Recent advances in *Botrytis* research

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Table of contents

Epidemiology

Biological Control

Chemical control

Preface

Since 1964, plant pathologists working on diseases caused by *Botrytis* spp. have met regularly in the so-called European *Botrytis* symposia and ten of these symposia have been held. At the last five symposia, specialists on this subject from other continents also participated in the discussions. At the symposium, held in 1976, it was suggested that a book on the state-of-the-art in *Botrytis* research should be produced. As a result, 'The Biology of *Botrytis*' was published in 1980, edited by J.R. Coley-Smith, K. Verhoeff and W.R. Jarvis.

Since 1980, more work on these fungi and the diseases they cause have been published and an up-dating of the book was needed.

In 1992, the 10th *Botrytis* symposium took place on Crete, Greece, and it seemed appropriate, to publish the Proceedings of this symposium. Therefore, one specialist of each section of the symposium was asked to prepare a review paper. These reviews were followed by shorter presentations and a number of posters were presented.

The text of almost all the oral presentations and of some of the posters, are presented in this book.

We are grateful to the authors for their cooperation in preparing this book. We would like to express our sincere thanks to Mrs. J.M . Mokveld, who did all the typing.

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N.E. Malathrakis
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Introduction

K. Verhoeff

The genus *Botrytis*, erected in 1729, contains a large number of host-specific pathogens (e.g. *B. fabae* on broad bean, *B. aclada* on onion, *B. tulipae* on tulip), and a single broad spectrum pathogen, viz. *B. cinerea*, which attacks a wide range of plants in temperate regions. *B. cinerea* attacks field-grown crops like grapes, but is also important in glasshouse-grown vegetables, flowers and fruits, during production or post-harvest during storage and transport. Due to its broad spectrum, *B. cinerea* attracted more attention than any of the host-specific species. It is difficult to assess the damage caused; in many crops, depending on prevailing weather, economic losses of more than 50% occur. In glasshouse crops, the losses also depend to a large extent on the climate in the glasshouse, although conducive climatic conditions can be reduced to some extent.

As a pathogen, *B. cinerea* has received attention for more than 100 years (de Bary, 1886; Ward, 1888) studies on its biology and host-pathogen interactions were started somewhat later (Brown, 1915,1916,1917; Blackman and Welsford, 1916). Since then, many papers have been published on aspects of the disease in various crops, most notably in grape, soft fruits, tomato and (other) glasshouse-grown crops. Although our knowledge of the fungus and the diseases caused by it has increased substantially, the work has be en hampered by one important factor: *in vivo*, only asexual multinucleate conidia are found and single-spore isolates always have shown phenotypic variation when subcultured. It was therefore impossible to compare directly results obtained with *B. cinerea* in grape from one region with those obtained in another region; this also holds for other crops.

Epidemiological studies were difficult because it was not possible to establish whether populations differed according to crops or geographical region. It was also impossible to know, whether a population present in a crop at the beginning of the season was still the same at the end of it.

With the introduction of systemic fungicides, effective control of *B. cinerea* was possible for a short period, but after a few years, reports were made of less effective control caused by the development of resistant isolates of the fungus (e.g. Bollen and Scholten, 1971; Jarvis and Hargreaves, 1973; Miller and Fletcher, 1974). This led to many publications which described methods for achieving adequate control in this new situation.

The development of resistant strains provided convenient genetic markers and led to the recognition, that different populations of *B. cinerea* do exist. This stimulated genetic studies of *B. cinerea*, but also work on alternative control methods, especially biological control (e.g. Dubos, 1992).

Breeding for resistance has generally not been succesful, as few genes associated with resistance have been identified. Defence mechanisms are however important in plants and several are elicited upon infection by *B. cinerea* (e.g. Glazener, 1982; Pezet and Pont 1992; Hoffman and Heale, 1987), but this knowledge has not been exploited in breeding programmes.

Studies on the sexual state of *B. cinerea* were performed for a number of years with limited succes, but recently methods were developed for mass production of ascospores of *B. fuckeliana* (Faretra and Antonacci, 1987). This progress, together with modern

techniques in molecular genetics developed for other fungi (e.g. Van Kan *et al.*, 1991) can now facilitate research on *B. cinerea*. It is anticipated that genes which code for enzymes essential for penetration and spread of mycelium within a plant will be identified and, in this way, pathogenicity determinants will be found and new control strategies will focus on this. The same approach also applies to genes responsible for the elicitation of defence reactions.

With such genetic information, epidemiological studies can be carried out more efficiently, and models refined to predict outbreaks of epidemics. This information will also support better strategies for chemical and biological control.

It can be expected that with the results obtained on the biology of *B. cinerea*, basic work on genetics of the fungus, as well as on host-pathogen interactions and epidemiology will lead to more effective and more environmentally friendly control strategies.

'Recent Advances in *Botrytis* Research' marks the beginning of a new era in research on *B. cinerea*, with a spin-off to work on diseases caused by other *Botrytis* species.

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GENETICS

Genetic studies of *Botryotinia fuckeliana* (*Botrytis cinerea*)

F. Faretra and M. Grindle

Summary

Botrytis cinerea is the pathogenic fungus responsible for 'grey mould' disease of numerous soft fruits, vegetables and ornamentals. *Botryotinia fuckeliana* is the teleomorph of *B. cinerea*; it produces sexual progeny (ascospores) by meiosis of heterozygous diploid nuclei in ascogenous hyphae derived from matings of sexually-compatible isolates. Ascospore progeny can be obtained reliably in the laboratory, and so isolates of *B. cinerea* are a menable to genetic analysis by classical Mendelian techniques. In this report, we describe materials and methods for growth and genetic manipulation of *B. fuckeliana* in the laboratory, review information from classical and molecular genetic studies, and propose a system of nomenclature to be used when communicating genetic information.

Introduction

Botrytis cinerea Pers.: Fr. and *Botryotinia fuckeliana* (de Bary) Whetz. are asexual (anamorphic) and sexual (teleomorphic) stages, respectively, in the life cycle of the same filamentous fungus. The asexual stage, often referred to as the 'grey mould' fungus, consists of vegetative hyphae, sclerotia, macroconidia and microconidia. The sexual stage consists of a reproductive body, the apothecium, containing ascospores in linear asci (Fig. 1). Thus, *B. cinerea* (= *B. fuckeliana*) is a member of the largest class of fungi, the Ascomycetes.

Field isolates of *B. cinerea* grown on synthetic media in the laboratory exhibit considerable variations in phenotype (Paul, 1929; Grindle, 1979; Coley-Smith *et al.*, 1980). Those differences which influence the biological fitness of *B. cinerea* populations are of practical importance in agriculture; for example, field isolates which combine vigorous growth on host plants with resistance to an important fungicide used in plant protection can seriously reduce crop yields and profitability. Knowledge of the genetic basis of such differences in phenotype might be useful in monitoring populations of *B. cinerea*, assessing the role of particular genes on biological fitness, and aiding the development of new strategies to combat mutant isolates.

The genomes of *B. cinerea* isolates can be investigated in the laboratory by classical Mendelian analysis of sexual progeny (Faretra and Antonacci, 1987; Faretra *et al.*, 1988a,b). Isolates of different mating type are cross-fertilised to initiate the sexual stage of the life cycle. Analysis of the sexual spores (ascopores) from apothecia has revealed the genetic basis for diff erences in mating type, size of apothecial stalks, and resistance to certain fungicides (Faretra and Pollastro, 1991, 1992a,b).

This report discusses techniques for the maintenance and genetic analysis of *Botryotinia fuckeliana*, reviews genetic knowledge of the fungus, and provides a framework for nomenclature to be used when communicating genetic information.

Sources of field isolates and laboratory mutants

Field isolates are readily obtained from diseased plants, plant debris and soils, and by trapping aerial spores on dishes of selective synthetic media (Kritzman and Netzer, 1978; Kerssies, 1990). It is advisable to obtain 'monoconidial' rather than 'mass-hyphal' o r 'mass-conidial' isolates for laboratory studies, since the first type of isolate is more likely to be genetically homogeneous (i.e . homokaryotic, rather than heterokaryotic). Monoconidial isolates are obtained by spreading conidia at low density on dishes of synthetic media and transferring individual germlings to tubes with fresh media. The chances of genetic homogeneity are increased by obtaining a succession of monoconidial isolates from monoconidial isolates, selecting the 'most typical' progeny on each occasion.

Laboratory mutants can be derived from field isolates or, preferentially, from well-documented laboratory strains of known phenotype. It is advantageous to derive most mutants from a particular wild type standard, so that the mutants are isogenic; that is, their genomes are virtually identical, except for particular mutant genes causing a specific change in phenotype.

Spontaneous mutants can be obtained by incubating hyphae or macroconidia on selective media which discriminate between particular mutant and normal phenotypes. For example, mutants resistant to a specific antifungal chemical can be obtained by incubating spores on media containing lethal or sub-lethal amounts of that chemical. Induced mutants can be obtained by deliberately exposing macroconidia to chemical or physical mutagens, before incubation on selective media. Since macroconidia are multinucleate, mutants derived from them frequently contain a mixture of mutated and non-mutated nuclei in heterokaryotic hyphae; such mutants are phenotypically unstable, producing mutant and non-mutant progeny during subculturing. The uninucleate microconidia might be better sources of mutants if they could be induced to germinate on synthetic media.

Field isolates and laboratory mutants of various phenotypes and genotypes are maintained in the culture collection of F. Faretra at the University of Bari, Italy. The collection includes standard laboratory strains of known mating type. Cultures in tubes of solid synthetic medium remain viable for several months at 5°C, but persistent storage under these conditions can lead to accumulation of mutations, reduction of aggressiveness and loss of ability to produce sclerotia and/or apothecia. Conidia or hyphal fragments can be stored for about 2 years at 5°C on anhydrous silicagel as described by Wilson (1986). Conidia and ascopores remain viable for at least 1 year in 10% glycerol at –70°C; this is probably the best method for storing strains.

Synthetic media for vegetative growth

Most isolates grow well on various defined and undefined synthetic media. Undefined media usually provide abundant organic nutrients for luxuriant growth, but their precise compositions are not known. Defined media contain known quantities of inorganic salts and supplements so that their ingredients can be reproduced or modified precisely; this is necessary for some genetic analyses, such as detection of genes affecting biosynthetic pathways. Our principal undefined media are malt extract agar (MEA; 20 g Oxoid malt extract and 20 g agar.l^{-1} water) and potato dextrose agar (PDA; 200 g peeled and sliced potatoes simmered for 1 h in water and filtered through cheesecloth; the filtrate volume made up to 1 litre, and pH adjusted to 6.5; 20 g glucose and 20 g agar added before

autoclaving). This home-made PDA is superior to commercial preparations. The defined media used for growth of *Aspergillus nidulans* (Pontecorvo *et al.*, 1953) or *Neurospora crassa* (Vogel, 1964; Davis and de Serres, 1970) also support good growth of *B. cinerea*. The composition of the media are given in Annex 1. These media vary with respect to their contents, preparation and storage. The effects of the differences in media composition on growth, conidiation and sclerotia development have not been quantified. For example, it is not known whether the phenotypes of strains are significantly affected by variations in trace elements or vitamins. Although *B. cinerea* grows reasonably well on all these minimal media, it grows considerably better on the complete media.

Sexual reproduction and classical genetics

Production of apothecia

Apothecia are the reproductive bodies which emerge from fertilised sclerotia (Figure 1). The procedure for obtaining apothecia has been described by Faretra and Antonacci (1987) and Faretra *et al.* (1988a,b): isolates to be crossed must have opposite mating ty pes; they are inoculated on to separate dishes of MEA and grown in the dark for 3 days at 21±1°C, followed by 4 weeks at 15±1°C and, finally, 4 weeks at 0°C to obtain sclerotia which are capable of carpogenesis; the sclerotia are transferred to modified test tubes containing sterile water, and fertilised with microconidia from cultures of the opposite mating type; fertilised (spermatised) sclerotia are incubated at 11±1°C with exposure to incandescent and fluorescent lights on a 12 h photoperiod. Successful crosses produce apothecia 3-13 weeks after spermatisation.

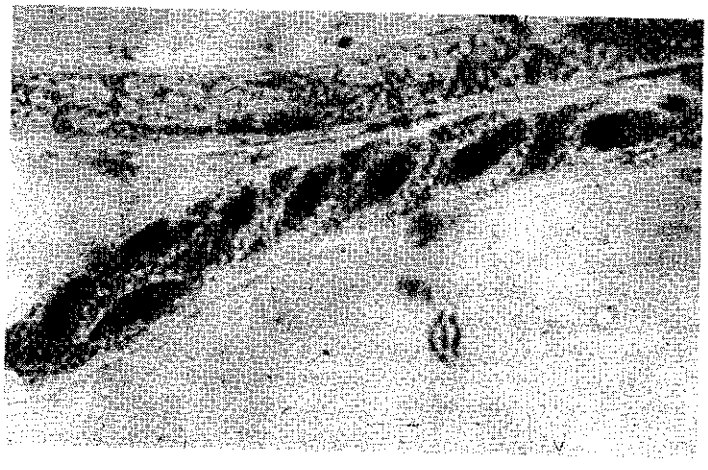
Isolates can usually function as the 'male' and the 'female' parent in reciprocal sexual crosses. For example, sclerotia of isolate A can be fertilised by conidia of isolate B (i.e. the cross is A ♀+ x B ♂) or sclerotia of B can be fertilised by conidia of A (B ♀+ x A ♂). Isolates can occasionally produce apothecia without cross-fertilisation; self-fertile isolates are probably heterokaryons carrying the two mating type alleles in different nuclei (Faretra and Pollastro, 1992a).

Isolation of ascospores

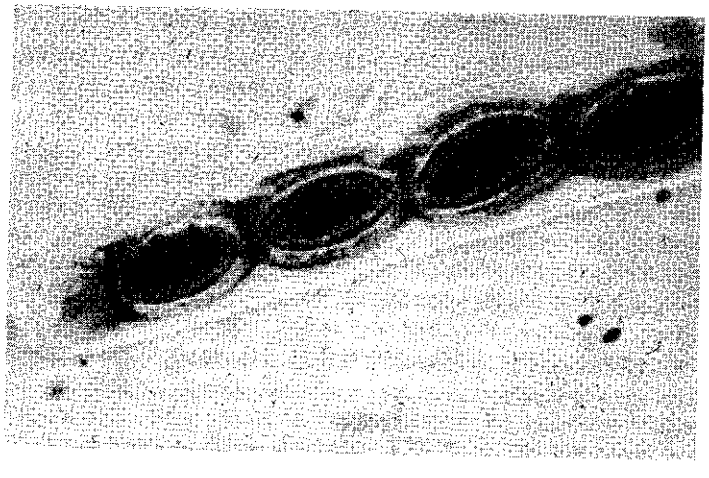
Ascospores are the sexual spores which eventually develop from the nuclei in spermatised sclerotia. Fusion of haploid nuclei from the two parents of opposite mating type produces heterozygous diploid nuclei, each of which undergoes meiosis and gives rise to eight haploid ascopores contained within an ascus (Fig. 1). An ascus comprises four pairs of ascospores, each pair having genetically identical nuclei, in a linear sequence; the spores probably develop in the same way as the ordered spores in linear asci of *Neurospora crassa* (Raju, 1980). Each apothecium usually contains several hundred asci.

Sets of eight ascospores for studies of ordered tetrads (Faretra *et al.*, 1992a) are obtained as follows: an apothecium is placed in a drop of sterile distilled water in a Petri dish, the hymenial layer is broken with a dissecting needle, and the asci are teased apart; individual asci are separated, and the ascospores in each ascus are dissected out in their correct sequence with a micromanipulator; the dissected spores are transferred to fresh media, where they germinate after 12-16 h at 21+1°C.

a



b



c

Fig. 1. Sexual reproduction of Botryotinia fuckeliana. a) apothecia produced by sclerotia in modified test tubes exposed to incandescent and fluorescent light at 11±1°C. b) a compl ete ascus containing eight ascospores. c) portion of an ascus, showing multinucleate ascospores

Samples of random ascospores are obtained by placing several apothecia in a vial of sterile distilled water and squeezing them with forceps or dissecting needles to obtain a suspension of spores. Alternatively, a single apothecium can be treated in 200-300 µl water in an Eppendorf tube; this is the recommended method, since transmission of genetic markers may not be identical in different apothecia. Spore suspensions are spread on dishes of water agar or growth medium and incubated at 21±1°C to obtain monoascospore colonies.

Cytology

The haploid number of chromosomes in vegetative hyphae of *B. cinerea* (Shirane *et al.*, 1988, 1989) and in developing asci of *B. fuckeliana* (Faretra and Contesini, unpublished) is sixteen.

Molecular genetics

We have commenced molecular studies in our laboratories to explore and manipulate the genome of *B. fuckeliana* using techniques such as those described by Leong (1988). Differences between field isolates and ascospore progeny have been identified by RFLP techniques (Grindle, unpublished). Electrophoretic karyotyping has revealed considerable variability among field isolates and laboratory strains (Faretra and Grindle, unpublished). Protoplasts can be obtained by using various enzymes and osmotic stabilisers (Faretra *et al.*, 1992a); they have been transformed with DNA from a plasmid carrying the hygromycin B phosphotransferase gene as a selectable marker (Chabani *et al.*, 1990), but transformation efficiency is very low.

Genetic nomenclature

There are various, conflicting systems of nomenclature for describing the genotypes of fungi. We propose that the following nomenclature (based on the suggestions of Yoder et al. (1986) be followed when communicating genetic information on *B. fuckeliana*.

The term **'strain'** can be used to distinguish cells or colonies of any particular isolate from those of other isolates, whether they originated in nature or in the laboratory. A strain does not have to be characterised genetically. Strains are assumed to be identical if they are asexual progeny (e.g. derived from conidial or hyphal inocula) of the same homokaryotic parent. A **field isolate** is any strain obtained from a natural source (e.g. from infected plants in the field, or from the air in a greenhouse) and not knowingly altered in the laboratory. A **wild type** strain has the 'normal' or 'standard' phenotypic traits of typical field populations (or of most laboratory strains, if the 'normal' phenotype of field isolates is not obvious). A **mutant** strain differs phenotypically from a normal strain due to one or more heritable changes caused by mutation(s) of genes or chromosomes; the mutations can be spontaneous or induced. Mutants which cannot grow on basal (minimal) medium unless it is supplemented with one or more specific chemicals such as an amino acid, are referred to as nutritional or **auxotrophic** mutants. Mutant strains which can be grown on basal medium, such as those which differ from normal strains in their pigmentation or response to fungicides, are **prototrophic** mutants.

When there is no obvious 'wild type' by which a 'mutant' can be judged, it might be better to describe phenotypically different strains as **variants**. For example, natural

variations in size of sclerotia among field isolates are probably caused by many alleles of the genes responsible for this phenotype. Since each allele would be a typical constituent of the total gene pool of a population, a variant would be a typical individual carrying one of those alleles. This phenomenon is known as genetic polymorphism. It can be argued that all naturally-occurring strains with different phenotypes caused by different alleles should be referred to as variants. However, some rare alleles may become prevalent due to man-made selection pressure, and strains carrying these alleles (e. g. fungicide-resistant strains) could perhaps be described as mutants since they can be distinguished from typical strains in normal populations.

Phenotypes are referred to by the same symbols used to designate genotypes (see below), except that the symbols are not italicised. For example, a biotin-requiring auxotrophic mutant might have the genotype *Bio1-* and the phenotype *Bio1-*.

The genotype of a strain can be identified by classical or molecular methods. Having identified a particular gene which affects a particular phenotypic character, the gene is given a unique, three-letter symbol which relates to that character. For example, mutants requiring adenine for growth can be designated Ade, and mutants resistant to fungicides containing the active ingredie nt methyl benzimidazole-2yl-carbamate can be designated Mbc. Appropriate symbols can be found in fungal genetics texts and review articles (e.g. Fincham *et al.*, 1979; Perkins *et al.*, 1982). The gene symbol is italicised, with a capital first letter.

Different genes which modify the same phenotype are numbered, the number following the gene symbol without a space or hyphen; for example, three genes affecting the ability to synthesise tryptophan could be *Trp1*, *Trp2* and *Trp3*. Genes which reside in the mitochondrial genome are enclosed in square brackets [ ] to distinguish them from nuclear genes.

Normal (wild type) and mutant alleles are distinguished by + and – signs, respectively, if the normal allele is obvious. In the special case of alleles of genes affecting a strain's response to antifungal chemicals, sensitive and resistance alleles are distinguished by the letters S and R, respectively. Alleles conferring different levels of resistance can be disinguished by the letters LR (low resistance), HR (high resistance), and so on.

The signs and letters for different classes of alleles follow the gene symbol, and are not superscripts. For example, normal and mutant alleles of a gene affecting melanin biosynthesis could be Mel1+ and Mel1-; sensitive and resistant alleles of a gene responsible for susceptibility to the antibiotic oligomycin could be *Oli1S*, *Oli1LR* and *Oli1HR*.

Dominant and recessive alleles of a gene are distinguished by upper case and lower case letters, respectively, for all letters of the gene symbol. For example, the dominant, normal and recessive, mutant alleles of a gene affecting histidine biosynthesis could be *HIS1+* and *his1–* respectively. Tests for dominance/recessivity of alleles are often problematical, and so many allel es may not be distinguished in this manner (Grindle, 1987, 1992).

Specific alleles of a gene are distinguished by numbers and/or letters in brackets, immediately after the allele designation . It is often useful to have a descriptive notation to indicate the source of the allele. For example, the symbols *His2*(UBF5) and *His2*(USG8) could distinguish two independent mutants carrying different alleles of the same gene affecting histidine biosynthesis, the former isolated at the University of Bari by Faretra, and the latter at the University of Sheffield by Grindle.

Genes can be assigned to linkage groups which are distinguished by Roman numerals (LGI, LGII, etc.). These are groups of genes which are probably on the same chromosome, since they tend to remain linked together during meiosis. Each gene occupies a particular site (locus) on a linkage group, which can be mapped in relation to other linked genes. The terms gene and locus are often used interchangeably, but gene is more specific. A locus could encompass two or three adjacent genes which are difficult to distinguish or separate by conventional Mendelian techniques.

When describing the genotype of a strain carrying several genes which have been identified and assigned to linkage groups, linked genes are separated by a single space and unlinked genes (on different linkage groups) by a semi-colon. For example, a strain having the genotype *his1*; *mel1 arg3*; *arg2* carries recessive alleles of four genes (on three linkage groups); *mel1* is linked to *arg3*, but the two *arg* genes are unlinked. When linkage information is not available, all genes are sep arated by single spaces; for example, two strains used in a sexual cross might have the genotypes *ade1- MbcS MAT1-1* and *ADE1+ MbcHR MAT1-2*, respectively.

Determining whether mutant genes are dominant, recessive, or allelic

Testing for dominance or recessivity can be done as follows. Two strains, one carrying the wild type allele and the other the mutant allele of a particular gene, are grown together so that their hyphae can anastomose to form heterokaryotic mycelium (Grindle, 1987, 1992). Heterokaryon formation in many species of fungi is restricted by nuclear genes controlling heterokaryon compatibility (Grindle, 1992), but the presence of such genes in *B. cinerea* has not been evaluated. It seems that the mating type gene does not influence vegetative compatibility, since pairs of strains with identical or different *MAT1* alleles can form heterokaryons (Chabani and Grindle, unpublished). It might be possible to overcome heterokaryon incompatibility problems by fusion of protoplasts. The mutant allele is completely (or partially) dominant if the heterokaryon is phenotypically identical (or similar) to the mutant parent; it is completely (partially) recessive if the heterokaryon is phenotypically identical (similar) to the normal parent.

Testing for allelism could be done in the following way. Heterokaryotic mycelium is derived from two different mutant strains with the same or related phenotypes (Grindle, 1987, 1992). For example, both mutants might require arginine for growth and are phe notypically Arg-. The mutants are allelic (i.e. they carry mutations in the same Arg gene) if the heterokaryon is phenotypically mutant (i.e. it still requires arginine for growth); they are not allelic if the heterokaryon is phenotypically normal (i.e. it grows on minimal medium).

The two mutant strains (e.g. two different Arg auxotrophs) are crossed to obtain sexual progeny. If there are very few proge ny which are phenotypically normal (e.g. less than 0.5% are prototrophic Arg+ recombinants) it is very likely that the two mutant genes occupy the same chromosomal locus and so the mutants are probably allelic.

Testing for mating types

The sexual process in *B. fuckeliana* is controlled by a single mating type gene with two alleles. The alleles show co-dominance in heterokaryons (Faretra *et al.*, 1988b), and are designated *MAT1-1* and *MAT1-2*. Thus, *B. fuckeliana* is a heterothallic fungus with only two mating types, and sexual progenies are obtained by cross-fertilisation of *MAT1-1* and

MAT1-2 isolates. Self-fertile isolates are designated *MAT-1/2*; most of these have been shown to carry the MAT-1 and MAT-2 alleles in separate nuclei in heterokaryotic hyphae, and are therefore pseudo homothallic isolates. The different mating types can be referred to informally as MAT1, MAT2 and MAT1/2 strains.

The parent of unidentified mating type (U) is used in reciprocal crosses with reference strains of known mating type (R1 being MAT-1 and R2 being MAT1-2), and it is also self-fertilized. Thus, the crosses are U♀ x U♂, U♀ x R1♂, U♀ x R2♂, R1♀ x U♂, R2♀ x U♂ (since some strains do not produce sclerotia, the U♀ crosses will not be possible). If apothecia are formed in crosses with only one reference strain, the unidentified strain has the opposite mating type of that reference strain. If apothecia are formed in crosses with both reference strains (or if U is self-fertile), the unidentified strain is given the mixed mating type, *MAT1/2*.

These recommendations should provide a framework for *B. fuckeliana* genetics. Changes in nomenclature can be introduced in the light of experience.

Taxonomy

By studying sexual reproduction of numerous field isolates of *B. cinerea*, Faretra *et al.* (1988b, 1992a) and Faretra and Pollastro (1992a,b) have shown that *B. fuckeliana* is undoubtedly the sexual stage in the life cycle of this fungus. Most taxonomists recommend that the scientific name of the sexual stage should be preferred to that of the asexual stage. For example, *Cochliobolus heterostrophus* has replaced *Helminthosporium maydis* as the scientific name for the fungus causing leaf blight of corn. Therefore, *Botryotinia fuckeliana* should replace *Botrytis cinerea* as the Latin binomial for the fungus causing the disease 'grey mould'. However, there are precedents for a more 'practical' solution: the name for the asexual stage of the life cycle may be retained because of its familiarity and widespread use over many years. For example, *Aspergillus nidulans* is the accepted name for the popular fungus studied by many geneticists, even though this refers to the asexual stage in the life cycle of *Emericella nidulans* (Eidam) Vuillemin.. Using this criterion, the familiar binomial recognised worldwide by numerous mycologists and plant pathologists – *Botrytis cinerea* – should be retained.

Until taxonomists agree on the most appropriate Latin name for the fungus causing 'grey mould', both *Botrytis cinerea* and *Botryotinia fuckeliana* should be included in publications concerning its genetics.

Acknowledgement

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Annex 1. Composition of media, used in studies of *Botryotinia fuckeliana*

Aspergillus medium

Recipe 1

(a) Minimal medium (MM) contains the following ($g.l^{-1}$): glucose (10.0); $NaNO_3$ (6.0); KH_2PO_4 (1.52); KCl (0.52); $MgSO_4.7H_2O$ (0.152); trace elements solution (5.0 ml). The pH is adjusted if necessary to 6.5. These ingredients (without glucose) are prepared as a 10 x concentrate (A), adjusted to pH 5.9, and stored over chloroform in 500 ml screwcapped bottles (add 5 ml chloroform to each bottle) at 5°C. To make 1 litre solid MM, add 100 ml A, 10 g glucose and 15 g agar to distilled water, and autoclave for 15 min at 120°C and 1.1 Pa.

(b) MM containing the above ingredients (without glucose and magnesium sulphate) is prepared as a 10 x concentrate (B): a 40% w/v glucose concentrate (C), and a 20% w/v $MgSO_4.7H_2O$ concentrate (D) are prepared separately. Concentrate B is stored over chloroform; concentrates C and D are filter-sterilised or autoclaved for 5 min at 120°C and 1.1. Pa; add 25 ml C and 2.5 ml D to each litre hot medium.

(c) Complete medium (CM) contains the following (g.l0<F128>1<F255>) in addition to minimal medium: peptone (2.0), casein hydrolysate (1.5); yeast extract (0.5); adenine hemisulphate (0.07); yeast nucleic acid hydrolysate solution (2.5 ml); vitamin solution (2.5 ml). These ingredients (without vitamins) are prepared as a 10 x concentrate (E), adjusted to pH 5.9, and stored over chloroform at 5°C. To make 1 litre solid CM, add 100 ml E to MM recipe (a) or (b) before autoclaving; the vitamin solution is added after autoclaving.

The trace elements solution contains the following ($g.l^{-1}$): ethylenediamenetetraacetic acid EDTA (5.0); $ZnSO_4.7H_2O$ (1.0); $FeSO_4$ (0.5); $CuSO_4.5H_2O$ (0.4). The solution is filter-sterilised or heated for 30 min at 100°C, and stored at 5°C.

The vitamin-solution contains the following ($g.l^{-1}$): nicotinamide (1.0); riboflavin (1.0); pyridoxine (5.0); thiamine (0.5); p-aminobenzoic acid (0.1); biotin (0.0002). The ingredients are dissolved in water, without heat, and stored over chloroform in bottles in the dark at 5°C.

The yeast nucleic acid solution is prepared by heating (20 min at 100°C) 2 g ribonucleic acid (RNA) in 15 ml NaOH and , separately, 2 g RNA in 15 ml NHCl. The two RNA samples are mixed, filtered hot, adjusted to 40 ml total volume and pH 6.0, and stored over chloroform in bottles in the dark at 5°C.

Recipe 2

(a) Minimal medium (MM) contains the following ($g.l^{-1}$): glucose (20.0); $NaNO_3$ (2.0); KH_2PO_4 (1.0); KCl (0.5); $MgSO_4.7H_2O$ (0.5); $FeSO_4$ (0.01); trace elements solution (1.0 ml). These ingredients (without glucose, trace elements and potassium phosphate) are prepared as a 100 x concentrate (A); a 100 x concentrate (B) of KH_2PO_4 is prepared separately. To make 1 litre solid MM, add 10 ml A, 10 ml B, 1 ml trace elements, 20 g glucose and 20 g agar to water, and autoclave as above.

(b) Complete medium (CM) contains the following ($g.l^{-1}$) in addition to minimal medium: peptone (10.0), case in hydrolysate (10.0); yeast extract (10.0); vitamin solution (1.0 ml). To make 1 litre solid CM, these ingredients (without vitamins) are added to MM recipe before autoclaving; the vitamin solution is added to autoclaved medium.

The trace elements solution contains the following ($g.l^{-1}$): $ZnSO_4.6H_2O$ (2.75); $CuSO_4.5H_2O$ (0.2); H_3BO_3 (0.03); $MnSO_4.6H_2O$ (0.33); $NaMoO_4$ (0.025); KI (0.006). The solution is stored at 5°C.

The vitamin solution contains the following ($g.l^{-1}$): inositol (4.0); choline chloride (2.0); nicotinic acid (2. 0); thiamine 1.0); p-aminobenzoic acid (0.5); pyridoxine (0.5); riboflavin (0.5); biotin (0.2); folic acid (0.002). The solution is filter-sterilised and stored at 5°C.

***Neurospora* medium**

Minimal medium (MM) contains the following ($g.l^{-1}$): sucrose (20.0); sodium citrate, $5H_2O$ (3.0); KH_2PO_4 (5.0); NH_4NO_3 (2.0); $MgSO_4.7H_2O$ (0.2); $CaCl_2.2H_2O$ (0.1); trace elements solution (1.0 ml); bi otin solution (0.05). It is prepared as a 50 x concentrate (without sucrose). The 50 x ingredients (e.g. 150.0 g sodium citrate) are added separately, in the order shown, to 750 ml water and dissolved with a magnetic stirrer at room temperature before adding the next. The 5.0 g calcium chloride is predissolved in 20 ml H_2O and added slowly to minimise precipitation (any precipitate can often be dissolved by leaving the medium overnight and stirring again). Total volume is finally adjusted to 1 litre, and the concentrate is stored over chloroform at 5°C.

To make 1 litre solid MM, add 20 ml concentrate, 20 g sucrose and 15 g agar to water, and autoclave for 15 min. at 12°C and 1.1 Pa. To make 1 litre complete medium CM, add 5.0 g caseine hydrolysate and 5.0 g yeast extract to MM ingredients before autoclaving.

The trace elements solution contains the following ($g.l^{-1}$): citric acid.$1H_2O$) (50.0); $ZnSO_4.7H_2O$ (50.0); $Fe(NH_4)_2(SO_4)_2.6H_2O$ (10.0); $CuSO_4.5H_2O$ (2.5); H_3BO_3 (0.5); $MgSO_4.7H_2O$ (0.5); $Na_2MoO_4.2H_2O$ (0.5). Each ingredient is added separately, in the order shown, to water and dissolved a t room temperature with a magnetic stirrer before adding the next. The concentrate is adjusted to 1 litre and stored over chloroform at 5°C; it is usually made in batches of 100 ml.

The biotin solution contains 5.0 mg biotin dissolved in 50 ml water. It is stored frozen, in batches of 5.0 ml.

Molecular genetic analysis of pathogenesis of *Botrytis cinerea*

J.A.L. van Kan, C.J.B. Bergmans and J.W. van 't Klooster

Summary

To study the role of putative pathogenicity determinants of *B. cinerea* by molecular methods, we are developing tools which enable us to disrupt key genes and produce site-directed mutants. We are also initiating a molecular characterization of genetic variation between different *B. cinerea* isolates by the use of RFLP and RAPiD markers.

Introduction

Knowledge about the molecular genetics of *B. cinerea* is poorly developed in comparison with other plant pathogenic fungi. Classical genetic analyses by Faretra have resulted in reproducible, although time-consuming, protocols for sexual crosses (Faretra and P ollastro, 1988; Faretra *et al.*, 1988a,b). In order to perform molecular genetic analysis of *B. cinerea*, several tools need to be developed. Firstly, genetic markers are required. It appears to be difficult to obtain stable morphological or auxotrophic markers for genetic analysis (Grindle, personal communication). Secondly, a transformation procedure needs to be established to allow over-expression, repression or disruption of specific genes. Thirdly, genetic variation can be studied by analysis of chromosome length polymorphism (CLP), restriction fragment length polymorphism (RFLP) or random amplified DNA polymorphism (RAPiD).

We aim to use all these tools in order to perform a molecular genetic analysis of *B. cinerea* genes involved in pathogenesis. Physiological studies have indicated roles in pathogenesis for hydrolytic enzymes such as cutinases, proteases and pectinases (McKeen, 1974; Van den Heuvel and Waterreus, 1985; Leone *et al.*, 1990). By making directed mutations in specific genes (gene disruption), we will be able to test the relevance of individual genes in pathogenesis. We are interested in genes encoding the above extracellular hydrolytic enzymes, and eventually also those involved in production of toxins. Here we present results obtained in the development of tools to study pathogenicity and genetic variation of *B. cinerea.*

Results and Discussion

Tools for genetic analysis

Eight isolates of *Botrytis cinerea* Pers.: Fr., obtained from the Netherlands were mated with two reference strains with defined mating types, SAS56 (containing with MAT1-1 allele) and SAS405 (containing the MAT1-2 allele) (Faretra *et al.*, 1988a). In each cross, mating partners were used both as male (micro-conidia) and female (sclerotia) (Table 1). Isolate Bc7, however, produced no sclerotia and could therefore only be used as male

partner. Self-fertilization controls are summarized in Table 1. Each Dutch isolate mated with at least one of the two reference strains. Five isolates Bc7, Bc12, Bc16, Bc21 and Bc26, mated with both SAS56 and SAS40 5, suggesting that these isolates could be homothallic. Isolates Bc12 and also Bc16 appeared to be self-fertilizing Self-fertilization g of Bc7, Bc21 and Bc26 was not observed.

Table 1. Sexual crosses of isolates of B. cinerea.

Sexual crosses of *Botrytis cinerea*

♀ \ ♂	SAS 56	SAS 405	Bc 7	Bc 12	Bc 16	Bc 18	Bc 21	Bc 25	Bc 26	Bc 29	USP[f]
SAS 56[a]	-[d]	+[e]	+	+	+	-	-	-	+	+	-
SAS 405[b]	+	-	+	+	+	+	+	+	-	-	-
Bc 7[c]											
Bc 12	-	-		-							-
Bc 16	+	+			+						-
Bc 18	-	+				-					-
Bc 21	-	+					-				-
Bc 25	-	+						-			-
Bc 26	+	-							-		-
Bc 29	+	-								-	-

a: SAS56 = MAT 1-1
b: SAS 405 = MAT1-2
c: Bc 7 produces no sclerotia
d: -, no apothecia produced
e: +, production of apothecia
f: USP = unspermatized sclerotia

In each successful cross, many apothecia were produced. In order to obtain progeny for molecular genetic tetrad analysis, we propagated subcultures of all eight ascopores from an ascus. For each cross, five apothecia where chosen. From each apothecium, two intact asci were taken, and gently hydrolysed with Novozym for a short period. Subsequently, the orderly released ascopores were spread apart over an agar plate, and left to germinate overnight. Germinated ascopores were transferred to individual agar plates and grown until sporulation occurred. In order to perform a good tetrad analysis, we usually carried out this procedure only when at least seven of the ascopores had germinated. Some of the crosses yielded progenies with distinct morphology, in a 1:1 or 1:2:1 ratio. Such mutant phenotypes might be used for genetic mapping in the future.

The ten parents will be analysed by RFLP and RAPiD analysis (Williams *et al.*, 1990) to determine polymorphic molecular markers. The segregation of individual markers will

be analysed in the progeny obtained from all the crosses, thus enabling us to establish molecular linkage maps between the different markers. By bulked segregant analysis (Michelmore et al., 1991) we might obtain markers which are linked to morphological characteristics, such as the inability of isolate Bc7 to produce sclerotia. The long term aim of these experiments is the construction of a molecular and genetic linkage map on which genes with known functions can be assigned to certain positions.

Tools for molecular analysis

To perform gene disruption experiments, it will be necessary to clone genes of interest from *B. cinerea*. From genomic libraries or cDNA libraries, genes encoding extracellular hydrolytic enzymes will be cloned by the use of antibodies or heterologous DNA probes. These clones will be mutagenized *in vitro* and the mutated gene will be reintroduced into the wild type *B. cinerea* by transformation. By homologous recombination, the mutation can be exchanged with the resident chromosomal gene, resulting in a mutant allele ('gene disruption'). Such a recombination event will occur with a certain frequency which can be scored by genetic or molecular analysis.

To perform gene disruption, one needs to be able to introduce foreign DNA into *B. cinerea* by transformation. A transformation protocol is currently being developed. We are attempting to obtain hygromycin-resistant transformants either by electroporation of fungal protoplasts or germtubes (Chakraborty *et al.*, 1991), or by the standard PEG/$CaCl_2$ treatment of protoplasts (Mishra, 1985). Results obtained sofar indicate that the medium on which the hygromycin selection is performed is extremely important. We have not obtained hygromycin-resistant colonies yet. Clearly, the transformation procedure needs further improvement before being used routinely. To allocate different molecular markers to specific chromosomes, electrophoretic karyotype analysis is being developed. Chromosome separation on agarose gels up to sizes of 10 Mbp can be achieved by CHEF gel electrophoresis (Mills and McCluskey, 1990). We have optimized the protocol for obtaining protoplasts of *B. cinerea* with high viability, but we were unable to release enough DNA from these protoplasts to visualise chromosome bands on CHEF gels. This technical problem will probably be resolved in the near future. Separation of chromosomes will enable us to analyse karyotypes of different *B. cinerea* isolates and their progenies, and will eventually allow the mapping of genes or genetic loci to specific chromosomes.

Conclusions and future prospects

Many basic molecular and genetic techniques which have been applied to plant pathogenic fungi in recent years were not developed for the analysis of *B. cinerea*. Therefore we are adapting the techniques to facilitate the molecular and genetic study of pathogenicity determinants in *B. cinerea*. Progenies from crosses have been obtained which are amenable to tetrad analysis with genetic or molecular markers. The crosses also provide means to study genetic instability unrelated to heterokaryosis. Genes encoding putative pathogenicity factors will be isolated, mutated and reintroduced into wild type *B. cinerea*. Transformants will be screened for disruption of particular genes of interest. The resulting mutants will be tested for pathogenicity on tomato and other host plants. Such an approach will provide a better insight into the relevance of various enzymes and other fungal products for pathogenesis of *B. cinerea*.

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Tools for molecular genetic analysis of *Botrytis cinerea*

C.J.B. Bergmans and J.A.L. van Kan

Summary

Sexual crosses were made between eight different field isolates of *B. cinerea* and two strains of known opposite mating type. Progenies of these crosses are now available for further molecular genetic analysis.

Introduction

Botrytis cinerea Pers.: Fr., the anamorph *Botryotinia fuckeliana* (de Bary) Whetzel, is a multinucleate fungus and due to anastomosis cells can contain nuclei of different genetic background. This heterokaryotic nature has seriously hampered molecular genetic studies of the fungus. To be able to study molecular genetics, sexual crosses between ten different isolates were performed and the homokaryotic progenies collected in an ordered way.

Sexual crosses

Sexual compatibility of *B. cinerea* is controlled by a single mating type gene with two alleles: MAT1-1 and MAT1-2.

To obtain a collection of homokaryotic progeny, eight different field isolates (prefix Bc) were each crossed with two strains (prefi x SAS) of known opposite mating type (Faretra et al., 1988a) according to a method described by Faretra *et al.* (1988b). Strains producing both 'male' (microconidia) and 'female' (sclerotia) structures were mated in reciprocal crosses. Controls included unspermatised and self-fertilised sclerotia. Table 1 shows all crosses and their success in producing apothecia. Apothecia emerged two to five months after spermatisation and arose from any part of the sclerotial tissue. Their formation started with the elongation of the stipe and a subsequent differentiation of the apex to a head. The occasional presence of conidiophores on the sclerotia did not prevent apothecia forming.

Of the 15 sclerotia used for each cross usually 25 to 100% produced apothecia of which the number varied between one to six per sclerotium. In two cases only, one apothecium was formed on the 15 sclerotia used.

Among the numerous apothecia formed, some were malformed, viz. reversion of an immature apothecium to the mycelial stage, an apothecium consisting of one stipe with two heads and malformations of the head structure.

Crosses which were able to form mature apothecia, are marked with a '+' in Table 1. Isolates Bc7, Bc12 and Bc16 were success fully crossed as 'male' with both SAS56 and SAS405, indicating that these *B. cinerea* isolates contain both mating type alleles, possibly due to their heterokaryotic behaviour. Bc16 also produced apothecia in the reciprocal crosses and in the self-fertilisation control. This was not observed for Bc12,

and sclerotia of Bc12 are therefore possibly defective in formation of apothecia. As no s clerotial structures for Bc7 are known (Salinas and Schot, 1987) reciprocal crosses could not be tested. All other Bc-isolates successfully crossed either with SAS56 or SAS405 in both reciprocal crosses. The results show that Bc18, Bc21, Bc25 have mating type MAT1 -1 and Bc26 and Bc29 have mating type MAT1-2.

Table 1. Sexual crosses of Botrytis cinerea isolates. Data were compiled a few months after the tenth Botrytis symposium.

