identified whose abundance is responsible for regulating gene expression in response to pathogens, including *Botrytis*. Interestingly, in another system, resistance to *B. cinerea* was found in tobacco transformed with the NC330 cDNA encoding an inhibitor of viral replication (IVR)-like protein that is resistant to tobacco mosaic virus (Akad et al., 2002).

The expression and activity of transcription factors is regulated through many signalling pathways. In suspension cultures of *Lycopersion peruvianum* cells, mitogen-activated protein kinase (MAPK) activity is induced by stress signals, systemin and oligosaccharide elicitors, that develop in pathogen-challenged plant tissues (Holley et al., 2003). Although their targets in plants have not been identified, MAPK substrates include transcription factors that can activate or inactivate multiple responses using transcription factor-regulated gene expression. Separate MAPKs are induced by systemin, chitin and chitin-derived oligosaccharides, β -glucan, polygalacturonic acid and by UV stress (Holley et al.,

2003). One would anticipate that different pathogens also will induce specific MAPKs or other signal-response systems in plants and these responses may differ throughout the plant and during development.

These multiple control points provide attractive targets for specifically altering resistance and susceptibility to *B. cinerea*. Manipulation of the expression of suites of genes through transcription factors and signals regulating transcription factor function will influence simultaneously many aspects of the plant tissue's answer to *Botrytis*.

5. Improvement of microbial control agents for better disease suppression

Many filamentous fungi, yeasts and bacteria have been found to be effective in controlling *Botrytis*-incited diseases (Elad and Freeman, 2002). The examples discussed below are drawn primarily from work with some isolates of filamentous fungi, yeasts and bacteria. Readers should keep in mind that the strategies discussed pertain also to enhancement of the biocontrol potential of all useful microorganisms (Chapter 13), recognizing that specific strategies must be compatible with the biology of the species being modified. Important modes of action of microbial control agents are induction of host plant defence factors, secretion of antibiotics and toxins, mycoparasitism and secretion of enzymes that degrade the pathogen's cell walls, reduction of the pathogen's saprophytic capabilities, reducing spore dissemination, and limiting the deployment or effectiveness of pathogenicity factors (Elad, 1996; Chapter 13). In the discussion that follows, the relevance of several (not all) of these mechanisms will be illustrated with examples. Biological issues relevant to maximization of the biocontrol impact of improved microorganisms are also discussed. In general, successful microbial control of plant diseases depends on an antagonist's ability to be active in the pathogen's niche and to use one or more of these mechanisms in the presence of the pathogen. Perhaps more important is the practical requirement that the antagonist must be able to proliferate and persist on the crop surface under a wide, often extreme range of microclimate conditions. Thus, enhancement of an antagonist's anti-*Botrytis* activity may result from improvements in its ability to use potential modes of action, increased responsiveness of the host plant to the antagonist's induction of pathogen defences, or increased persistence and survival of the antagonist on plant surfaces.

5.1. Enhanced production of enzymes and antibiotics

Enhanced production of fungal cell wall-degrading enzymes and antibiotics has been associated with increased antagonism of *B. cinerea*. The data supporting a role for endo-chitinase in the biocontrol shown by *Trichoderma* spp. are equivocal. Disruption of the *ech42* (endo-chitinase-encoding) gene in *T. harzianum* reduced both enzyme activity and activity against *B. cinerea* in culture (Woo et al., 1999). Furthermore, a *T. harzianum* mutant with two to four times more chitinase, β -1,3- and β -1,6-glucanase activities than the WT and producing 3-fold increase of the inhibitor α -pyrone provided better protection for grapes against *B. cinerea* (Rey et al., 2001). However, chitinase over-expressing and deletion *T. atroviride* mutants

(*chit42*) showed the same level of control as the WT (Carsolio et al., 1999). A similar absence of differential control of *B. cinerea* was shown by mutant strains of *Serratia plymuthica*, with high and low chitinolytic activity relative to controls (Kamensky et al., 2003).

Combinations of anti-pathogen factors having different mechanisms of action in a single agent should have particular utility. The synthesis of both peptaibols and hydrolytic enzymes by *T. harzianum* was induced in the presence of *B. cinerea* cell walls. When purified peptaibol antibiotics and hydrolyases were added singly or together in tests of antagonism of *B. cinerea* and *Fusarium oxysporum* hyphal elongation and spore germination, the combination was synergistic relative to the effects of antibiotics or enzymes added singly (Schirmböck et al., 1994). Culture filtrates of *Serratia marcescens* contain different chitinolytic enzymes, including a 58-kDa endo-chitinase and a 98-kDa chitobiase, as well as a red pigment, prodigiosin. Individually, the pigment and chitinases inhibited *B. cinerea* spore germination. However, when applied in concert, a synergistic inhibitory effect was observed (Someya et al., 2001). In antifungal bioassays, the purified lipodepsipeptide toxins, syringomycin E (SRE) and syringopeptin 25A (SP25A), of *Pseudomonas syringae* pv. *syringae* interacted synergistically with chitinolytic and glucanolytic enzymes purified from the same bacterial strain or from the biocontrol fungus *T. atroviride* (Fogliano et al., 2002). Thus, combination of proteins and antibiotics can enhance “antagonism potential”.