Sexual crosses of *Botrytis cinerea*

♀ \ ♂	SAS 56	SAS 405	Bc 7	Bc 12	Bc 16	Bc 18	Bc 21	Bc 25	Bc 26	Bc 29	USP[f]
SAS 56[a]	-[d]	+[e]	+	+	+	-	+	-	+	+	-
SAS 405[b]	+	-	+	+	+	+	+	+	-	-	-
Bc 7[c]											
Bc 12	+	+		+							-
Bc 16	-	+			+						-
Bc 18	-	+				-					-
Bc 21	-	+					-				-
Bc 25	-	+						-			-
Bc 26	+	+							-		-
Bc 29	+	-								-	-

a: SAS56 = MAT 1-1
b: SAS 405 = MAT1-2
c: Bc 7 produces no sclerotia
d: -, no apothecia produced
e: +, production of apothecia
f: USP = unspermatized sclerotia

For most of the successful crosses a defined collection of progenies was collected by isolating ten asci from five different apothecia. The head of an apothecium was squashed in water in order to release ripe asci. A mature ascus was transferred to a droplet of Novozym (5 mg.ml^{-1} in 10 mM MES buffer, pH 6.0) on a wateragar plate. After an incubation of 30 sec at 37°C the ascus was rinsed in a droplet of water. The Novozym treatment weakened the natural opening of the ascus and ascospores could then be released by forcing them from the ascus in the natural sequence. The spores were separated on the agar and germinated for two days. Subsequently the young colonies were transferred to fresh tPDA plates (PDA containing 300 g crushed tomato leaves per litre) and cultured until they sporulated. Conidia were collected and stored in a 10% glycerol solution at -80°C. Regularly it was observed that not all eight spores of one ascus

were viable. As incomplete sets were not regarded useful for genetic studies they were discarded. Mature apothecia were also stored in a 10% glycerol solution at -80°C.

Monoascosporic cultures from a complete set of ascospores of the same ascus usually showed great uniformity in morphology. Occasionally sets showed a segregation of morphological characters (1:1 or 1:2:1) giving rise to different cultures.

Further work will be carried out using parental isolates and their progenies. Parental isolates will be used for karyotyping and RAPiD analysis. Segregation of polymorphic markers in the progeny will be analysed and results will be used for the construction of a molecular linkage map.

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Genetics of cream mutants of *Botryotinia fuckeliana* (*Botrytis cinerea*) with aberrant production of laccase enzyme

F. Faretra and A.M. Mayer

Summary

Two UV-induced albino mutants of *B. fuckeliana* (*B. cinerea*), with conidia and conidiophores of a cream colour, showed altered production of laccase enzyme. Genetic defects of mutants were investigated through genetic analysis of ascospore progeny of sexual crosses with a wild-type strain. In both mutants, single Mendelian genes were responsible for the aberrant morphology. Laboratory tests of agressiveness on cucumber cotyledons and grape berries showed that ascospore progenies with cream phenotypes were no less aggressive than wild-type progenies.

Introduction

In mutagenesis experiments aimed at obtaining genetic markers, two mutants of *B. cinerea* were found yielding sporulating colonies with a cream colour rather than the grey-brown colour of the wild-type strain.

Fungi produce a variety of dark pigments known generically as melanins, although their synthetic pathways are different (Bell and Wheeler, 1986). It is known that for some fungi, such as *Colletotrichum* and *Magnaporthe* spp., melanin has an essential role in the penetration of host plants (i.e., Chumley and Valent, 1990; Kubo et al., 1987). Studies of several fungi suggested that laccase is involved in melanin biosynthesis (Bell and Wheeler, 1986). In *B. fuckeliana*, there is evidence that laccase is also involved in pathogenicity (Bar Nun and Mayer, 1989; Bar Nun *et al.*, 1988; Hodson *et al.*, 1987; Marbach et al. , 1984, 1985).

This paper deals with genetic studies of cream mutants and the evaluation of their laccase production and aggressiveness.

Material and Methods

The two mutants of *Botryotinia fuckeliana* (de Bary) Whetz., teleomorph of *Botrytis cinerea* Pers.: Fr., BAM28 and BAM19, were obtained from strain SAS56 after UV-irradiation. They were mated with the strain SAS405 as described (Faretra *et al.*, 1988a, b). Random ascospores from individual apothecia were collected singly and grown on malt extract agar (MEA) as described by Faretra and Grindle (this volume).

The aggressiveness of B. fuckeliana isolates was tested on cucumber (cv. Beta Alfa) cotyledons floating on a thin layer of water in Petri dishes, as well as on grape berries (cvs. Italia and Regina) kept in a humid chamber. Conidial suspensions (10 µl containing 10^5 spores.ml^{-1} in 0.05% Tween 20) or mycelial plugs (1 mm^3 blocks or 3-mm diameter disks) were used as inoculum. Conidia were collected from seven day-old colonies

Table 1. Analysis of random ascospores from crosses between the wild-type strain SAS405 (MAT1-2 Mbc1HR Daf1LR) and cream mutants, which were both MAT1-1 Mbc1S Daf1S[a)]

Parent in cross		Total ascospores examined	No. and phenotypes of ascospores progenies		χ^2 value for 1:1 segregation	Recombination (%) between pairs of genes (χ^2 values for independent segregation)		
Reference strain	Mutant		wild-type	Cream		Crm/Mbc1	Crm/Daf1	Mbc1/Daf1
SAS405	BAM18	130	66	64	0.03	45 (1.51)	56 (1.96)	37 (8.89)
SAS405	BAM19	94	51	43	0.68	55 (1.06)	55 (1.06)	45 (1.06)

[a)] χ^2 values for 1 degree of freedom are 3.84 at p=0.05 and 6.63 at p=0.01 levels of probability

Table 4. Aggressiveness of wild-type and cream progenies (selected among those normally sensitive to benzimidazoles and dicarboximides) from crosses of strain SAS405 with cream mutants BAM18 or BAM19[a)]

Mutant parental of isolate	Phenotypes tested ascospores	Cucumber cotyledons			Grape berries			
		Conidia	Mycelial plug		Conidia (wound)		Mycelial plug (1 mm³)	
			1 mm³	3 mm³	'Italia'	'Regina'	'Italia'	'Regina'
BAM18	wild-type	1.3 ±0.7	1.4 ±1.1	1.0±0.7	3.0 ±0.8	2.7 ±0.7	1.1 ±0.5	1.4 ±0. 7
	cream	2.3 ±0.8	2.9 ±0.3	1.9 ±0.6	3.5 ±0.6	4.0 ±0.6	0.9 ±0.9	0.8 ±0.8
BAM19	wild-type	1.2 ±1.0	2.2 ±0.9	0.6 ±0.5	3.5 ±0.6	3.5 ±0.8	0.7 ±0.3	0.7 ±0. 6
	cream	1.8 ±1.2	2.7 ±0.8	2.2 ±0.8	3.4 ±0.4	4.0 ±0.6	1.6 ±0.6	0.9 ±0.9

[a)] Figures represent averages (+ SD) of data obtained from 10 ascospore progenies with similar phenotypes. See Table 3 for details.

Table 2. Intracellular (endo) and extracellular (exo) laccase enzyme activity estimated after culturing for 21 days.

Strain	Enzyme activity ($\mu l\ O_2.min^{-1}.ml^{-1}$ medium)		Mycelial growth (mg dry weight)	Specific enzyme activity ($\mu l\ O_2.min^{-1}.g^{-1}$ dry weight)	
	endo	exo		endo	exo
SAS56	0.65	0.44	576	8.45	99.30
SAS405	0.76	0.54	629	9.66	111.60
BAM18	0.11	0.71	266	1.02	346.99
BAM19	1.20	0.00	626	10.53	0.00

Table 3. Aggressiveness of cream mutants and wild-type reference strain[a]

Strain	Conidia	Cucumber cotyledons[b]		Grape berries[c]			
		Mycelial plug		Conidia (wound)		Mycelial plug (1 mm^3)	
		1 mm^3	3 mm^3	'Italia'	'Regina'	'Italia'	'Regina'
SAS56	0.7	2.0	1.3	3.0	3.0	0.7	1.7
SAS405	2.0	1.7	2.3	2.7	2.0	1.0	1.0
BAM18	2.7	0.3	1.3	3.0	2.7	0.7	0.7
BAM19	0.7	2.7	3.0	3.0	3.0	0.0	0.0

[a]) Figures represent classes of empirical scales ranging from 0 (healthy) to 3 (symptom spot > 11 mm) for cucumber cotyledons and from 0 (healthy) to 6 (berry rotten) for grape berries.

[b]) Survey carried out after two, four and six days of incubation for conidia, 1 mm^3 and 3-mm diameter plugs of mycelium, respectively.

[c]) Survey carried out after six days of incubation.

grown on potato dextrose agar and exposed to white and near UV-radiation on a 12 h photoperiod; mycelium was collected from the margin of actively growing colonies on MEA. Grape berries were wounded with a needle immediately before inoculation with conidia. In experiments with cucumber leaves conidial suspensions were prepared in solutions containing 25 $g.l^{-1}$ glucose and 50 mM KH_2PO_4 (Akutsu *et al.*, 1987). Symptoms were evaluated at two-day intervals by using the following infection scales: a) cucumber leaves, 0 = healthy leaf, 1 = rot spot diameter < 5 mm, 2 = 6-10 mm, 3 = > 11 mm; b) grapes: 0 = healthy berry, 1 = rot spot diameter 1-5 mm, 2 = 6-10 mm, 3 = rot on 25% berry surface, 4 = 50%, 5 = 75%, 6 = berry entirely rotten. All experiments were carried out twice with three replicates.

For tests of laccase enzyme production, the fungus was grown in 1 L flasks containing 130 ml medium at 20°C (static culture). The culture medium contained (per 1000 ml) 20 g malt extract and 16 g citrus pectin in 0.01M KPi-citrate buffer pH 3.5; 1 g gallic acid was added after autoclaving. Enzyme activity was measured by using an oxygen electrode with 10 mM quinol as substrate, pH 4.6 at 25°C.

Results and discussion

The two mutants were crossed with MAT1-1 strain SAS56 and MAT1-2 strain SAS405. They were fertile only with SAS405, thus retaining the mating type of the parental isolate SAS56.

Wild-type and cream phenotypes segregated in a 1:1 ratio in ascospore progenies from crosses of strain SAS405 with both cream mutants (Table 1). This indicates that mutant phenotypes were due to single chromosomal genes, which were both provisionally coded Crm. Both mutants produced dark sclerotia but were unable to produce apothecia; therefore it was not possible to make crosses for testing allelism.

Crm genes segregated independently during meiosis from either the Mbc1 gene of resistance to benzimidazoles and the Daf1 gene for resistance to dicarboximides (Faretra and Pollastro, 1991, 1992). The genes Mbc1 and Daf1 showed a loose linkage in the cross SAS405 x BAM18 but were independently reassorted in the cross SAS405 x BAM19.

Strains SAS56 and SAS405 produced similar amounts of endo- and exo-laccase. In comparison, BAM18 produced less endo-laccase but more exo-laccase; and BAM19 was almost normal in endo-laccase production, but deprived of exo-laccase (Table 2). Preliminary acrylamide gel electrophoresis indicated differences in enzyme mobility among mutants. The enzyme produced by BAM18 appeared as one band while that of BAM19 separated into two bands, one of which was very close to starting point. The control (an INRA strain) had 1-2 bands corresponding to the faster band of BAM19.

Evaluations of aggressiveness yielded data varying with the assay systems used. For example, BAM18 was the most aggressive isolate in tests with conidia on cucumber, but the least aggressive in tests with mycelium on the same host (Table 3). Thus, no conclusive evidence of correlation between production of laccase and aggressiveness could be obtained. Meiotic progenies of mutants were also evaluated. In all tests, cream progenies were consistently more aggressive than wild-type progenies (Table 4). This suggests that aberrant phenotypes do not necessarily cause loss of aggressiveness.

Meiotic progenies derived from the mutants are now being investigated to ascertain whether, as the preliminary results indicate, the aberrant production of laccase and aberrant pigmentation co-segregate during meiosis; that is to say, whether they are effects of the same genetic defect.

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Pectin degradation by *Botrytis cinerea*: a molecular genetic approach

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Summary

The homokaryotic strain *B. cinerea*, SAS56, was investigated by iso-electric focusing in combination with overlay staining and Western analysis of samples of the growth medium to determine its pectinolytic activity. It was shown that this strain produced several isoforms of polygalacturonase (PG) and pectinesterase (PE), and evidence was found that one or more pectin lyases (PL) were also produced. Using pectinolytic genes from *Aspergillus niger* as probes, it was possible to isolate genes in *B. cinerea* by heterologous hybridisation. A gene library of *B. cinerea* SAS56 has been constructed for this purpose. There were indications of a polygalacturonase and pectin lyase gene family in *B. cinerea*.

Introduction

Botrytis cinerea Pers.: Fr. produces several extracellular enzymes which are implicated in pathogenesis. Cutinases may play a role in the primary penetration of the host plant; cellulases, hemicellulases and pectinases degrade the plant cell wall, and proteases and phospholipases are able to degrade the cell membrane. Pectinases have received particular attention, since these enzymes are the first to be produced by several phytopathogenic fungi when grown on cell wall material. Their role in the infection process has been established in bacteria (Collmer and Keen, 1986) as well as in fungi such as *Verticillium albo-atrum* (Durrands and Cooper, 1988), *Fusarium solani* f.sp. *pisi* (Crawford and Kolattukudy, 1987) and B. cinerea (Leone, 1990; Movahedi and Heale, 1990). Although multiple forms of polygalacturonase are described for *B. cinerea*, it is not clear whether these are all encoded by different genes. Over the past 5 years, our group has isolated and characterised several pectin lyase and polygalacturonase genes from the saprophytic fungus *Aspergillus niger* and studied the regulation of these genes (Gysler *et al.*, 1990; Harmsen *et al.*, 1990; Bussink *et al.*, 1991,1992; Kusters-van Someren *et al.*, 1991,1992). We also have purified and characterised the encoded pectinolytic enzymes. Now we have investigated which pectinolytic enzymes are produced by *B. cinerea* SAS56 and whether, using the *A. niger* genes as probes, we might be able to isolate the homologous genes from *B. cinerea*. Analysis of the genes and especially of the encoded enzymes, will lead to a better understanding of the physiology of the infection process.

Material and Methods

Strains and plasmids

B. cinerea homokaryotic strain SAS56 was kindly provided by Dr. F. Faretra (Bari, Italy)

and used for the construction of a gene library in the lambda vector EMBL3 from Promega. Plasmid pGW820 contains the *A. niger* N400 pectin lyase A (*pel*A) gene (Harmsen *et al.*, 1990), plasmid pGW1800 contains the *A. niger* N400 polygalacturonase II (*pga*II) gene (Bussink *et al.*, 1990) and plasmid pIM305 contains the *niger* N400 pectinesterase gene (pme) (Kusters-van Someren *et al.*, unpublished results). *Escherichia coli* LE392 (F^-, *hsd*R514(r_k^-, m_k^-), *sup*E44, *sup*F58, lacY1 or delta (*lac*IZY) 6, *gal*K2, *gal*T22, *met*B1, *trp*R55, *lambda*$^-$) was used as the lambda host strain.

Growth conditions and analysis of pectinolytic enzymes

The medium used for pectinolytic enzyme production contained per litre distilled water 5 g KNO_3, 2.5 g KH_2PO_4, 0.25 g $MgSO_4$, 0.4 g $FeCL_3$ with 1% pectin (from apple, degree of esterification 72.8%, Obi-pektin, Bischofszell) and 1% glucose as carbon sources. The medium was inoculated with 10^6 spores.ml^{-1} and incubated in a rotary shaker at 20°C for varying time periods. PG and PE zymograms were made by isoelectric focusing of culture filtrates (10 µl), followed by overlaying the focusing gel with a pectin-containing agarose gel, incubating it for 30 min and staining it with ruthenium red. For Western analysis, proteins from the growth medium were separated on a 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were blotted onto nitrocellulose and detected using the alkaline phosphatase assay (Biorad). Polyclonal antibodies raised against *A. niger* PLI and PLII or PGII were used to probe the Western blots.

DNA manipulations

Standard methods were used for plasmid DNA isolation, random primed labelling, plaque screening and Southern analysis (Sambrook *et al.*, 1989). DNA fragments were isolated from agarose gels using the GeneClean kit from Bio101.

Gene library construction

A medium containing per liter distilled water 2.0 g KH_2PO_4, 2.5 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4.7H_2O$, 2.0 g yeast extract (Gibco-BRL) and 10.0 g glucose was inoculated with *B. cinerea* spores (10^6ml^{-1}) and grown for 2 days at 20°C in a rotary shaker. The mycelium was collected by centrifugation (10 min, 10.000 rpm in a GSA rotor) and lyophilised. DNA was isolated as described for *Aspergillus* chromosomal DNA (de Graaff *et al.*, 1988), taking care to shear the DNA as little as possible. To isolate fragments of 14-20 kb, 30 µg of the DNA was partially digested for 1 h at 37°C using 0.075 units Sau3A. After addition of EDTA to a final concentration of 20 mM, the DNA fragments were separated on a 0.4% agarose gel in TAE (50 x TAE consists of 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA [pH8.0] per litre) and the fragments of interest were cut out of the gel. The DNA was recovered by electro-elution. For ligation, 125 ng *B. cinerea* chromosomal DNA fragments were used with 500 ng EMBL3 BamHI-digested, dephosphorylated lambda arms (Promega) in a 10 µl reaction mix, further containing 1 unit T4 ligase (Gibco-BRL) and ligase buffer (Gibco-BRL). After 4 h incubation at room temperature, 50 µl packaging mix (Packagene, Promega) was added. Packaging and titration was done as recommended by Promega (Promega Protocols and Applications Guide). The gene library was amplified as described by Sambrook et al (1989).

Hybridisation conditions

Hybridisation was done for 16 h at 60°C in standard hybridisation buffer containing 6 x SSC (1 x SSC consists of 0.15 M NaCl, 0.015 M sodium citrate). The blots or filters were then washed twice for 30 min with 4 x SSC, 0.5% SDS and twice for 30 min with 2 x SSC, 0.5% SDS at 60°C.

Results and Discussion

Pectinolytic enzyme production

B. cinerea SAS56 was grown on various carbon sources (polygalacturonic acid, pectin/ sugar beet pulp, pectin/glucose) for 5 days. Each day a sample of the culture medium was taken and analysed by iso-electric focusing and activity staining. With all three media PG and PE bands were visible after several days. At least two forms of polygalacturonase were detected and four forms of pectinesterase (not shown). From these preliminary results we concluded that the best condition for production of these enzymes was growth for 5 days in a medium with 1% pectin and 1% glucose as carbon source. The Western analysis showed that polyclonal antibodies raised against *A. niger* pectin lyase are useful to detect the corresponding *B. cinerea* enzymes (Fig. 1). The *A. niger* PGII antibody was not very specific, and detected some other proteins, besides several *A. niger* polygalacturonases.

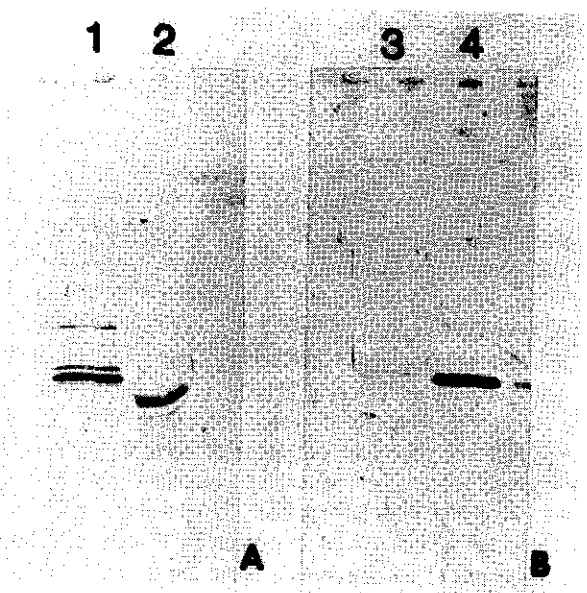

Fig. 1. Western analysis of proteins, produced by B. cinerea in a culture medium after 5 days of growth.

Lane 1: growth on 1% pectin/1% glucose; lane 2: *A. niger* PLII; lane 3: growth on 1% pectin/1% glucose; lane 4: *A. niger* PG I. A: a-PLI and a- PLII polyclonal antibody; B: a-PGII polyclonal antibody.

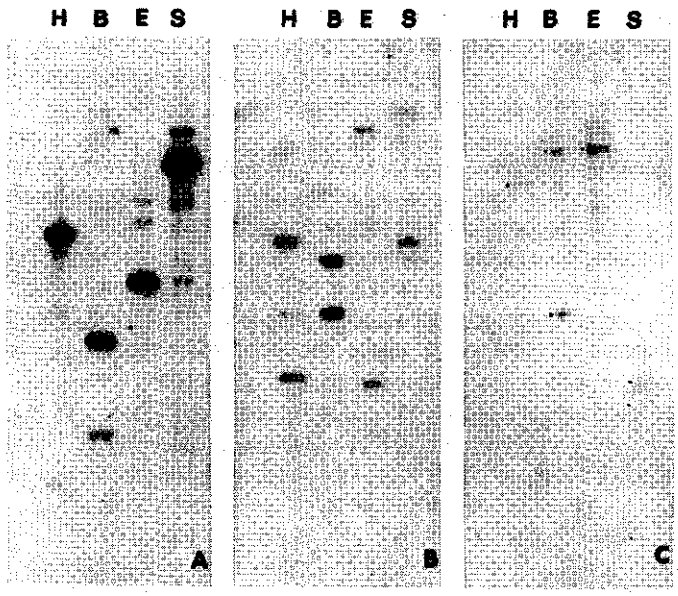

Fig. 2. Southern analysis of B. cinerea SAS56 chromosomal DNA using A. niger pectinolytic genes as probes.

H: HindIII; B: BamHI; EcoRI; SalI. Probes used: A: *A. niger* N400 pelA 1.6 kb ClaI fragment; B: *A. niger* N400 pgaII 1.2 kb BamHI/BglII fragment; C: *A. niger* N400 pme 0.7 kb SalI fragment. The hybridisation conditions were as described in Material and Methods.

Southern analysis

Chromosomal DNA was isolated from *B. cinerea* SAS56 and digested with *Hin*dIII, *Bam*HI, *Eco*RI and *Sal*I. After Southern blotting onto nitrocellulose, DNA fragments containing the *A. niger* genes encoding pectin lyase A, polygalacturonase II, and pectinesterase were used as probes to detect the homologous *B. cinerea* genes. Fig. 2 shows that hybridising bands could be detected using these genes as probes. The fact that multiple bands of different intensity are visible in Fig. 2A and 2B is indicative of a gene family for pectin lyases and endo-polygalacturonases. In *A. niger*, gene families for these functions have also been described and cloned (Harmsen *et al.*, 1990; Bussink *et al.*, unpublished data). There is probably only one gene encoding pectin esterase, although a second hybridising band was seen in Southern blots using this gene as a probe (Khanh *et al.*, 1991; Kusters-van Someren *et al.*, unpublished results).

Construction of the *B. cinerea* gene library

High molecular weight chromosomal DNA (30 μg) from *B. cinerea* SAS56 was partially digested using 0.075 units Sau3A. Fragments of 14-20 kb were isolated and used for subcloning in the lambda vector EMBL3 digested with BamHI. After ligation and

packaging c. 2.5 x 10^4 plaques were found, thus containing c. 3-4 x 10^5 kb DNA in total. Assuming that the genome size of *B. cinerea* approximates that of *A. niger* (5 x 10^4 kb), the complexity of the gene library is 6-8 times the genome size.

Screening of the gene library with *A. niger* genes as probes

Five plates with 3 x 10^4 plaques each were used for the plaque screening. The same pectin lyase and polygalacturonase probes were used as previously in the Southern analysis. Fig. 3 shows that strongly, as well as weakly, hybridising plaques could easily be detected against a completely negative background. Differences in signal strength may reflect differences in plaque size, differences in the size of the hybridising fragment cloned in a particular phage or differences in the extent of homology with the probe. The latter reason is very probable considering the results of the Southern blots (Fig. 2).

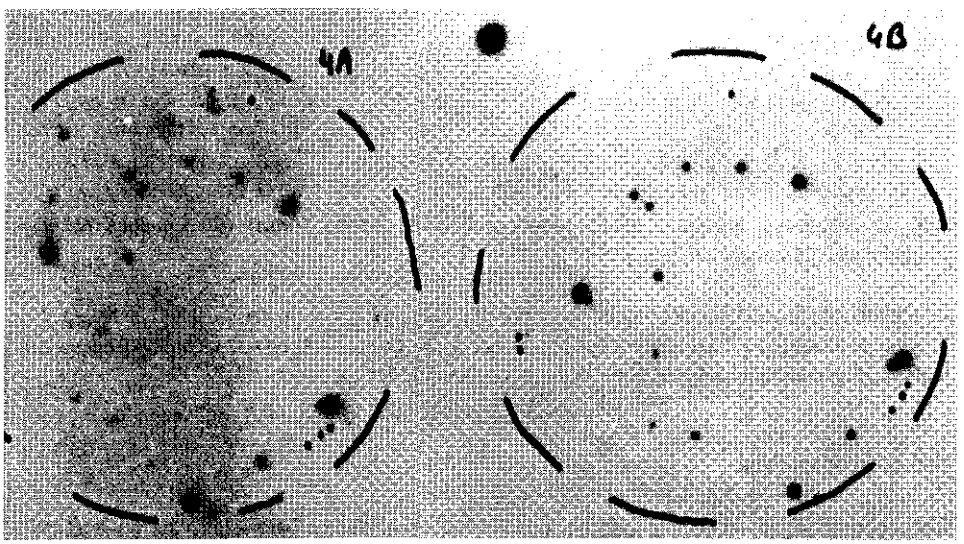

Fig. 3. Plaque screening of the B. cinerea SAS56 genomic library using the A. niger as a probe.

Duplicate filters are shown.

DNA from strongly and from weakly hybridising phages will be further characterised by restriction analysis, subcloning and sequencing. With the cloned genes we will be able to study the role of pectinolytic genes and enzymes of *B. cinerea* in pathogenesis with emphasis on the following three aspects:

Characterisation of the enzymes which are encoded by these genes

The enzymes may be produced in *Aspergillus*, after multiple copies of the genes have been introduced into the genome by transfor mation, until a transformation system of

B. cinerea becomes available. It is important to characterise the enzymes to investigate whether they differ for instance in substrate specificity, pH optimum or in their degradation products. This will lead to a better understanding of the role of the various pectin degrading enzymes in the infection process.

Study of the regulation of pectinase encoding genes in *B. cinerea*

So far, nothing is known of the way the pectinolytic genes are regulated: whether they are all regulated by the same activator and/or repressor or whether different genes are regulated in different ways.

Construction of a *B. cinerea* strain with reduced virulence by inactivation of pectinolytic genes

We are especially interested in cloning and disrupting the gene encoding a constitutively produced endo-polygalacturonase with a high iso-electric point, which is thought to be very important early in infection (Leone, 1990; van der Cruyssen and Kamoen, 1992; Johnston and Williamson, 1992). Gene disruption to study the relevance of pectinolytic enzymes in pathogenesis has been used with the bacterium *Erwinia chrysanthemi* and with *Cochliobolus carbonum*, the fungal pathogen of maize. In the case of *E. chrysanthemi*, inactivation of all five pectatelyase genes led to a reduction in virulence, but the strain could still cause soft rot (Ried and Collmer, 1988). Only a secretion-defective strain was non-virulent. In *C. carbonum*, only one endo-polygalacturonase gene is present; its inactivation had no effect on the virulence of the pathogen on maize (Scott-Craig *et al.*, 1990). These results show that pectinolytic enzymes are highly unlikely to be the only pathogenicity factors, and it is unlikely that inactivation of the pectinolytic genes in *B. cinerea* have a drastic effect on its pathogenicity. Nevertheless, they do play an important role in infection, and it is therefore well worth studying the complex process of pectin degradation by *B. cinerea* at a molecular level.

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HOST-PATHOGEN INTERACTION AND PHSYSIOLOGY

Botrytis cinerea: host-pathogen interactions

O. Kamoen

Summary

The interactions of *Botrytis cinerea* with a compatible host are considered here as three consecutive stages: (1) germination, (2) initial colonisation during penetration of the host and (3) expansion of the lesion. Incompatible combinations are not discussed. The morphologocial, physiological and biochemical factors described in the literature as involved in these stages, are reviewed.

Introduction

This review of different aspects of the host-pathogen interaction will be restricted mainly to successful fungal infections that result in spreading lesions. It is meant to provide a better understanding of the infection process from published data, but some hypotheses are also made which should be tested.

The review commences from the emergence of germtubes from conidia. Essentially the same growth processes occur in mycelium g rowing from sclerotia as from a successful penetrated germtube of a conidium. Therefore, the former will not be discussed. Infections derived from ascospores of *Botryotinia fuckeliana* or from microconidia of *Botrytis cinerea* Pers.: Fr. have not been described in the literature.

Three phases can be distinguished during fungal infection of a succeptible host plant and consequently will be discussed in three sections.

1. Germination of conidia on the plant surface.
2. Penetration of the epidermis.
3. The expansion of fungal hyphae in the host tissue.

In each section morphological, physiological and biochemical aspects of infection are discussed.

Germination

Inhibition of germination in *B. cinerea* may be due to toxic substances released by the plant, inhibition by the phylloplane microflora, competition with the phylloplane flora for nutrients, the age of the conidia and the number of conidia in the inoculum droplet. Self-inhibition of germination occurs when spores are present in high density; more than 10^5 conidia.ml^{-1} leads to a reduction in the percentage of germination and older conidia also show reduced germination (Last, 1960). A loss of endogenous nutrients also occurs and this can be reversed by addition of nutrients (Blakeman, 1980). However, infection using droplets containing high spore concentrations may be more effective than low inoculum densities, as was shown by Deveral and Wood (1961). They suggested that some of the conidia germinated and penetrated in the first instance; this resulted in nutrient leakage and subsequent stimulation of the germination of the other conidia.

Stimulation of germination of conidia can be achieved by addition of nutrients and presence of pollen in the infection droplets or by presence of senescent or dead plant tissues or wounds (Mansfield, 1980).

Sugars have been examined for their ability to supplement for loss of endogenous reserves. The stimulation of germination by pollen, wounds or senescent or dead plant tissues could be attributed to nutrients release, but not entirely (Van den Heuvel, 1987). Spatial isolation of the fungus and absorption of the inhibitors can perhaps partly explain these effects.

Conidia of specialised *Botrytis* spp. like *B. fabae* on broad bean (Mansfield, 1980) and *B. squamosa* on onion (Clark and Lorbeer, 1976) germinated better than conidia of *B. cinerea*. Some of these species were less sensitive to the inhibitors of their hosts than *B. cinerea* (Rossall and Mansfield, 1978).

Germtubes of *B. squamosa* grow towards stomata or anticlinal wall junctures (Clark and Lorbeer, 1976) whereas germtubes of *B. cinerea* follow anticlinal wall junctions before penetration of petals. Appressoria often develop at the apex of a germtube before penetration. The shapes of appressoria varies from simple swollen tips to many-branched structures, e.g. on tomato leaves (Verhoeff, 1980). Appressoria are mostly formed on anticlinal wall junctions (Ogawa and English, 1960; Clark and Lorbeer, 1976).

A gel, sometimes with microfibrils, often surrounds the hyphae and appressoria of *B. cinerea* and is probably composed of a β(1-3) (1-6) glucan (Dubourdieu, 1978). The largest molecules are insoluble compounds probably derived from the cell wall which may be partly seen as a gel; the smaller molecules ($< 10^6$ Da) are more soluble. McKeen (1974) suggested, that the gel is necessary for adhesion of hyphae to the plant surface. Because a gel makes good contact with the cell surface it might also affect the tropism towards anticlinal cell wall junctions to offer protection against desiccation or attract toxins and enzymes produced by the fungus to assist in penetration. This might be the case in infections at high relative humidity in the absence of waterdrops on the leaf surface (e.g. Salinas *et al.*, 1989).

Glucans from *B. cinerea* are not particularly toxic to plant cells, neither do they seem to act as elicitor for antifung al compounds produced by plants (Kamoen et al., 1980).

Degradation of the cuticle under germtubes has often been observed by scanning electron microscopy (e.g Pie and De Leeuw, 1991; Verhoeff, 1980). Whether this degradation of the cuticle is due to enzymes, to mechanical pressure, a combination of both, or to other factors deserves further examination.

When inoculum droplets are used, the plant cell turgor under the drops usually remains intact until penetration occurs. We may conclude, therefore, that degradation of the epidermal cell does not occur by the action of enzymes or toxins in the inoculum droplets on the surface of the plant, though some compounds secreted by the fungus at, or shortly after, the germination of conidia may be important.

Cutinases seem to be essential for penetration of the cuticle by degradation the cutin in some hostplants. Cutinolytic activity was detected in conidia of *B. cinerea* and in culture media in which the fungus had grown (McKeen, 1974; Salinas, 1992) and monoclonal antibodies raised against purified cutinase gave protection of gerbera flowers to infection by *B. cinerea* (Salinas, 1992). Pectin degrading enzymes are also present in the germination fluid and are secreted early by conidia and young germtubes and both these enzyme activities do play a role during the penetration of germtubes into plant tissue.

Glucans and low molecular weight polysaccharides have been detected in the germination fluid *in vitro* (Bowen and Heale, 1987; Dubourdieu, 1978). Their influence is mainly visible later during hyphal expansion.

Penetration

Penetration can be considered as the phase between surface growth and lesion formation. The fungus penetrates the intact surface of the epidermis by a narrow infection peg (Clark and Lorbeer, 1976) which may result from an appressorium or the tip of a germtube.

On onions, *B. squamosa* and *B. cinerea* showed an intermediate swelling in the intracellular spaces after penetration, or the infection hyphae ramified within the outer periclinal cell walls of the epidermis (Tichelaar, 1967). Similar hyphal swellings have been observed in other hosts, e.g. tomato fruit (Verhoeff, 1970; Rijkenberg *et al.*, 1980), and gerbera ray florets (Salinas, 1992).

The infection peg mostly penetrates via anticlinal wall junctions (Ogawa and English, 1960; Mansfield and Richardson, 1981). This may be due to a lower wax content (Lamarck, pers. comm.) perhaps by a thinner cuticle, or by the proximity of the vertically oriented middle lamella as a preferred pectin-containing path into the tissue. Penetration in epidermal cells through the outer periclinal walls in epidermal cells seems to occur less frequently.

During the penetration process, the following changes can be seen by electron microscopy: the cuticle is further degraded (Verhoeff, 1980), the cell walls softened by the degradation of pectin (Pucheu-Planté and Mercier, 1983) and a thickening of the epidermal cell walls occurs (Pie and De Leeuw, 1991). During the first hours of penetration, plasmolysis of cells adjacent to the infection peg may still occur (Geeraert and Kamoen, unpublished data).

The thickness of the cuticle and epidermal cell layers has an effect upon penetration of grape epidermis by *B. cinerea* (Prudet *et al.*, see page 99).

Botrytis spp. can penetrate the plant through natural openings. *B. tulipae* on tulips (Hopkins, 1921), *B. gladiolorum* on gladiolus (Bald, 1952), *B. squamosa* on onions (Clark and Lorbeer, 1976) and *B. cinerea* on broad bean and dahlia can penetrate stomata (Louis, 1963). In strawberries and raspberries, penetration of stigmas occurs and hyphae enter transmitting tissues of the carpels (McNicol *et al.*, 1985, Breston *et al.*, 1986).

Many types of wounds generally favour the penetration of *B. cinerea* and the subsequent development of lesions. Some of these have important agronomic consequences, e.g. in grapes (Gärtel, 1970; Bessis, 1972; Fermaud and Le Menn, 1989) and tomatoes (Wilson, 1963). Wounds favour the fungus in two ways: it represents a ready access to the internal tissues; and it contains necrotic cells which may have a lower resistance to the fungus and provide a saprophytic growth base from which fungal enzymes and toxins may diffuse into adjacent healthy tissues.

Nutrients in the infection droplets also stimulate the penetration and the subsequent lesion expansion. Phosphates increase the pectic enzymes in infection droplets (Van den Heuvel and Waterreus, 1985) and purines (Kō *et al.*, 1981) stimulate infection.

During penetration, *Botrytis* spp. secrete a number of compounds. Tables 1 and 2 give a list of known secretions with comments about their possible importance for the host-pathogen relationships.

Pectin degradation associated with the first cell necrosis seems to be the most marked phenomenon during the penetration. These aspects of early pectin degradation have been

Table 1: List of enzymes secreted by Botrytis spp. with possible importance for the host-pathogen interaction.

Substrate	Enzymes	Selected references
cutin (cuticle)	cutinase	Salinas 1990, 1992
pectin (cell wall)	polygalacturonases	Leone 1990
	pectin lyases	Heale 1992
	pectin methyl esterases	Marcus and Schejter 1983
protein (cell wall)	acid proteinase	Movahedi and Heale 1990
phenols	laccase	Marbach *et al.* 1984
β(1-3)glucans	β(1-3)glucanase	Dubourdieu 1978
cellulose	cellulases	Verhoeff and Warren 1972
phospholipids (membranes)	phospholipase	Shepard and Pitt 1976
	phosphatidase, lipases	Tseng *et al.* 1970
		Trofimenko *et al.* 1975

Table 2: Possible toxins produced by B. cinerea.

Class	Secreted substances	Selected references
polysaccharides	glucans	Dubourdieu 1978
	rhamno-galacto-mannans	Aksenova 1962
		Kamoen and Dubourdieu 1990
organic acids	citric acid	Ladygyna 1962
	oxalic acid	Ladygyna 1962
		Verhoeff *et al.* 1988
other substances	thiourea	Ovcarov 1937
	urea	Ovcarov 1937

reviewed previously. The classification of the pectic enzymes (Rombouts and Pilnik , 1972), their relationship with cell necrosis (Basham and Bateman, 1975; Movahedi and Heale, 1990), host defences against pectic enzymes by inhibitors (Collmer and Keen, 1986, Schlösser, 1983) and the role of pectic cell wall fragments in host defence elicitation (Collmer and Keen, 1986) have been adequately described.

In *B. cinerea* a pectin-degrading enzyme is present in the conidium (Verhoeff and Warren, 1972) and in the germtube (Van den Heuvel and Waterreus, 1985; Leone, 1990). This first secreted isozyme does not require induction by pectin fragments (Van der Cruyssen and Kamoen, 1992).

Expansion in the host tissues

The number of penetrations or infections may be rather low in relation to the inoculum present in the air, but once established, lesions can expand rapidly in compatible host-pathogen combinations.

In our institute we have been studying the border zone of expanding lesions and from this we concluded that there are two possibilities for expansion. The hyphal tips may grow between the living cells and the cells are killed only after hyphae have grown beyond them. This has been observed in strawberry petals. The other possibility is that host cells are killed ahead of the hyphal tips and the fungus grows within the necrotic tissue, e.g. in leaves, stems, and green fruits. The assumption in the latter case is that the fungus was unable to grow in living tissue.

To elucidate the killing of the cells ahead of the growing hyphae we are attempting to answer two questions; viz. which secretions (or substances) kill the cells and how do they move through tissues. Examining Koch's postulates for the different secretions (Dimond and Waggoner, 1953), firstly the substances which may be responsible must be isolated from infected tissues showing characteristic symptoms. Two polysaccharides (PS) were isolated from infected grapes (Kamoen and Dubourdieu, 1990); and pectic enzymes (PE) were obtained from inoculum droplets as described by Leone (1990) and from infected tissue (Balasubramani *et al.*, 1971). Secondly, the secretions isolated from the infected tissues must be purified and identified. This was partly done for glucans and rahmno-galacto-mannan-polysaccharides and a number of pectic isozymes have been purified partly (Leone, 1990). Thirdly, each of the (partly) purified secretions must evoke a characteristic symptom. With respect to the last of Koch's postulate, the following conclusions can be drawn: with partly purified rahmno-galacto-mannans, subcellular disorganisation was observed and the ability of cells to plasmolyse was lost only later (Kamoen *et al.*, 1978); with glucans, hardly any toxicity was observed (Kamoen *et al.*, 1980); with partly purified pectic enzymes, membrane rupture was the first symptom (inability to plasmolyse) and afterwards subcellular disorganisation occurred (Kamoen *et al.*, 1978). One conclusion is, therefore that fractions of both the PS and PE complex are partly responsible for cell necrosis.

Other secretions also have been studied: an acid proteinase (Movahedi and Heale, 1990); organic acids (Kamoen, 1972), laccas es (Dubourdieu *et al.*, 1984; Pezet *et al.*, 1991; Viterbo *et al.*, 1992) and a phospholipase (Shepard and Pitt, 1976).

For killing of cells ahead of the hyphae a signal must preceed the hyphae. This signal could be transferred either via the symplast (Kahl, 1982) or via the apoplast by intercellular diffusion. Arguments in favour of intercellular diffusion are: the hyphal tips are mainly growing in the intercellular spaces and toxins and enzymes are secreted there (Kamoen, 1972); the expansion increases at low water vapour deficit, which increases intercellular water content; and similar symptoms can be obtained with intercellular infiltration of fungal secretions (Kamoen *et al.*, 1978). The diffusion must result in a gradient of toxic secretions. At the boundary of the lesion the following zones can often be distinghuished (Kamoen, 1989): zone 0 close to the necrotic center of the lesions with the hyphal tips secreting toxins and enzymes; zone I: diffusion zone with toxic concentrations of toxins and enzymes; zone II: diffusion zone with sublethal concentrations of both types of compounds, and zone III: healthy tissue.

A model for expansion of lesions based on diffusion has been proposed by Kamoen (1972). Diffusion of the secretions may occur via intercellular spaces; at the same time,

the secretions may be taken up in the cell walls and cytoplasm. From diffusion laws (Jacob, 1962), it appears that the distance over which the cells are killed ahead and the expansion of the lesions mainly depend on the intercellular water content, and to a lesser extend on the concentration of toxins and enzymes produced by the hyphal tips. The intercellular water content depends on the relative humidity or water vapour deficit, the stomatal aperture, etc. An alternative hypot hesis for the expansion of lesions is based on dilution and has been proposed by Harrison (1980, 1988). An alternation of humid nights and drier days may cause the typical zonation of expanding *Botrytis* lesions.

Concluding remarks

There is some agreement about the morphological aspects of *Botrytis* diseases, but less is known about the biochemical and physiological aspects. We need strong evidence about the role of the different enzymes and toxins secreted by the pathogen in the disease process. For example:

- Which pectin degrading isozymes are essential and are produced in sufficient quantities for expansion of lesions?
- Is the proteinase and the phospholipase essential for pathogenesis?
- What is the role of other enzymes secreted by *Botrytis* spp.?
- Are acids and rahmno-galacto-mannans important for necrosis or for host defence elicitation ?

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Activation of host defence mechanisms in response to *Botrytis cinerea*

J.B. Heale

Summary

Evidence from a range of host-parasite interactions shows that fragments, often of a carbohydrate origin, released from fungal cell walls, can elicit active defence mechanisms in host cells. There is some evidence for such direct (primary) elicitors from *B. cinere* a, but of more significance probably is indirect (secondary) elicitation caused by host cell wall degradation and/or host cell necrosis, releasing endogenous elicitors. An aspartic proteinase (APr) as well as endo-PG and endo-PL enzymes secreted by *B. cinerea* are implicated in both latter processes, although the mechanisms involved are distinct. Mannan polysaccharides may function either as primary elicitors or by causing cell death leading to secondary, endogenous elicitor release. In carrot root slices, elicitation of induced resistance responses causes activation of the enzyme ACC synthase, a key step in ethylene biosynthesis; accumulation of the phytoalexin 6-MM is triggered by ethylene but induced resistance is not; both 6-MM accumulation and induced resistance are i nhibited by free radical scavengers, thus inducing susceptibility; fatty acid peroxidation products generated during ethylene biosynthesis are important in this type of induced resistance response.

Introduction

As a necrotrophic plant pathogen, *Botrytis cinerea* Pers.: Fr. has a critical requirement for dead plant tissue, or exogeneous nutrients supplied in an aqueous environment e.g. 'thin' water films on wound sites, a favoured entry route. Direct penetration involving cutin esterase may also occur (Shishiyama *et al.*, 1970; Salinas, 1992). Rapid multiplication of the pathogen leads to high inoculum levels with increasing extracellular release of phytotoxic components from spores and growing hyphal tips (Heale, 1987).

The macerating activity of the pectic enzyme complex in *B. cinerea* is well understood, but the primary mechanisms under lying the necrosis-inducing activity of the pathogen are still a controversial area, largely because of the lack of critical studies. These activities are usually ascribed to pectic enzymes of the endo-PG type in *B. cinerea*, but in recent studies we have also implicated an endo-pectin lyase (PL) in both maceration and cell death in *Botrytis* infection of carrot tissue (Movahedi and Heale, 1990a,b). Di Lenna *et al.* (1981) and more recently Kaile *et al.* (1991) have also reported on the role of endo-PL in pathogenesis for *B. cinerea*. More significantly, we have demonstrated for the first time a widespread role for an aspartic proteinase (APr), both in early host cell death and in initial infection stages for *Botrytis* interactions involving eight different isolates of the pathogen with grape berries, strawberry, raspberry, cabbage, broad bean leaves and carrot slices (Movahedi and Heale, 1990a,b; Movahedi *et al.*, 1991). Phytotoxic mannans contribute to host cell-killing in spreading lesions (Kamoen *et al.*, 1978; Kamoen, 1984).

Many of these pathogen-produced components are associated with both necrosis and with subsequent elicitation of resistance responses, but only a few studies have attempted a comprehensive analysis of the role of such individual components (Kamoen *et al.*, 1978; Kamoen, 1984; Bowen and Heale, 1987a). This review will therefore attempt to clarify the role of different types of elicit or in *Botrytis*-host interactions in relation to the current interest in plant cell-signalling systems, and the mechanisms involved in active defence responses 'triggered' under these conditions. Finally, 'noble rot' of grape berries, 'ghost spotting' of tomato fruits, as well as latency in general, will be briefly considered in relation to such mechanisms.

Conditions which favour elicitation of active host defence mechanisms

If for convenience, we simplify the outcome of a potential Botrytis-plant interaction, we can consider just two possible results: localised infection and spreading lesions.

Due to a combination of two or more infection-negative conditions [e.g. low initial levels of inoculum, low humidity or absence of free water, absence of very juvenile (or senescing) host tissue, absence of wounds or exogeneous nutrients], a localised infection occurs, with relatively little host cell necrosis. These conditions favour elicitation of defence mechanisms.

Due to the combination of several infection-positive, predisposing factors [e.g. high initial inoculum, high humidity or presence of free water, juvenile or senescent tissue, wound tissue or exogeneous nutrients], the rate of release of phytotoxic components and their increasing effects overwhelm all responses by surrounding healthy cells, i.e. the latter are killed before they can react to the presence of potential elicitors and spreading lesions develop.

Mechanisms of elicitation

In highly characterised systems, interactions between plant host and pathogen may be classed as recognition, signal transduction and response. We do not yet have such clear-cut observations for *B. cinerea*-host interactions. For the present, we can consider first: (a) direct elicitation by primary fungal elicitors, and secondly: (b) indirect elicitation via either cell wall degrading enzymes (CWDEs) or other necrosis-inducing components from the pathogen, causing (c) the release from the host cell of constitutive (endogenous) elicitor(s), which act(s) as 'second messenger(s)', triggering active responses in adjacent healthy cells. In order to dissect the various mechanisms, most researchers have usually investigated single components, while ignoring temporal aspects and the multiplicity of interactions between molecular components of pathogen and host. Particularly important here are the primary stages in the interaction between *B. cinerea* and its potential host, i.e. germination and the immediate 12 to 24 h post-germination period which usually decide the outcome between infection leading to spreading lesions or active resistance leading to localised lesions. Also considered here is the possible role of ethylene whose synthesis invariably results after infection by *B. cinerea*.

Direct elicitation

Evidence from a range of host-parasite interactions suggests that fragments, often of carbohydrate origin, released from fungal cell walls, can elicit active defence

mechanisms in plants and cell suspension cultures (Templeton and Lamb, 1988). Elicitors include gl ycoproteins, simple and complex carbohydrates, certain fatty acids such as arachidonic acid, and glutathione. The best-known charact erised elicitors derived from fungal cell walls include a β-linked heptaglucoside from *Phytophthora megasperma* f.sp. *glycinea* active in soybean, and chitosan oligosaccharides (Templeton and Lamb, 1988). The fact that only 1 of 300 similar hepta-β-glucosides was active as an elicitor of PA accumulation suggests the presence of a specific receptor for this molecule (Hahn *et al.*, 1989). We can speculate that such a receptor exists in the plasma membrane of the host cell and that following a specific recognition event, a signal is somehow transduced to the nucleus and coordinated gene regulation results. Transcriptional activation of host 'disease response' genes can be detected within 5 min after addition of elicitor to plant cell suspension cultures (Templeton and Lamb, 1988). How elicitors act at the nuclear level to regulate gene expression is still not understood, although chitosan is reported to interact directly with plant DNA and chromatin (Kendra *et al.*, 1987). Whether such primary elicitors are important in *B. cinerea* interactions with its hosts is not clearly established.

Our previous studies with the carrot-*B. cinerea* interaction have revealed that heat-killed conidia or cell-free germination fluid induced various active defence responses including suberisation, lignification, and accumulation of phytoalexins including the polyacetylene falcarinol and the isocoumarin, 6-methoxymellein [6-MM] (Harding and Heale, 1981). Many of these responses can probably be explained by a secondary elicitation mechanism, as discussed below. Kurosaki *et al.* (1987) on the other hand have shown that fungal mycelial preparations, chitin or chitosan, will induce chitinase in a strain of cultured carrot cells, without any loss of host cell viability. The host chitinase degraded the hyphal walls of the pathogen *Chaetomium globosum* and the liberated soluble fragments stimulated PAL activity and enhanced the biosynthesis of phenolic acids in the carrot cells.

Other components possibly acting as primary elicitors in host interactions with *B. cinerea* at sub-toxic levels include particular polysaccharides (mannan) fractions (Kamoen *et al.*, 1978; Kamoen 1984), but if shown to possess even slight phytotoxicity, they may be better regarded as indirect elicitors (see below).

Indirect elicitation via host cell wall degradation and/or host cell necrosis

In studies relating the time course of cell death in carrot root slices infected with *B. cinerea* spore suspensions with the time of first detection of necrosis-inducing components in planta, it appeared that two cell layers were killed after 3 h, increasing to eight layers after 12 h. Up to 8 h, the only in planta necrosis-inducing fraction was identified as having aspartic proteinase (APr) activity. Endo-PG and endo-PL activity were first detected at 8 and 10 h respectively, followed by pectin methyle sterase (PME) at 12 h, and the phytotoxic glycoprotein and polysaccharide components were not identified before 18 h after inoculati n (Movahedi and Heale, 1990a,b).

In the same investigation, we showed that at relatively low concentrations, both purified endo-PL and the APr from *B. cinerea*, caused cell death and induced resistance to the pathogen in surface cell layers of carrot root slices. At higher concentrations, both caused extensive cell death in carrot tissue and of carrot suspension cells. Neither enzyme lysed osmotically balanced carrot protoplasts unless carrot cell wall preparations were also present in the same suspension. Plasmolysed carrot cells were protected from the

cytotoxic activity of the endo-PL, but not from APr-induced damage. It was concluded that cytotoxicity of both endo-PL and APr was indirectly caused by toxic wall components released separately by each enzyme, with evidence that different components were involved in each case.

The APr apparently releases a low molecular weight cytotoxic component from the host cell wall which can diffuse to the host cell plasma membrane, even when the latter is no longer in direct contact with its wall (i.e. when the cell is plasmolysed). For the endo-PL, we have proposed a cytotoxicity mechanism based upon free radicals released from enzyme-treated carrot cell walls, which have a very short half-life and require close contact between the host cell wall and the vulnerable polyunsaturated fatty acids of its plasma membrane for peroxidation-type reactions to occur. We have also shown that free radical scavengers inhibit the resistance responses of carrot tissue to this pathogen (Hoffman and Heale, 1989).

Alternatively, Kaile *et al.* (1991) have suggested that pectic enzymes from *B. cinerea* release a high level of Ca^{2+} ions normally bound to pectic substances in the middle lamella during host maceration (in this case Swede bulb tissue). As regards the mechanism of cytotoxicity operating here, they proposed an increased flux of Ca^{2+} into cells from the apoplast, resulting in the lowering of H^+/K^+ATPase activity and the high K^+ efflux observed from diseased tissues.

That indirect elicitation can occur in the *B. cinerea*-carrot interaction was demonstrated by the fact that triggering of the pathway to accumulation of the isocoumarin 6-MM could be induced by soluble, non-cytotoxic elicitors, released from damaged carrot cells by endo-PL and APr enzymes when tested separately (Movahedi and Heale, 1990a,b). Treatment of carrot cell walls with APr reduced their protein content by c. 30% and increased the macerating activity of endo-PL by 40%. When APr activity was specifically inhibited in spore suspensions by 'Pepstatin', necrosis and infection were completely prevented in most cases, even though there were no effects on germination or growth *in vitro* (Movahedi and Heale, 1990a,b; Mohavedi *et al.*, 1991).

Kurosaki *et al.* (1984, 1985), investigating carrot suspension cells, reported that pectinase or trypsin released host cell wall fragments that elicited the production of the carrot phytoalexin 6-MM. The elicitors purified from pectinase-treated carrot cell walls were distinct from those released by trypsin. The results are consistent with our findings regarding APr-treated as compared with endo-PL-treated carrot cell walls, which both resulted in the release of non-cytotoxic elicitors of 6-MM accumulations (Movahedi and Heale, 1990b). The phytotoxic and elicitor fractions of cell wall components released both by APr and endo-PL were distinct from each other. The former are heat-labile since autoclaving the soluble fraction of cell wall APr- or endo-PL hydrolysates to inactivate the enzymes also removed the toxic activity, whereas the elicitor fractions were stable to autoclaving for 20 min.

De Lorenzo *et al.* (1991) have shown that whereas an endo-PL from the bacterium *Erwinia carotovora* caused maceration in potato tissue only at high pH (above 7.0, with an optimum at 9.0), there was an extended half-life for oligogalacturonides with elicitor activity at low pH (5.75) causing a variety of disease responses in potato. Such active oligogalacturonides, which have been shown to be liberated generally by many different microbial endo-PGs and PLs have 10 to 13 galactosyluronic acid residues. They are active at micromolar concentrations, but their activity can be completely destroyed by endo-PG and PL under optimal conditions fo r enzyme activity (Darvill *et al.*, 1989). This is likely to be a 'fine-tuning' mechanism for the interaction between host and *B. cinerea*,

based upon apoplastic pH levels which change during development and maturation. During active infection by *B. cinerea* in carrot tissues, the pH value falls from c. 6.0 in healthy tissue to c. 3.5 over 3 days (Movahedi and Heale, 1990a), thus changing to the optimal value for APr activity. The possibility that endo-PL from *B. cinerea* releases effective elicitors from the host cell wall at low pH requires thorough investigation both here and in other systems involving this pathogen.

PG-inhibiting proteins are known to be present in a wide variety of dicotyledonous plants. Cervone *et al.* (1989) showed that over a 24 h period, such inhibitor-limited, endo-PG activity results in the liberation of 10 to 12 residue galactosyluronic acid fragments, thus essentially turning a virulence factor of the pathogen (the PG enzyme) into an avirulence factor, determining the release of a highly active elicitor from the hosts'own cell wall. It was already known from earlier work (Deverall and Wood, 1961a, b) that such elicitation resulted in activation of a host phenolase enzyme, with the resultant oxidised phenols causing further inhibition of the macerating pectic enzyme activity.

The differential rates of release and diffusion of phytotoxic components and elicitors, as well as rates of response are highly critical here. Furthermore, there is evidence for a synergistic disease resistance response by the plant in the mutual presence of both direct fungal elicitors as well as its own cell wall oligosaccharins (De Lorenzo *et al.*, 1989). It is still not generally recognised that cell death caused by *B. cinerea* occurs before maceration (Tribe, 1955; Movahedi and Heale, 1990b), and that it can be caused indirectly by the toxic effects of host cell wall components released by pectic enzymes (endo-PG and -PL) or the A Pr as described above. There is clear-cut evidence that injury to the membranes of pectic enzyme-treated host cells occurs almost immediately after exposure, and before any significant loss of wall structural integrity could possibly occur (Hislop *et al.*, 1979; Byrde, 1982; Cooper, 1984). Thus, the hypothesis put forward by Bateman (1976) that the death of plant tissues treated with pectolytic enzymes results from plasma membrane physical damage, caused by bursting of the protoplasts through the enzyme-degraded and weakened cell wall under osmotic stress, i.e. 'the bursting hypothesis', can no longer be held.

Kamoen (1984) has described elicitation of phytoalexins by *B. cinerea* in the outer zone (zone III) of restricted lesions on leaves at low humidity, occurring as a result of sub-toxic concentrations of mannan polysaccharides (10-50 x 10^3 Da) diffusing to this zone. Inhibition of plasmolysis in dying cells in zone I, just ahead of leading hyphae in zone 0, was attributed mainly to slow-diffusing pectic enzymes (e.g. PG: 34 and 56 x 10^3 Da for two isoenzymes respectively), whereas chloroplast degeneration in cells still capable of plasmolysis was attributed to the more rapidly-diffusing, low molecular weight mannans, which reached relatively high concentrations in zone II. Thus, the level of water or high humidity in determining diffusion of cytotoxic components, and the particular concentrations reached in different zones, will decide if active defence responses are sufficiently 'in place' before even more damaging pathogen components reach the same location, should high water conditions resume.

An endogenous elicitor released as a consequence of cell death in carrot root cells, induces resistance to *B. cinerea*.

Low molecular weight components described as constitutive or endogenous elicitors have been obtained from plant tissues without empl oyment of any CWDE-treatment e.g. from damaged French bean (Hargreaves and Selby, 1978) and carrot root cells (Bowen

and Heale, 1987b). The latter authors reported that a peptide (c. 5 x 10^3 Da) extracted from carrot root tissues damaged by slicing or freeze-thawing, induced active defence mechanisms in carrot root slices against *B. cinerea*. Endogenous elicitor activity was present in homogenates of fresh and freeze-thawed tissues, but was absent in autoclaved tissue. A similar peptide fraction was also obtained from carrot tissue treated with cell-free germination fluid of the pathogen. The detection of this peptide elicitor within 2 h of cell damage indicates that it is released or activated during the early stages of cell necrosis. The possible relationship between this type of endogenous elicitor, released as a direct result of host cell death (irrespective of causation, biotic or abiotic) which may result from host cell wall or plasma membrane degradation by a self-induced (autolytic) host enzyme on the one hand, and other indirect or secondary elicitors in the form of host cell wall fragments released by the pathogen's pectic enzymes described above on the other, has not been investigated.

Activation of disease responses and the role of ethylene

Whether primary or secondary elicitors (or both, acting synergistically) are involved, healthy carrot root cells undergoing induced resistance responses to *B. cinerea* accumulate a complex of phytoalexins (including 6-MM). We have concentrated in recent studies on the relationship between the induced resistance response, 6-MM accumulation and the role of ethylene (Hoffmann and Heale, 1987; Hoffman *et al.*, 1988; Hoffman and Heale, 1989). Induction of active responses here is associated with activation of a controlling step (ACC synthase) in ethylene biosynthesis, but whereas 6-MM accumulation is triggered by ethylene itself, induced resistance is not, i.e. ethylene-treated slices accumulate the phytoalexin but remain susceptible to an invasive level of spore inoculum. Both the phytoalexin accumulation and induced resistance are inhibited by the free radical scavengers salicylhydroxamic acid and propyl gallate, thus inducing susceptibility. This is explained by the fact that the conversion of the precursor 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene by the ethylene-forming enzyme (EFE), is known to involve a free radical. The fatty-acid composition of carrots includes about 70% linoleic acid; both growth, and germination of conidia of *B. cinerea* are inhibited in the presence of lipoxygenase and linoleic acid. It appears therefore that the fatty-acid peroxidation products generated during ethylene biosynthesis are important in the induced resistance response.

Research in other host-parasite interactions has yielded findings which possibly implicate fundamental underlying mechanisms . Esquerré-Tugaye *et al.*, (1989) working with tobacco and *Phytophthora parasitica nicotianae*, reported that a pathogen-derived glycoprotein elicitor which caused slight browning in tobacco cell suspension cultures, induced lipoxygenase after only 1.5 h. In intact plants, lipoxygenase induction was maximal 2 days after inoculation of the resistant cultivar. They suggested that the hydroperoxides produced from linoleic and linolenic acids (polyunsaturated fatty acids) in tobacco by the increased lipoxygenase activity could play a multiple role in a series of cascade reactions leading to hypersensitive cell death, as well as participating in secondary signals to adjacent healthy cells. This elicitor also induced the same pathway of ethylene biosynthesis as described above for carrot root tissue, with an increase in ACC synthase activity within only 15 min of elicitor treatment, followed by ACC and ethylene production.

These workers proposed the following steps operating in a cascade involving:

recognition at the plasma membrane level leading to membrane depolarisation, quickly followed by ethylene synthesis; ethylene and membrane depolarisation could play the part of rapidly exported cell-to-cell signals with intracellular signals including phospholipid metabolites such as compounds resulting from lipoxygenase action and/or inositol phosphates; ion fluxes, particularly Ca^{2+} and phosphorylation involving protein kinases (Farmer *et al.*, 1989) could operate as part of the intracellular signal transduction system leading to disease response gene activation (Esquerré-Tugaye *et al.*, 1990).

Expression of resistance

The various types of active resistance responses in plants operating against *Botrytis* have been considered in detail by Mansfield (1980) and are beyond the scope of this review. They include phytoalexins (PAs) which perhaps are better considered as having a role in lesion restriction, as well as host cell wall changes involving formation of callose, suberisation, lignification, and papilla formation. In most interactions, there is evidence of a complex of such responses implying coordinated regulation by the type of signals previously discussed. Just one example will be quoted here; in carrot root slices undergoing active defence responses induced by cell-free germination fluid, heat-killed conidia, or sub-invasive levels of live conidia (1×10^5 conidia.ml^{-1}), several different phytoalexin pathways are induced (including those producing isocoumarins, and polyacetylenes), and both suberisation and lignification are strongly developed (Heale and Sharman, 1977; Harding and Heale, 1981; Heale *et al.*, 1982a). In the upper four cell layers of induced slices, we observed more frequent nuclear migration, and an increase in both nucleolar volume and uptake of tritiated uracil, as compared with control, wounded-only slices (Heale *et al.*, 1982b). The cell wall modifications were also seen in control, wounded-only slices, but the level of expression was greatly elevated in the induced slices as a result of the fungal invasion and increased endogenous signals discussed earlier. Thus, there is evidence of a synergistic elevation of response in the presence of a complex of both host- and pathogen-derived signals.

Latency, 'ghost-spotting' and 'noble rot'

A discussion of signal type responses in hosts invaded by *B. cinerea* would be incomplete without a brief consideration of several well-known examples where host necrosis is absent or at least is strongly reduced. Latency ('quiescence') in fruit crops such as raspberry, strawberry and grape is a commonly occurring phenomenon (Verhoeff, 1980). It can be initiated in a number of ways including colonisation of necrotic flower parts after blooming, without any further development of obvious symptoms until fruit maturity, when extensive rotting often occurs in wet or damp environments. Here, the long period of 'balanced parasitism' without host damage is likely to be partly explained by a highly-suppressed APr- and pectic enzyme activity in the pathogen, possibly accompanied by release of elicitor-active signals. McNicol and Williamson (1989) described germination of *B. cinerea* spores on the wet stigmatic surfaces of blackcurrant flowers followed by their systemic invasion of the whole style tissue without any obvious necrosis, leading to premature abscission of blooms and developing fruits. One possible mechanism suggested here to explain flower shedding was the in duction of ethylene in the host tissues as a result of the infection by *B. cinerea*.

Ghost-spotting caused by the invasion of young tomato fruits by conidial germtubes

of *B. cinerea* is associated with localised lignification (van Maarschalkerweerd and Verhoeff, 1976; Glazener, 1982), again evidence for a signal-mediated defence response.

It is established that a glycoprotein inhibitor of endo-PG activity in grape berries is an important factor in resistance to *B. cinerea*. The factors determining 'Noble rot' of grape are certainly complex, involving climatic and edaphic factors, but we can at least speculate that reduced water availability within the fruit tissues at critical periods significantly reduces the diffusion of several necrosis-inducing components from the pathogen.

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Production in vitro of polygalacturonases by *Botrytis cinerea*

D.J. Johnston and B. Williamson

Summary

Four polygalacturonases (PGs) were produced by *B. cinerea* in a modified Czapek-Dox liquid medium containing citrus pectin. Two endo-PGs and two exo-PGs were purified to homogeneity from 10-day-old cultures and polyclonal antisera were raised to both endo-PGs. In Western blots both the antisera reacted positively with either endo-PG isozyme, but not with the exo-PGs or other extracellular proteins. The antiserum to endo-PGI was used to study the production of endo-PGs in liquid media containing various carbon sources. Endo-PGs were found to be constitutively expressed, whereas when the fungus was grown on 0.01% galacturonic acid a novel peak detected by preparative isoelectric focusing was attributed to exo-PG induction. The roles of these four isozymes in pathogenesis of raspberry fruits is being examined by molecular and ultrastructural techniques.

Introduction

Botrytis cinerea Pers.: Fr. attacks flowers, fruits and vegetables of a wide range host of plants in temperate regions as a necrotroph, but it commonly establishes latent infections which culminate in post-harvest grey mould (Coley-Smith *et al.*, 1980; Williamson *et al.*, 1987).

This pathogen produces several enzymes which macerate plant cell walls. Polygalacturonses (PGs) are the first cell wall-degrading enzymes produced by several fungi when grown on isolated cell walls (Cooper and Wood, 1975) but little attention has been given to the induction of these PGs in *B. cinerea*. In many plant pathogens the synthesis of PGs is regulated by galacturonic acid (Lorenzo *et al.*, 1987). In *B. cinerea* biochemical studies have shown that endo-PGs are expressed constitutively in ungerminated and germinated conidia (Verhoeff and Liem, 1978), and *in vitro* both constitutive and inducible PGs have been described (Leone and Van den Heuvel, 1986). This paper outlines the purification of two endo-PGs and two exo-PGs from a pectin-enriched liquid medium which was fully described elsewhere (Johnston and Williamson, 1992) and also briefly provides new information about the production of these enzymes derived from immunological techniques.

Material and methods

Fungal isolate and growth conditions

Sporulating cultures of *B. cinerea* isolate 347 (IMI 339491) were maintained according to Harrison (1978). For purification of PGs, forty 250 ml conical flasks, each containing 100 ml of a modified Czapek-Dox liquid medium supplemented with 1% glucose and 1%

citrus pectin were inoculated with mycelium on an agar disk. The flasks were incubated with orbital shaking at 120 rpm at 20°C for 10 days. The filtrate was dialysed against the appropriate buffer before purification. For production studies, the fungus was grown as above in a modified Czapek-Dox liquid medium, but lacking pectin. After 6 days, the mycelium was recovered and transferred to similar flasks containing either an identical medium or one in which glucose was substituted with galacturonic acid at various concentrations for a further 48 h.

Purification, separation and concentration of PGs

Endo-PGI, endo-PGII, exo-PGI, exo-PGII were purified to homogeneity as described by Johnston and Williamson (1992) and endo-PGI was used to produce polyclonal antisera from a rabbit. The gamma-globulin fraction from the antisera was used in Western blots and PTA-ELISA. For unambiguous detection and quantification of endo-PGs from culture filtrates, it was first necessary to separate and concentrate the isozymes. Endo-PGI was concentrated by loading culture filtrate previously dialysed against 20mM acetate buffer at pH 5 .2 on to an S-Sepharose Fast Flow column. The column was eluted with the same buffer containing 1 M NaCl. The unbound material containing endo-PGII was applied to an identical column containing Q-Sepharose Fast Flow and eluted similarly. Further analysis of isozyme production involved preparative isoelectric focusing (prep. IEF) in the pH range 3.5 to 9.5.

Results

Enzyme characteristics

Purification revealed two endo-PGs (endo-PGI and endo-PGII) with pI values of 8.8 and 4.9 and two exo-PGs (exo-PGI and exo-PGII) with pI values of 4.9 and 3.5. Endo-PGI and II both had apparent M_r values of 36 kDa; exo-PGI and II had M_r values of 6 5 and 70 kDa respectively. Each protein consisted of a single polypeptide chain. Exo-PGI and II were shown to be glycoproteins by their affinity to concanavalin A. Their pH and temperature optima have been determined (Johnston and Williamson, 1992).

PG induction with galacturonic acid

Filtrate from cultures grown in a modified Czapek-Dox liquid medium containing galacturonic acid ranging from 0.01% to 1.0% revealed no consistent increase in total PG activity, relative to mycelial dry weight after 48 h. Subjection of the filtrate to prep IEF revealed a novel isozyme peak with a pI value between 3.7 and 6.3 when the fungus was grown on a medium supplemented with galacturonic acid, but not with 1% glucose.

Antiserum raised against endo-PGI reacted positively with both endo-PGI and II in Western blots but no reaction was detected against any other extracellular protein. Fractions from prep IEF subjected to Western blot analysis showed the presence of endo-PGI in the medium when the fungus was grown on either 1% glucose or 0.01% galacturonic acid. Endo-PGII was not detected in either case. Following separation and concentration of endo-PGI and II by ion exchange chromatography, both enzymes were identified on blots from media supplemented with either 1% glucose of 0.01% galacturonic acid.

B. cinerea grown in duplicate flasks supplemented with either galacturonic acid or glucose produced endo-PGI and endo-P GII to similar amounts regardless of carbon source, confirming the results obtained for Western blots.

Discussion

Most fungal pathogens studied show induction of endo-PGs when grown in the presence of low levels of galacturonic acid (Cooper and Wood, 1975; Keen and Norton, 1966; Lorenzo *et al.*, 1987). A recent study of PG-production of *B. cinerea* revealed both constitutive and inducible endo-PGs using gel electrophoresis (Leone and Van den Heuvel, 1986). In our work, the new peak of activity, produced only when the fungus was grown in the presence of galacturonic acid, could only be accounted for by the induction of either endo-PGII, exo-PGI or exo-PGII because the pI of the isozymes resides within this pH range (Johnston and Williamson, 1992).

Western blots of the prep IEF fractions corresponding to endo-PGI revealed a band from filtrates from cultures grown with both galacturonic acid and glucose. The presence of these bands is indicative of constitutive production of this isozyme. Although endo-PGII was not detected in that test, its presence was later proved after further concentration of filtrate from either medium, suggesting that constitutive production of endo-PGII occurs in *B. cinerea* under these conditions. Constitutive production of both endo-PGs suggested by Western blots was confirmed by PTA-ELISA.

The appearance of the novel PG activity observed during prep IEF can therefore not be accounted for by endo-PGII which was constitutively produced. We suggest that this peak can only be explained by the induction of one or both exo-PG isozymes. The significance of exo-PG induction and constitutive expression of endo-PGs is not understood. Endo-PGI will be positively charged after release of acidic vacuolar fluids from mesocarp cells in ripening raspberry fruits and results in the binding of this isozyme to the negatively charged pectin within cell walls, hence facilitating its actions. Endo-PGII has a relatively low specific activity compared with endo-PGI when using polygalacturonic acid as a substrate. It is possible that this isozyme degrades particular pectin moieties in the cell wall with higher efficiency. The monomers produced by these isozymes would probably induce exo-PGs and the resulting combination of PGs may then act synergistically to efficiently degrade cell walls and produce a carbon source for fungal growth. Further studies are required to determine the extent of synergism between various combinations of these purified isozymes *in vitro* and *in planta*.

Few fungal PGs have been shown to be glycoproteins and we are the first to report the presence of exo-PGs from *B. cinerea*; the phytopathogenic significance of these glycoproteins has not been elucidated but it has been suggested that their ability to form complexes with host proteins is likely to depend on the carbohydrate moiety of the PGs.

Our current investigations are concerned with the ultrastructural localisation of endo-PGs during infection of raspberries and blackcurrants by *B. cinerea*. We are also cloning the genes encoding these isozymes with a view to negating each of these isozymes by site-directed mutagenesis to assess their effect on pathogenicity.

Acknowledgement

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Significance of polygalacturonase production by *Botrytis cinerea* in pathogenesis

G. Leone

Summary

During infection of plants *B. cinerea* produces different kinds of cell wall-degrading enzymes, including the pectic enzymes polygalacturonases (PGs). To establish the role of PGs in infection, the significance of total PG production by the fungus is distinguished from the activity of a single isoenzyme, PG2. It is proposed that the primary significance of the sequential total PG production is related to the digestion of the pectin portion of the plant cell wall. The physiological and biochemical properties of PG2 indicate that this enzyme has a double role supporting the penetration of primary cell walls and the onset of the chain production of other isoenzymes involved in pectin catabolism.

Introduction

The fungus *Botrytis cinerea* Pers.:Fr. is a saprophyte ubiquitous on weakened or dead plant material (Blakeman, 1980). As a pathogen, it usually first becomes established on dead or moribund parts of a host and then spreads into adjacent healthy tissues (Mansfield, 1980). Direct penetration of germtubes into undamaged tissues has also been observed on different hosts, among these are grape berries (McClellan and Hewitt, 1973) and gerbera flowers (Salinas *et al.*, 1989). *B. cinerea* is also known as a secondary invader, attacking plants already infected by other pests or weakened by senescence or stress factors (e.g. Leone and Tonneijck, 1990).

The most common symptom induced by *B. cinerea* is the decay of infected tissues. Cell wall-degrading enzymes secreted by the fungus, have long been known to be involved in the infection process (Hancock *et al.*, 1964). Pectic enzymes, in particular the polygalacturonases (PGs), have been associated with the penetration and colonization of French bean leaves by *B. cinerea* (Van den Heuvel and Waterreus, 1985).

The actual role of PGs during infection by *B. cinerea* has not been fully established but the significance of PG production by this fungus in pathogenesis has been examined (Leone, 1990; Leone and Van den Heuvel, 1987; Leone et al., 1990a,b). Some of the results are discussed briefly here.

Results and Discussion

Significance of total PG production

Polygalacturonases produced by *B. cinerea* can be separated into a number of electrophoretic variants referred to as isoenzymes. To understand the significance of PG production in pathogenesis, it is important to distinguish between total PG production resulting from the combined activity of multiple isoforms, and single PG isoenzymes.

This approach helps to establish the role of each enzyme at an early stage (e.g. penetration) or in more advanced stages (e.g. colonization) of infection.

Several studies showed that *B. cinerea* produces a number of pectic isoenzymes belonging to the PG group (Cruickshank and Wade, 1980; Drawert and Krefft, 1978; Di Lenna and Fielding, 1983; Magro *et al.*, 1980). A clear relationship between growth of the fungus on a cell wall-related polysaccharide (isolated bean cell walls, citrus pectin or sodium polygalacturonate) as the sole C source, and PG production was reported by Leone and Van den Heuvel (1987). The type and concentration of the substrate, and the concentration of conidia in the inoculum, influenced growth and total PG activity. Pectic enzymes were produced in a consistent sequence. Isolate BC1 first produced the constitutive isoenzyme PG2. This degrades sodium polygalacturonate with an endo-mode of action (Leone *et al.*, 1990a). After PG2, other pectic isoenzymes were produced, including PG1 which is induced. The pectic enzymes are numbered according to Van den Heuvel and Waterreus (1985); this notation was based on the location of PG bands in gels after electrophoresis and not on the sequence of production of pectic enzymes. The regulation of two enzymes, PG1 and PG2, was found to be mediated both by the C sources, most likely through enzyme inhibition by pectic oligomers and/or feedback repression by D-galacturonic acid, and by the adenylate pool, probably through the metabolic energy status of the fungal cell (Leone *et al.*, 1990b).

Catabolite repression was not involved in the regulation of some pectic enzymes of *B. cinerea* (Leone and Van den Heuvel , 1987); the production of PG2, PG3 and PG4 was little affected by the presence of D-glucose. This characteristic may be important in the colonization of host tissues. The production of certain pectic enzymes will not be influenced by the increasing release of free sugars caused by the coordinated action of the whole array of cell wall-degrading enzymes of *B. cinerea*. Also PG1 did not undergo catabolite repression (Leone *et al.*, 1990b). Its synthesis could never be derepressed by addition of cAMP to a medium containing D-glucose as the only C source, as would be expected when an inducible enzyme is governed by catabolite repression (Cooper, 1983; Pall, 1981).

The points discussed above indicate that the significance of the sequential production of PGs by *B. cinerea* lies in the provision of nutrients from pectin-related polymers of plant cell walls. The presence of multiple isoforms of PGs is related to an advanced stage of infection when plant material is degraded and digested by the fungus. This might suggest that *B. cinerea* evolved from a saprophytic ancestor whose ecological task was the recycling of dead plant material.

Role of inorganic phosphate in PG production

The infection of healthy green leaves by germtubes of conidia of *B. cinerea* is usually dependent on the presence of external nutrients, probably because of the scarcity of available nutrients on leaves, compared with the surfaces of flowers or fruits, which are more commonly infected (Blakeman, 1980). Inorganic phosphate (KH_2PO_4) and purine nucleotide derivatives stimulate infection (Kō *et al.*, 1981). The phosphate-stimulated penetration of bean leaves by isolate BC 1 of *B. cinerea* reported by Van den Heuvel and Waterreus (1983) was correlated with a concomitant phosphate-stimulated increase in PG activity, particularly PG1 and PG2 (Van den Heuvel and Waterreus, 1985).

Twelve different isolates of B. cinerea were strongly dependent on KH_2PO_4 for PG production (Leone, 1990). The involvement of KH_2PO_4 and/or adenine nucleotides in the

regulation of PG1 and PG2 was studied in detail with isolate BC1 (Leone *et al.*, 1990b). Experiments were performed with cultures lacking cell wall-related substrates to establish whether this regulation acted independently of that for carbohydrates. Cyclic AMP, ADP and ATP stimulated the constitutive production of PG2 as well as fungal growth, whereas AMP induced production of PG1 but not growth. The effects of KH_2PO_4 on the two isoenzymes and on growth were intermediate between those of AMP and of the other nucleotides. The KH_2PO_4 or adenine nucleotide-dependent PG production involved *de novo* enzyme synthesis. Inhibition of the ATP production in the fungal oxidative phosphorylation resulted in a rapid detection of PG1 in the culture medium. Experiments affecting the intracellular concentrations of adenine nucleotides also gave indications that the KH_2PO_4-dependent PG1 and PG2 synthesis by BC1 was mediated by adenine nucleotides. It was therefore postulated that PG1 synthesis was controlled by a pectic inducer and a low metabolic energy status of the fungal cells, a situation likely to occur when germinating conidia initiate infection. Some factors and their interaction involved in the regulation of the synthesis and activity of PG1 and PG2 of isolate BC1 are illustrated in Fig. 1.

Although the importance of phosphate for PG biosynthesis has been established, the significance of a phosphate source for the fungus to become infective is still unclear. It might be possible that inorganic phosphate mimic somehow the effect of dead plant material, one of the primary substrates for *B. cinerea*, triggering the metabolic activities of the fungus necessary for plant tissue degradation and transformation.

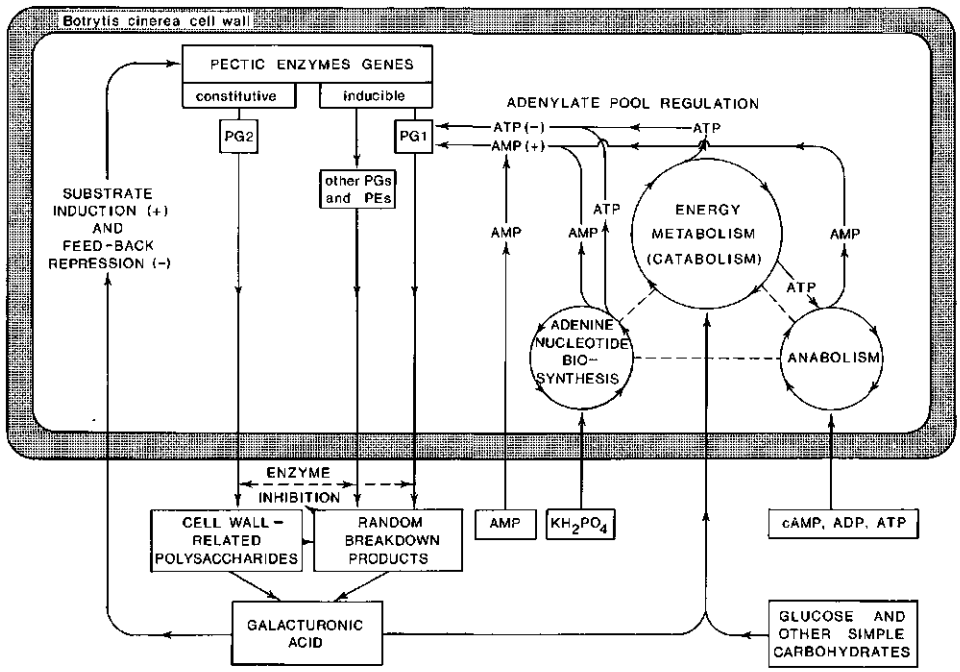

Fig. 1. Schematic representation of the regulation by carbohydrates and adenine nucleotides of the synthesis of PG1 and PG2 of isolate BC1 of Botrytis cinerea.

Origin of variability of PG production

B. cinerea is known for its phenotypic variability, possibly caused by heterokaryosis and/or diploidy (Grindle, 1979). Variability between isolates also occurs in PG isoenzymes (Magro *et al.*, 1980). The origin of this variation was studied with 12 isolates differing by source of host plant, year of isolation and geographical origin (Leone, 1990). The cultural conditions used to study regulation by KH_2PO_4 of PG production in isolate BC1, were used to compare the isozyme composition of these isolates. Electrophoresis showed that all isolates produced basic isoforms of PGs (pI above 8) *in vitro* and *in vivo* on bean and tomato leaves, which always included at least one PG 1- or one PG2-like isoenzyme.

Two sources of variability were distinguished: variation within, and between, isolates. The former is related to pectin catabolism by the fungus and is influenced by the time of sampling and cultural conditions *in vitro* and/or *in vivo* (Leone, 1990; Leone and Van den Heuvel, 1987).

Variability between isolates was small when cultural conditions known to prevent variability within single isolates were used. In previous studies it is probable that at least part of this variability could have been based on differences due to the metabolic control of the enzymes. Some of the causes of the remaining variation in PG production between isolates (Leone, 1990) may be found in the genetical instability for specific characters (e.g. the differences found in AMP involvement in PG1-like isoenzyme synthesis), to mutations and/or to modifications of proteins at translational or post-translational level (reflected, for instance, in the small differences in the relative electrophoretic mobilities of some PGs).

When conidia were suspended in a solution containing nutrients and used to inoculate bean and tomato leaves, all *B. cinerea* isolates were able to infect and showed no host specificity and no isoenzymatic host adaptation (Leone, 1990). These results, coupled to a lack of association between the rate of PG production and the level of pathogenicity, indicate that PG production should be regarded as only one of a complex of factors contributing to the expression of pathogenicity of *B. cinerea*.

PG2 and its properties

In the phosphate-stimulated infection of French bean leaves by *B. cinerea*, PG2 accumulated in inoculum droplets, with PG1, 6 to 12 hours after inoculation (Van den Heuvel and Waterreus, 1985). PG activity remained negligible unless penetration occurred. The same occurred during the infection of tomato leaves (Leone, 1990). Because of the probable importance of PG2 in infection, the enzyme was purified to study its biochemical properties (Leone *et al.*, 1990a).

PG2 is a basic (pI 9.1) endo-enzyme able to degrade pectin and sodium polygalacturonate. Its affinity for sodium polygalacturonate was three times higher than that for pectin. These results indicate that PG2 is able to break down the pectic portion of the plant cell wall. The enzyme was produced constitutively, it was present in ungerminated conidia and was first detected during the sequential pectic enzyme production *in vitro* and *in vivo* (Leone, 1990; Leone and Van den Heuvel, 1987). Therefore, PG2 seems to show a double role in the infection process; firstly by assisting penetration and secondly, in triggering the onset of the chain production of other isoenzymes for pectin catabolism (see also Fig. 1). The latter function could be mediated by the release of monomers and

oligomers from the pectic portion of the cell wall (Leone *et al.*, 1990a). The rapid softening of the primary cell wall by the endo-activity of PG2 will facilitate mechanical pressure exerted by the growing hyphal tip during penetration.

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Polygalacturonases of *Botrytis cinerea*

G. van der Cruyssen and O. Kamoen

Summary

Polygalacturonase (PG) enzymes produced by one homokaryotic strain of *B. cinerea* and expressed in the presence of different carbon sources were examined. The time course of the expression of the PG isozymes was studied and their zymogram patterns compared.

One isozyme was constitutively expressed but subjected to end-product repression; the other isozymes were more or less subjected to catabolite repression, end-product repression and/or induction. A classification into four 'expression groups' is proposed for the different detected isozymes.

Introduction

A comparison of the number of polygalacturonase (PG) isozymes secreted by the fungus *B. cinerea* revealed that between one and thirteen isozymes were produced by different strains. The complexity of the PG profiles could not be attributed entirely to heterokaryosis, as we observed similar high numbers of PGs in heterokaryotic and homokaryotic strains.

The main factor influencing the expression of the separate isozymes seems to be the growth medium. Our hypothesis was that groups of PG isozymes could be defined which were similarly regulated. The following experiments describe the effect of catabolite repression, substrate induction and end-product repression on the expression of single PG isozymes of *B. cinerea.*

Material and Methods

All experiments were done with cultures of the homokaryotic *Botryotinia fuckeliana,* (de Bary) Whetz. (*Botrytis cinerea* Pers.: Fr.) strain R16, supplied by dr. F. Faretra, Bari, Italy (strain hereafter called B.c. FR16).

Cultures and Samples

Experiment 1

Erlenmeyer flasks (100 ml) containing 50 ml Richards' medium were inoculated with 10^6, 10^5 and 10^4 conidia.ml^{-1}, and incubated on a rotary shaker (100 rpm) for 4 days at room temperature in darkness. The medium contained per 1000 ml Richards' medium either 20 g glucose, 20 g galacturonic acid, 20 g peptone, 5 g pectin or 5 g sodium polygalacturonate and the medium was adjusted to pH 5. The assays lasted 24, 48, 72 or 96 h.