Increased activity of cell wall-degrading enzymes can be obtained by inclusion of additives to product formulations. Polysaccharides from host cell walls (Sivan and Chet, 1989; Schickler et al., 1998; Mach et al., 1999) may function as “primer” substrates whose breakdown generates elicitors of host defence genes, additional enzyme synthesis, or allosteric activators of key enzymes. Basal amounts of wall-degrading enzymes have been detected under non-inducing conditions. The low enzyme activity may release and “activate” host cell wall polysaccharides to elicit stronger enzyme production and additional defence responses.

The elicitation of biocontrol genes suggested above may be useful for management of antagonistic microorganisms in the field. Integration of multiple copies of the *T. atroviride prb1* proteinase gene into the *T. atroviride* genome increased biocontrol of *Rhizoctonia solani* five-fold relative to the WT (Flores et al., 1997). *prb1* is expressed in response to chitin, a non-substrate (Geremia et al., 1993) and, in *T. atroviride* and *T. hamatum*, by glycerol, suggesting carbon source-related regulation (Cortés et al., 1998). The inducer of *ech42* and *prb1* was probably a polysaccharide in *R. solani* (Cortés et al., 1998; Zeilinger et al., 1999; Kullnig et al., 2000). Possible inducers from *R. solani* cell walls have been tested for biocontrol activity and impacts on enzyme production, with the intent of commercial exploitation (Kubicek et al., 2001). Whether these inducers act in their polymeric form or after degradation by constitutive enzymes to generate active oligosaccharides is not known. In *T. atroviride*, *prb1* expression is also controlled by nitrogen catabolite repression (Olmedo-Monfil et al., 2002). Repression may be mediated through interaction of regulatory proteins with promoter GATA motifs, such as those in other mycoparasitic genes from *Trichoderma* spp. (Cortés et al.,

1998; Donzelli et al., 2001). Elucidation of the regulatory pathways involved in eliciting these molecular responses may enable greater prediction, manipulation and maximization of biocontrol potential (Steyaert, 2003).

When hydrolases contribute to biocontrol, their secretion and diffusion to the pathogen target are prerequisites for mycoparasitism, including impacts resulting from induction of host genes (Cortés et al., 1998; Zeilinger et al., 1999; Kullnig et al., 2000). Thus, increased hydrolase production may enhance antagonism and improve biocontrol. Post-translational events, such as those related to the secretory pathway and membrane permeation, may be altered in a *T. harzianum* mutant with increased protein secretion and biocontrol activity (Palmarezyk et al., 1998; Rey et al., 2001). Enhanced secretion by a proteinase-producing *T. atroviride* transformant contributes to its enhanced effectiveness as a control agent (Flores et al., 1997). Enhanced control may result from selection for variants with small increases in several extracellular enzymes and antibiotics. This should reduce self-toxicity while enzyme cooperation in digestion of pathogen-localised substrates would likely improve antagonism. While the accumulation of individual hydrolytic enzymes *in vivo* at far below the levels needed for effective fungicidal activity may be insufficient for pathogen control, combinations of enzymes with different activities may increase the net antifungal impact (Steyaert, 2003).

5.2. Induction of plant defences

Inhibition of *B. cinerea* pathogenicity enzymes (e.g., pectinases and cutinase) by serine protease is one of the modes of action used by *T. harzianum* T39 (Elad et al., 1998; Elad and Kapat, 1999). The effect on disease severity appears to be both direct (i.e., on the enzymes' activities) and indirect (i.e., activating leaf defence mechanisms; Elad et al., 1998). The PG of *B. cinerea* elicits defence mechanisms in bean leaves (Urbanek et al., 1991), perhaps because of accumulation of elicitor-active OGA products, as discussed in Sect. 3.1.1. and in Cervone et al. (1989). This mechanism could be activated if *T. harzianum* on bean leaves can act to alter the pectin breakdown catalysed by *B. cinerea* PGs so that larger OGAs that are defence elicitors accumulate in its presence, but not when *T. harzianum* is absent (Zimand et al., 1996). The use of control microorganisms that can facilitate the induction of plant defences, by whatever means, should be further explored for the protection against plant diseases, including *Botrytis*-incited diseases.