Experiment 2

In a 'two step culture' experiment the fungus was pre-grown in a medium with a high concentration of glucose (20 $g.l^{-1}$) during 3 days, the mycelium then washed and grown on a fresh medium containing 20 $g.l^{-1}$ or 1 $g.l^{-1}$ glucose or 5 $g.l^{-1}$ pectin for another 2 days.

Three Erlenmeyer flasks of each medium were sampled every 24 h and the culture filtrates obtained and the mycelial dry weigh ts determined. The filtrates were dialysed for periods of 24 to 48 h and centrifuged (8000 rpm/10 min) to remove insoluble material. They were then frozen and lyophilysed, dissolved in 1 ml water, passed through a gel filtration column (PD-10 Pharmacia Biosystems), lyophilysed again and finally redissolved in 1 ml water.

IEF-PAGE and PG-activity staining

Iso-electric focusing (IEF) was performed on ampholine gels (broad-range pH 3.5 - 9.5; Pharmacia Biosystems) using the Multiphor II system (LKB Pharmacia). Applied amounts ranged between 0.004 and 0.2 units, one unit was defined as the activity which released 1 mm oligalacturonic $acid.min^{-1}$. The three samples from the same medium were used as distinct IEF samples in different amounts. The zymogram technique of Cruickshank and Wade (1980) was modified in some aspects. The IEF gels were rinsed several times with McIlvain buffer (pH 5.2) and incubated in the substrate solution (prepared by dissolving polygalacturonic acid in buffer, at pH 5.2) at 37°C for 15 min. Following thorough rinsing with tap water, the gels were immersed in 0.02% ruthenium red solution for 2 h or longer, and rinsed with tap water. The gels were dried under vacuum (Bio-Rad Gel Dryer), the relative distances and intensities of the different spots of PG activity were determined and compared to the patterns obtained in other gels.

Results

Examination of glucose as a catabolite repressor

Because the PG activities measured in the glucose-containing medium were relatively high, repression was not expected to reside in many PG isozymes.

In 4 day-old cultures with an excess of glucose only one PG (pI 9) was consistently present (Fig. 1, row 2) and this we called PG-A. In some cases of high loading of gels, one or two acid isozymes (pIc. 4.8) and one or two with basic pI values, could be detected in small amounts.

The control cultures grown on peptone showed marked differences by the first day as additional alkaline isozymes and two acidic isozymes (pI 4.9 and 4.7) were observed. The choice of peptone as a reference carbon source was therefore justified.

To assess whether catabolite repression by glucose occurs, the second medium of the 'two step medium', either supplemented with a high or low level of glucose was examined after 1 and 2 days growth in these media. No PG-A was detected. Two acidic isozymes pI 4.7 and 4.9 but no other isozymes were detected, but these were only present at the lower glucose concentration (Fig. 2, row 1 and 2).

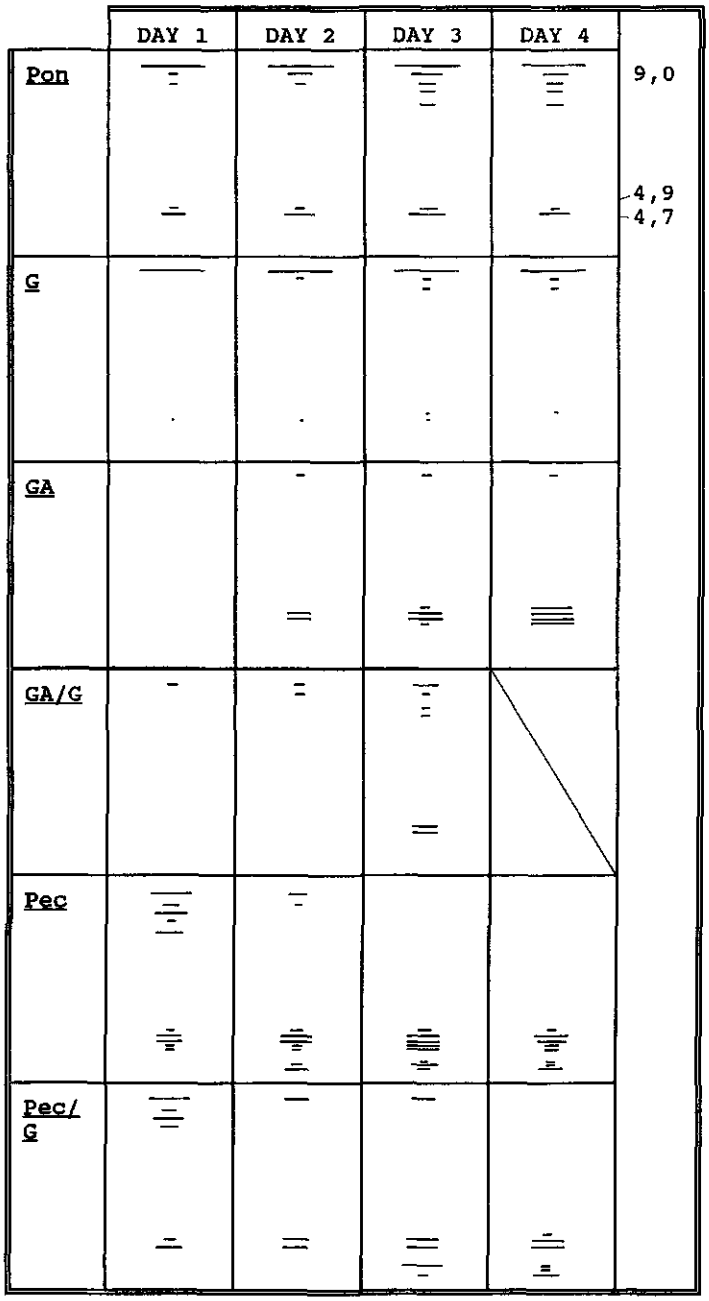

Fig. 1. Profiles of PG isozymes produced by B. cinerea daily in basal salt medium supplemented with different carbon sources at high concentrations. Dashes of different width correlate with the different intensities of activity. The relative distances along the length of each column are in proportion to the distances of the isozymes along the pH gradient.

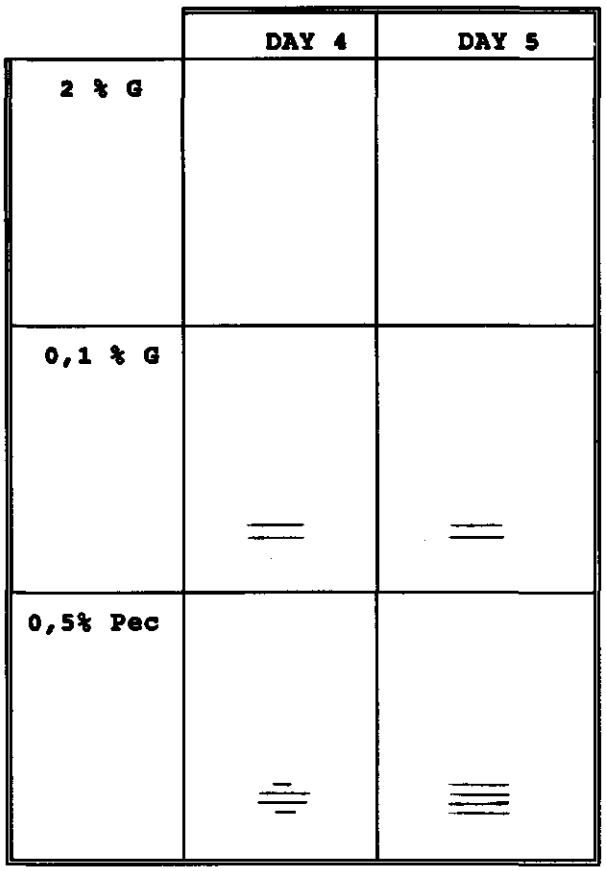

Fig. 2. Profiles of B. cinerea PG isozymes produced during 2 days in the second medium of two-step cultures.

Examination of pectin and polygalacturonate as inducers

By the first day, more isozymes, alkaline as well as acidic, were detected in cultures from pectin than in those on peptone (Fig. 1 compare lanes 1, 4 and 5). After day 2, the alkaline forms disappear and the number and concentration of the acidic forms increased.

When the fungus was grown on the combination pectin/glucose (Fig. 1, row 6), two acidic PGs seemed to be controlled by substrate induction, rather than by catabolite repression. The PG-A again disappeared, after a delay i.e. one day later than on pectin alone (Fi g. 1, rows 5 and 6). A possible explanation is the delayed uptake of the pectin degradation products in the presence of glucose. Similar results were obtained using sodium polygalacturonate as substrate.

Compared to the pattern obtained with glucose, here again the PG-A was not detected. More acidic enzymes, of higher activity , were detected in these cultures compared to the culture on a small content of glucose (Fig. 2, row 3). Here again the PG-A was notdetected.

Examination of galacturonic acid as end-product repressor

We hypothesized that the abolished expression of the alkaline PGs on the pectin after day 2 could be mediated by pectin degradation products. To check this possibility, we studied the PG isozymes produced on galacturonic acid. No PG-A was detected during the 4 days (Fig. 1, row 2) of the experiment. The acidic isozymes then appeared in the medium. The combined effect of galacturonic acid and glucose in the medium resembled that of galacturonic acid, except for the presence of alkaline isozymes in small quantities. Therefore the results obtained with pectin and galacturonic acid are similar.

Temporal regulation

A sequence in the PG expression can be defined by comparison of the profiles during the successive days of incubation (the four lanes of Fig. 1). One alkaline and two acidic isozymes were generally expressed earlier than the other isozymes. No PG-A was produced by mycelium in the second medium of the two-step culture system (Fig. 2).

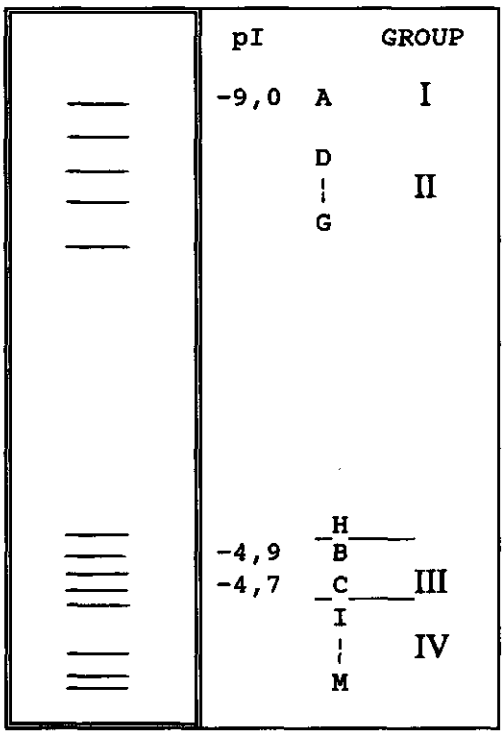

Fig. 3. Schematic representation of all PG isozymes detected in media containing a variety of carbon sources. Roman numerals indicate the group number, PG isozymes are indicated by letters (see also Table 1). The pI values of three important isozymes are shown.

Table 1. Proposed grouping and designation of the PG isozymes of B. cinerea according to the regulatory response to different carbon sources (G = glucose, GA = galacturonic acid, Pect = pectin).

Group	Designation	pI	Description
I	A	9,0	no catabolite repression (G) no induction end-product repression (GA,Pect) early expression
II	B C	4,9 4,7	catabolite repression (G) induction (GA,Pect) but induction not necessary early expression
III	D - G	8,9 to 8,5	catabolite repression (G) induction not so clear end-product repression (GA,Pect)
IV	H - M	4,9 to 3,5	catabolite repression (G) substrate induction necessary no end-product repression (GA)

Grouping and designation of the different isoenzymes

We concluded that the isozymes could be placed in one of four groups according to their regulatory responses on the different substrates and to their temporal expression. The groups are identified by Roman numerals (Fig. 3, Table 1). Within the groups, the PG isozymes are named by means of a letter analogous to the classification of pectin lyases used for *Aspergillus niger* (Harmsen *et al.* , 1990). In this way the three isozymes most often observed are called A, B and C. The characteristics of each group are summarised in Table 1.

Discussion

Based on the differences in the temporal expression and in the expression on the different carbon sources, four 'expression groups' could be distinguished.

A single isozyme called PG-A was detected generally first; and this was the only isozyme detected on media containing high glucose. It is probably analogous to the PG2 detected in *B. cinerea* strain BC-1 by Leone and Van den Heuvel (1987). We interpret this 'glucose effect' as catabolite repression of some of the PGs at the genetic level. Further evidence could be obtained by measu ring the amounts of mRNA derived from different PG-genes, as was done for the PL-A of *Aspergillus nidulans* by Dean and Timberlake (1989).

The absence of PG-A production by pre-grown mycelium transferred to fresh culture fluids (containing 20 g or 1 $g.l^{-1}$ glucose or 5 $g.l^{-1}$ pectin) cannot be readily explained.

Possibly this isozyme is related to the germination of conidia, and it may be released from the conidia and then it is no longer synthesised.

Pectin was shown to be an inducer for the isozymes of groups II, III and IV. This induction seemed even to be more effective than the repression by glucose. As pectin degradation progressed, isozymes in the groups I and III were no longer detected, while higher activities were detected for the isozymes of groups II and IV. We suggest that there is strong end-product repression of the basic isozymes; even PG-A was highly subjected to end-product repression.

The grouping we propose reflect the phenotypes. Perhaps it is unlikely that all of these forms constitute true isozymes encoded by different structural genes. A variety of post-transcriptional events and post-translational modifications can alter the isoelectric point of a protein. The question as to whether there could be a concerted regulation of some PGs (belonging to the same group) requires further research.

The production sequence for PG isozymes by *B. cinerea* during infection of a plant may be as follows: PG-A which is immediately present at high activity *in vitro* could be the first 'acting element' *in vivo*. Depolymerisation of pectin in cell walls would then induce other PGs. After some time, the basic isozymes would be repressed and the acid ones take over as 'acting elements' during lesion expansion.

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The function of laccase from *Botrytis cinerea* in host infection

A. Viterbo, N. Bar Nun and A.M. Mayer

Summary

Invasion of plant tissue by a pathogenic fungus involves the secretion of 'attack' enzymes which allow the fungus to penetrate its host. The host responds with various defence mechanisms, which include the formation of phenolics and lignification. Pathogenic fungi , such as *B. cinerea* have developed mechanisms to overcome or by-pass such defences, one of which may be the secretion of oxidative enzymes, such as laccases. Some fungal laccases, including that of *B. cinerea* have been characterised in great detail. Appearance of laccase is rigourously controlled by the level of specific phenolics, by pectins or their degradation products and by copper. We have shown that factors which prevent or repress laccase formation can also afford protection of a host against attack by *B. cinerea*. The appearance of laccase activity, but not that of other enzymes, is prevented by cucurbitacin. The prevention by cucurbitacins of laccase formation by *B. cinerea*, without affecting fungal growth of other fungal attack enzymes should therefore afford protection of the host to *B. cinerea*. Cucurbitacins which are able to repress appearance of laccase can also prevent invasion of the host by the pathogen, probably because lignin formation can occur. We would like to suggest that laccase should be considered as part of the complex of 'attack' enzymes formed by *B. cinerea*.

Introduction

Invasion of plant tissue by fungal hyphae is accompanied by secretion of a number of enzymes by the fungus which permit it to penetrate its host and to overcome some of the host plant defence mechanisms (Hall and Wood, 1973; Dickman *et al.*, 1982; Marcus and Schejter, 1983; Van Etten and Kistler, 1984). Fungal attack is often very specific, e.g. attacking some cultivars but not others (Bell, 1981). The host responds by the formation of various compounds which defends it against the invader, including toxic compounds, enzyme inhibitors, phytoalexins (Ouchi, 1983; Darvil and Albersheim, 1984; Horsfall and Cowling, 1984; Mayer, 1989) or by the formation of defensive barriers, such as lignin and cutin (Horsfall and Cowling, 1984).

Some of the plant defense mechanisms are based on the formation of specific chemical substances such as phenolics and tannins (Weinhold and Hancock, 1980; Horsfall and Cowling, 1980). Many other plant secondary metabolites, with marked biological activity are present in plant tissues (Cutler, 1988; Mayer, 1989), and these may find uses in agriculture as protectants against pathogens. Secondary metabolites have generally been regarded as being defensive agents against herbivores (insects, predators), but little attention has been paid to the function of such compounds against fungi.

Fungi are metabolically very versatile and show a considerable ability to detoxify or to overcome plant defense mechanisms, e.g. by inactivating phytoalexins (Smith *et al.*,

1982; Van Etten, 1982). One of the ways in which fungi inactivate plant defens es is by oxidizing compounds which have a protective role using various enzymes (Smith et al., 1982) one of which is laccase. Laccase belongs to a group of enzymes generally referred to as the polyphenol oxidases present both in higher plants and in fungi.

Laccases are enzymes which catalyse the oxidation of ortho- or paradihydroxyphenols to the corresponding quinones in a reaction using molecular oxygen. The oxidation is a two step process, involving apparently free radical formation. A number of properties of fungal laccases are given in Table 1.

Glycosylation seems to be important for enzyme activity, as even partial deglycosylation reduced activity and caused loss of copper, at least in *Rhus vernicifera* (Graziani *et al.*, 1990) and the same seems to be true for *B. cinerea.*

Table 1 Some properties of fungal laccases

Composition	glycoproteins
Molecular weight	38000 - 350000 Da
Number of subunits	variable two to four, apparently equal
pH optimum	3.5-7.5, usually with a fairly sharp peak
Copper content	1 atom Type I Blue copper (ESR signal) 1 atom Type II Copper (not blue, ESR signal) 2 atoms Type III copper (ESR silent)
Substrates	monophenols, o-diphenols, p-diphenols, syringaldazine, related compounds, lignin
Latency	not reported
Presence	usually low level of constitutive enzyme enzyme induction by various compounds
Induction	often highly specific, and inducers differ in different species or genera of plants
Location	cytoplasmic and perhaps in or on the cell wall

Table 2 Suggested functions for fungal laccases

- Involved in spore formation, possibly increasing resistance of spores, e.g. against bacterial or other attack.
- Degradation, solubilisation and depolymerisation of lignin.
- Oxidative polymerisation of lignins.
- Oxidation of various substrates, possibly for utilisation by the fungus.
- Oxidation of phytoalexins.
- Detoxification of plant defence compounds.
- Prevention of lignification, by oxidation of phenolics required for the process.
- Part of the attack system of pathogenic function (our hypothesis).

The proposed functions for laccase in different fungi are shown in Table 2.

Except for the assumed function of laccase in the degradation of lignin (Higuchi, 1989) other functions of this widespread fungal enzyme remain undetermined or unproven. We wish to present evidence for our hypothesis of a laccase function.

Some fungal laccases have been well characterised and their genes cloned (Yelton *et al.*, 1985; German *et al.*, 1988; O'Hara and Timberlake, 1989), but homology between the genes is poor and enormous differences appear to exist between laccase proteins from different organisms. The enzyme may have different functions in different fungi.

B. cinerea is a widespread plant pathogen, capable of attacking most plants and almost all plant tissues (Coley-Smith *et al.*, 1980) and causes 'grey rot' in grapes which impairs wine quality (Dubernet *et al.*, 1977) although 'noble rot' results in the formation of high quality wines. This lead us to a detailed study of the fungal laccase and its characterisation (Gigi *et al.*, 1980, 1981; Marbach *et al.*, 1983, 1984, 1985).

Results and Discussion

We have purified laccase of *B. cinerea* using precipitation, gel filtration and native acrylamide gel electrophoresis followed by elution, to give a homogeneous preparation (Viterbo and Mayer, unpublished). Some of the properties of these laccases are shown in Table 3.

Laccase formation in *B. cinerea* is under very tight control and appears to be regulated by compounds originating in the host tissue such as certain phenolic compounds and by pectins as well as by the level of copper ions, by Ca^{2+} and by the addition of EDTA to the growth medium. The effect of pectins is a highly specific one. Low molecular weight derivatives of pectins (obtained from Professor C.W. Nagel, Washington State University) were tested for their ability to induce laccase formation in the presence of gallic acid. Dimers or tetramers derived from pectin as well as dimers, trimers or tetramers of galacturonic acid suppressed fungal growth and there was no laccase formation. A methylated pectin trimer was a good inducer of laccase and permitted fungal growth, while the corresponding unsaturated trimer suppressed growth.

Table 3. Properties of laccases produced by B. cinerea, in the presence of gallic acid or grape juice as inducers.

	Gallic acid	Grape juice
pH optimum	3.5	4-4.5
Isoelectric point	about pH 2.0	various forms
Relative activity towards substrates:		
quinol	100	100
p-coumaric acid	50	4
p-cresol	87	12
Sugar % of total in glycoprotein:		
Mannose	39	55
Glucose	24	13
Arabinose	7.4	2.7
Amino acid composition:		
% Basic	6.0	7.7
% Acidic	20.4	16
Molecular weight	37-39kDa & 67kDa	– [1]
Bands on SDS Page	2	2
Subcellular location	cytoplasmic	– [1]

[1]) not determined

Laccase formation and secretion are entirely distinct from the growth and development of the fungal mycelium. EDTA can repress laccase formation and this effect is related to copper availability, since it can be reversed by copper ions. Although copper is required for enzyme formation, this requirement is small. Addition of 1 mM copper as the chloride raised the copper content of the mycelium fiftyfold, yet enzyme activity only doubled. An indication of the importance of laccase in the infection process was detected, by the use of EDTA. Pretreatment of cucumber fruit with 12 mM EDTA, a concentration which still permitted fungal growth, prevented infection of such fruits by mycelium of *B. cinerea*.

It is possible to extract fractions from cucumber fruit which inhibit the formation of laccase under inducing conditions (Bar Nun *et al.*, 1988). Similar compounds were isolated from a common weed *Ecballium* and identified as cucurbitacins (Bar Nun and Mayer, 1989a,b, 1990). We showed that purified cucurbitacins (tetracyclic triterpenoids-lanosterol derivatives) can prevent laccase formation by *B. cinerea* under conditions favouring enzyme formation (Table 4).

Table 4: Effect of different cucurbitacins I, D, B and E, and partially purified extracts (PPE) on gallic acid and pectin-induced extracellular laccase activity

	Length of culture period			
	7 days		14 days	
	Activity	% Inhibition	Activity	% Inhibition
Control	1.96	–	1.09	–
EtOH	1.41	–	0.98	–
I	0.54	62	0.27	72
D	0.54	62	0.31	68
B	0.38	73	0.21	78.5
E	0.24	82.7	0.1	78.5
PPE	0.38	73	0.27	72

Total activity ($\mu l\ O_2.ml^{-1}.min^{-1}$), was measured using an oxygen electrode. Partially purified extract or 3 mg of purified cucurbitacins dissolved in 0.2 ml EtOH, were added to 30 ml culture medium at the onset of the growth period.

The cucurbitacins had no effect on the development of the fungal mycelium, and the same final fresh and dry weight of mycelium were obtained.

Laccase formation in *B. cinerea* shows a very strict dependence on culture conditions. In experiments on the mass culture of the fungus on orange peels as the source of carbon, we have shown that control of laccase and of polygalacturonase are distinct (Karmona *et al.*, 1991). Formation of polygalacturonase, pectin methylesterase, cellulase, aspartic proteinase and carboxy peptidase were not in any way affected by cucurbitacins.

Our hypothesis suggests that the ability of *B. cinerea* to secrete an extracellular laccase is an essential part of its infective mechanism, although insufficient alone to cause infection. We postulate that in addition to the secretion of a variety of lytic enzymes which are required to penetrate into the host tissue and to lyse, decompose and macerate it, *B. cinerea* also secretes laccase. The function of this laccase is to inactivate the host defence mechanisms by oxidation of some of the compounds present in the host as protectants. This is supported by the work on *Cryphonectria* (Rigling, 1989; Rigling

et al., 1989), which showed that its hypervirulence correlates with reduced laccase activity.

We have tested this hypothesis indirectly. Factors preventing laccase formation by the fungus e.g. EDTA, also protected the host against infection by *B. cinerea* (Bar Nun *et al.*, 1988; Bar Nun and Mayer 1989a, 1990). The applications of cucurbitacin I or plant extracts enriched in cucurbitacins, to cucumber fruit, leaves of carrots, prior to their inoculation by *B. cinerea* afforded total protection against the invading fungus.

Under the test conditions used, the host tissue was able to form a lignified layer, which seems to prevent fungal penetration. In the absence of the cucurbitacins, lignification was apparently delayed and the fungal penetration, accompanied by laccase secretion, occurred before formation of a protective barrier. When protection was afforded by cucurbitacins, which are pharmacologically active (Johnson *et al.*, 1989; Lavie and Glotter, 1971; Nes and Patterson, 1981; Rhem *et al.*, 1977), no laccase secretion by the fungus could be detected (Table 5). A previously unsuspected role of cucurbitacins in plant tissue may be that of antifungal agents, which assist the host plant to ward off the invading fungus. Apparently no case of specific inhibition of the biosynthesis of a single recognised defined enzyme involved in pathogenicity has yet been reported.

Table 5. Infection of cucumber fruit with B. cinerea, laccase formation and the effect of cucurbitacin I.

Treatment	Presence of lignin and laccase after						
	24 hr		48 hr		96 hr		
	Lignin	Fungus	Lignin	Fungus	Lignin	Fungus	Laccase
Control	±	–	+	–	+	–	–
Infected	++	+	++	+	++	+	+
Cucurb.	–	–	±	–	±	–	–
Infected + Cucurb.	±	–	+	only on surface	+	only on surface	–

Cucumbers were pretreated with EtOH or with EtOH + cucurbitacin (cucurb.) for 24 h and then inoculated. At the end of each period tissue was removed, stained for presence of lignin and mycelium and tested for laccase activity. ± = very little lignification; + lignification of a cell layer of at least 2 cells; ++ marked lignification, several layers thick. Fungus: mycelium present in the cucumber tissue: + present; – absent.

Based upon our results, it is hypothesised that by inhibiting the formation of an enzyme or enzymes, produced and secreted by the fungus, it is possible to protect plants against the pathogen. Laccase might constitute such an enzyme in the case of *B. cinerea*, although it is clear that the attack of *B. cinerea* on a host involves a number of enzymes, of which laccase is only one.

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Relationship between grey mould of grapes and laccase activity in the must

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Summary

In 1991, a correlation existed between the laccase activity in the must and the percentage of berries, infected by *B. cinerea*. This correlation is highly significant during the last 10 days before harvest.

Introduction

Grey mould of grapevine is a serious disease not only because it affects the quantity of the harvest but also, and even more important, the quality of the wine produced from grapes with infection by *Botrytis cinerea* Pers.: Fr.. It is known, that in must of infected grapes, laccase activity is high and the activity of this enzyme detiorates wine quality. Visual observation of grey mould in the vineyard does not allow evaluation of the potential damage to the quality of the wine. Therefore we determined the relationship between the development of grey mould and the concentration of laccase present in musts. Development of grey mould was followed by visual observation from véraison to harvest on bunches of grapes treated with fungicides to control *B. cinerea* and on non-treated ones.

The activity of laccase was determined for each bunch at harvest. Laccase activity was measured spectrophotometrically, with syringaldazine as substrate (Harkin and Obst, 1973a,b; Harkin *et al.*, 1974). The results concerning the development of grey mould and the activity of laccase measured were analysed statistically.

Table 1. Correlation between infection of grapes by B. cinerea and laccase activity in the must at three different sampling dates.

Sampling date	% Infection activity	Laccase
September 10	9	0.216
17	36	0.461
20	57	0.522

Significance threshold of correlation coefficient (Fisher) at 1% for 300 bunches observed: 0.148.

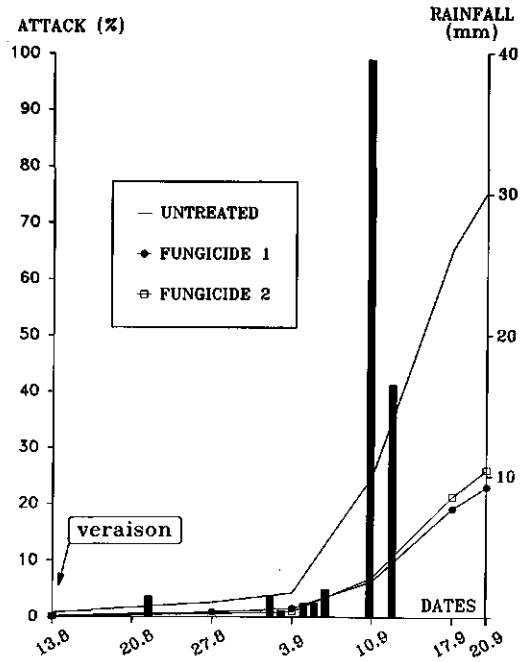

Fig. 1. Development of grey mould from véraison till harvest

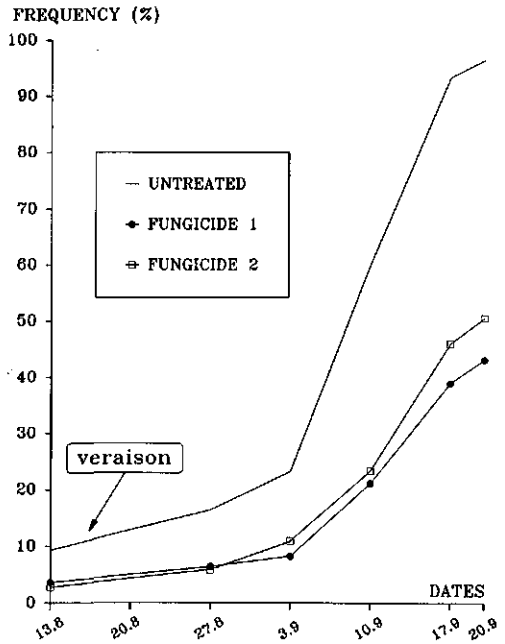

Fig. 2. Development of the number of bunches infected by grey mould

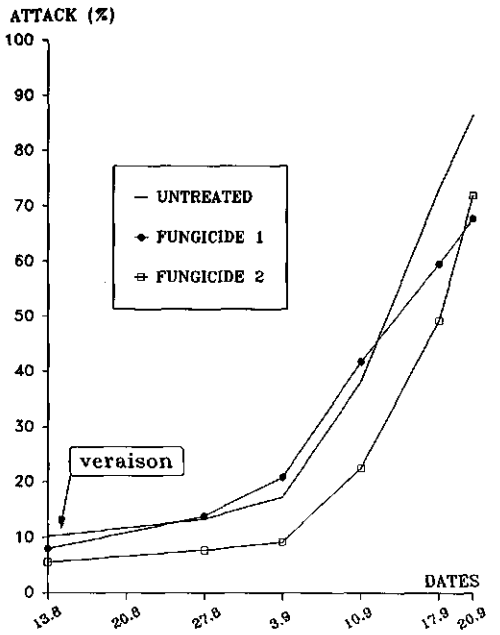

Fig. 3. Development of grey mould in bunches already showing symptoms on August 13.

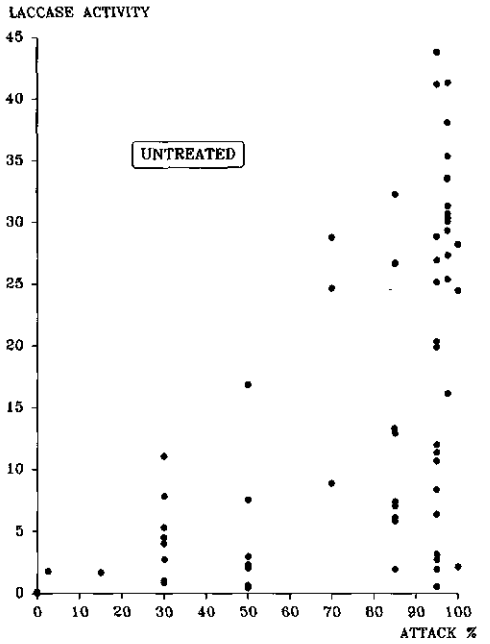

Fig. 4. Estimation of damage by grey mould. Comparison of visual observations and determination of laccase activity.

Results and Discussion

The year 1991 was characterised by an explosive development of the disease during the three weeks preceding harvest. There were two distinct phases in the development of grey mould, which was correlated to rainfaill and maturity of the berries (Fig. 1). Fungicide treatments reduced the number of bunches affected and delayed the appearance of the effects of grey mould (Fig. 2). On the other hand, once established on the bunch, *B. cinerea* developed at the same rate on treated and non-treated bunches (Fig. 3).

A significant correlation was observed between the level of grey mould at harvest and the activity of laccasse in the must (Fig. 4).

A good correlation between rot, caused by *B. cinerea* and laccase activity in the must was obtained. However, the findings of 1991 were different from those of previous years. So more experiments, to be carried out under different climatic conditions have to be done.

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Enzymatic detoxication of stilbenes by *Botrytis cinerea* and inhibition by grape berries proanthocyanidins

R. Pezet, V. Pont and K. Hoang-Van

Summary

On a pectin-containing medium, *B. cinerea* produces a hydroxystilbene-degrading enzyme, stilbene oxidase, identified as a laccase. Stilbene oxidase oxidizes both proanthocyanidins and resveratrol. This oxidation detoxifies the hydroxystilbenes. Stilbene oxidase was partially purified on Sephadex G-100, DEAE-Sepharose FF and on Syringyl EAJ-Sepharose4B. It binds on Con A Sepharose 4B indicating a glycoprotein nature. On IEF-PAGE (pH 3-10) the pI values were found to be between 3.5 and 4.7 for the different isoenzymes. The relative molecular weights (SDS-PAGE, 10%) of the three major bands of stilbene oxidase detected after IEF-PAGE purification were 97.6, 86,2 and 65.4 kDa. Proanthocyanidins from skin of grape berries inhibit competitively the activity of stilbene oxidase. The level of this inhibition seems to depend on the constitution of the tannins.

Introduction

Pterostilbene (trans 3,5-dimethoxy-4^1-hydroxystilbene) and resveratrol (trans 3,5,4^1-trihydroxystilbene) are constitutive components of the woody parts of many species of the *Vitaceae* (Hart, 1981; Pool *et al.*, 1981). However, in the leaves and fruits these compounds are only produced after exposure to UV-radiation or after fungal infection and so they could act as phytoalexins. If phytoalexins are important factors in the resistance of a plant, the ability of a pathogen to detoxify these compounds could be an important feature in the success of a fungal pathogen (Van Etten *et al.*, 1989). On the other hand, proanthocyanidins of grape berries are potent inhibitors of stilbene oxidase produced by *B. cinerea*. These tannins could contribute to the resistance of grapes to infection by *B. cinerea* by inhibiting stilbene oxidase, and preventing detoxification of the phytoalexins as suggested by Nyerges *et al.* (1975).

Results and Discussion

Botrytis cinerea Pers.: Fr. isolate P-69, used in the experiments was obtained from diseased grape berries (cv. Pinot). The methods used in this work were described previously (Pezet *et al.*, 1991).

Proteins precipitated from liquid culture filtrate, as well as the IEF-PAGE purified stilbene oxidase possess the ability to modify the UV spectra of hydroxystilbenes (Fig. 1). This modification is linked to to detoxification of pterostilbene (Table 1). The enzymatic transformation of resveratrol was demonstrated but not its detoxification, because it is not toxic in the concentration used in this work.

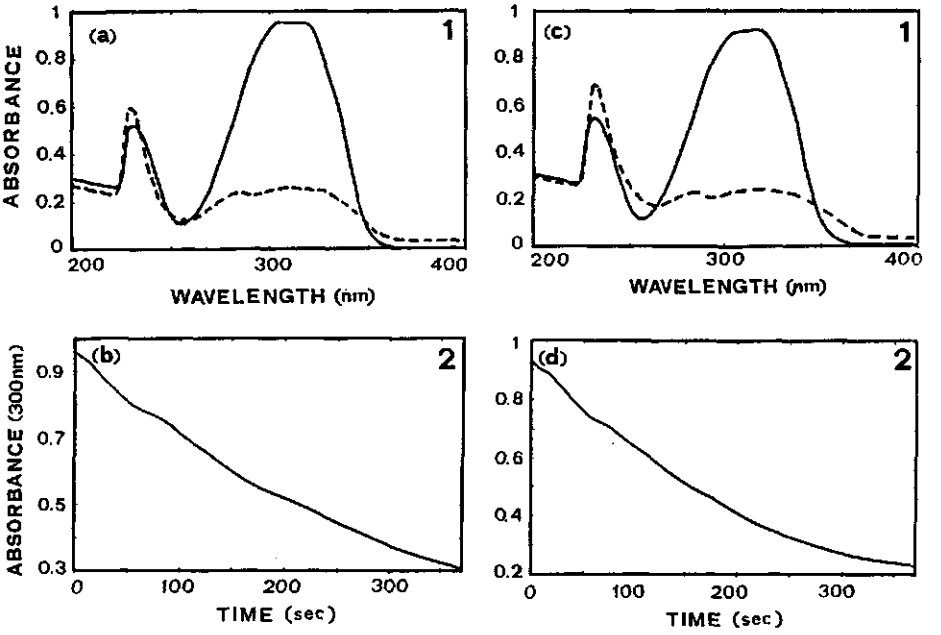

Figure 1. UV spectra of the degradation products of resveratrol and pterostilbene produced by the stilbene oxidase activity in culture filtrate of B. cinerea. The basic reaction mixture consisted of 30 µl of ethanolic solution of stilbene (1mg.ml^{-1}); (a) and (b) pterostilbene; (c) and (d) resveratrol in 3 ml citrate-phosphate buffer (0.1 M, pH 5.2) (a) and (c) spectra without enzyme (----) as after the addition of stilbene oxidase (—) (b) and (d). Kinetic studies were done at an absorbance of 300 nm during enzymatic reaction.

Stilbene oxidase in culture filtrate of *B. cinerea* depends on the age of the culture (Table 2).

The crude enzyme solution was partially purified through several steps described in Table 3.

The purified active fraction obtained from the laccase affinity gel Syringyl-EAH Sepharose 4B, was applied on a IEF-PAGE (pH 3-10). Proteins degrading hydroxy-stilbenes and having laccase activity were stained by incubating with p-phenyl-enediamine. Six important bands were visualized between pH 3.5 to 4.7. The positions

Table 1. Detoxification of pterostilbene by a crude protein extract of the culture filtrate of B. cinerea

Stilbene	Inhibition of germination[a)] (%)	Dead conidia (%)
pterostilbene[b)]	100	100
pterostilbene[b)] + CE[c)]	0	0

[a)] Percentage inhibition was determined from observations on 300 conidia
[b)] Concentration 5 x 100^{-4}M
[c)] CE, crude extract of proteins secreted by *B. cinerea* in a liquid culture medium

Table 2. Stilbene oxidase in culture filtrates of B. cinerea as a function of the age of the culture

Age of the culture (days)	stilbene oxidase (units[a])	activity Protein ($\mu g\ ml^{-1}$)
1	0.43	n.d.
2	0.64	9.85
3	0.68	11.18
4	0.80	19.80
5	1.83	25.33
6	3.27	25.90
7	7.69	144.48
8	27.76	1175.12

[a]) determined with 50 ml of a crude enzyme solution (5 ml).

Table 3. Partial purification of stilbene oxidase produced by B. cinerea

Steps	Total activity[a)]	Proteins[b)]	Specific activity[c)]	Factor
Culture filtrate	4421.06	15.66	352.12	1.00
$(NH_4)_2SO_4$ fraction	7804.94	323.20	689.77	1.95
Conc. to 5 ml	8235.00	1175.12	1401.70	3.98
DEAE-Sepharose	16552.00	378.80	5591.89	15.88
Syringyl EAH	28550.00	49.92	11653.06	33.09

[a]) units min^{-1} (= change in absorbance per min (x -100) to convert to a positive number)
[b]) $\mu g\ ml^{-1}$
[c]) units $min^{-1}mg^{-1}$ proteins

of the proteins were located on an unstained IEF gel, the bands (1 to 6) were then removed and suspended in 3 ml of citrate-phosphate buffer (0.1 M, pH 5.2). The activity of the proteins was determined using pterostilbene or syringaldazine as substrate (Fig. 2). The major bands 4 to 6 were also transferred to an SDS-PAGE (10%) in order to determine the molecular weight. Fraction 4 showed an important band situated at 86.2 kDa, fraction 5 at 64.5 kDa and fraction 6 at 97.6 kDa.

Hydroxystilbenes were oxidized into several unidentified products. One of them, separated by HPLC from oxidized pterostilbene and analyzed by mass spectroscopy has a molecular weight of 512 Da compatible with a dimeric structure of this stilbene at an oxidation stage.

Stilbene oxidase activity can be inhibited by several known laccase inhibitors, such as sodium diethyldithiocarbamate and sodium azide. Recently we have shown that proanthocyanidins (tannins) extracted and purified from the skin of grape berries, using the method of Porter (1989), are strong inhibitors of stilbene oxidase and laccase (Fig. 3). The Lineweaver-Burke plot with pterostilbene as substrate indicates a competitive type of inhibition (Fig. 4). The Michealis-Menten constant (K_m) of *B. cinerea* of stilbene oxidase for pterostilbene was calculated as 66.6, 80 and 111.1 (μmoles pterostilbene ml^{-1}) in the presence of 0, 5 and 15 mg ml^{-1} inhibitor proanthocyanidin, respectively.

The importance of phytoalexins in the resistance of a plant to pathogenic fungi can be indicated by the ability of a pathogen to detoxify these compounds as it invades the host.

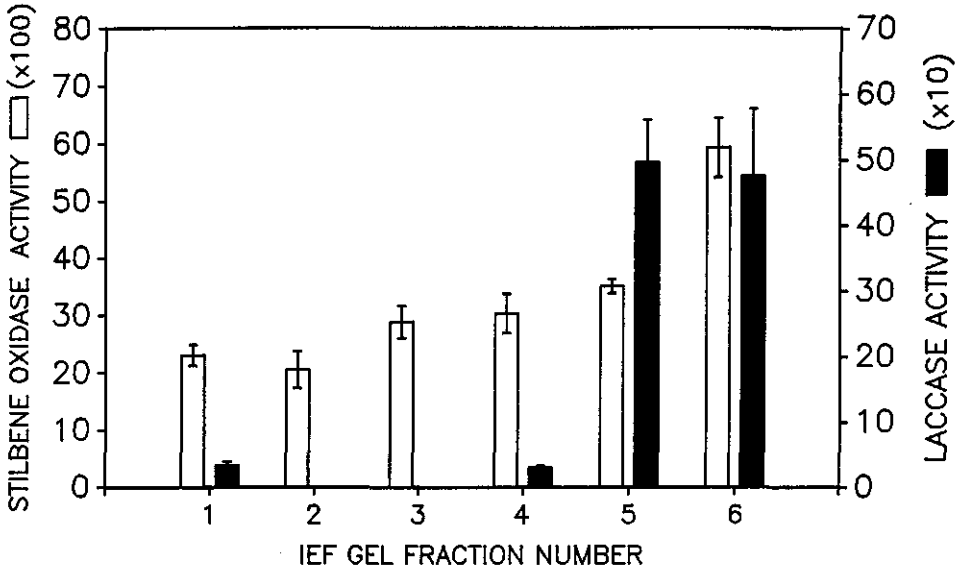

Figure 2. Evidence of simultaneous oxidase and laccase activity of protein bands separated by IEF. Bands corresponding to stilbene oxidase isoenzymes (IEF gel fraction number 1 to 6) were removed and suspended in buffer. Stilbene oxidase activity was measured with pterostilbene (□) and laccase activity with syringaldazine (■) as substrate.

B. cinerea is known to metabolize and thus detoxify phytoalexins from a number of plants (Van Etten *et al.*, 1989). A pathogen like *B. cinerea* might be expected to regulate the secretion of its enzymes in response to the nature of the host resistance mechanisms. *B. cinerea* can adjust the molecular structure of its extracellular laccase to the pH of the host and the nature of phenolics present in this host (Marbach *et al.*, 1985). Such effects could explain the heterogenicity of polyphenol oxidase purified from culture filtrates of *B. cinerea*. The pI value, molecular weight and affinity of polyphenol oxidase for laccases produced by *B. cinerea* on various substrates given in the literature differ considerably according to the methods used to cultivate the fungus (Marbach *et al.*, 1984). If laccase is an integral part of the infection process of *B. cinerea* in some hosts, it does not appear to be the case for infection of unripe berries of *Vitis* spp. The poor development of *B. cinerea* in unripe grape berries indicates the natural resistance of those organs to the fungus, and probably the inactivation of exoenzymes of the parasite. Proanthocyanidins are important constituents of grape berries. They complex strongly with carbohydrates and proteins (Porter, 1989), and thus inhibit enzymes. The competitive inhibition of proanthocyanidins with stilbene oxidase (laccase) and pterostilbene as substrate shows that tannins extracted from grape berries do not complex strongly with oxidase but have an affinity for the enzyme. The inability of proanthocyanidins to inhibit polygalacturonase of *B. cinerea* (results not shown) confirms that the inhibitory activity of these tannins is specific to some enzymes and is not the result of a protein denaturation.

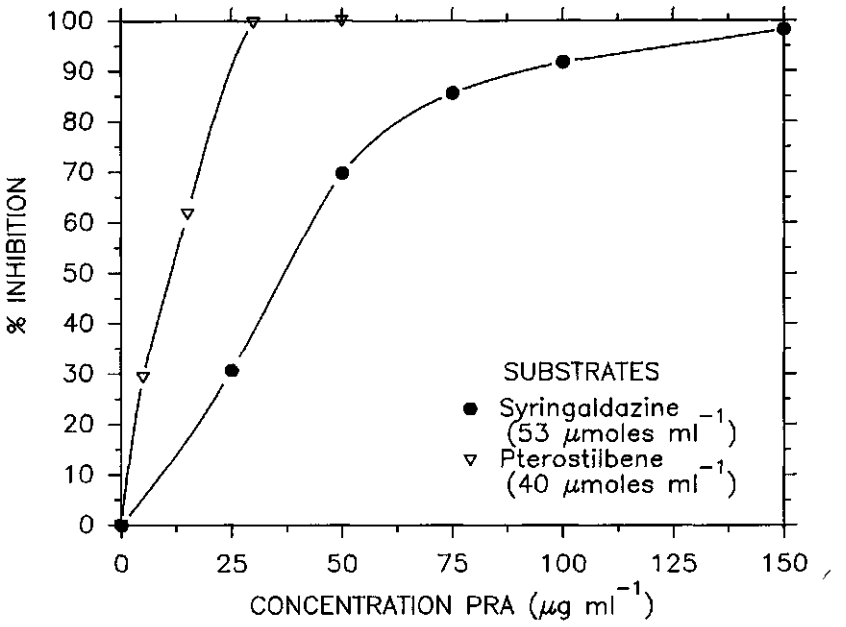

Figure 3. Inhibition of stilbene oxidase activity by various concentrations of proanthocyanidins (PRA) extracted from the skin of grape berries.

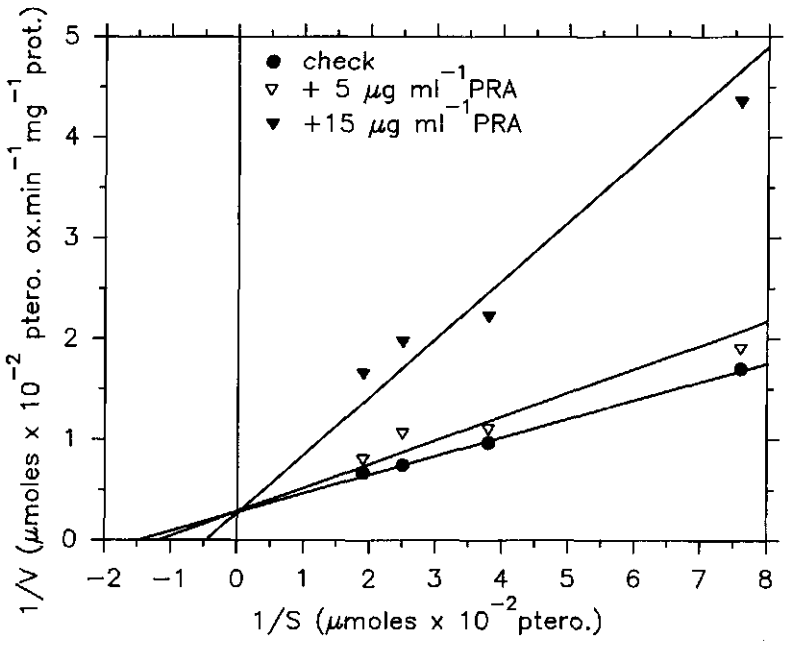

Figure 4. Lineweaver-Burke plot of the enzyme kinetics of stilbene oxidase in the presence of 0.0, 5 and 15 μg ml⁻¹ of PRA. K_m values were calculated from this plot.

Proanthocyanidins, present in the skin of grape berries are mainly cyanidin and delphinidin. The proportion of each differs according to the developmental stage of the berries from which the tannins were extracted. The inhibitory activity of these proantho cyanidins extracted from mature berries to stilbene oxidase is lower that that from unripe berries. Therefore, proanthocyanidins could play an important role in the processes involved in resistance of grape berries to the development of *B. cinerea.*

Acknowledgements

We thank Mrs. I. de Groote for her essential technical assistance.

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Differing biochemical and histological studies of two grape cultivars in the view of their respective susceptibility and resistance to *Botrytis cinerea*

R. Pezet and V. Pont

Summary

Studies of resistance of grapevines to *B. cinerea* should consider all known parameters involved in the process, rather than focus on one aspect. With this perspective we studied two contrasting Swiss grapevine cultivars, cv. Gamay which is susceptible and cv. Gamaret resistant to *B. cinerea*. Our analyses showed histological and biochemical differences between berries of these two cultivars. Their respective ability to synthesise phytoalexins, the relative concentrations of glycolic acid, phenolic compounds and proanthocyanidins and the thickness and structure of the skin are some of the parameters which provide an approach to the understanding of the causes of resistance of grape to *B. cinerea*.

Introduction

In recent years the mechanisms of resistance of grape berries to *Botrytis cinerea* Pers.: Fr. have been studied intensively . Jeandet and Bessis (1989) reviewed this subject and concluded that numerous mechanisms were probably involved in active and passive defence of grapes. The synthesis of phytoalexins (Langcake and Price, 1977), the inhibition of exoenzymes of *B. cinerea* by proanthocyanidins (Bachmann and Blaich, 1977; Pezet and Pont, 1992) and by unidentified glycoproteins (Grassin, 1987) or the toxicity of glycolic acid (Pezet and Pont, 1988), anthocyans and other penolic compounds (Nyerges *et al.*, 1975) towards *B. cinerea* are some of the active defence systems involved. Passive defences could also be effective through the synthesis of lignified barriers produced from oxidized phenols (Mansfield, 1980), the structure of the epidermis (Bernard, 1976) and thickness and composition of the cuticle (Radler, 1968). This paper compares two grape vine cultivars, cv. Gamay which is susceptible and cv. Gamaret which is resistant to *B. cinerea* in the light of some of these parameters.

Results and Discussion

Synthesis of resveratrol

After UV radiation (256 nm), grape berries were peeled and the fresh weight of the skins determined. They were then extracted according to the method of Southerton and Deverall (1990) for free phenolic acids. Of the final methanol extracts, 30 µl was analyzed by HPLC in the system described by the same authors, except that the solvent system was a gradient of methanol in formic acid (50 mM) (4 5% MeOH to 80% MeOH in 25 min). In this system resveratrol had a retention time of 5.65 min. Resveratrol

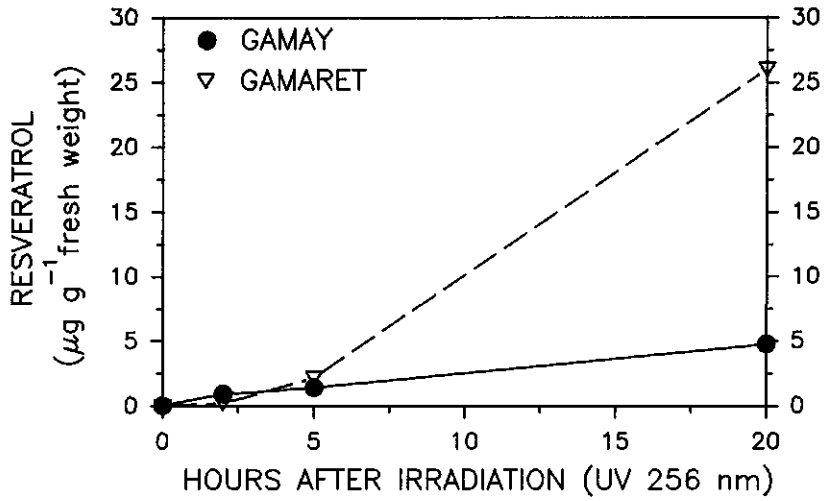

Fig. 1. Synthesis of resveratrol after UV irradiation (256 nm) by the berry skin of cv. Gamay (susceptible) and cv. Gamaret (resistant). The skin was separated from the pulp at different times after UV irradiation and resveratrol was extracted immediately.

content of berries was determined from 0 to 20 h after irradiation. It appeared that cv. Gamaret synthesised more resveratrol than cv. Gamay after 20 h (Fig. 1).

Natural amounts of resveratrol in berry skins at véraison and at harvest time are determined by the same method. The grapevine cv. Gamaret contained more resveratrol than cv. Gamay (Table 1).

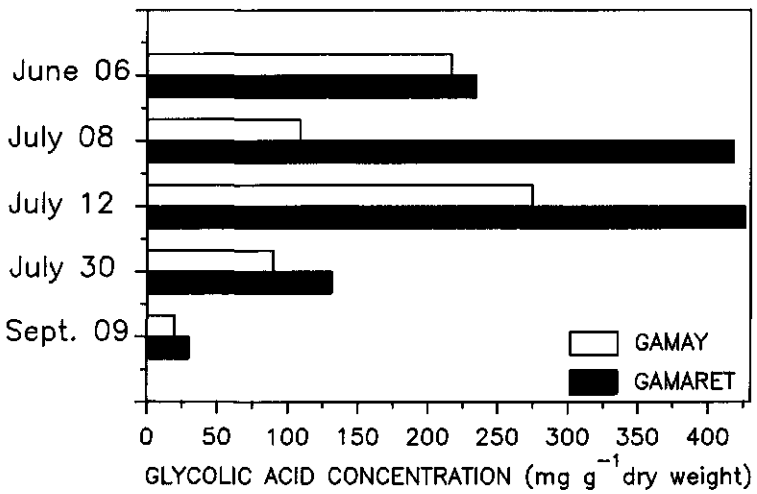

Fig. 2. Glycolic acid concentration in berries of cv. Gamay and cv. Gamaret at five developmental stages. Measurements were made on extract of whole berries.

Table 1. Content of resveratrol in the skin of grape cvs Gamay and Gamaret at véraison, and at the end of the season.

	Resveratrol ($\mu g.g^{-1}$ dry weight)	
	Gamay	Gamaret
Véraison	1.72	3.53
Harvest	3.24	16.66

Concentration of glycolic acid

Organic acids were extracted with water from freeze-dried and powdered grape berries (500 mg powder in 50 ml water); 5 ml of this extract was applied to a polyvinyl polypyrolidone (PVPP) column (1 x 6 cm), while 10 µl of the eluate was analysed by HPLC isocratically (H_3PO_4, 0.1%) on a Supelcogel C-610H column (300 x 7.8 mm, detection at 210 nm). The retention time of glycolic acid was 16.62 min. Periodically, at different developmental stages, the content of glycolic acid in berries was determined in both cultivars. The concentration of glycolic acid decreased substantially in September, but the concentration was always higher in cv. Gamaret than in cv. Gamay (Fig. 2).

Epicuticular waxes

The amount of epicuticular wax on berries of both cultivars was determined after measuring their surface area. Berries were soaked in chloroform for 30 s, the solvent was evaporated to dryness under vacuum and the residue weighed. Higher densities of wax were extracted from cv. Gamaret than cv. Gamay (Table 2).

Table 2. Density of epicuticular waxes on grape berries of cvs Gamay and Gamaret in late September.

	Cuticular waxes (mg/cm^2)	
Dates, 1991	Gamay	Gamaret
September 25	0.11	0.14
September 30	0.08	0.17

Histological observation of epidermis

At the véraison stage, halves of berries were fixed, embedded in paraffin and cut into thin sections. After staining they were studied by light microscopy. There were no fundamental differences visible between the two cultivars in the first layers, but the inside layer of the epidermis was much thicker in cv. Gamaret and the thickness of the epidermis as a whole was greater in cv. Gamaret than in cv. Gamay.

Phenolic acids

Phenolic acids were extracted and separated into four classes according to Southerton and Deverall (1990). Extractions were made from entire berries until July 12 1991 and

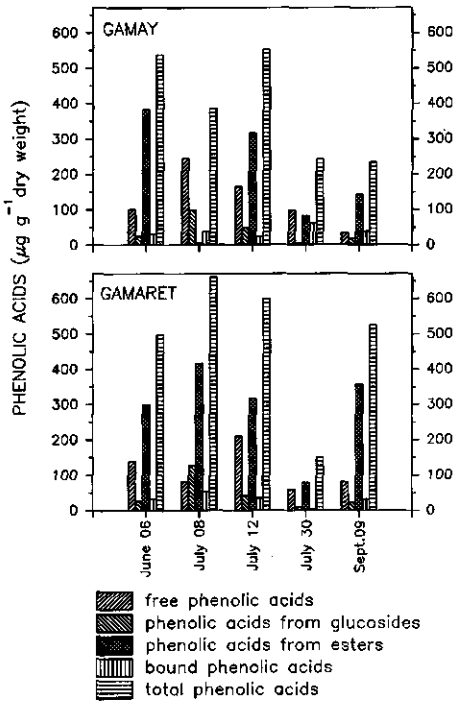

Fig. 3. Concentrations of phenolic acids in grape berries of cvs Gamay and Gamaret, present as free phenolic acids, glycosides, este rs and bound forms, determined at five developmental stages.

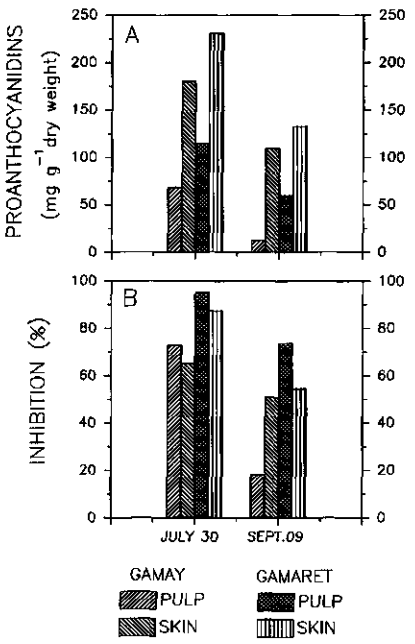

Fig. 4. A. Proanthocyanidin concentrations in pulp and skin of two grapevine cultivars, Gamay and Gamaret.
B. Inhibition of B. cinerea oxidase by proanthocyanidins extracted from skin and pulp of grape berries of Gamay and Gamaret (enzymatic reaction medium: 3 ml citrate-PO_4 buffer [0.1 M, pH 5.2]; 30 µl crude enzyme [1.49 mg prot.ml^{-1}]; 20 µl pterostilbene [1 mg.ml^{-1} EtOH solution]; 20 ml proanthocyanidins [1.5 mg.ml^{-1}, aqueous solution])

from the epidermis from July 30 1991. Predominant phenolic acids of each class were identified and quantified by HPLC analysis (gradient I) according to the same authors. The total phenolic acid content was determined by adding concentrations of identified standard compounds which absorb at 275 nm in each class. Six compounds, mainly predominant in the free phenolic acid class but also present in other classes, were identified as stilbenes by their UV spectra and these were subsequently quantified using external peak standardisation for resveratrol at 275 nm. The level of phenolics was highest in the esterified form, except for cv. Gamay at the end of flowering time (July 8 1991). At the end of the season (September 9 1991), the total phenolic acids were twice as high in cv. Gamaret than in cv. Gamay (Fig. 3).

Proanthocyanidins

Condensed anthocyanidins are important components of both the skin and the pulp of grape berries. They were extracted by the methods of Czochanska *et al.* (1980) and Porter (1989) and their concentrations determined gravimetrically. Delphinidin and cyanidin we re released from grape proanthocyanidins by acid hydrolysis. These anthocyanidins represent c. 2% of the weight of the proanthocyanidins extracted from grape berries. According to Czochanska *et al.* (1980) a still unsolved problem is the separation of polymeric proanthocyanidins from hydrolysable tannins. We concluded that the tannins extracted from the skin and the pulp of grape berries were composed of condensed tannins and hydrolysable tannins (Fig. 4A). Is was clear that the concentration of proanthocyanidins was always greater in the skin than in the pulp. Highest concentrations were found in cv. Gamaret and they decreased in this period from July 30 until September 9 1991. The inhibition by these tannins of stilbene oxidase from *B. cinerea* is shown in Fig. 4B. For the same concentration of proanthocyanidins, inhibition of oxidase was always greater for those extracted from cv. Gamaret. At véraison, the tannins from pulp of cv. Gamaret showed the most oxidase inhibition. These results indicate that the quality of tannins in berries is important for the inhibition of oxidase.

Conclusions

All parameters examined in berries of cv. Gamaret showed conditions less favourable for development of *B. cinerea* than those in cv. Gamay. These results accord with the observed resistance of cv. Gamaret to *B. cinerea* in the field. The importance of each parameter in the resistance process is relative. In this model it is impossible to designate a single resistance factor as essential; all contribute towards unfavourable conditions for growth of *B. cinerea*, and are exhibited at different levels related to the developmental stages of the berries, climatic condition and grapevine cultivars. Aggressiveness of *B. cinerea* populations, as yet ill defined, was not taken into account in this study.

Acknowledgements

We thank Mrs. I. de Groote for proficient technical assistance.

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Some characteristics of resistance of grape berries to grey mould caused by *Botrytis cinerea*

S. Prudet, B. Dubos and R. Le Menn

Summary

A comparison by light and electron microscopy of the development was made of the skin in the two grapevine cvs Arriloba and Sauvignon, which differ in susceptibility to *B. cinerea*.

The skin of cv. Arriloba (resistant) was thicker because of a higher number of cell layers, and had more dispersed cuticular striations than that of cv. Sauvignon (susceptible).

Development of these structures at the end of fruit maturation seemed to be related to their susceptibility to *B. cinerea*, particularly the development of deep hypodermal cells into mesocarp parenchyma (pulp) cells. In cv. Arriloba, the thickness of the skin is maintained longer at the end of maturation owing to less degradation of the number of cell layers.

The continued presence of an endo-polygalacturonase inhibitor in resistant cultivars after véraison may also be involved. The pectic substances of the skin become more susceptible to degradation by pectolytic enzymes of *B. cinerea* during maturation and this trend is greater in cv. Sauvignon than in cv. Arriloba.

Introduction

The susceptibility of grapes to *Botrytis cinerea* Pers.: Fr. varies according to the stage of development of the berries; immature grapes become susceptible to fungal development after véraison.

The discovery of an inhibitor of endo-polygalacturonase produced by *B. cinerea* in grapes (Grassin, 1987) gave a fresh impetus to studies on the resistance of the vine to this pathogen. The probable location of this inhibitor in the epidermal and hypodermal layers (hereafter called 'skin') which constitute the first barrier to fungal penetration, raises the question of the role of these layers in resistance of the grape berries to *B. cinerea* and the relationship between decreases in the level of the inhibitor and the modification of these structures during ripening.

We therefore performed a histological study of the 'skin' in cultivars Sauvignon (very susceptible) and Arriloba (resistant). First, we used light and electron microscopy to examine development of the 'skin' during maturation. Secondly, we investigated the changes in digestibility of pectic substances from the 'skin' by pectinolytic enzymes of *B. cinerea* and *Aspergillus niger*.

Material and Methods

From flowering until maturation of the berries, samples of the berries of the cvs Arriloba and Sauvignon were taken weekly.

By light, scanning and transmission electron microscopy, the following parameters were studied: the surface pattern and thickness of the cuticle, the shape of the epidermal cells, number and thickness of cell layers under the epidermis and the thickness of the inner and outer pecto-cellulose walls of epidermal cells.

The patterns on the cuticle surface of cvs Sauvignon and Arriloba were compared when the berries had a diameter of 3, 11 and 16 mm.

To study the breakdown of pectins from the 'skin' of berries of both cultivars, pectinolytic enzymes produced by *B. cinerea* and by *A. niger* were used. The pectin was obtained after crushing the berries and dipping them in ethanol at 100°C. The ethanol-insoluble material containing the pectins were used as substrate. The quantity of pectin was determined by the metaphenyl-phenol methods, as described by Blumenkrantz and Abroe-Hansen (1973). Neutral sugars were determined by the sulphuric phenol method (Montreuil and Spik, 1963).

Pectinolytic enzymes of *B. cinerea* (polygalacturonase and pectinases) were extracted from a liquid culture medium of the fungus and assayed by the method of Somogyi-Nelson (Somogyi, 1952).

Polygalacturonase from *A. niger* was obtained commercially and similarly assayed.

Digestion was performed by placing the same quantity of pectin with each enzyme for 2 h at 37°C.

Results and Discussion

No differences were observed between the two cultivars in cuticle surface patterns at these stages of berry development using a scanning electron microscope. This parameter probably plays no role in the differences in susceptibility of cultivars to *B. cinerea*.

Both cultivars had similar patterns in the increases of cuticle thickness during muturation. By light microscope (Fig. 1) it was shown that the cuticle in cv. Arriloba was 4.75 μm thick and that of cv. Sauvignon only 3.5 μm. This greater thickness in cv.

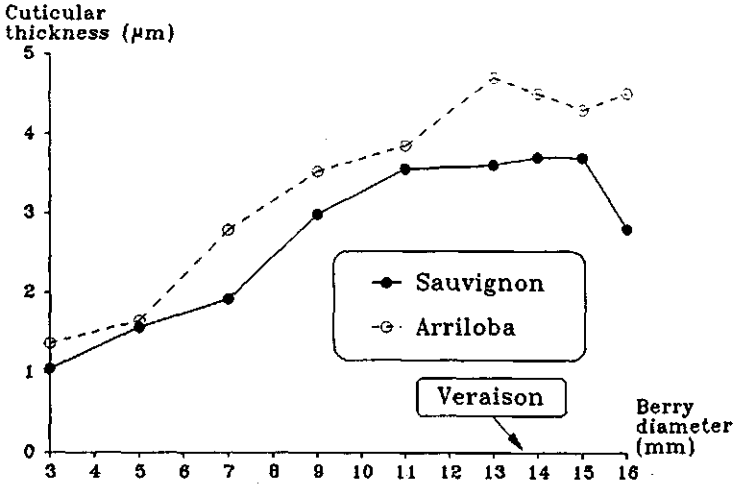

Fig. 1. Changes in thickness of the 'skin' in berries of the cvs Arriloba and Sauvignon.

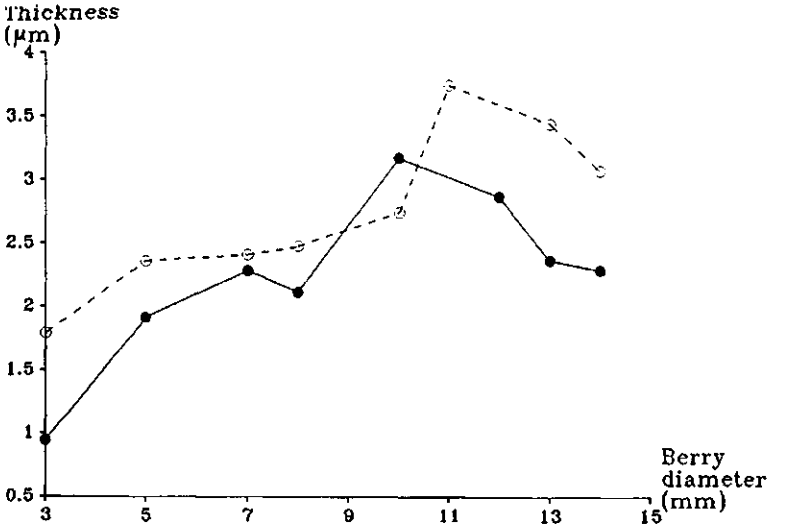

Fig. 2. Changes in thickness of the cuticle.

Arriloba was visible when the berries had attained a diameter of 9 mm. However, when the berries were 16 mm in diameter, the cuticular thickness in cv. Arriloba was reduced to 4.4 µm and that of cv. Sauvignon to 2.65 µm.

'Skin' thickness developed similarly in both cultivars; until véraison, its thickness was relatively high, but then decreased. In cv. Arriloba, the final 'skin' thickness was 62 µm and that of cv. Sauvignon 33 µm.

The changes in thickness of the 'skin' may be related to the change in the number of cell layers. Before véraison, cv. Sauvignon had c. 14 cell layers compared to 12.5 in cv. Arriloba, but after véraison, the layers decreased in both cultivars, but was still greater (6) in cv. Arriloba at the last two stages of maturation than in cv. Sauvignon (3.5).

Using transmission electron microscopy (TEM), we studied thickness of the cuticle and the inner and outer walls of epidermal cells. The change in size of these cells was expressed by ratio R (length L to height H of cells).

The TEM studies of the cuticle confirmed the results from light microscopy that the cuticle of cv. Arriloba was thicker than in cv. Sauvignon (Fig. 2).

The outer cell walls of the epidermis were of the same thickness in both cultivars, with a steady increase to 1.45 µm when the berries had a diameter of c. 16 mm, for the inner walls, there was a similar development in both cultivars with a thickness of c. 0.75 µm in berries of > 9 mm diameter.

The development of the epidermal cell ratio R, as shown in Table 1, indicated the tendency for the cell pattern to change. R increased from 1.33 to 4.43 in cv. Sauvignon and from 1.30 to 2.68 in cv. Arriloba. This indicates that these cells flatten during maturation, and contribute to the reduction found in the final 'skin' thickness, a phenomenon more pronounced in cv. Sauvignon than in cv. Arriloba (Table 1).

On the basis of these observations, the change in 'skin' thickness at the end of maturation probably plays an essential role in the varying degrees of susceptibility to *B. cinerea*. Clearly, the physical barrier offered by the 'skin', decreases during

maturation, particularly in cv. Sauvignon. These changes in cell wall structure indicated that there may be chemical changes in the pectin composition which may affect their degradation by pectinases.

Table 1. Development of the epidermal cell ratio R (= length to the height of the cells) in berries of cvs Sauvignon and Arriloba.

Diameter of the berries (in mm)	R	
	cv. Sauvignon	cv. Arriloba
3	1.33	1.30
5	0.99	1.22
7	1.48	1.64
10	1.56	1.78
12	3.52	2.14
13	3.70	2.43
14	4.43	2.68

The quantitative changes in pectic substances present in the 'skins', and their levels of neutral sugars and acids in the period of July 10 to September 14 1990 are shown in Fig. 3.

The changes in pectic substances during maturation of 'skins' of cvs Sauvignon and Arriloba were similar, particularly regarding acid pectins which were examined for their digestibility.

An increase in the digestibility of 'skin' pectins during maturation was found in both cultivars (Fig. 4) when pectinesterases and polygalacturonases of *B. cinerea* and polygalacturonase of *A. niger* were used. There was no difference before véraison, but

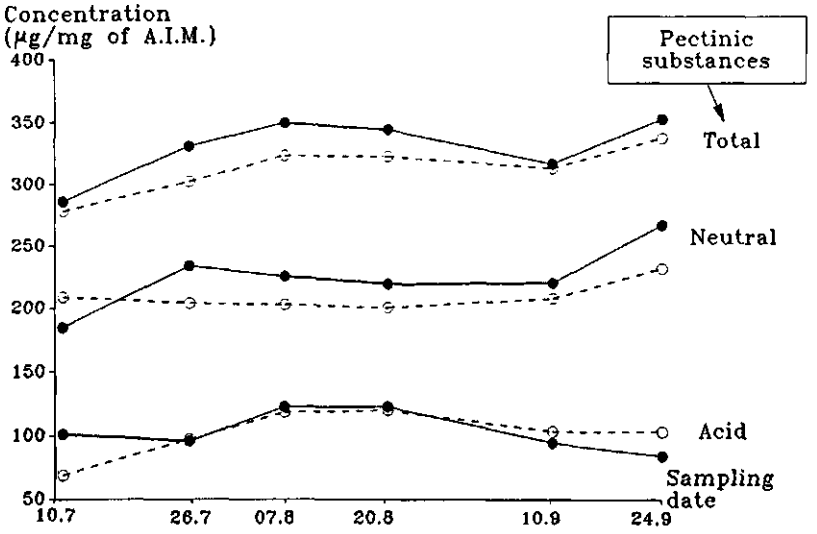

Fig. 3. Quantities of neutral and acid sugars in grape berries of cvs Sauvignon and Arriloba.

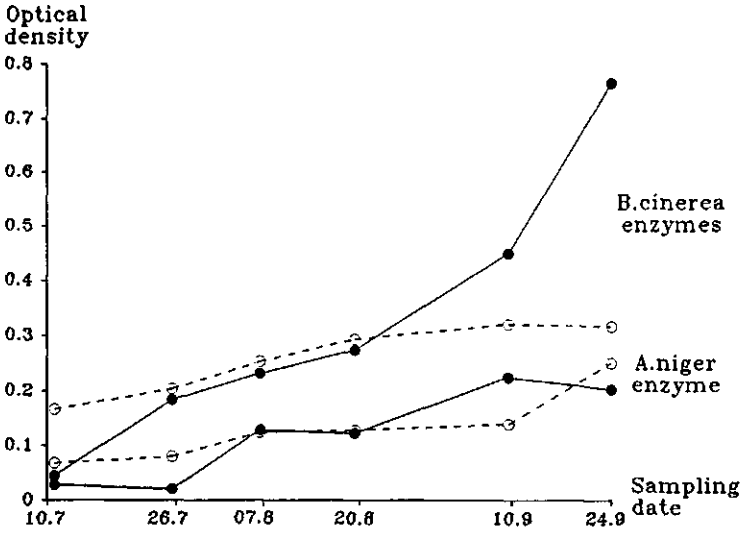

Fig. 4. Changes in digestibility of pectin during maturation of berries of the cvs Arriloba and Sauvignon.

thereafter pectin from cv. Sauvignon was more digestible by pectinases from *B. cinerea* than pectin from cv. Arriloba and this difference increased until harvest.

In both cultivars, pectin digestibility increased during maturation; but was greater in cv. Sauvignon than in cv. Arriloba. Biochemical analysis of these grape pectins should now be performed to improve knowledge of this resistance mechanism.

In the cv. Sauvignon susceptible to *B. cinerea*, the decrease in the mechanical and biochemical performance of the 'skin ' during maturation was significantly greater than in the resistant one. The various patterns of development of the 'skin' complex may affect the polygalacturonase inhibitor present in these cells.

While these results to some degree explain the increased susceptibility of cv. Sauvignon berries at véraison, they examine only one aspect of fruit resistance. Therefore they should be considered only as part of the complex defence system of the grapeberries against *B. cinerea.*

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In vitro bioassays to evaluate the relationship between grapevines and *Botrytis cinerea*

R. Bessis, D. Vannel and M. Barbier

Summary

Culture filtrates from media in which *B. cinerea* was grown contain substances that are toxic to grapevine *in vitro*. The level of toxicity varied with cultivar and the species of *Vitis*. The heteropolysaccharide-containing fraction was found to be the most toxic. The screening of *Vitis* germplasm for susceptibility to toxic metabolites of *B. cinerea* can now be performed rapidly *in vitro* under controlled conditions.

Introduction

Cultivars of grapevines differ in susceptibility to *B. cinerea*; some are so sensitive that they are being grown less (e.g. cv. Folle blanche), others are more resistant (e.g. cv. Cabernet-Sauvignon). Variation also occurs in the development of *B. cinerea* during the growing season on different *Vitis* spp. Grape berries must be wounded, split open, or being fully ripened to be infected. The time of ripening of bunches is important. On the same vine, only the bunches of the first generation are susceptible at harvest, while in the other bunches, although under the same climatic conditions, disease symptoms may not appear.

Therefore the factors responsible for grape resistance are complex and depend on the species, stage of fruit development, and cultural conditions.

Consequently, it is difficult to screen grapevine cultivars for resistance to *B. cinerea* in the field and an *in vitro* approach for these evaluations was sought.

Material and Methods

Plants were cultivated *in vitro* in controlled conditions (Barbier and Bessis, 1987) which provided reproducible results throughout the year. The plant cultures used in this work were derived from a single budded micro-cutting taken from the middle part of the plant. Cell suspensions were prepared from leaves as described elsewhere (Barbier and Bessis, 1990).

Four isolates of *Botrytis cinerea* Pers.: Fr. with similar growth rate and toxicity of their culture filtrates were provided by M. Leroux (INRA, Versailles). Only isolate N was used in our study. It was maintained by subculturing a conidial suspension on solid or liquid malt-extract media. The medium used for micropropagation of grapevine also proved excellent for growth of the fungus.

After 4 weeks incubation the medium was filtered and the protein fraction precipitated by ammonium sulphate according to the technique of Kamoen (1984) and Kamoen *et al.* (1978). The polysaccharide fractions were then precipitated in two fractions according to the methods described by Dubourdieu (1982); the first fraction ($P_{0.5}$) was precipitated by

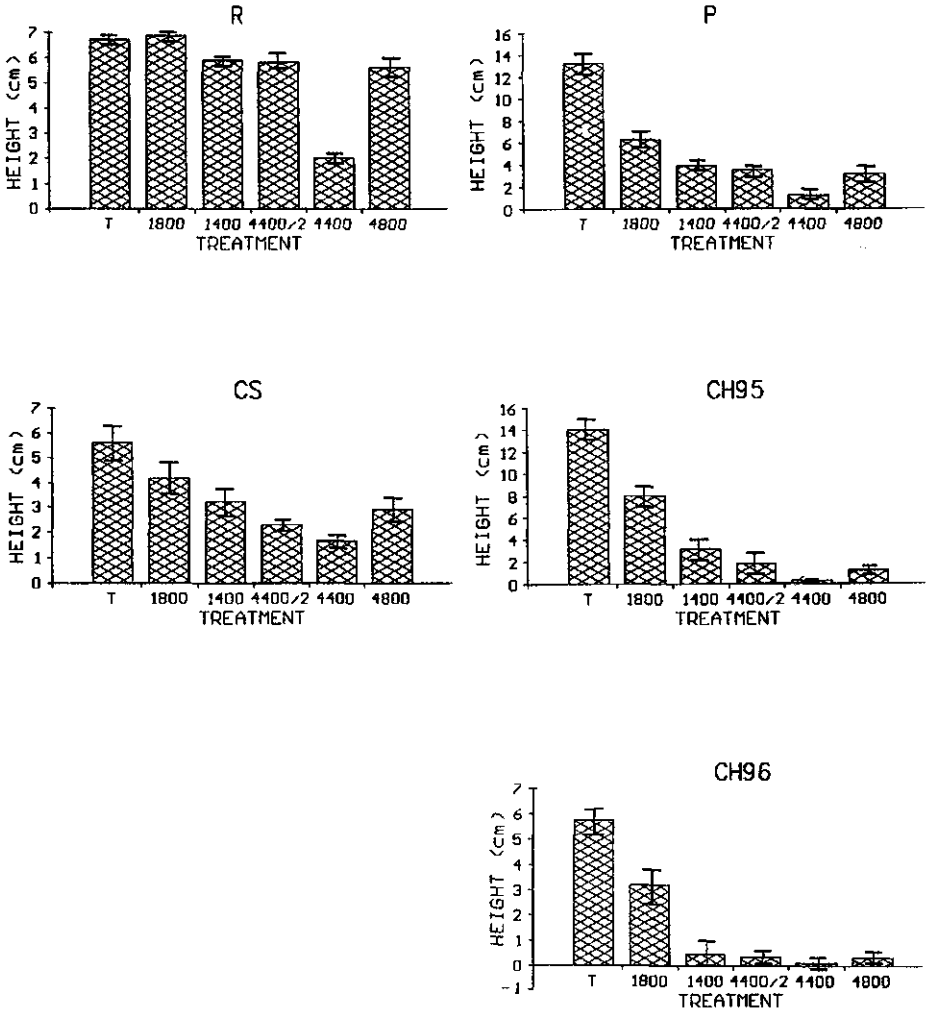

Fig. 1 Height (cm) of micropropagated plants of V. riparia (R), cvs Cabernet-Sauvignon (CS), Pinot (P) and Chardonnay clones 9 5 (CH95) and 96 (CH96) after 2 months culture on control medium (T) or on media containing filtrates obtained after 1 week of incuba tion in 800 ml (1800) and 400 ml (1400) or after 4 weeks of incubation in 800 ml (4800) and 400 ml (4400). The medium 4400/2 equals the filtrate 4400 twice diluted.

one half volume of ethanol and the second (P_4) was obtained by adding 3.5 volumes of ethanol to the supernatant.

Results and Discussion

After 2 weeks incubation on the medium used for grapevine micropropagation (Barbier and Bessis, 1987), *B. cinerea* produced toxic compounds causing necrosis on micro-cuttings of cv Chardonnay.

The phytotoxicity of the culture medium after growth of *B. cinerea* differed for different species and cultivars. All the Chardonnay plantlets showed necrosis, whereas the selection 41 B (*V. vinifera* x *V. berlandieri*) grown under the same conditions grew satisfactory.

We concluded from these two experiments that the fungus released substances in the medium which were toxic to grapevine and that germplasm of grapevine differed in sensitivity to these compounds.

Subsequent tests examined the relationship between the length of exposure to various phytotoxic materials from *B. cinerea* and the species of the grapevine. The results are given in Fig. 1.

This bioassay allowed rapid discrimination between species and cultivars: *V. riparia* showed little or no sensitivity to the filtrate; *V. vinifera* cvs. Pinot and Chardonnay were very sensitive, whereas the cv. Cabernet-Sauvignon was slightly less sensitive. This result is important because in vineyards, cv. Cabernet-Sauvignon is known to be one of the cultivars least affected by *B. cinerea*.

Toxicity of the culture medium increased with the length of the incubation and after 1 week the toxicity pronounced easily (Fig. 1).

The fraction containing heteropolysaccharides (Dubourdieu, 1982) was highly toxic (Fig. 2), but the glucan fraction was hardly toxic. After dialysing the filtrate, its toxicity was reduced slightly.

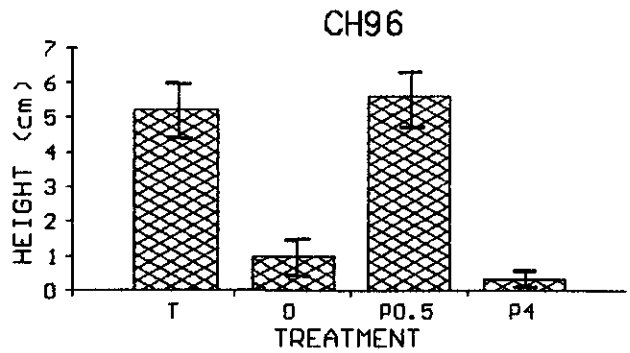

Fig. 2 Height (cm) of micropropagated plants of V. riparia (R), cvs Cabernet-Sauvignon (CS), Pinot (P) and Chardonnay clones 9 5 (CH95) and 96 (CH96) after 2 months growth on control medium (T) or on media with dialysed filtrate (D) or filtrate fractions containing glucan ($P_{0.5}$) or heteropolysaccharides (P_4).

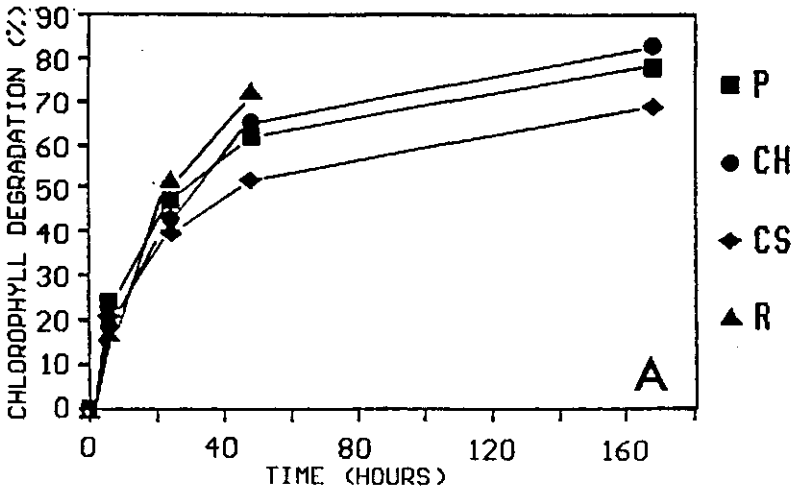

Fig. 3 Destruction of chlorophyll pigments in leaves of micropropagated plants of V. vinifera cvs Pinot (P), Chardonnay (CH), Cabernet-Saugvignon (CS) and V. riparia (R) incubated for 6, 24 and 48 h or 1 week in undiluted fungal culture filtrates. Results are expressed as percentages of the chlorophyll content of control leaves. The points represent the mean of three experiments with two samples each. Variability for each point was always less than 7%.

This bioassay allowed us to establish a link between the susceptibility of grapevines in the vineyard to *B. cinerea* and the sensitivity of micropropagated plants to the culture filtrates of the fungus. It was shown that the heteropolysaccharide fraction was highly toxic. However, this plant bioassay requires large volumes of filtrate and a 2 month cultivation period. Therefore, more rapid assays were evaluated. Excised leaves of micropropagated plants of various grapevines were incubated with culture filtrate of *B. cinerea*. The leaves turned brown rapidly and this alteration was measured quantitatively by destruction of chlorophyll (Fig. 3). The control leaves submerged in uninoculated medium showed no decrease in the chlorophyll content. No discrimination was found between grapevine germplasm. For example *V. riparia*, which is resistant in the field, was very susceptible under these conditions.

The study of the activity of the various fractions on excised leaves showed that the fraction $P_{0.5}$ was never phytotoxic, the fraction FP was phytotoxic to all cultivars and the fraction P_4 destroyed chlorophyll in all *V. vinifera* cultivars, but not in *V. riparia*. This fraction therefore contains a factor which may be useful in discrimination between genotypes.

Naked protoplasts from *Vitis vinifera* cv. Chardonnay and from *V. riparia*, or protoplasts with regenerated cell walls exposed to crude culture filtrate of *B. cinerea* died rapidly. With *V. riparia*, protoplasts were more sensitive than cells. Using selected fractions of the culture filtrates with protoplasts or cells it was shown that the heteropolysaccharide-containing fraction was the most toxic.