5.3. Compatibility with diverse abiotic conditions

Effective suppression of plant diseases by microbial agents is largely affected by the external biotic and abiotic conditions (Elad, 1996; Elad and Freeman, 2002). A strategy to reduce variability in biocontrol while increasing field efficacy is based on simultaneous application of several biocontrol agents (BCAs), each having different ecological requirements for survival, growth and activity (Guetsky et al., 2002b). *Pichia guilliermondii* and *Bacillus mycooides* were tested separately and together for suppression of *B. cinerea*. The combination resulted in additive activity, as

compared to separate application, over a wide range of microclimate conditions. The benefit was due to the summation of biocontrol mechanisms of both agents (Guetsky et al., 2002b), suggesting that the search for compatible BCA mixtures should include a consideration of the possibility of niche “complementation” for mixture applications.

The efficacy of biocontrol may be influenced by the availability of nutrients in the phyllosphere, and by the interaction and competition between the microorganisms present. Thus application of nutrient supplements together with microbial agents can improve establishment, survival, and activity and, thus, efficacy, under natural environmental conditions. Guetsky et al. (2002a) found that additions of citrulline, leucine, pantothenic acid, thiamine, choline chloride and *myo*-inositol improved the biocontrol of *B. cinerea* by *P. guilliermondii* and *B. mycooides*. Additives may be used in biocontrol formulations provided they do not promote pathogen growth or negatively affect the plant.

The development of individual microbial agents that are effective under a wide range of biotic and abiotic conditions has proved to be difficult. This is not surprising since they are living organisms and affected by the environment, as is the pathogen. *Ulocladium atrum* in liquid cultures responded to water-stress by accumulating increased concentrations of sugar alcohols (polyols), particularly glycerol, that are normally present in the cells. The inoculum from these stressed cultures retained viability during storage better than that from unstressed cultures (Frey and Magan, 2001). Trehalose accumulates in a large number of organisms in response to stress conditions. It mediates desiccation tolerance by affecting both proteins and lipid membranes, apparently by replacing the shell of water around macromolecules, thus preventing damage during drying. Transformation of tobacco with a gene encoding the trehalose phosphate synthase (Romero et al., 1997) increased the production of trehalose and thereby improved the drought tolerance of tobacco plants. Viability of the post harvest BCA, *Candida sake* was improved by addition of trehalose to the liquid formulation (Abadias et al., 2003). It seems that engineering trehalose or glycerol biosynthetic capacity into microorganisms might confer improved survival under adverse conditions.

5.4. Microorganisms as sources of anti-fungal products

Microorganisms may be sources of novel *Botrytis*-inhibiting compounds. If so, the “source” organism or other microbes can be engineered for high-level expression of genes responsible for inhibitor production. The transgenics can then be used directly as BCAs or cultured for “factory” production of anti-fungal proteins or enzyme products. Chaetoatrosin A, a chitin synthase II inhibitor, was isolated from *Chaetomium atrobrunneum* culture broth. It showed antagonistic activity against *B. cinerea* and other fungi (Hwang et al., 2000). Enzymes that degrade fungal cell walls may be produced by antagonistic microorganisms, isolated from the producer, formulated and used for control of *Botrytis*. Such isolated enzymes were effective in plant-pathogen bioassays performed in the absence of the enzyme-producing biocontrol organism (Y. Elad, unpubl.). Microbial genes encoding products that

induce plant resistance to *Botrytis* (e.g., Chapter 13; Elad and Kapat, 1999; Elad and Freeman, 2002; El Ghaouth et al., 2003) could also be engineered into biocontrol microorganisms or into crop plants. In the latter situation, however, gene expression would have to be placed under the control of pathogen-responsive promoters. For instance, it is thought that cell wall surface components or other secreted products are responsible for control agents' initial induction of defence.

5.5. The perfect microbial agent

What we define as microbial control of plant diseases is the result of the aggregate impact of an array of interacting factors that lead to disease control under field conditions. An important target for development of the super microbial control agent for limiting *Botrytis* disease is the optimisation of combinations of "activities" that give a multi-pronged attack on the fungus with a minimum of disturbance to the productivity (quality and quantity) of the crop plant. Included in such an optimised combination should be production of an array of resistance inducers, enzymes and inhibitory compounds that are stable and act synergistically on plant surfaces. The control organism must compete well with the pathogen and sustain itself in the desired plant niches while utilizing compounds secreted by the plant or supplied externally and may even selectively inhibit the pathogen. It must withstand severe conditions of temperature, relative humidity and osmotic pressure and be able to expand its population and colonize the plant it is to protect while experiencing these extremes. It must survive both on plant surfaces and on the shelf and be able to switch from dormant to active stages quickly. Optimally, such a microorganism will have expanded pathogen host range and be compatible with many host plants. A substantial variety of genes and gene products that negatively impact pathogen growth have now been described. Many of these are potentially useful in optimised biocontrol microbes. Thus, efforts for developing the next generation of control microbes should probably be focused on manipulations aimed at enhancing the microbes' ability to tolerate the abiotic environmental stresses that limit expansion of the agents' population in agricultural settings.

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