This study demonstrated the possibility of devising rapid screening tests for measuring the sensitivity of grapevines to *B. cinerea*, but for practical use more work is needed to examine more cultivars, the correlation of symptom development and the

susceptibility of the cultivars under field conditions and to identify the toxic compounds involved. It should now be possible to screen new cultivars of zygotic or somatic origin and establish test procedures for use in vitro. Other technical aspects of this work have been published elsewhere (Vannel *et al.*, 1991a,b).

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The use of phytoalexin induction and of *in vitro* methods as a tool for screening grapevines for resistance to *Botrytis cinerea*

Ph. Jeandet, M. Sbaghi and R. Bessis

Summary

The ability of *in vitro* cultures of grapevine (*Vitis vinifera* L.) to synthesise the phytoalexin precursor resveratrol in response to ultraviolet light irradiations was studied to develop methods for screening for resistance to grey mould caused by *B. cinerea*. The concentration of resveratrol in leaves of 150-day-old plantlets cv. Cabernet Sauvignon exposed to 400 $\mu W/cm^2$ irradiation at 254 nm for 7 min was twice that for cvs Pinot Noir and Chardonnay. This ranking accords with the relative susceptibility of these cultivars to grey mould after leaf inoculation in the field.

Introduction

Grey mould caused by *Botrytis cinerea* Pers.: Fr. is an important disease which causes serious losses to a wide range of plants. In vineyards the disease affects both the yield of grape berries and the quality of wine. Because of its lack of host specificity and of the difficulty of protecting the crop, we describe here procedures devised for screening for resistance to *B. cinerea* in grapevines produced by somatic embryogenesis.

In vitro methods using vegetative multiplication and somatic embryogenesis (Bessis, 1986) are necessary because conventional hybridisation techniques are not permitted in traditional wine producing areas where the production of great wines is based upon a limited number of cultivars which have assured the reputation of these vineyards. Indeed, *in vitro* techniques presently used on a large scale for clonal propagation of a wide range of crop plants have also proved to be of interest in grape breeding programmes where greater variabilities can be obtained and used in the selection of improved disease resistant cultivars (Bessis, 1986). However, no method is available to identify soma-clones derived by somatic embryogenesis with increased levels of resistance to *B. cinerea*. Traditional field screening is unsuited to the selection of a large number of somatic embryos; to reduce the time taken to identify resistant individuals, we describe here a technique devised to use the assessment of the phytoalexin precursor, resveratrol as a selection criterion to aid the screening of in vitro-grown plantlets for grey mould resistance.

Previous results (Langcake, 1981; Pool *et al.*, 1981) have shown that the speed and intensity of resveratrol synthesis are positively correlated with resistance of grapevine cultivars to grey mould. Therefore the production of resveratrol, induced by various stress factors, has been used as a screening procedure for host resistance to *B. cinerea* in the field (Jeandet and Bessis, 1989). Unfortunately, resveratrol formation by field-grown leaves was highly sensitive to environmental changes (Barlass *et al.*, 1987), thus limiting its effectiveness. Use of *in vitro* techniques can overcome this type of variation.

This paper describes experiments designed to measure synthesis resveratrol *in vitro* and to compare three cultivars of *V. vinifera* showing differences in susceptibility to grey mould, namely cvs Pinot Noir and Chardonnay, which are considered to be very susceptible to *B. cinerea*, and cv. Cabernet Sauvignon, which is least affected by grey mould.

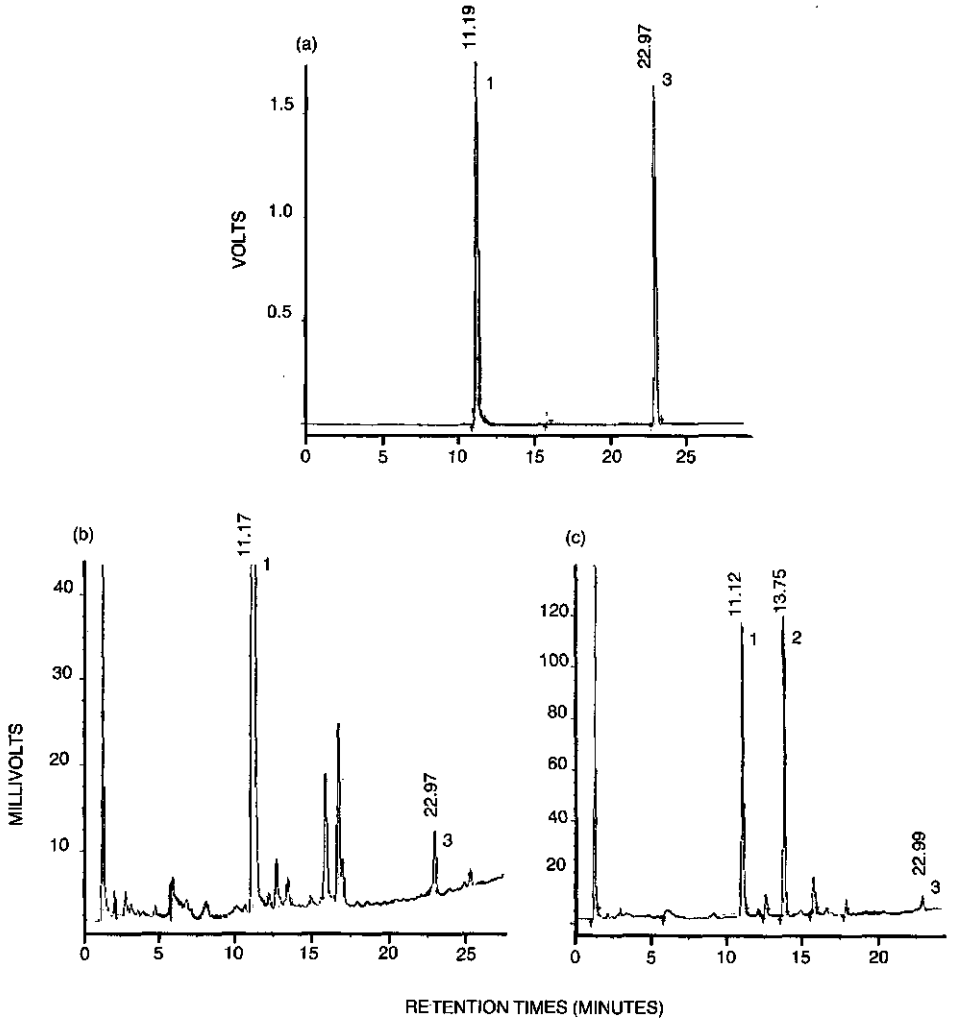

Fig. 1: HPLC analysis of stilbene-type phytoalexins

Chromatographic conditions were as follows: linear gradient elution within 25 min from 40% to 100% methanol in water at a flow rate of 1 ml.min^{-1}. The separation was performed on a Beckman C18 column (Ultrasphere ODS, 4.6 mm x 25 cm, 5 μm). Detection was at 307 nm. (a) Synthetic standards; (b) UV-irradiated leaves (cv. Cabernet Sauvignon); (c) Photochemical isomerisation (within 1 h of daylight exposure) of trans-resveratrol to the cis-isomer in a grape leaf extract; 1, trans-resveratrol; 2, cis-resveratrol; 3 , trans-pterostilbene.

Results and Discussion

The efficiency of three different procedures for elicitation of resveratrol in plantlets grown *in vitro* were tested: viz. inoculation with conidial suspensions of *B. cinerea*; induction by galacturonic acid; and induction by short wave UV-irradiation (254 nm).

The abaxial leaf surface of the three cultivars were tested by inoculating a single drop (50 µl) of a conidial suspension of *B. cinerea* containing c. 4 x 10^4 conidia.ml^{-1}. In all material this resulted in the development of large necrotic areas and dense sporulation over most of the leaves after 72 h of incubation in darkness. No fluorescence indicative of the presence of resveratrol was detected in the apparently healthy tissues adjacent to the infected zones 48 h after inoculation. This suggested that *in vitro* plantlets were very susceptible to infection even when a low density conidial inoculum was used.

Galacturonic acid, which is released by pectinolytic enzymes from pectic polymers in cell walls may stimulate phytoalexin accumulation (Bachmann and Blaich, 1980). Higher galacturonic acid concentrations (0.5 and 1%) were phytotoxic, inducing large necroses on the leaf surfaces. Solutions containing 0.1% galacturonic acid showed potent elicitor activity for resveratrol formation, but this result was not reproducible. Our observations therefore indicate that these first two procedures are unsuitable for studies of the elicitation of resveratrol by grapevine *in vitro*.

Radiation with a wavelength of 260-270 nm has a selective and reproducible effect on resveratrol synthesis by grape leaves (Langcake and Pryce, 1976) and by grape berries (Jeandet, 1991; Jeandet *et al.*, 1991) under field conditions. In contrast Barlass *et al.* (1987) reported that in vitro-grown plantlets showed a general necrosis and no associated resveratrol formation when irradiated under the same conditions, i.e. at 254 nm with a fluence rate of 600 µW/cm^2 for 10 min (0.36 J/cm^2). These authors therefore implied that the technique could not be used with *in vitro*-grown leaves. In order to avoid undesirable effects of UV-irradiation on this material, our experiments were performed with the energy fluence rate and exposure time reduced. A fluence rate of 400 µW/cm^2 with

Table 1. Production of resveratrol in leaves of different grape cultivars grown in vitro.

	Trans-resveratrol (mg.g^{-1} fresh weight + SE)[a]		
	Pinot Noir	*Chardonnay*	*Cabernet Sauvignon*
N1[b]	88.6 ± 5.3	174.8 ± 10.8	213.0 ± 13.7
N2	102.3 ± 6.1	not done	192.7 ± 10.6
N3	97.0 ± 5.9	63.2 ± 4.2	202.2 ± 12.1
N4	89.7 ± 5.5	39.7 ± 2.9	110.0 ± 6.5
N5	37.0 ± 2.9	70.0 ± 3.9	138.6 ± 5.9
N6	42.2 ± 3.1	70.0 ± 4.5	77.0 ± 3.5
N7	48.2 ± 3.4	78.2 ± 4.9	138.4 ± 7.1
N8	35.0 ± 2.8	79.6 ± 4.1	138.4 ± 6.5

[a]) Each value represents the resveratrol production of three leaves taken at the same leaf position on different shoots of the same vine variety. All measurements were done in triplicate. SE = Standard Error of the three measurements. Resveratrol concentration in leaves was estimated by GLC (see Jeandet *et al.*, 1991).

[b]) Leaf position on shoot numbered from base.

exposure of 7 min (0.17 J/cm^2) was sufficient to induce stilbene production in leaves of the three cultivars tested (Fig. 1) (Jeandet *et al.*, 1992).

The results obtained from assaying resveratrol from 150-day-old plantlets showed differences between cultivars evaluated by their ability to synthesize resveratrol in response to UV-irradiation, as shown in Table 1. The concentration of resveratrol in the leaves of cv. Cabernet Sauvignon, when expressed as a mean of the values given in Table 1 (151 $\mu g.g^{-1}$ fresh weight), was twice as high as that for cvs Pinot Noir and Chardonnay (67 and 82 $\mu g.g^{-1}$ fresh weight, respectively). This accords with the grey mould resistance of the corresponding cultivars assessed by leaf inoculation in the field in which cv. Cabernet Sauvignon has intermediate field resistance while cvs Pinot Noir and Chardonnay are both more susceptible.

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Ethylene production and growth of *Botrytis cinerea* in kiwifruit as influenced by temperature and low oxygen storage

N.D. Niklis, C.C. Thanassoulopoulos and E.M. Sfakiotakis

Summary

The effects of temperature and low O_2 concentrations on the development of grey mould caused by *B. cinerea*, on ethylene production and tissue softening in kiwifruit were examined.

Kiwifruit stored at 0°, 5° and 10°C produced significant amounts of ethylene 20-30 days after inoculation with *B. cinerea*, but only trace amounts were detected in healthy controls. In another experiment in which fruits were inoculated with *B. cinerea* and exposed to a range of temperatures (–2°, –1°, 0°, 5° and 10°, 20°, 30°C), low temperatures reduced ethylene production and softening of flesh. Infection was delayed for c. 24-48 days and fungal growth rates were reduced at 0°, –1° and –2° compared to higher temperatures. Storage of the inoculated kiwifruit at low oxygen (1% O_2) and 0°C strongly reduced the growth rate of the fungus and ethylene production.

Introduction

Serious losses in kiwifruit production occur because of grey mould caused by *Botrytis cinerea* Pers.:Fr. (Sommer, 1982; Sommer *et al.*, 1983). Several postharvest treatments have been used to control fungal growth during storage of fruits. Low temperature suppressed the pathogenicity of *B. cinerea* (Brooks and Cooley, 1928) and elevated CO_2 concentrations may suppress other fungi (El-Goorani and Sommer, 1981). Lowering the O_2 concentration of the atmosphere from c. 21% of air to 2 to 3% suppressed the pathogen little and only after the O_2 had been lowered to < 1% was the fungus suppressed (Couey and Well, 1970; Foolstad, 1966). These near anaerobic storage conditions may risk injury to the fruit. Several postharvest pathogens produce ethylene at high rates during growth on citrus fruits or *in vitro* (Chou and Young, 1973; DaSilva *et al.* , 1974; Tzeng and Devay, 1984)

This paper examines, a) the possible involvement of *B. cinerea* in ethylene production, b) the effect of temperature and c) the effect of low oxygen on grey mould development, ethylene production and softening of the infected fruit.

Material and Methods

Kiwifruit (*Actinidia deliciosa*) cv. Hayward harvested from commercial orchards or taken from refrigerators of Pieria county of Central Macedonia in Northern Greece were used. For inoculations, an isolate of *B. cinerea*, obtained from kiwifruits in this county was used in all experiments. The fungus was maintained on potato dextrose agar (PDA). Two parallel punctures were made in the stem end area of the fruit; the fruits were inoculated here with a piece of agar (c. 5 mm) bearing mycelium of *B. cinerea*.

Experiment 1
Kiwifruits were harvested during October 1989 from a commercial orchard when their average firmness and soluble solids content were 5.5 kg and 8.7% respectively. Half of the fruits were inoculated and the other half were kept as control. The fruits were placed into 5 L jars, each containing 18 fruits, representing one plot. One jar with healthy fruits and one with inoculated were used for each temperature treatment. The jars were placed in waterbaths adjusted to 0°, 5° or 10°C. Continuous aeration (90 $ml.min^{-1}$) was used and the ethylene concentration in the gas-phase was measured in the air flow. Samples were taken 45 (10°C) and 175 (0°, 5°) days after inoculation and the experiment was replicated three times.

Experiment 2
Kiwifruits were taken from the commercial refrigerator of the Union of Agricultural Cooperatives-Pieria with average firmness and soluble solids content of 3.5 kg and 13% respectively were used to study fungal growth, ethylene production and flesh firmness.

Inoculated kiwifruits were placed in 5 L jars, 18 fruits in each jar, and the jars were placed in waterbaths of 30°, 20°, 10°, 5°, 0°, –1°, –2°C in six replications. Anti-freeze was used in the water for –1 and –2°C treatments. Measurements were made in four sampling periods, four fruits each of the first three samples and six for the last.

Experiment 3
Fruits from the same source as in Expt. 2, with firmness of 3 kg and 12.7% soluble solids were used. Inoculated fruits were placed in sealed 400 L steel chambers at O_2 concentrations of 1% and 21% (0°C and 97% RH), 20 fruits per plot. The O_2 concentrations of the storage atmosphere was monitored with a paramagnetic gas-analyzer. This experiment was replicated four times. Measurements were taken six times in six subsequent sampling periods, five fruits per plot in each period.

For analysis of gas-phase ethylene concentration, the 'head-space' gas samples were removed by 5 ml syringe and ethylene concentration was analyzed by Varian 3300 GC equipped with FID in samples taken from 1 ml syringe.

The soluble solids content was evaluated by portable electronic refractometer (Atago, model PR-1). The flesh firmness (two measurements per fruit) was measured after removal of skin, using a Chatillon tester fitted with a 7.9 mm plunger. Infected flesh area was measured by a ruler from the point of inoculation to the end point of the rotted flesh. Statistical analysis was performed by M-STAT (Micro-static) IBM program and figures were plotted using the CRICKET Apple program.

Results and Discussion

Expt. 1. Ethylene production from infected and healthy kiwifruits at temperatures 0°, 5° and 10°C. The ethylene concentration in the gas-phase in the non-inoculated fruit was low (Fig. 1a, b, c). Ethylene production at 10°C was c. twice that at 0°C, but in the latter the decline in ethylene levels was slower. Infected kiwifruits showed significant increases in ethylene concentration in the gas-phase in the period from 20 to 30 days after inoculation at all temperatures (Fig. 1), but then decreased as the fungus colonized the tissue and softened it.

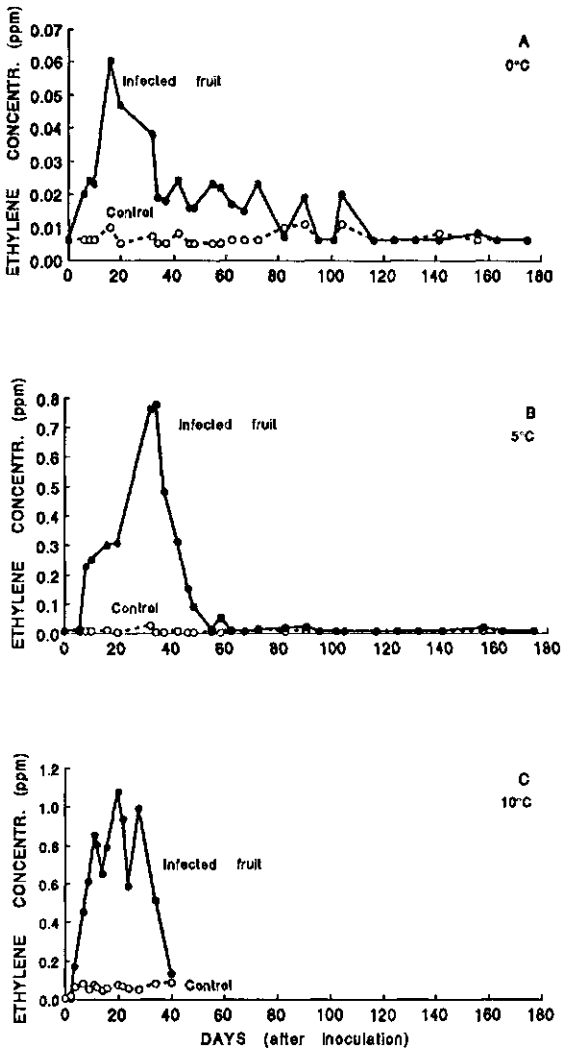

Fig. 1. Ethylene concentration in the gas phase of the control (non inoculated kiwifruit) and with Botrytis cinerea infected fruit, kept under continuous air flow (90 ml.min^{-1}) at temperatures 0°C (A), 5°C (B) and 10°C (C).

Expt. 2. The influence of different temperatures in ethylene production and the growth of grey mould. At temperatures of 30°, 20° and 10°C a rapid growth of the fungus occurred and by the 15th day the fruit was completely destroyed. Ethylene production, firmness and the infected area are shown in figure 2. At 30°C there was still significant growth of mycelium, and production of spores and sclerotia inside the fruit. At 5°, 0°, –1° and –2°C the lag phase was c. 20 days but the growth rates were lower than at higher temperatures. The ethylene production decreased at -2°C but the small amount of ethylene at –2°C originated from the grey mould infection. At –1° and –2°C, mycelial growth did not

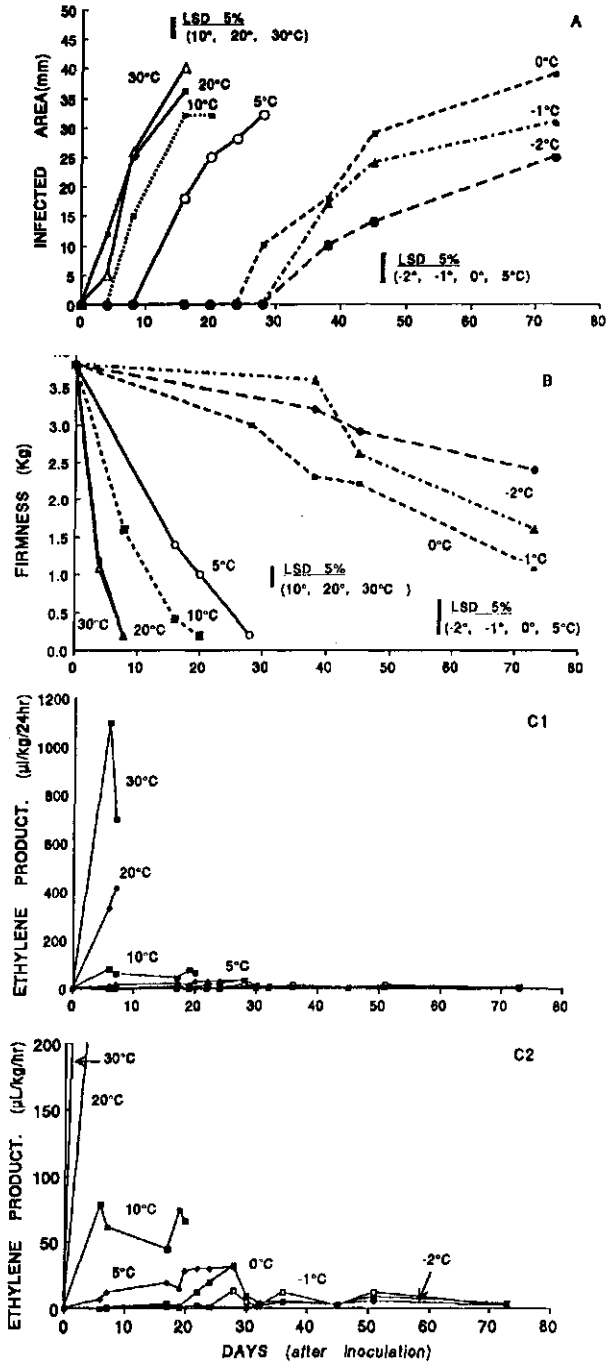

Fig. 2. Growth of Botrytis cinerea in kiwifruit (A), softening (B) and ethylene production of the infected kiwifruit at constant temperatures of –2°, –1°, 0°, 5°, 10°, 20° and 30°C (C1 and C2).

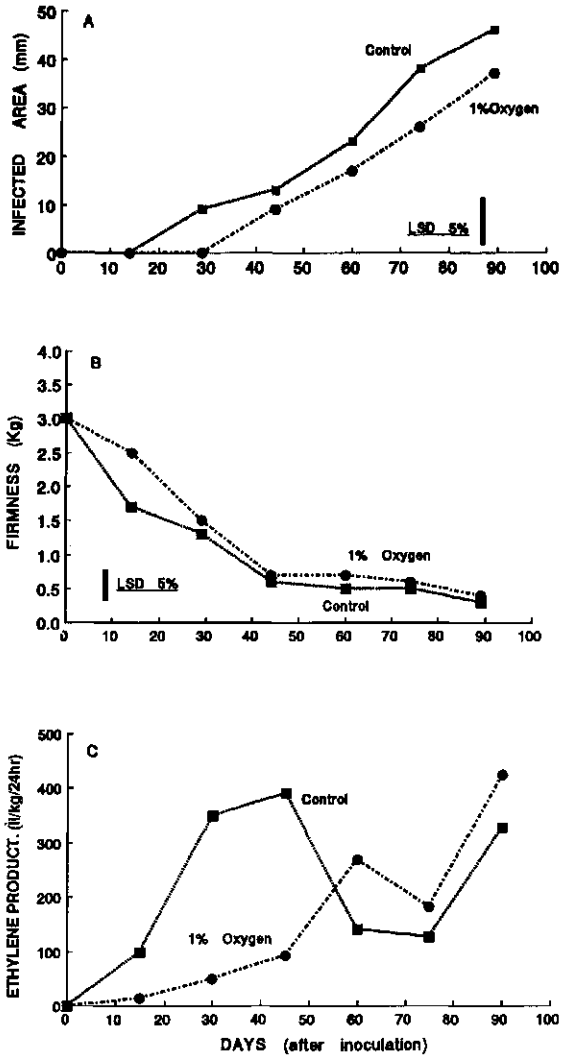

Fig. 3. Growth of Botrytis cinerea in kiwifruit (A), softening (B) and ethylene production (C) of the infected fruit under normal and low oxygen (1%) atmosphere storage at 0°C.

appear on the fruit surface, but inside the flesh; both flesh and skin were infected at 0°, 5° and 10°C.

Expt. 3. Effect of O_2 concentrations on ethylene production and fungal growth. Low O_2 concentrations reduced production of ethylene and fungal growth for 45 days, but had no effect on firmness of the fruit (Fig. 3).

It is evident from this study that by storing kiwifruit at low temperatures (0°, 5° and 10°C) ethylene production was negligible. However, the fruit infected with *B. cinerea* produced

significant amounts of ethylene 20-30 days after inoculation at these temperatures. In subsequent experiments fruits inoculated with *B. cinerea* and exposed to 5°, 10°, 20° and 30°C showed that the growth rate of fungus was similar but with an increased lag phase. This trend was most marked at lower temperatures where rotting was delayed for > 24 days, but the growth rate of the fungus was lower. Ethylene production was reduced at low temperatures; even at –2°C infected fruit produced enough ethylene to induce ripening. Storage of the inoculated kiwifruit at low oxygen concentration (1% O_2) and at 0°C, reduced the growth rate of fungus and ethylene production, but did not stop the pathogen completely and thus did not reduce softening of the fruit.

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Influence of plant growth regulators on mycelial growth, germination of conidia and pathogenicity of *Botrytis cinerea*

S. Benlioğlu and D. Yilmaz

Summary

The effect of the plant growth regulators 4-CPA, gibberellic acid, Na-salts, N-m tolypthalamic acid and ethephon on mycelial growth, spore germination and pathogenicity of three *B. cinerea* isolates was investigated.

Five concentrations of each growth regulator were tested *in vitro* and pathogenicity tests were performed on tomato cv. Supermarmande. The recommended concentration of 4-CPA at 10 $\mu g.ml^{-1}$ inhibited mycelial growth and conidial germination but gibberellic acid, Na-salts, and N-m tolyphtalamic acid had no effect. Ethephon slightly affected mycelial growth of *B. cinerea*. On the basis of these results *in vitro*, the effect of plant growth regulators on pathogenicity of *B. cinerea* isolates is discussed.

Introduction

Turkey produces annually c. 5.6 million tons of tomatoes in greenhouses and in the field; 74% of the total production comes from southern and south-western coastal areas. Despite intensive spraying, tomatoes grown in greenhouses in cooler and humid seasons suffer from several diseases, especially from grey mould caused by *Botrytis cinerea* Pers.: Fr.. Growers apply growth regulators (PGRs) to increase fruit setting and yield and these may influence the severity of the disease.

The effects of some widely used plant growth regulators on mycelial growth, spore germination and pathogenicity of *B. cinerea* are described in this paper.

Material and Methods

Three isolates of *B. cinerea* from the provinces of Antalya and Mugla were used in the experiments.

The sensitivity of these isolates to the PGRs was tested on potato dextrose agar (PDA) amended after autoclaving with 0.3, 1 , 3, 10, 30 $mg.ml^{-1}$ of 4-CPA (Tomatone 0.145%); 0.5, 1, 10, 50, 100 $\mu g.ml^{-1}$ of gibberellic acid (Berelex 9.6%); 0.1, 0.3, 1, 3, 10 $\mu g.ml^{-1}$ of Na-salts (sodium ortho-nitrophenolate 0.2%, sodium para-nitrophenolate 0.3% and sodium 5-nitroquacalate 0.1%; Atonik); 1, 2, 10, 20, 100 $\mu g.ml^{-1}$ of N-m tolyphtalamic acid (Tomaset 20%) and 10, 50, 100, 500, 1000 $\mu g.ml^{-1}$ of ethephon (Ethrel 48%).

The sensitivity of isolates to PGR was determined by transferring mycelial plugs (4 mm in diameter) from 3 to 4 day-old cultures on PDA to fresh PDA plates containing the above concentrations of PGRs, three plugs per isolate. Unamended PDA plates were inoculated as controls. After 3 days incubation at 20°C in darkness, colony diameters were measured and the growth of isolates calculated as a percentage of rates on unamended PDA plates (Georgopoulos and Dekker, 1982).

Table 1. The effect of five growth regulators in five different concentrations on mycelial growth of B. cinerea isolates after 3 days of incubation at 20°C. Concentrations are given in ppm a.i. Untreated control = 100. (is the concentration recommended)*

Isolates	Mycelial growth rate (% of control) 4-CPA (ppm a.i.)				
	0.3	1	3	10*	30
90/3-D	90	90	82	13	0
90/3-T	99	90	79	38	0
90/8-I	102	94	84	31	0
	gibberellic acid				
	0.5	1	10	50*	100
90/3-D	107	109	112	112	106
90/3-T	95	95	97	94	90
90/8-I	98	102	100	100	96
	Na-salts				
	0.1	0.3	1	3*	10
90/3-D	102	98	95	90	83
90/3-T	99	103	97	95	82
90/8-I	101	98	93	88	78
	N-m tolyphtalamic acid				
	1	2	10	20*	100
90/3-D	95	96	89	88	88
90/3-T	106	97	98	97	94
90/3-I	102	102	104	105	98
	Ethephon				
	10	50	100	500*	1000
90/3-D	104	102	99	87	63
90/3-T	99	99	97	91	66
90/8-I	102	104	102	94	68

To determine the effect of PGRs on germination of conidia a suspension was prepared from sporulating colonies on PDA and adjusted to 10^3 conidia.ml^{-1} with sterile distilled water and 100 ml of suspension was spread on plates in each treatment. After 24 h incubation at 20°C, the percentage germination was determined.

The effect of PGRs on the pathogenicity of *B. cinerea* was evaluated using tomato plants cv. Supermarmande at 5-6 true leaf stage. Plants were sprayed with the following

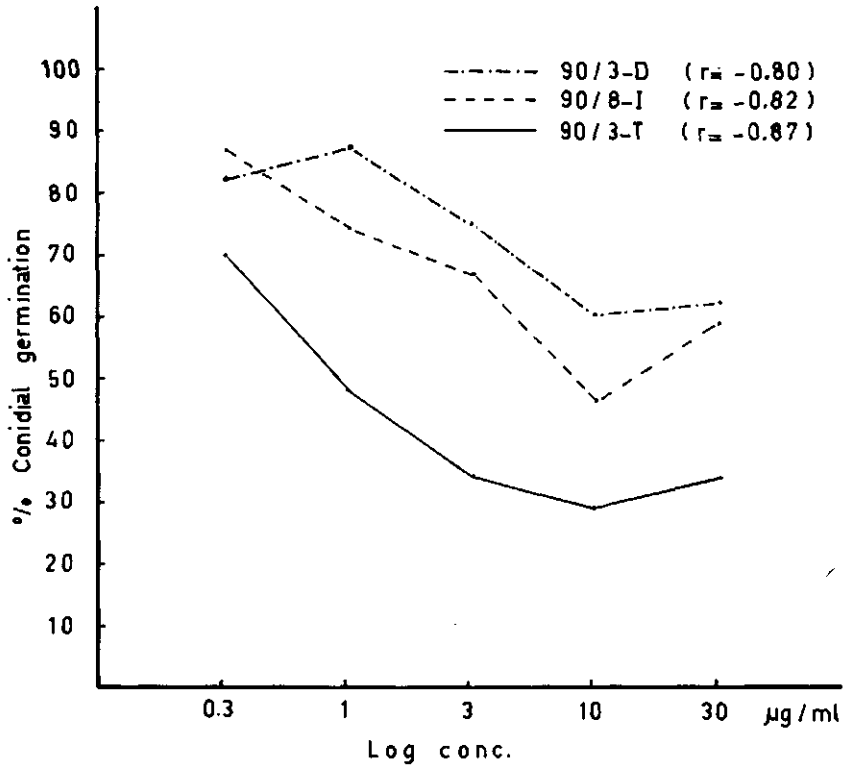

Fig. 1 The effect of 4-CPA on conidial germination of three B. cinerea isolates

commercially advised concentrations of PGRs: 10 µg.ml^{-1} a.i. 4-CPA; 20 µg.ml^{-1} a.i. N-m tolyphtalamic acid; 3 µg.ml^{-1} a.i. Na-salts; 50 µg.ml^{-1} a.i. gibberellic acid and 500 µg.ml^{-1} a.i. ethephon. A conidial suspension of *B. cinerea* (10^5 conidia.ml^{-1}) produced on autoclaved carrot bouillon (300 g grated carrot in 500 ml distilled water + 1% gelatin) was sprayed on plants after 24 h. The plants were then covered with plastic bags and incubated at 20 ± 1°C. The bags were removed 48 h later. Disease severity was evaluated by means of a scale (Delen *et al.*, 1986) where 0 is no infection and 5 the whole plant covered with sporulating lesions 6 days after inoculation.

Results

The effects of different concentrations of 4-CPA, gibberellic acid, Na-salts, N-m tolyphtalamic acid and ethephon on mycelial growth of three isolates of *B. cinerea* are shown in Table 1.

The percentage inhibition of growth of the three isolates on PDA containing 10 µg.ml^{-1} 4-CPA were 87, 62 and 69. Gibberellic acid, Na-salts, N-m tolyphtalamic acid and ethephon had no significant effect on these isolates, even when applied at recommended and higher rates. Only ethephon at 1000 ppm inhibited growth of *B. cinerea* by 40%. Similar results were obtained in the experiments which examined the

effect of PGRs on conidial germination. Only 4-CPA influenced the germination at concentrations in the range 0.3 $\mu g.ml^{-1}$ to 30 $mg.ml^{-1}$. A negative correlation between concentration of 4-CPA and percentage germination of conidia was found (Fig. 1).

In pathogenicity experiments, plants sprayed with 4-CPA, gibberellic acid, Na-salts, N-m tolyphtalamic acid and ethephon showed 50%, 62%, 62%, 64% and 80% infection respectively, compared to 60% of the control.

Discussion

In our assays, gibberellic acid had no effect either on the mycelial growth or conidial germination of *B. cinerea*, whereas Ozbek and Delen (1989) reported that gibberellic acid stimulated the mycelial growth of this fungus *in vitro*. Brecbuhler (1982) and Pearson (1982), however claimed that application of gibberellic acid to vineyards reduced *B. cinerea* infections. In our pathogenicity studies, plants sprayed with the recommended concentrations of PGRs showed similar levels of disease severity as the control. Except for 4-CPA these findings confirmed our *in vitro* results. Nevertheless, growth regulators may decrease or in crease the susceptibility of different plant species and cultivars to *B. cinerea*. Further investigations in the field or in greenhouses are required to clarify the effect of PGRs on this fungus and the development of grey mould in glasshouse-grown tomatoes.

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The physiology of conidiation in *Botrytis squamosa*

F.J. Balis and J.W. Lorbeer

Summary

Onion foliar extract agar supplemented with glucose (4 $g.l^{-1}$) as a carbon source and $Ca(NO_3)_2.4H_2O$ (4 $g.l^{-1}$) and termed calcium nitrate agar provided a suitable medium for the production of conidia of *B. squamosa*. When isolate BS82-1 was grown on calcium nitrate agar in Pyrex Petri dishes in an incubator at 18°C under 14 h photoperiods of near UV radiation massive conidiation occurred. This system consistently provided conidia of that isolate of *B. squamosa* for epidemiological investigations and studies on the control of this fungus in New York.

Introduction

Botrytis squamosa Walker, the causal agent of *Botrytis* leaf blight of onion, is one of the most destructive pathogens of onion grown on organic soils in New York (Hancock and Lorbeer, 1963; Lorbeer, 1992). Epidemiological studies of the fungus have been complicated by the difficulties of producing high levels of conidial inoculum with regularity and the factors regulating the production of conidia of this fungus *in vitro* are not well understood (Bergquist *et al.*, 1972). This study had two objectives: firstly, to develop a reliable technique for the consistent production of conidia, and secondly, to identify and elucidate those factors which play major roles in regulating the reproductive behaviour of *B. squamosa in vitro*. The first part of the study involved an *in vitro* investigation of the effects of near-ultraviolet (NUV) radiation and moisture stress on the production of conidia of the fungus. The second part examined *in vitro* the effects of carbon-nitrogen nutrition on conidiation and the role of Na^+, K^+ and Ca^{2+} cations in the production of conidia and sclerotia. Isolate BS82-1 of *B. squamosa* was used in all experiments.

Results and discussion

Effects of NUV radiation

Effects of NUV radiation were studied with cultures of *B. squamosa* grown on onion foliar extract agar (OEA) in Pyrex Petri dishes and exposed to 0, 8, 14, 16 or 24 h of NUV radiation. This was supplied by a Sylvania F20T12/BLB, black light blue, 20 W bulb emitting radiation between 310-420 nm, with a peak emission at 356 nm suspended 45 cm above the cultures. Pyrex glass is an efficient transmitter of NUV radiation. All experiments were conducted in incubators at 18°C for 12 days.

The OEA was prepared by resprouting onion bulbs (cv. Downing Yellow Globe) for 6 to 7 weeks at which time the foliage was harvested, washed and dried at 50°C. Desiccated foliage was stored in the laboratory at room temperature until used. To prepare the medium, 15 g of desiccated tissue was steamed in 1 l of deionized water for

1 h; the resultant slurry was passed through four layers of cheesecloth and centrifuged at 300 rpm for 3 min to remove leaf debris. Bacto-Difco agar (2% w/v) was added to the centrifuged extract to produce the OEA.

A similar study made use of onion leaf sections infected with *B. squamosa* at high humidity in Pyrex Petri dishes. The leaf sections were exposed to continuous darkness, a 14 h photoperiod of NUV radiation, or to continuous NUV radiation at 18°C for 12 days.

As photoperiods of exposure to NUV radiation were increased from 0 to 24 h, it was found that the production of conidia was increased when the fungus was grown on either OEA or the onion leaf sections. Production of conidia was greatest after continuous exposure of cultures to NUV radiation.

Effects of moisture stress

The effect of transient drying (10 h) of 4 day-old cultures of the fungus grown on OEA and by similar transient drying of infected onion leaf sections was studied. Leaf sections were prepared by inoculating and incubating them in a moist chamber for 4-5 days, during which time lesions and blighting developed. Then they were dried for 14 h under a coincident photoperiod of NUV radiation in chambers at 100, 87, 76 or 44.5% relative humidity. These humidities were established by using 20 ml of deionized H_2O (100%), or 20 ml of a saturated slurry of KNa tartrate (87%), NaCl (76%) or K_2HPO_4 (44.5%) in each chamber. Following drying, the leaf pieces were placed in a 100% relative humidity chamber for 36 h before the production of conidia was measured.

In another series of experiments, *B. squamosa* was grown in Pyrex Petri dishes containing 7 ml of OEA adjusted from –1.5 bar to –9, –27, –36 or –45 bar water potential with KCl, $NaNO_3$, sucrose, or PEG 4000. The cultures were incubated at 18°C with a 15 h photoperiod of NUV radiation and conidia were quantified after 12 to 14 days.

Although conidiation was induced by transient drying of cultures of *B. squamosa* growing on OEA, transient drying of blighted leaf segments did not increase conidiation compared to the non-dried control. No significant increase in conidiation occurred on OEA adjusted from –1.5 to –45 bar water potential.

Effects of carbon-nitrogen nutrition

Production of conidia and sclerotia was examined when the fungus was grown on OEA, with or without enrichment with nitrogen as $NaNO_3$ at 4 $g.l^{-1}$. These media were then supplemented with carbon as glucose at 0, 2, 4 or 6 $g.l^{-1}$. The cultures were grown at 18°C under a 14 h photoperiod of NUV radiation for 12 to 14 days.

Conidiation by *B. squamosa* on OEA enriched with 0 or 4 $g.l^{-1}$ nitrogen, using $NaNO_3$, and supplemented with 0, 2, 4 or 6 $g.l^{-1}$ carbon, using glucose, was stimulated in media containing both nutrients. On OEA alone 4.06×10^6 conidia per dish were produced, but with the addition of either 4 $g.l^{-1}$ nitrogen or 2 $g.l^{-1}$ carbon, conidiation was reduced to a mean of 2.6×10^6 or 3.05×10^6 conidia per dish, respectively, and conidiation decreased further with increasing supplements of carbon. On the addition of both 4 $g.l^{-1}$ nitrogen and 2 $g.l^{-1}$ carbon, conidiation increased to 4.63×10^6 conidia per dish, and continued to increase with greater supplements of carbon.

Production of sclerotia increased greatly from zero on OEA alone to 356.8 sclerotia per dish on OEA supplemented with 6 $g.l^{-1}$ carbon. Sclerotial production was depressed at all levels of supplemented carbon, in the presence of 4 g of nitrogen, compared to

treatments with equal carbon supplements but without a nitrogen enrichment. Increases in numbers of sclerotia with increases in carbon supply were less in the presence of nitrogen enrichment.

Because the addition of nitrogen using $NaNO_3$ also depressed the water potential of each of the OEA media, it was necessary to determine if $NaNO_3$ was acting merely as an osmoticum. Therefore, an experiment was devised in which OEA was either adjusted or not adjusted to –15 bar water potential using either $NaNO_3$, KCl, a 5:3:2 molar ratio mixture of NaCl:KCl:Na_2SO_4, or PEG 4000. All media were then supplemented with 2.5 $g.l^{-1}$ carbon as glucose. PEG 4000 containing medium was solidified with 1% Gelrite gellam gum because PEG 4000 inhibits solidification of standard laboratory agars.

In the presence of $NaNO_3$, sporulation was twice that in any other treatment but there were no significant differences in sporulation on unadjusted OEA and the media adjusted to –15 bar with KCl and the NaCl:KCl:Na_2SO_4 mixture. Sporulation was depressed significantly on the medium adjusted to –15 bar with PEG 4000. Sclerotial production was greatest when KCl was used.

These results provided evidence for a strong $NaNO_3$ effect on *B. squamosa* reproductive behaviour, most likely as a result of providing nitrate nitrogen, and not because of osmotic effects. When KCl was present in high concentration, sclerotial production was induced.

Conidial and sclerotial production were also studied on an enriched defined medium. The medium consisted of 15 g glucose (6 g carbon), 0.55 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.02 g $CaCl_2.2H_2O$, 0.02 g NaCl, 0.97 mg $FeCl_3.6H_2O$, 0.15 mg $MnSO_4.H_2O$, 0.88 mg $ZnSO_4.7H_2O$, 3 mg H_3BO_3, 2 mg $H_2MoO_4.4H_2O$, 4 mg $CuSO_4.5H_2O$, 20 g Bacto-Difco agar in 1 l deionized water. In these experiments which also concerned the role of carbon:nitrogen ratios on the reproductive behaviour of *B. squamosa*, $NaNO_3$ was used to provide 0, 1, 2, 3, 4, 5 or 6 $g.l^{-1}$ nitrogen. Culture media with 0 to 6 g of nitrogen per litre had water potentials of –2.4, –5.7, –9.2, –12.7, –16.4, –19.0 and –22.9 bar, respectively, and pH readings of 5.98, 5.87, 5.79, 5.72, 5.68, 5.63 and 5.60, respectively.

When the defined medium, containing 6 $g.l^{-1}$ carbon was supplemented with 0 to 6 $g.l^{-1}$ nitrogen, sclerotia were produced at high carbon:nitrogen ratios and conidia were most abundant at low carbon:nitrogen ratios. When the medium contained no nitrogen, conidia were not produced and sclerotia were minute, poorly delimited and only weakly pigmented. With 1 and 2 g. l^{-1} nitrogen and 6:1 and 3:1 carbon nitrogen ratios, conidiation was minimal while normal sclerotia were abundant. At 3 $g.l^{-1}$ nitrogen, a carbon:nitrogen ratio of 2:1, sclerotial production was sharply depressed while conidiation was stimulated. At 4, 5 and 6 $g.l^{-1}$ nitrogen, sclerotia were absent while conidiation increased to a maximum of 3.69×10^5 conidia per dish at 5 $g.l^{-1}$ nitrogen, and then decreased to 2.15×10^5 conidia per dish at 6 $g.l^{-1}$ nitrogen.

The effect of different cations associated with nitrate nitrogen on reproduction was also studied. OEA was used as the base medium and was supplemented with 4 $g.l^{-1}$ carbon as glucose for all treatments. The carbon-supplemented OEA was enriched with 4 $g.l^{-1}$ nitrogen using either $NaNO_3$, KNO_3 or $Ca(NO_3)_2.4H_2O$ (CNA = Calcium Nitrate Agar), or with it lacked nitrogen; a concentration of each cation equal to that provided with each nitrate source, respectively, using NaCl, KCl or $CaCl_2.2H_2O$ was provided. Two identical experiments with 3 x 2 factorial designs with three cations (Na^+, K^+ or Ca^{2+}) and two anions (NO_3^- or Cl^-) were conducted. The medium enriched with $Ca(NO_3)_2.4H_2O$ supported the greatest conidiation. Conidiophores were exceptionally elongated on the media enriched with Ca^{2+}. On the Na^+ and K^+ enriched media,

conidiophores were short with large terminal clusters when NO_3^- was used, but elongate with smaller but more numerous intercalary condial clusters when the Cl^- was used. The difference in sporulation on the $CaCl_2.2H_2O$ and $Ca(NO_3)_2.4H_2O$ enriched media was greater than for the NaCl and $NaNO_3$ or the KCl and KNO_3 enriched media, with extremely heavy conidial production on CNA. On this medium, a dense covering of conidiophores developed a reddish-brown coloration with 12.68 and 16.84 x 10^6 conidia produced per dish in trial 1 and trial 2, respectively. This was more than double the conidial production on any other treatment in each trial. Comparing the monovalent Na^+ and K^+ cations to the divalent Ca^{2+} cation, was significant (at P = 0.0001), providing further evidence for the strong conidiation stimulus provided by CNA. Sclerotial production in both trials was most numerous in the K^+ enriched media. In addition, on K^+ enriched media, sclerotia were mostly 1-1.5 mm in diameter, while on media enriched with either the Na^+ or Ca^{2+} cation, sclerotia were mostly slightly larger at 1-1.5 x 1.5-2 mm in diameter.

In conclusion, the two parts of the study gave the following results.

The first part of the overall study showed that NUV radiation stimulates the production of conidia of *B. squamosa in vitro* while moisture stress does not. Although *B. squamosa* produces conidia primarily on dried, necrotic foliage tissue in the field, it is unlikely that drying of the tissue per se is an important stimulus for conidiation.

For the second part of the overall study, the following relationships were revealed. (1) On the variously supplemented OEA, carbon and nitrogen, provided as glucose and $NaNO_3$ interacted in a manner that when both nutrients were provided at favourable levels (high carbon, high nitrogen = low carbon:nitrogen ratios) an increase in sporulation by *B. squamosa* occurred. Conversely, on amended OEA, sclerotial production was inhibited by $NaNO_3$ supplements at all levels of carbon. (2) Similarly on the defined medium at high carbon:nitrogen ratios, sclerotial production was favoured, while at low ratios, conidiation was favoured. (3) The Ca^{2+} cation specifically interacted with nitrate nitrogen to result in the highest levels of conidiation. The K^+ cation favoured sclerotial formation by *B. squamosa*.

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Detection of *Botrytis cinerea* on gerbera flowers using monoclonal antibodies

J. Salinas and A. Schots

Summary

Monoclonal antibodies (MAbs) have been developed against whole conidia and extracellular proteins from conidia of *B. cinerea*. Both types of MAbs react in an immunofluorescence test. The MAbs show no reaction with healthy gerbera flowers and spores from other common airborne fungi. The reactivity with other *Botrytis* species has been tested. More than 75% of conidia from ten selected isolates of *B. cinerea* react in the immunofluorescence test. On the basis of these results a routine test will be developed for the detection of *B. cinerea* conidia on gerbera flowers.

Introduction

Botrytis cinerea Pers.: Fr. has become an important limiting factor for the production and export of gerbera flowers in the Netherlands. Conidia produced by the fungus spread easily through the air. After landing on the flowers, conidia remain dormant until a thin water film, for instance through condensation, is available for conidial germination and flowers become infected within a few hours (Salinas *et al.*, 1989). Especially in autumn and in spring, lesions caused by the fungus lead to serious economic losses (Bakker, 1986). Symptoms can be visible in greenhouses during the growth of the flowers, but can also develop during storage, transport and shipment of the flowers, when changes in temperature occur leading to high humidity conditions and subsequent infection when conidia of *B. cinerea* are present (Salinas *et al.*, 1989; Verberkt, 1986). Therefore, a serological test is now being developed based on specific monoclonal antibodies to monitor the presence of dormant conidia on flowers at any stage of production and handling.

Material and Methods

Fungal cultures

Isolates of *B. cinerea* were obtained from field infected plants (Table 1). The isolates were kept as sporulating cultures on X-medium at 4°C in darkness (Salinas *et al.*, 1989). Dry conidia were harvested from fungal cultures grown on tomato agar (Salinas, 1992).

Antigen preparation and immunisation

Two sources of antigen were used. Whole conidia were washed eight times with 20 mM piperazine-HCl, pH 6.0 to ensure that most of the extracellular proteins were removed. Mice were injected four times, with 4 week-intervals, using about 5.10^6 conidia in PBS

Table 1. Isolates of Botrytis cinerea used in the experiments.

Isolate number	Isolated from
Bc- 7	tomato
Bc- 8	taxus
Bc- 9	carnation
Bc-10	cyclamen
Bc-12	gerbera
Bc-13	gerbera
Bc-14	gerbera
Bc-15	gerbera
Bc-18	gerbera
Bc-25	rose

using Freunds Incomplete Adjuvant (FIA) for the first three immunisations and no adjuvant for the last immunisation. The supernatant from the first wash step, containing most of the extracellular proteins, was concentrated on an Amicon YM10 ultrafilter until a protein concentration of 1 mg.ml^{-1} was reached. Mice were immunised, four times with 4 week-intervals, with 100 μg of these extracellular proteins in PBS. For the first immunisation Freunds Complete Adjuvant was used, for the second and third immunisation FIA, while adjuvant was omitted for the last immunisation.

Production of monoclonal antibodies

Monoclonal antibodies were produced as described by Schots *et al.* (1992).

Immunofluorescence

Millipore Multi Screen-HV 96-well filtration plates, pore size 0.45 μm, were blocked using 5% donor horse serum in PBS + 0.1% Tween 20 (PBST) for two hours at 37°C. The plates were washed four times with PBST whereafter 100 μl of a suspension of conidia (5. 10^6 conidia.ml^{-1} in PBS) and 100 μl of hybridoma culture supernatant was added to each well. The plate was incubated for 1 h at 37°C. The plate was washed four times with PBST. Subsequently 50 μl of a goat anti mouse IgG(H+L)-FITC conjugate (Sigma), diluted 1:100 in PBST + 0.1% BSA was added to each well and incubated for 1 hr. at 37°C. The plate was washed twice with PBST and once with ultrapure water; 15 μl antiquench solution (Johnson and Nogueira Araujo, 1981) were added to each well, the conidia were resuspended and 2 μl from each well was taken and put on a 24 well multi test slide (Nutacon). Subsequently, the reaction of the conidia was monitored using a fluorescence microscope.

Isotype determination

The subclass of the immunoglobulins produced by the hybridomas was determined using an ELISA as described by Schots *et al.* (1992).

Protein concentration

Protein concentrations of the extracellular proteins were determined according to Bradford (1976) using bovine serum albumin as a standard.

Results and Discussion

Fusion experiments carried out with splenocytes from mice immunised with whole conidia or extracellular proteins resulted in eight hybridomas producing monoclonal antibodies (MAbs, Table 2) which were reactive in the immunofluorescence assay (Fig. 1 and 2). To ensure that we would only obtain MAbs which were specific for the genus *Botrytis* we used a mixture of spores from saprophytic fungi isolated in greenhouses (*Penicillium* spp., *Aspergillus* spp., *Alternaria* spp., *Cladosporium* spp., *Trichoderma* spp. and *Mucor* spp.) as negative controls. None of the MAbs reacted with spores from these fungi (Table 2). A further characterisation was carried out with conidia from other *Botrytis* spp. and healthy flowers. Several MAbs (Table 2) reacted with conidia from *B. aclada* and *B. squamosa*. However, the reaction pattern with conidia from *B. squamosa* differed considerably from that observed with *B. cinerea* as only patches on the surface of the conidia fluoresced (Fig. 2) and not all MAbs reacted with this *Botrytis* species. No reaction of the eight MAbs was observed with healthy gerbera flowers.

The mice used in these fusion experiments were immunised with a mixture of conidia or extracellular proteins extracted from conidia of ten *B. cinerea* isolates (Table 1). This was done to avoid too specific MAbs, as was observed by Hardham *et al.* (1986) with MAbs against zoospores and cysts of *Phytophthora cinnamomi*. Indeed, all our MAbs

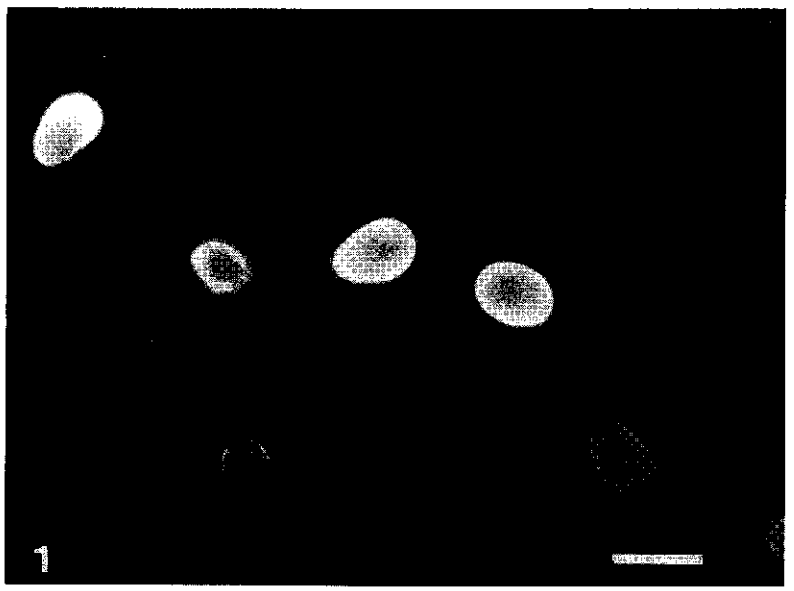

Fig. 1. Reaction of Botrytis cinerea conidia with monoclonal antibody 9E11. The immunoreaction was visualised using a goat anti-mouse-FITC-cojugate. Bar = 10 µm.

reacted with conidia from the ten isolates used. In most cases at least 75%, and often more than 90%, of the conidia were positive (Table 3). The absence of a reaction can be a consequence of either changes in the cell wall or disappearance of the extracellular proteins from dead or dying conidia.

Most of the prerequisites for the development of a routine test have been met. The MAbs did not react with other fungi and microorganisms generally present in greenhouses (data not shown). Also, no reaction with tissue from gerbera flowers was observed. The final prerequisite is that MAbs have to react with all *B. cinerea* isolates to avoid false negatives. Concerning this point the reaction was quantified with conidia from isolates used for immunisation only. A test with other isolates is presently in progress.

A routine test based on monoclonal antibodies can replace tests based on selective media (Kerssies, 1990) or the use of polyclonal antisera. Polyclonal antisera (e.g. Ricker *et al.*, 1991) are not specific, while it has often been shown that cross absorption to improve specificity, decreases sensitivity dramatically. Disadvantages of the use of selective media are that most selective media are not completely selective and working with such media is usually rather time consuming. Furthermore, some taxonomical knowledge is required. A test based on MAbs is specific and can be performed within a few hours and the reaction pattern of the MAb used, concerning specificity and sensitivity, is exactly known and will not vary.

The test for the detection of *B. cinerea* on gerbera flowers will be based on the immunofluorescence assay described in this paper. The advantages of an immunofluorescence assay are the sensitivity and the specificity; even a single conidium can be detected. Surprises which can occur because of presence of cross-reacting spores of other fungi, which structure and reaction pattern most likely differ from conidia of *B. cinerea*,

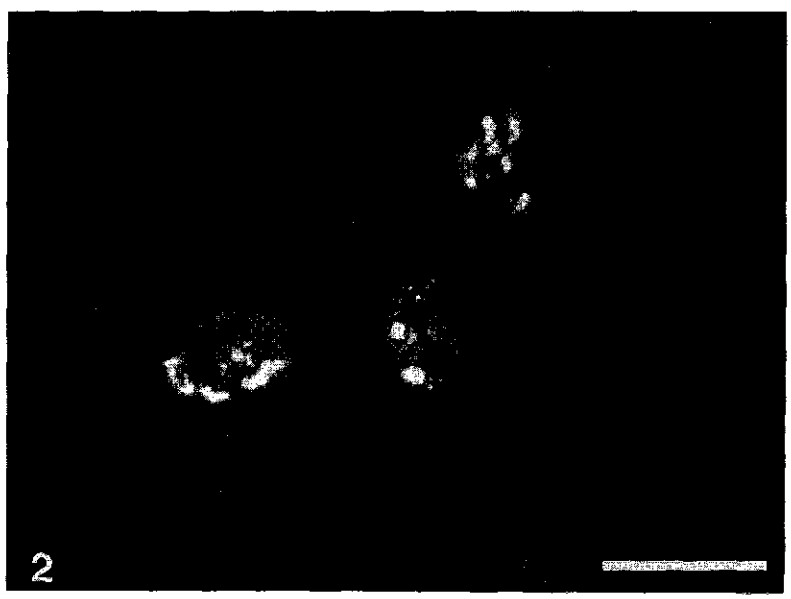

Fig. 2. Reaction of Botrytis squamosa conidia with monoclonal antibody 4H10. The immunoreaction was visualised using a goat anti-mouse-FITC-cojugate. Bar = 20 µm

Table 2. Immunofluorescence reactions of monoclonal antibodies with saprophytes, B. aclada and B. squamosa.

Antibody	Isotype	Negative control[1]			*B. aclada*[2]	*B. squamosa*[3]
		1-4-'91	1-10-'91	1-1-'92		
9E11	IgG1	–[4]	–	–	+[5]	–
4H10	IgM	–	–	–	–	+
4A3	IgM	–	–	n.d.[6]	+	n.d.
9C7	IgM	–	–	n.d.	+	n.d.
10G1	IgM	–	+	n.d.	+	n.d.
7B9	IgM	–	–	n.d.	–	n.d.
9A12	n.d.	–	–	n.d.	+	n.d.
2G11	IgM	–	–	n.d.	+	n.d.

[1]) mixture of saprophytes isolated from gerbera plants in the 91-92 season.
[2]) mixture of four *B. aclada* isolates
[3]) mixture of two *B. squamosa* isolates
[4]) no visible immunofluoroscence
[5]) immunofluorescence visible
[6]) not done

Table 3. Immunofluorescence reactions of monoclonal antibodies with ten different isolates of B. cinerea.

Antibody	Isolates									
	Bc7	Bc8	Bc13	Bc15	Bc16	Bc17	Bc21	Bc25	Bc27	Bc29
4H10	99[1]	67	83	70	99	99	70	80	95	80
9E11	83	99	74	99	99	96	89	86	99	99
4A3	82	70	99	99	99	94	70	75	80	99
9C7	93	42	54	99	99	99	99	99	90	99
10G1	99	79	93	99	91	75	99	90	99	99
7B9	79	75	75	79	99	73	99	90	92	91
9A12	91	50	80	56	86	92	80	78	57	58
2G11	99	73	50	50	90	90	89	80	80	80

[1]) percentage of conidia showing fluorescence

are excluded. Trials of this test are presently in progress using gerbera flowers obtained from a flower auction. Preliminary results show that more than 90% of the conidia of *B. cinerea* on infected flowers can be washed off and reacted positive in the immunofluorescence assay.

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The effect of gamma irradiation on *Botrytis cinerea* and *B. aclada* causing rot of pear and onion respectively

M. Gürer and O. Tiryaki

Summary

Doses of 3 kGy delayed rotting of pear cv. Ankara caused by *Botrytis cinerea*, but failed to inhibit the process completely. Controls developed rots with an average diameter of 25.6 mm, in irradiated rots pears had an average diameter of 13.2 mm after irradiation with 3 kGy 10 days after inoculation. Irradiation of *B. cinerea* with 3 kGy *in vitro* caused heterogenous sporulation and mycelial growth. Similar results were obtained with *B. aclada* in onions.

Introduction

Botrytis cinerea Pers.: Fr. on pear fruits and *Botrytis aclada* Walker on onion bulbs are the main causes of storage rot in Turkey. Nelson *et al.* (1959) stated that gamma irradiation of the doses of 1.10^5 rep and 2.10^5 rep had a fungistatic effect on *B. cinerea* of grape, while with doses of 4.10^5 rep (= 3.7 kGy) or higher fungicidal effects occurred.

Tiryaki (1990) described the sensitivity of a number of fungi to gamma irradiation *in vitro* at 3-4°C and *B. cinerea* was the most sensitive of the fungi tested.

Material and Methods

B. cinerea was isolated from pear cv. Ankara, and *B. aclada* was isolated from onion bulbs and these fungi were cultured on potato dextrose agar (PDA, Difco) for irradiation studies. A ^{60}Co gamma irradiator (10 kGy) was used in all experiments.

Agar plates inoculated with *B. cinerea* and *B. aclada* were incubated at 22±1°C under fluorescent light during a 12 h photoperiod for 7 days. Actively growing cultures were irradiated with 1, 2 and 3 kGy and then 6 mm agar disks removed and transferred to fresh PDA. Agar plates inoculated with *B. cinerea* were incubated at 3-4°C after pre-incubation at 23°C for 4 h, while *B. aclada* was incubated at 22±1°C. These exposure treatments were replicated four times for each isolate. The diameter of colonies was measured and the most effective irradiation dose determined after 7 days.

For *in vivo* studies, fruits were surface-sterilised with 0.5% NaOCl for 2 to 3 min and rinsed with water. Two tissue disks with a diameter of 6 mm were removed from either side of each fruit. The holes were filled with disks of 7-day-old cultures of *B. cinerea*. After incubation at 23°C for 21 h (Beraha *et al.*, 1959) fruits were exposed to 1, 2 and 3 kGy irradiation and stored at 0°C and 85-90% relative humidity for 60 days. The diameter of rot on each fruit was measured and the most effective dose determined. The same inoculation method was used for *B. aclada* on onion bulbs. To test the possible long-term effects of irradiation on pathogenicity of *B. aclada*, 7-day-old culture of the fungus irradiated previously with the most effective dose *in vitro*, was used as inoculum and

compared to non-irradiated inoculum. Inoculated onion bulbs were covered with polyethylene bags and incubated at 22±1°C. After 7 days incubation, the lesion diameters were measured. Ten fruits or bulbs were used in each of the three irradiation and inoculation treatments in these experiments.

Results and Discussion

After irradiation with 3 kGy, *B. cinerea* showed initially no growth after 7 days incubation at 3-4°C, compared to 24.5 mm in the control, *B. aclada* was much less affected, even at 3 kGy (Table 1). These results were similar to those of Barkai-Golan *et al.* (1967). Nelson *et al.* (1959) reported that mycelial growth of *B. cinerea* irradiated with the equivalent of 3.7 kGy, was slight after 12 days at 3 to 4°C whereas mycelial growth was inhibited completely with the equivalent dose of 7.4 kGy.

Table 1. Colony diameters of irradiated and non-irradiated cultures of B. cinerea and B. aclada on PDA 7 days after inoculation.

Irradiation dose (kGy)	Colony diameter (mm) *B. cinerea*	*B. aclada*
0	24.50 a*)	63.5 a
1	7.00 b	60.0 ab
2	6.75 b	56.0 bc
3	6.00 b	53.0 c

*) figures followed by different letters differ significantly at $p < 0.001$

Table 2. Development of mould on pear fruits wound-inoculated with B. cinerea, exposed to irradiation doses and cold-stored.

Irradiation dose (kGy)	Rot diameter (mm) Days after irradiation				
	10	20	30	35	40
0	25.63 a*	35.04 a	40.58 a	46.29 b	48.58 b
1	22.83 b	34.54 a	49.46 a	53.33 a	57.21 a
2	15.79 c	25.88 b	43.58 b	50.63 ab	59.04 a
3	13.21 d	20.88 c	32.13 c	37.54 c	44.54 b

* figures followed by different letters differ significantly at $p < 0.001$

Irradiation (1 kGy) stimulated sporulation of *B. cinerea*.

B. cinerea re-isolated from pears after irradiation with 3 kGy, showed atypical sporulation and mycelial growth. The most inhibitory dose for pear rot after 10 days was 3 kGy (Table 2).

The most inhibitory irradiation dose for mycelial growth of *B. aclada* was 3 kGy but irradiated *B. aclada* isolates still caused rot of onions, viz. irradiated 27.6 mm and unirradiated, 31.4 mm.

Nelson *et al.* (1959) observed that the mycelial growth of *B. cinerea* did not start until 12 days after irradiation , but we found pronounced effects after 10 days (Table 2). Our results were similar as those described by Behara *et al.* (1960).

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Impedance mycology, a new rapid method for the evaluation of metabolic activity of *Botrytis cinerea* and *B. aclada* as influenced by environmental factors

P.V. Nielsen and J. Kampp

Summary

Studies of spoiling capacity of fungi as affected by environmental factors, such as substrate composition, temperature, humidity and atmosphere, can advantageously be performed by impedance mycology, as this method is easy, rapid and produces information unattainable by traditional methods. Results from a study of the effect of storage conditions on growth of *Botrytis* sp. are given.

Introduction

The use of rapid methods in microbiology has increased substantially during the last two decades, especially in the area of clinical and food microbiology. The objectives of these methods have been to obtain fast and reliable results in clinical diagnosis and in control during food manufacturing. These methods are now being used in bacteriological and mycological research, to obtain new infomation which would not have been possible by traditional methods.

Impedance mycology is probably the most promising new method for studies of the effect of environmental factors on fungal growth. The method is based on the measurement of metabolic activity rather than secondary characteristics such as colony diameter, mycelial dry weight or inhibition zones. The method is simple, rapid and a high number of tests can be performed simultaneously.

The technique has been established as a tool for rapid detection of bacterial contamination of a wide range of foods, and now it is entering the disciplines of food technology and general mycology. Besides the fundamental work by Williams and Wood (1986), most emphasis has been directed towards detection of yeasts in fruit mixtures (Fleischer *et al.*, 1984), orange juice (Zindulis, 1984) and wine (Henschke and Thomas, 1988). A recent study of impedance changes induced by moulds causing food spoilage resulted in the development of a medium for general impedimetric examination of food products (Watson-Craik *et al.*, 1990).

This paper describes the principles of impedance mycology and then gives the results of a study of the combined effect of temperature, humidity and controlled atmosphere storage on growth of *B. cinerea* and *B. aclada.*

Results and Discussion

Microbial metabolism alters the electrical properties of the growth medium as large molecules are degraded, other excreted and some substrate components assimilated from the medium. These electrical changes are monitored by an impedimeter by sending an

alternating electrical current through the medium and measuring the impedance this flow encounters. Impedance (Z) is the resistance towards flow of an electrical current in the substrate. It consists of two components, conductance (G) which is associated with the mobility and number of ions in the substrate, and capacitance (C) which is associated with changes in close proximity to the electrode. A detailed explanation of this is given by Firstenberg-Eden and Eden (1984).

Several studies have shown that the capacitance signal is superior to conductance and impedance for monitoring fungal growth , whereas conductance is preferable for most bacterial studies (Nielsen, 1992; Watson-Craik *et al.*, 1989; Zindulis, 1984).

The impedance readings are taken by the Bactometer and presented as curves showing the change in impedance with time (Fig. 1). Detection time for growth (DT) is recorded when a significant change in impedance has occurred.

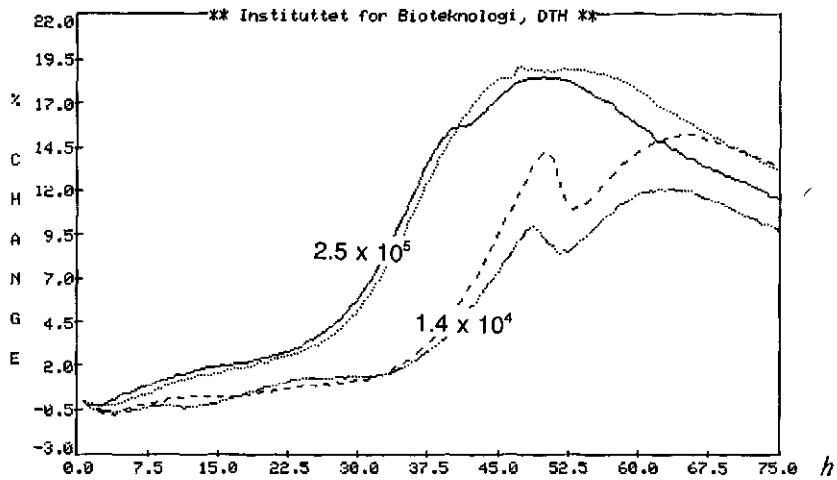

Fig. 1. Impedence values obtained when Botrytis cinerea isolate IBT 7036 from an Italian peach was cultured on PYG, a medium specially designed for the Bactometer. PYG contains 10.0 g potassium phosphate; 7.5 g yeast extract; 30.0 g glucose and 20.0 g agar per 1000 ml distilled water.

Within a certain range of the initial spore number (10^2 - 10^6 cfu.measuring cell^{-1}) a good correlation exists between log(cfu) and the detection time (DT), as shown in Fig. 2. This relationship is species-specific due to differences in growth rate (Nielsen, 1991). The metabolic growth rate, μ, may be calculated from the slope of the right line portion of the curve. Growth rate: $\mu = 1n(10) \cdot \Delta log(cfu)/\Delta DT\text{-}2{,}30/\alpha$.

Low numbers of cfu result in increased scattering, while detection at higher levels will occur immediately after germination. The curve will level off and the detection time converge to a common point as seen in Fig. 2. Consequently an estimate of the length of lag phase is the intercept between regression line and the line log(cfu) = 7.0

As part of a study of the role of storage conditions and antagonists on the keeping quality of onions, apples and carrots in Denmark, the combined effect of temperature and humidity, controlled atmospheres on growth of *B. cinerea* and *B. aclada* was studied. The experimental domain was: temperature (5-25°C), humidity (a_w 0.970-0.996), O_2 (1-

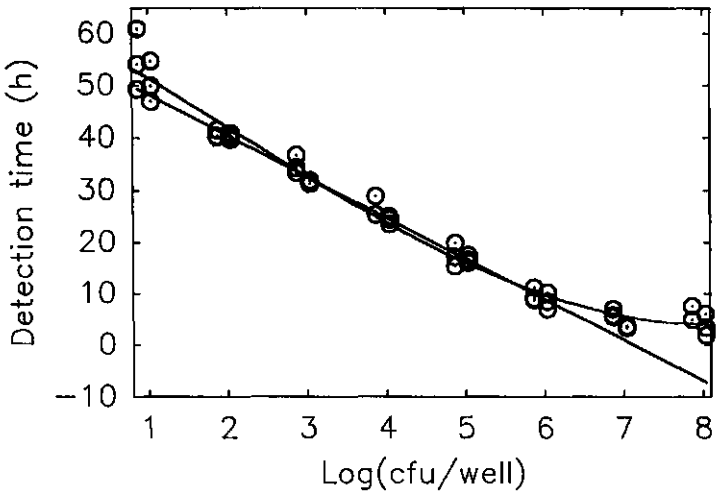

Fig. 2. Relationship between detection times (h) and the logarithm of colony forming units (cfu).

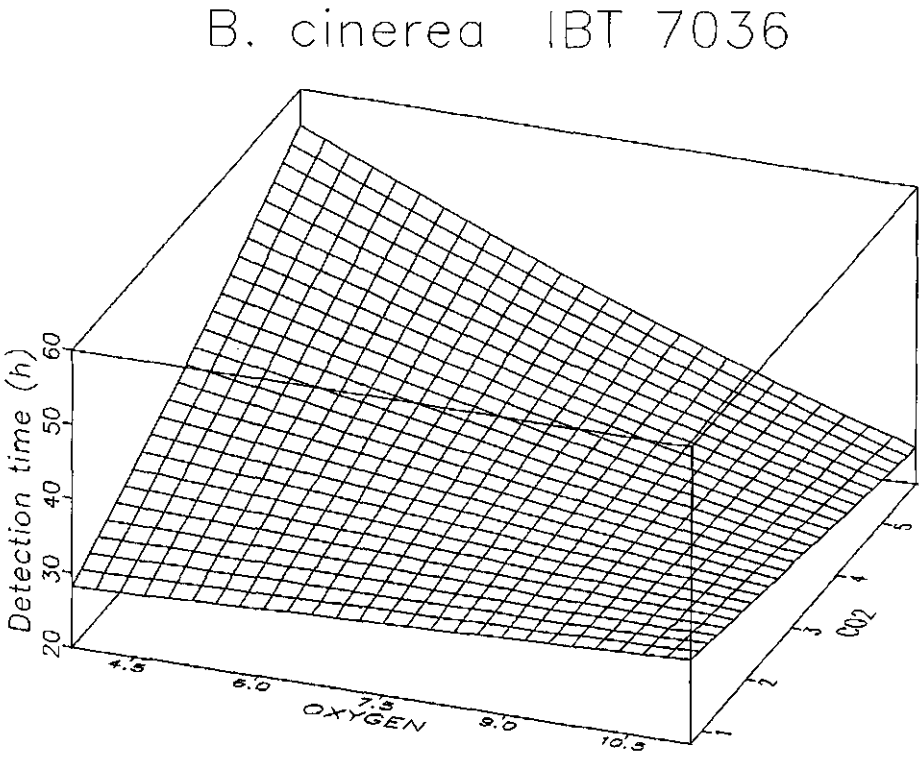

Fig. 3. Response surface plot of detection time for growth of B. cinerea IBT 7036 cultures on PYG, a_w 0.983, pH 5.0 at 19.2°C. The multiple linear regression is based on 30 measurements, and $R^2 = 0.93$.

21%), CO_2 (0-10%), N_2 (balanced), pH (3-6.5). For these studies, a special chamber was developed.

The response surface plot in Fig. 3 shows the predicted values of detection time for *B. cinerea* isolate IBT 7036 cultured on phosphate yeast glucose agar (PYG), at 19.2°C with water activity a_w 0.983 at pH 5.0.

Other results from this study (data not shown) indicated that *B. aclada* is much more inhibited by decreased water activity. As a linear relationship existed between the logarithm to cfu and detection time, a response surface model should detect if the fungal growth rate was affected by the factors examined. In this experiment growth rates were generally not affected; only that of *B. aclada* was somewhat decreased when the oxygen level was decreased. Consequently the difference in detection time is primarily due to changes in lag time.

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Nuclear magnetic resonance (NMR) microimaging of soft fruits infected by *Botrytis cinerea*

B. Williamson, B.A. Goodman, J.A. Chudek and D.J. Johnston

Summary

Healthy tissue and that infected by *B. cinerea* have been distinguished in both raspberry and blackcurrant fruits by NMR microimaging using a gradient echo pulse sequence. It was possible to follow non-invasively the progressive development of infection in both fruits over a 4-day period.

Introduction

Fruits of red raspberry (*Rubus idaeus* L.) and blackcurrant (*Ribes nigrum* L.) are prone to grey mould caused by *Botrytis cinerea* Pers.: Fr. (Williamson *et al.*, 1987; McNicol and Williamson, 1989; McNicol *et al.*, 1990). Mature fruits are particularly difficult subjects for conventional histological studies; artifacts caused during fixation, dehydration and embedding occur because the mesocarp consists mostly of extremely large, thin-walled parenchyma cells which surround the tough endocarp of lignified sclereids. The tissue changes associated with the onset of disease caused by release of fungal enzymes, toxins and organic acids further exacerbate attempts to visualise events as infection proceeds. NMR micro-imaging now offers an alternative approach to the study of infected fruits.

The recent development of microimaging attachments for conventional 'high field' NMR spectrometers (≥ 7 T field strength) has made it possible to perform non-invasive histology in plants and fruits (e.g. Walter *et al.*, 1989; Bowtell *et al.*, 1990; Williamson *et al.*, 1992).

By making the correct choice of imaging pulse sequence and experimental parameters, it is possible to discriminate between water molecules in different chemical or structural environments (Wehrli *et al.*, 1988; Faust *et al.*, 1991). This paper describes how the technique can be used to follow infection of raspberry and blackcurrant fruits by *B. cinerea*. Part of this work has been reported briefly elsewhere (Goodman *et al.*, 1992).

Material and methods

Inoculation of fruits

Glasshouse-grown fruits of raspberry cv. Autumn Bliss and blackcurrant cv. Ben Alder were harvested by cutting the pedicel. Single fruits were incubated at high RH in polyethylene boxes at 20°C before and after inoculation to follow the progress of infection by NMR imaging.

An inadequately fertilized raspberry fruit, which developed only three drupelets attached to the central receptacle, provided material for initial inoculations. One drupelet

was inoculated with dry conidia from a culture of *B. cinerea* isolate 347 grown on medium-X plus 10% sucrose (Harrison, 1978); one drupelet was pierced with a sterile needle as a wound control; and the third was left intact as a healthy control.

In a completely fertilized ripe raspberry consisting of c. 100 drupelets, a single drupelet was similarly wound-inoculated and the fruit examined over 4 days.

A ripe blackcurrant was wound-inoculated in the median transverse plane (equatorial) and left until it was completely mouldy and then examined in comparison to that of a healthy unwounded fruit of the same age.

Another ripe blackcurrant was wound-inoculated similarly and used for a time course study of the infection.

NMR microimaging

NMR images were acquired using a Bruker microimaging unit fitted with a 25 mm coil attached to a Bruker AM300/WB Fourier Transforms pectrometer (7.2 T; 300 MHz 1H). Data were collected with a standard gradient echo sequence as 256 x 512 unit matrices, which were transformed into 256 x 256 pixel images. Accumulation parameters for the raspberry measurements were: pulse angle, 10° (Hermit e pulse); time of echo, 13.24 ms; repetition time 220 ms; dephasing gradient, -0,3 mT/cm; read gradient, 0.3 mT/cm; phase encoding gradient, 0.25 mT/cm (2 x 10^{-3} mT/cm increment); slice select gradient, 0.2 mT/cm. These yielded pixel dimensions of 70 x 70 mm with a slice thickness of 500 μm. Thirty-two accumulations were used for the generation of each image, which thus

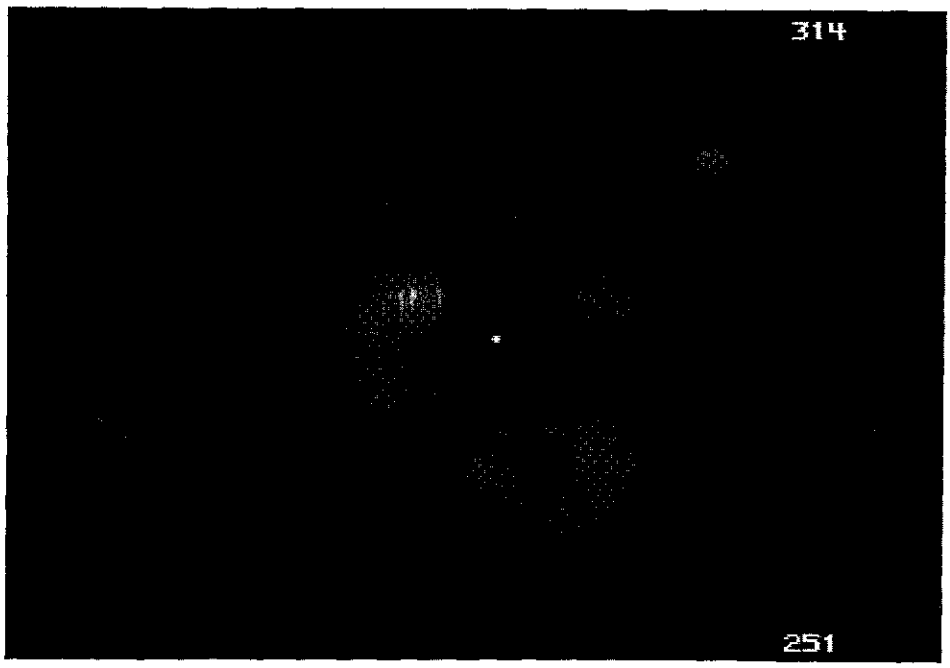
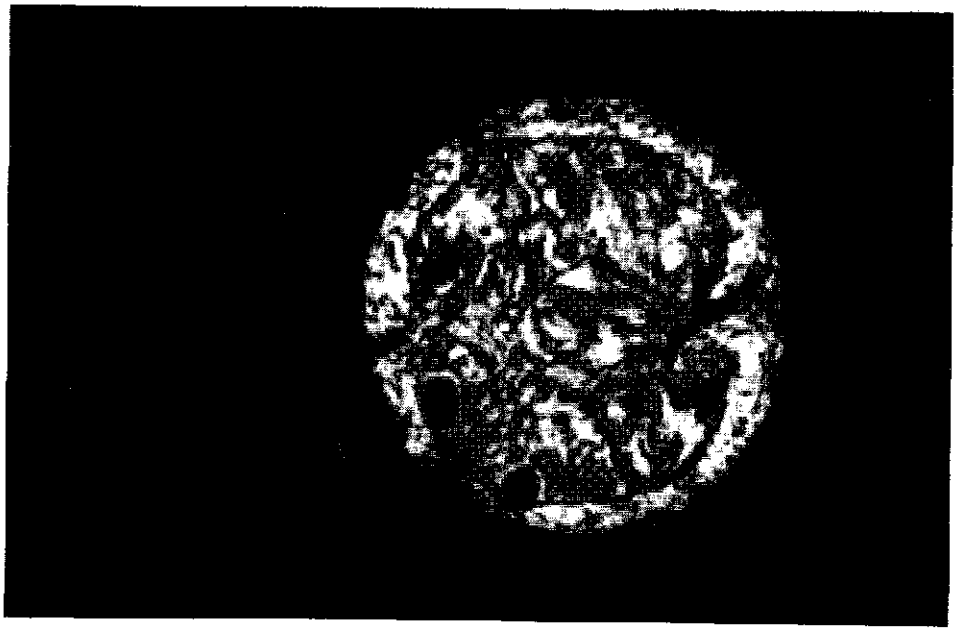


Fig. 1. Gradient echo NMR image of a three-drupelet raspberry fruit.
Drupelet A was inoculated with conidia in a single wound; drupelet B was wounded but not inoculated; drupelet C was a healthy unwounded control.



a

b

Fig. 2. Gradient echo NMR images of blackcurrant fruit.
(a) Healthy
(b) Badly infected with *B. cinerea*

required approximately 30 min for total acquisition. Accumulation parameters for the blackcurrant measurements were: dephasing gradient, 0.28 mT/cm; read gradient, 0.28 mT/cm; phase encoding gradient, 0.21 mT/cm (1.8×10^{-3} mT/cm increment); slice select gradient, 0.18 mT/cm; all other parameters were the same as for the raspberry measurements. These produced pixel dimensions of 60 x 60 μm and a slice thickness of 300 μm.

Results and Discussion

In NMR images of healthy raspberry fruit using a gradient echo pulse sequence, pronounced radial striations are observed in the mesocarp (Williamson *et al.*, 1992). Similar structures have been explained by Bowtell *et al.* (1990) as being caused by the large differences in magnetic susceptibility that exist at the junctions between the water-filled cells and the gas-filled intercellular spaces. The NMR image of the undamaged and wounded drupelets in the three-drupelet raspberry fruit showed similar structure, where as this was completely lost from the infected drupelet (Fig. 1).

NMR images produced from a single transverse slice of a fully-formed raspberry fruit in the plane of the wound inoculation showed boundaries between the infected and uninfected tissue with a steady movement of these boundaries across the fruit during a 4-day period. Even after 4 days there was a distinct boundary between infected and uninfected tissue. This experiment was reported briefly by Goodman *et al.* (1992), who confirmed from histological studies that the boundaries between striated and non-striated components in the NMR images correspond to the limits of progression of the internal fungal mycelium.

A similar set of experiments was performed with blackcurrant fruits and images from healthy and severely infected fruits are shown in Fig. 2. The ovary wall, the parenchyma derived from the placenta and the seeds were clearly delimited in the healthy fruit. Each seed was surrounded by a bright layer which corresponded to the gelatinous epidermis of the seed coat. Dark fibrillar structure was present throughout the parenchyma of the healthy fruit, but was absent from the images of the infected fruit.

In the final blackcurrant experiment, images from eight slices were acquired simultaneously, so that the development could be followed essentially in three dimensions. As with raspberries, the spread of infection could be seen clearly over a 4-day period, but the boundaries between infected and uninfected tissue were less well defined.

Conclusions

NMR microimaging represents a powerful method for non-invasive observation of the development of plant disease processes and promises to facilitate significant advances in understanding disease processes in plants at the tissue level.

Acknowledgements

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EPIDEMIOLOGY

Epidemiology of grey mould, caused by *Botrytis cinerea* in vegetable greenhouses

Y. Elad, D. Shtienberg, H. Yunis and Y. Mahrer

Summary

Control of grey mould depends on fungicide applications, because severe epidemics can be expected when conditions in greenhouses favour the disease. Microclimatic conditions which affect epidemics were characterised in unheated polyethylene greenhouses to investigate the possible introduction of non-chemical control methods for use with cucumber crops. From 14 to 9 days before the appearance of symptoms, plants were predisposed to the disease by low (<9°C) and high (>24°C) temperatures and by dryness. Infection occurred 7-8 days before symptoms were visible and was promoted by high humidity (>91% r.h.) and temperatures in the range of 9-24°C. After infection took place, the infective mycelium was relatively protected by the host tissue and temperature and r.h. requirements were less stringent. A model based on qualitative analysis of the relationships between microclimatic factors and fruit or stem infections was developed. The two important parameters associated with outbreaks of epidemics were the duration of leaf wetness and the duration of night time temperatures between 9° and 21°C. The daily averages for the thresholds were 7 h per day for the wet period and 9.5 h per day for the duration of specific temperatures.

The nutritional and hormonal status of host plants also affected their susceptibility to development of grey mould epidemics. High humidity restricted movement of calcium to upper plant parts and calcium enrichment of host tissue reduced the susceptibility of pepper, tomato and eggplant to grey mould. Application of auxin to enhance fruit setting of eggplant reduced their susceptibility to the disease.

In order to reduce dependence on fungicides to which the pathogen has become resistant, alternation with the biocontrol agent *Trichoderma harzianum* with fungicide is suggested. This method should reduce exposure of the pathogens' population to fungicides. The survival of *B. cinerea* in infected stems and the effect of grey mould on cucumber production were studied during high summer temperatures. The outcome of these epidemiological studies has been the introduction of a workable control programme for the integration of non-fungicidal measures into the production system to reduce the proportion of fungicide-resistant isolates and to make better use of available chemicals.

Introduction

In warm regions production of many vegetable crops can be done in non-heated greenhouses during winter. Parthenocarpic cultivars of cucumber (*Cucumis sativus* L.), tomato (*Lycopersicon esculentum* Mill.), pepper (*Capsicum annuum* L.) and eggplant (*Sola num melongena* L.) are examples of these crops and they are all seriously affected by grey mould caused by *Botrytis cinerea* Pers.: Fr. Control of grey mould in vegetable crops depends on frequent fungicide applications, but resistance of the causal agent to common

fungicides is widespread. At the beginning of the 1980s resistance of the *B. cinerea* population to benzimidazoles was already widespread in Israel, and resistance to dicarboximide fungicides had been identified in some greenhouses (Katan, 1982). In recent years resistance to dicarboximides became common (Yunis and Elad, 1989). The mixture of diethofencarb with carbendazim was tested recently for grey mould control, but unfortunately resistance was already found (Katan *et al.*, 1989). Since resistance to currently available fungicides is common in greenhouses, alternative methods to suppress *B. cinerea* are of great importance to commercial growers.

Microclimatic conditions which affect grey mould

Information on the effect of microclimatic conditions on grey mould is available from many studies with various crops, but it is highly variable. Hunter *et al.* (1972) found that infection of racemes of macadamia by *B. cinerea* was correlated with duration of leaf wetness at temperatures between 18° and 22°C or >95% r.h.. Jarvis (1980) reported that optimum temperatures favouring infection of other hosts were between 15° and 20°C, but infection could occur even at 2°C. Similar ranges of temperatures suitable for grey mould development were reported for flower crops, but the r.h. requirements varied in the different studies. For gerbera and rose flowers the optimal r.h. was >95%, but disease developed on the flowers at 80% r.h. (Marois *et al.*, 1988; Elad, 1989; Salinas *et al.*, 1989).

In Israel non-heated growth structures are covered with a new type of polyethylene covers which are opaque to infrared (IR-thermal) radiation; differences between night time leaf and air temperatures are now usually smaller than under the polyethylene cover previously used (Elad *et al.*, 1988). It was suggested that high temperatures, near the optimum for development of grey mould, compensate for the reduced duration of wetness.

Microclimatic parameters were monitored in cucumber crops grown in non-heated greenhouses during the winter of 1987/88. Air and leaf temperatures, r.h., rainfall, and radiation, were recorded hourly by C21X data loggers (Campbell Scientific Inc., Logan, UT, USA). Air and leaf temperatures were measured using copper-constantan thermocouples and r.h. with PCRC-11 electro-humidity sensors (Yunis *et al.*, 1990). The 1987/88 winter season was characterised by a relatively large number of rainy days. In the greenhouses r.h. is usually high for several hours of the day, depending on outside humidity, e.g. during and after rain showers. Air temperatures inside the greenhouses ranged between 3° and 38°C and exceeded the outside temperature by 2-10°C throughout the day. At night, air temperatures inside the greenhouse were equal to 1°C or higher than those outside. Location, orientation and position of the main entrance dictated the conditions inside each greenhouse. Persistance of temperature in defined ranges, r.h. in certain ranges, and the leaf wetness were calculated. Multiple regression and correlation analyses (according to R^2 values) between these temperatures, r.h. and wetness duration parameters in certain ranges, and disease incidence (square root transformed), were conducted using SAS procedures.

Disease was characterised by two stages, according to the rate of its development and the microclimatic conditions influencing it. In the first phase of the epidemic, a high correlation was found between infected fruits and air temperature in the range of 11-25°C, and r.h. in the range of 97-100% or leaf wetness. In the second phase, disease incidence was better correlated with air temperature in the range of 11-16°C and r.h.

Table 1. Ranges of microclimatic parameters having a significant ($P<0.05$) effect on cucumber grey mould in winter.

Days before disease assessment	Ranges of microclimatic variables		R^2	
	Temp (°C)	r.h. (%)	1989	1990
FRUIT INFECTION		Disease establishment period		
1 - 2	9-18	61-91	0.8415	0.9234
2 - 4	18-21		0.9568	
4 - 5		76-82		0.8912
6 - 7	9-24	>91	0.9734	0.8683
5 - 8	>24	79-85	–0.8929	–0.8928
10 - 14	27-30	61-85	0.9127	0.8378
		Epidemic outbreak period		
7 - 9	15-21	79-94	0.8489	0.8277
9 - 12	24-30	61-94	0.8511	0.9296
11 - 14	0-9	82-88	0.8699	0.8528
		Epidemic decline period		
3 - 5	24-27	52-79	0.944	0.831
5 - 7	6-12	70-99	0.802	0.91
9 - 14	18-24	61-79	0.8827	0.8829
10 - 14		79-91		0.8977
STEM INFECTION		Epidemic outbreak period		
1 - 2	9-24	76-91	0.8469	0.9255
4 - 7	12-24	79-97	0.9375	0.799
8 - 11	>30	61-67	–0.894	–0.8157
8 - 11		82-91		0.8157
8 - 11		wetness		0.8157
14		58-76		0.9255
		Epidemic outbreak period		
1 - 4		70-90, 49-58	0.8677	0.8318
3 - 5	24-27	52-79	0.944	0.831
7 - 8	15-21	91-100	0.8401	0.8281
7 - 8		wetness		–0.9326
10 - 14		61-85, 79-91	0.8977	0.8409
10 - 14		wetness		–0.8328

>85% (R^2=0.681); there was no correlation between disease and leaf wetness at this stage. Development of stem infections was correlated with air temperature in the range of 11-16°C during the first phase of the epidemic. By contrast, the second phase was characterised by a close correlation between stem infections and r.h. in the range of 80-

100%, but also with air temperature in the range of 11-16°C, or with air temperature in the range of 11-25°C and r.h. 80-100%, and leaf wetness (Yunis *et al.*, 1990). In addition, it was revealed that in a 'wet' winter season, r.h. and leaf wetness influence the disease less than temperature in the optimal range, that is, in a 'wet' winter the limiting factor for disease development is temperature (Elad *et al.*, 1988; Yunis *et al.*, 1990). An attempt was made to determine the conditions which are most important for disease development in greenhouses. An example of the microclimatic conditions on certain dates before disease assessment, which were positively correlated with fruit or stem infections of cucumber, is presented in Table 1. The data reflect the conditions which affect various stages of disease development. The conditions which prevail 7 to 8 days before symptom appearance and which are significantly correlated with grey mould incidence are high humidity and temperatures around the optimum for germination of conidia. Infection probably takes place during that time, before grey mould is observed macroscopically. During the week between infection and symptom appearance the factors which are correlated with incidence are lower r.h. (91%) and leaf wetness, probably because at that time the mycelium of *B. cinerea* is located inside the water-soaked tissue, where it is protected from external dryness. An interesting finding is the correlation between high temperature (>24°, 27° or 30°C), low temperature (<9°C), or low r.h. on the one hand, and, on the other hand, disease incidence 9-14 days after the occurrence of such conditions. These conditions do not affect germination, infection or mycelium development inside the host tissue, but rather influence the host susceptibility to grey mould. Indeed, it was found in controlled experiments in growth chambers that predisposition to infection of cucumber plants by grey mould occurred when the plants were incubated under these conditions for one week before inoculation. During the next stages of an epidemic, once disease is established in the greenhouse, the microclimatic conditions which are correlated with disease incidence are more broad and compensation probably occurs between humidity and temperature. Compensation for reduced exposure to high humidity by temperatures around the optimum for grey mould was suggested in tests of different greenhouses (Elad *et al.*, 1988).

Development of a model for predicting outbreaks of epidemics of grey mould

Empirical forecasting systems are developed from the observation and analysis of data on disease and other biotic and abiotic factors. Such systems can be developed in one of two ways. The first is a quantitative approach, which is based on statistical analysis of observed data and model development of epidemics. The second is a qualitative approach, involving the development of prediction criteria (rules) without any formal statistical analysis (Madden and Ellis, 1988).

The next step of our research was aimed at characterising the microclimatic conditions inducing outbreaks of grey mould epidemics. Disease incidence of infected fruits or nodes and microclimatic parameters recorded in the 1989 and 1990 seasons were used to develop models for predicting outbreaks of grey mould epidemics. The precision and predictive ability of the model were verified and validated. Verification was accomplished using the same data sets (1989 and 1990) that had been utilised for constructing the models. Models that produced reasonable predictions were examined further, and validated with the 1988 data set. The 1988 data set was considered as an independent one, because it was not used in the construction of the models. The precision of the regression models was determined using several statistics, including the coefficient

of determination (R^2), the error variance (MSE) and the significance of the regression model as determined by the F-test. The precision of the qualitative model was determined via careful examination of the model's predictions with actual disease observations.

Temporal progress curves of grey mould epidemics were quite different between the 1989 and 1990 seasons. In 1989 the disease appeared in the second part of the season, whereas in 1990 it developed very rapidly early in the season and the percentage of infected fruits diminished gradually thereafter. The population size of the pathogen was not a reliable indicator of disease incidence, because there was no coincidence between the time of peaks or the number of trapped spores and the percentage of infected fruits or nodes.

Initial efforts were devoted to developing quantitative, statistically-based prediction models. Simple, multiple and stepwise regression analysis techniques were used to quantify the relationship between disease incidence and microclimatic parameters. In some analysis non-linear regression techniques were used. The percentage of infected fruits was the dependent variable, and various microclimatic parameters were used as the independent variables in all analyses. The following parameters, alone or in combination, were used as independent variables in different runs: mean, minimum or maximum air temperature; mean, minimum or maximum r.h.; duration of wet foliage and of temperature ranges of <6°, <9°, 9-15°, 15-21°, 9-21°, >21° and >2 4°C. The duration of parameters were calculated in different analyses over a single night (18.00 to 08.00 h), 1 day, 3 days or 7 days. The duration of the above parameters was also averaged over the following time intervals, prior to the dates of disease observation: 0-3, 0-7, 3-5, 5-7, 7-9, 9-12 or 12-15 days.

Although a vast amount of time and effort was devoted to constructing quantitative models using formal statistical techniques, the overall results were very disappointing. Statistically significant, biologically sound predictions were not obtained for even a single regression model. The very few significant regression equations (based on the 1989 and 1990 data sets) produced insignificant, insufficient predictions when utilised for the 1988 data set.

Alternatively, a qualitative approach was employed for developing a model to predict grey mould epidemics. The development procedure consisted of the following steps: (i) disease incidence and microclimatic parameters were delineated on the same time scale (i.e. disease progress curves and microclimatic parameters curves, respectively); (ii) the time of occurrence of marked changes in the rate of disease development was indicated on all graphs; and (iii) microclimatic parameter curves were examined carefully in an attempt to elucidate the causes for the marked changes in the rate of disease development.

The qualitative approach yielded an accurate, biologically sound prediction model. Marked changes in the rate of disease development occurred twice in each growing season: during weeks 14 and 17 in 1989, and weeks 10 and 17 in 1990. Vertical lines were drawn at these time points on the disease progress curves. Among the microclimatic parameters that were examined in this study, the coincidence of two of the parameter curves was observed at these time points: the duration of persistence of wet foliage, and the duration of temperature in the range of 9-21°C during the night (18.00 to 08.00 h). For both parameters, these thresholds were 7 h per day for the wet period and 9.5 h per day for the duration of optimal temperature. The values used were daily averages over the week that preceded each disease observation. These microclimatic parameters will be referred to hereafter as the wet period and the duration of optimal temperature, respectively.

The next step was to validate this model with an independent set of data. In 1988 there

were two disease outbreaks, during the first half and at the end of the season. The two occasions were predicted accurately by the model. The occurrence of node infections was recorded in all experiments. Peaks in the number of infected nodes per plant appeared 5-7 weeks after the outbreak of infected fruits. A similarity was found between microclimatic conditions which were correlated with fruit infection, and those correlated with node infection.

The germination of spores of *B. cinerea*, tested in controlled environment experiments, was markedly influenced by the persistence of free water. Germination occurred only when spores were exposed to free water. In this case germination was initiated at 22°C after 4 h incubation. Drying the spores at that time halted the germination process. When free water was eliminated, germination was not observed for at least 24 h, regardless of the r.h.. However, germination did occur in the absence of water after 30 h of incubation at 100% r.h., At < 95% r.h., germination was delayed until at least 52 h of incubation.

The infection process involves three consecutive phases: germination, penetration and establishment (Goodman *et al.*, 1986). During the two first processes the conidia and newly germinated conidia are extremely sensitive to microclimatic influences. In the last process, however, the mycelium is exposed to the conditions prevailing within the host tissue. The main difference between these two environments is the accessibility of free water. Whereas free water is formed on the foliage surface only occasionally, green host tissue may be considered as constantly water-soaked.

The process following germination is penetration. This process in *B. cinerea* lasts c. 2-3 h. Thus, the pathogen is exposed to environmental microclimatic influences for 9-10 h (7 h for germination plus 2-3 h for penetration). This figure resembles the duration of the optimal temperature threshold in the field. Once the pathogen has penetrated the host tissue, a shortage of free water is no longer a limiting factor for infection. Similar results (7 h) were reported for germination and penetration of conidia of *B. squamosa* in onion (Alderman and Lacy, 1983) and of *B. cinerea* (5 h) in gerbera flowers (Salinas *et al.*, 1989)

We are confident of the potential benefits of the model proposed here as a tool for preventing outbreaks of epidemics. Similarly, Vincelli and Lorbeer (1988) based their prediction system for *B. squamosa* of onion on temperature and leaf wetness duration in order to predict infection potential when inoculum is present. In Israel, a recommendation to growers as an outcome of the present study is as follows: on nights when temperatures between 9 and 21°C are expected, the duration of the wet period should be reduced to <7 h. This can be done by forced ventilation or by ventilation through openings in the greenhouse. In heated structures, heating can also be beneficial in suppression of grey mould.

The effect of fertilisation on severity of epidemics

Nutritional regimes can influence the susceptibility of plants to diseases (Goodman *et al.*, 1986). The effect of the nitrogen source depends on the plant species and on the conditions under which it is grown. For instance, Verhoeff (1965) found a decrease in the susceptibility of tomato to grey mould when the crop was grown in soil with a high level of N. Hobbs and Waters (1964) reported the opposite results with chrysanthemum flowers under such conditions. Sol (1967) found that plants of *Vicia faba* fertilised with a ammonium source of N were more susceptible to grey mould than plants fertilised with a nitrate source. In experiments to study the effect of nitrogen source on grey mould, it

was found that the N source did not influence the susceptibility of eggplant or pepper plants to grey mould. In contrast a higher proportion of nitrate in the fertiliser was associated with reduced incidence of the disease in cucumber plants.

One of the significant environmental features in greenhouses is the high r.h. under which crops are grown. Decreased transpiration due to high r.h. reduced the translocation of some ions from the roots to the canopy (O'Leary and Knecht, 1972). Calcium is generally immobile in the phloem and moves almost exclusively in the xylem. The amount of calcium entering an organ is therefore dependent on the volume of water flow through the xylem. Environmental factors such as atmospheric humidity which directly influence transpiration can cause or aggravate calcium shortage in young tissues and fruits. Calcium can also be translocated in response to root pressure, which is sensitive to atmospheric humidity and to the salinity of the root solution (Hobbs and Waters, 1964). Negative effects of high humidity have been reported for crops such as cucumber, tomato, pepper and eggplant (Adams, 1984; Bakker, 1988,1990). Calcium nutrition was shown to reduce grey mould of rose flowers (Volpin and Elad, 1991), tomatoes (Elad, unpublished; Stall, 1963), pepper and eggplant, but its effects on cucumber grey mould was not obvious (Yunis *et al.*, 1991).

Calcium is usually found in high concentrations in the plant cell wall (Ressignol *et al.*, 1977). It is responsible for the integrity of membranes and production of the cell wall (Simon, 1978; Baydoun and Northcote, 1981). Calcium is involved in stabilisation of the cell walls because of the high binding of calciumions to pectin (Demarty *et al.*, 1984). The presence of this element is associated with delayed senescence of plant tissues by reducing respiration rate and ethylene production, and delaying the softening of fruit flesh (Ferguson, 1984), thus making it also less susceptible to degradation by the enzymes of *B. cinerea*. Prevention of membrane deterioration can result also in reduced leakage of nutrients to the surface of the host tissue, thereby rendering them less available to the pathogen (Volpin and Elad, 1991).

The presence of calcium in the root zone solution does not necessarily ensure high assimilation in the canopy. Provided that calcium is present in the root zone, transpiration rate as well as root pressure are responsible for its movement to the canopy (Palzkill and Tibbitts, 1977; Kirkby, 1979). Calcium-related disorders usually occur when there is interference with the uptake and distribution of the ion (Kirkby, 1979). In our work calcium fertilisation of the cucumber crop resulted in higher concentrations of the element in the upper, young leaves. However, the r.h. dictated the differences between calcium and other treatments. The effect of calcium on grey mould reduction was more pronounced in cucumber plants grown under high, than under lower r.h., in spite of the fact that higher r.h. is associated with more severe epidemics of grey mould in cucumber (Yunis *et al.*, 1990).

The use of calciumnitrate is not the only way to enrich plant tissues with the Ca^{2+} ion. Gypsum can be useful for supplying the plant with calcium and for reducing the severity of grey mould. It can be an inexpensive way of supplying the ion when plants are grown in soil or in soilless substrates.

Effect of hormone treatments on grey mould development

Hormone treatments can affect the development of an epidemic in greenhouses. Auxins are applied to ensure fruit setting and development in eggplant or tomato greenhouses. Auxin translocation from meristems, flowers and young fruits is associated with

movement of calcium towards these organs and its incorporation into cells. Auxins seem to affect the susceptiblity of young fruits to grey mould.

The effect of sprays of $Ca(NO_3)_2$ with or without auxin, on grey mould of eggplant fruits was tested in non-heated and heated greenhouses. In the latter, the incidence of fruit rot was reduced by sprays of calcium and N-M-T (Tomaset, N-metatolyphthalamic acid) but not by the auxin 4-CPA (Tomatoton, 4-chloro-phenoxyacetic acid); the combination of calcium with either of the auxins was better than either treatment alone. The only treatment effective in a non-heated greenhouse was 4-CPA. In another experiment, 2,4-D triethanol amine salt [Albar Super] was effective under non-heated conditions (Yunis *et al.*, 1991).

Integration of chemical and biological control

Biocontrol of plant pathogens in integrated pest management programmes is a worthwhile goal, but despite considerable research, only rarely have workable strategies for consistant biological control of plant diseases have been implemented. There are many reasons for this, but most of the difficulties in devising biological controls are related to the complexities of the systems being managed. Nevertheless, there are increasing economic and social pressures to develop usable biological control strategies (Elad, 1990). *Trichoderma harzianum* has proved to be a reliable biocontrol agent of grey mould in cucumber. In most of the experiments the preparation significantly decreased the severity of the disease. In some cases, when the biocontrol was applied with the dicarboximide fungicides iprodione or vinclozolin, there was a trend toward better control in comparison with either agent alone. The slightly improved efficacy of the mixture of *T. harzianum* with these fungicides may be due to the presence of resistant *B. cinerea* strains in the greenhouse (Elad and Zimand, 1991).

The ultimate aim of our work was to reduce the application of fungicides during the growing season without loss of control. For this reason we alternated *T. harzianum* application with fungicides, which resulted in a level of control similar to that obtained with the fungicide treatments recommended for grey mould control without any failures as occurs commonly with *Trichoderma* treatments alone. Alternation of chemical and biological control had two effects: first, there was a reduction in fungicide residues on the fruits. Second, the pathogen was less exposed to the fungicides and therefore under less pressure to develop resistance. Similar results were obtained by Gullino *et al.* (1991), who sprayed dicarboximide + thiram in alternation with *Trichoderma* for control of grey mould in tomatoes and strawberries.

The level of the *T. harzianum* population on leaves and fruits of cucumber remained high in plants treated with the biocontrol preparation. The survival of *Trichoderma* on the phylloplane is a key factor for achieving effective control (Elad and Zimand, 1991). Similarly, McKenzie *et al.* (1991) concluded that the poor activity often shown by *Trichoderma* against grey mould under severe disease pressure, can be partly explained by its poor survival in the phylloplane under field conditions. Our preparation ensured satisfactory survival of an efficient population for 1 month under commercial greenhouse conditions, and an adequate population of the antagonist was also established in the non-treated control plots. This may be due to secondary distribution of the propagules of the fungus in the air. The secondary dispersal may be important for the establishment and survival of the biocontrol agent, but under our conditions it did not reduce grey mould in the control plots sufficiently.

Calculations of relationships between microclimatic conditions and disease control achieved by *Trichoderma* revealed a negative effect of wetness and a positive effect of temperatures above 20°C. This may explain the failure of *T. harzianum* to control grey mould in one experiment. A search could be conducted for isolates of *Trichoderma* which effectively control the disease under a wide range of conditions. Meanwhile, effectiveness is achieved by a combination of the biocontrol agent with fungicides.

Effect of reduced populations of *B. cinerea* on yield of non-infected fruit

To test whether the disease caused yield loss, experiments were conducted in tomato and cucumber greenhouses with fungicides applied weekly. Significant control was achieved with tebuconazole (with or without dichlofluanid), fenbuconazole and diethofencarb + carbendazim.

Linear regressions were produced to determine the relationship between grey mould in cucumber and fruit yield. Data on disease incidence and yield of individual plots were analysed in all experiments. The following correlation were examined for each sampling date: (*a*) % infected fruit versus yield of cucumbers in the following week (the week following disease assessment); (*b*) number of infected fruits versus yield in the following week; (*c*) cumulative number of diseased fruits versus cumulative yield in the following week; (*d*) number of infected stem nodes versus yield in the following week; and (*e*) cumulative number of infected stem nodes versus cumulative yield in the following week.

No significant negative correlations were obtained between any disease parameters and yield. The lack of correlation between diseased senescening female flowers of cucumber and decreased yields is due to the nature of the development of fruits in parthenocarpic cultivars of cucumber (Yunis *et al.*, 1991). Abortion of the senescing female flowers is a natural phenomenon and compensation in fruit development occurs when some flowers abort in the greenhouses. Only some of the flowers develop to produce a full-size cucumber even when there are no disease epidemics. The plants react similarly when senescing flowers become infected by *B. cinerea*; in both cases, other healthy flowers develop. It appears that the optimum temperatures for grey mould epidemics in cucumber are in the range (11-25°C) in which fruit production is not affected. An r.h. >85% which promotes epidemics of grey mould, is also a condition under which satisfactory cucumber yields are produced.

Since there was no correlation between low disease level and cucumber yield under these experimental conditions, it can be recommended that fungicides aimed against *B. cinerea* should be applied less frequently. Under severe epidemic conditions, usually at the end of the season, grey mould lesions may occlude translocation in the stems causing wilt and death of upper parts of the plant. Therefore, there is only a need for frequent sprays of effective fungicides when a severe epidemic is expected. Plant compensation for lost fruits does not seem to occur in tomato and the quality and yield of this crop were significantly reduced by grey mould epidemics. Similar results have also been obtained with pepper, eggplant, strawberry, bean and cut-flowers.

Survival of *B. cinerea* during the warm summer

Surveys in greenhouses have been conducted since the first detection of dicarboximide-resistant isolates in Israel. At the beginning of each season, the relative incidence of

resistant isolates was lower than that at the end of the previous season. An increase of resistant isolates occurred in response to sprays with iprodione or vinclozolin during the season, a phenomenon reported also from covered crops in other areas of southern Europe. We have noted failures in grey mould control in cucumber greenhouses even at the beginning of the season.

Although considerable information is available about grey mould, limited attention has been given to the survival of *B. cinerea* in a warm climate. In regions in Europe where the climate is cool and humid, the pathogen is active during summer in vineyards and greenhouses and in greenhouses during winter and the limiting factor for survival is therefore low temperatures. In warm regions with hot, rainless summers grey mould epidemics occurs in greenhouses during winter. We found that *B. cinerea* survives in plant debris, particularly in infected stems. For the fungus'survival infected hosts were the best ecological niche, the pathogen survived in up to 18% of stem samples. Although survival was poor outside the infected tissue and in fruits and flowers. Conidia on the surface of debris probably cannot survive during the summer. Coley-Smith (1980) found that under less adverse conditions, the conidia did not survive more than 53 days. Internal stem tissues perhaps provide an isolated niche which may protect the mycelium from extreme conditions or from other microorganisms. There is also a possibility that dicarboximide-resistant isolates have undergone selection for survival under high temperatures and dry conditions. Therefore, there now may exist in greenhouses a population of *B. cinerea* different from that in the early 1980s when resistance first appeared in Israel. *B. cinerea* could be isolated f rom various susceptible crops and weeds under summer conditions and these plants may offer another means of summer-survival for the pathogen. Grey mould is seen occasionally in rose greenhouses during the summer. In these cases there are probably borderline conditions of humidity and temperature in which *B. cinerea* can develop. Removal of infected plants at the end of each growing season is important to reduce the amount of surviving inoculum and delay in the onset of epidemics in greenhouses.

The main reason for the decline in *B. cinerea* in debris during summer is that under such conditions survival of *B. cinerea* might be poor, as was shown for *B. convoluta* (Mass, 1969). According to earlier work and our results, no importance should be attributed to survival of *B. cinerea* as sclerotia; the latter cannot survive at high temperatures under dry conditions. Moreover, in Israel, sclerotia are found rarely on plants infected by *B. cinerea* and are never produced during summer (Yunis and Elad, 1989).

Concluding remarks

Use of the measures dictated by the developed model to reduce or delay grey mould epidemics of fruits or stems will decrease the need for fungicide applications. Future work on the prediction of grey mould epidemics should be aimed at the development of models for other crops grown in non-heated or heated greenhouses, such as tomatoes.

The nutritional status of plants, which influences the physiological balance of tissues and their susceptibility to grey mould, should be studied further to achieve a better understanding of the differential role of important elements in the system.

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Epidemiology of *Botrytis cinerea* in gerbera and rose grown in glasshouses

A. Kerssies

Summary

The dispersal of conidia of *B. cinerea* and their horizontal and vertical distribution was studied in gerbera and rose crops, grown under glass. Conidia were caught in simple traps based on impaction placed at different heights in each glasshouse and their numbers derived from colony counts after incubation. Lesions due to conidial infection were also counted on the flowers. No seasonal pattern was found in the numbers of colonies developed from trapped conidia, but the numbers of lesions on the flowers were seasonal. In spring and early summer few lesions were produced, whereas many lesions appeared in other seasons. Linear regression accounted for 77 and 81% of the variation in the number of lesions on gerbera flowers in the two glasshouses in terms of relative humidity (positively correlated), total incident solar radiation outside the glasshouse (negatively correlated) and age of the crop (positively co rrelated).

The role of wax and cuticular membrane in the susceptibility of flowers was unclear. No correlation was found between the amount of wax and cuticular membrane per cm^2 and the number of lesions on gerbera and rose flowers.

Introduction

Botrytis cinerea Pers.: Fr. causes damage to ornamentals like gerbera, rose, chrysanthemum and several other flower species such as Saintpaulia grown as pot plants (Kerssies, unpublished data). Conidia, ascospores, mycelial fragments and sclerotia are important for dispersal (Jarvis, 1980), although only conidia seem to play a role in disease dispersal in glasshouses. Conidia are dispersed by air currents, water droplets and insects. Necrotic lesions ('spotting') on flower buds and petals are caused by early infections of plant tissue. Studies on dispersal of plant pathogens in glasshouses are scarce (Frinking and Scholte, 1983). Hirst (1959) was the first to monitor densities of airborne spores, and *B. cinerea* conidia were amongst those trapped. Frinking *et al.* (1987) studied the dissemination of particles in a glasshouse divided into three bays using *Lycopodium* sp.

Little is known about the factors influencing dispersal of spores of *B. cinerea* in ornamentals in glasshouses. Frinking and Scholte (1983) showed that the complex dispersal process involves aspects of the pathogen, host, environment and the activity of man. The aim of this study was to investigate the patterns of conidia dispersal in a gerbera and rose crop growing in glasshouses and the effects of environmental factors on dispersal and infection of the harvested flowers during storage and transport.

Materials and methods

Measurement of environmental conditions

The relative humidity (r.h.) and the temperature in and above the crop were measured and meaned over 1 h periods using psychrometers coupled to a data logger. Incident radiation outside the glasshouse ($Jcm^{-2}day^{-1}$) was measured by a Kipp solarimeter 8 m above the ground at the Research Station for Floriculture in Aalsmeer. The windspeed outside the glasshouse in Aalsmeer was measured and meaned over 1 h periods using a tachometer 8.5 m above the ground.

Dispersal and distribution of *B. cinerea*

The dispersal and distribution of *B. cinerea* in a gerbera crop grown on rockwool was studied in two glasshouses, one of 100 m^2 in Aalsmeer (glasshouse A; 480 plants, cv. 'Terrafame') and one of 350 m^2 in Vleuten (glasshouse V; 2000 plants, cvs. 'Rosamunde' and 'Maria'). The densities of *B. cinerea* conidia in the air of the glasshouse was studied between April 1988 and October 1989 using two methods. In method 1, spore traps were distributed within and above the crops in a regular pattern (Kerssies, 1990); in method 2, the number of lesions on petals of gerbera flowers was counted after harvest.

In glasshouse A, spore traps were exposed 63 times over a period of 545 days at

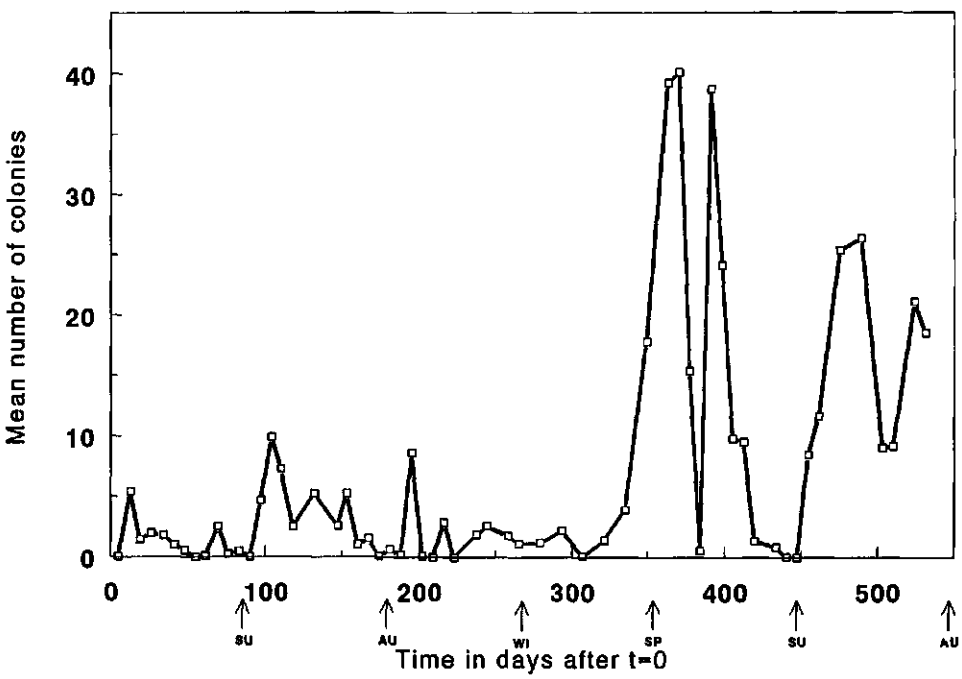

Fig. 1. Number of colonies per spore trap (n=24) placed within the crop from 8.30-16.30 in glasshouse A in 1988-1989. SP, SU, AU, WI : beginning of spring (21 March), summer (21 June), autumn (21 September) and winter (21 December), respectively.

daytime and 55 times over a period of 545 days at night. Flowers were harvested and lesions were counted 46 times over a period of 405 days. In glasshouse V spore traps were exposed 49 times over a period of 439 days, at daytime only. Flowers were harvested and lesions were counted 45 times over a period of 371 days.

Statistical analysis

The mean numbers of colonies on 53 trapping occasions over 545 days and mean numbers of lesions on gerbera petals in 36 samples over 371 days (glasshouse A) were the dependent variables [ln(N+1) transformed] in regressions against environmental variables. The variables used in the best equation determined with the data of glasshouse A were also used for the data from glasshouse V (only for the numbers of lesions), where lesions on petals were counted 37 times over a period of 390 days.

Results

Colonies

Figure 1 shows the number of colonies per spore trap within the gerbera crop between 08.30 and 16.30 over a period of 545 days in glasshouse A. The number of colonies on

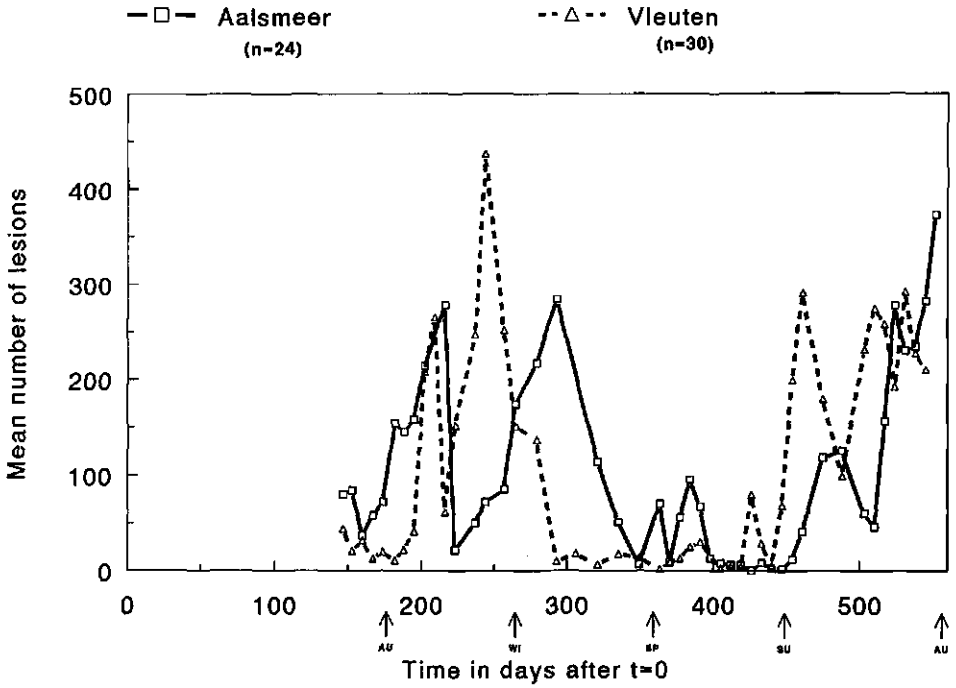

Fig. 2. Number of lesions per flower formed on ten petals of gerbera flowers in glasshouse A (n=24 flowers) and V (n=30 flowers, t=t -110days), in 1988-1989. SP, SU, AU, WI: beginning of spring (21 March), summer (21 June), autumn (21 September) and winter (21 December), respectively.

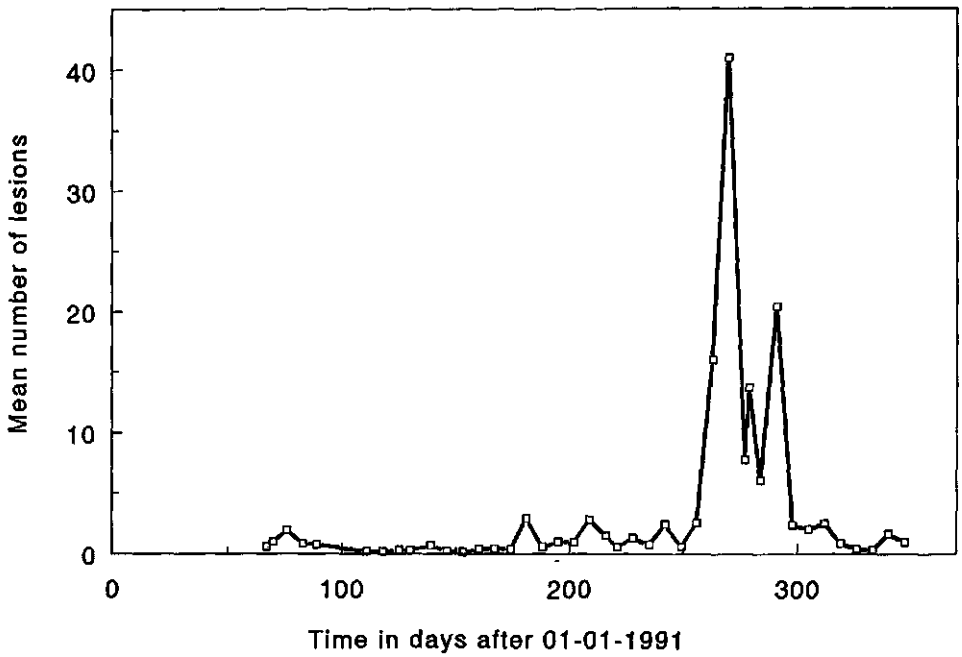

Fig. 3. Number of lesions formed on rose petals (n=100 flowers) in 1991.

spore traps 0.5 m and 1.5 m above the crop showed the same patterns. High and low numbers of colonies alternated rapidly over time. The number of colonies in the daytime was significantly correlated with those at corresponding nights, but about 3 to 4 times higher. The mean number of colonies over time trapped in glasshouse V between 8.30 and 16.30 was low compared to those in glasshouse A. Peaks could hardly be distinguished. Mean numbers fluctuated over time from 0 - 7 colonies per spore trap.

Lesions

The number of lesions formed on ten petals of a single gerbera flower varied from 0 - 375 in glasshouse A (n=24 flowers, Fig. 2) and from 0 - 450 in glasshouse V (n=30 flowers, Fig. 2). During spring and early summer (t=350 - t=460 in glasshouse A and t=200 - t=330 in glasshouse V) very few lesions occurred on petals, though many lesions appeared at other times. Towards the end of the recording period the number of lesions was still increasing and the pattern was similar for the two glasshouses. The mean number of lesions over time was less variable than the mean number of colonies.

Regression analysis

A good linear regression model expressing fluctuations in the number of colonies could not be made with the independent variables used. The adjusted R^2 values were all below 0.4. The best linear regression model for the number of lesions (glasshouse A) utilised three variables: MRH, MS and t and gave an adjusted R^2 of 0.81 ($P \leq 0.05$). MRH is the

mean RH for days 6, 7 and 8 before the day of harvesting gerbera flowers; MS is the mean incident radiation for days 1, 2 and 3 before the harvest day; while t is the age of the crop. This model for the number of lesions on gerbera petals explained 77% of the variation in glasshouse V (adjusted R^2 of 0.77; $P \leq 0.05$). The levels of the two linear regression models are significantly different at $P \leq 0.05$. This suggests that the type of glasshouse had a significant effect on the linear regression model. The best linear regression model for the number of lesions on gerbera petals for the combined glasshouses is (adjusted R^2=0.77; $P \leq 0.05$):

$$Y = -6.6(\pm 1.5) + 0.12(\pm 0.03)*MRH - 0.00074(+0.00017)*MS + 0.0042(\pm 0.001)*t + 0.98(\pm 0.23)*G(3),$$

in which G is the glasshouse effect ($J^{-1}cm^2$), G is 0 or 1. This is the effect of differences between glasshouse A and V in size, height, production system, location etc.

Preliminary experiments with rose flowers

A study of the dispersal and distribution of *B. cinerea* in a rose crop, where crop structure is totally different from a gerber a crop on rockwool was started in January 1992 in a glasshouse of 300 m^2 in Aalsmeer (2400 plants, cv. 'Sonia') and ends in January 1993. Observation done so far, showed that in the rose crop high and low numbers of trapped spores also alternated rapidly. The numbers of lesions on rose petals are low compared to those on gerbera petals (<3, Fig. 3) and only in September and October (1991) 10 to 40 lesions were counted on petals.

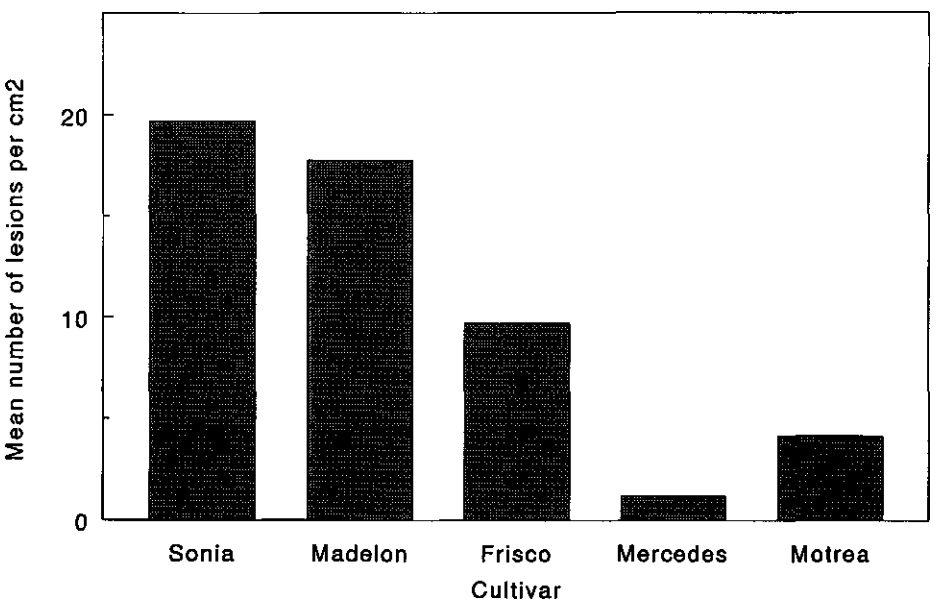

Fig. 4a Number of lesions per cm^2 on rose petals (n=20) of five cultivars. (Experiment performed by Mr. A. Hazendonk).

Discussion

In the spore traps of glasshouse A, the increasing number of colonies over time was attributed to the increasing quantity of dead plant tissue which accumulated as the gerbera crop aged (> 6 months) and the rise in r.h. in the crop to between 85 and 100% when the canopy closed. Changes in the numbers of colonies over time could be caused by changes in environmental conditions in the glasshouse . Differences in the numbers of colonies between samples taken by day and night in glasshouse A could be explained by increased air movements in the glasshouse in daytime due to labour, open windows, open doors and higher temperatures. The differences in the numbers of colonies between glasshouse A and V were due to the difference in production systems. The exposure time of gerbera flowers (7 - 14 days) was longer than for the spore traps (< 16 hours) and therefore the latter was more sensitive to rapid changes in conditions. Although Salinas *et al.* (1989) showed that one conidium can induce a lesion on a gerbera petal, significant correlations ($P \leq 0.05$) between the colonies and lesion numbers were not found in this study. The lack of correlation may be due to differences in exposure time between traps and flowers, to differences in the position of traps(vertical) and flowers (horizontal) and to variation in unfavourable conditions for germination. This is in accordance with the findings of Salinas *et al.* (1989).

In both glasshouses, the numbers of lesions depended more on the season than the numbers of colonies obtained (Fig. 1 and 2) . The numbers of lesions from the same days of the year in glasshouses A and V at locations thirty kilometres apart were significantly correlated and not significantly different ($r>0.30$ at $P<0.05$ and $n=41$). Frinking *et al.*

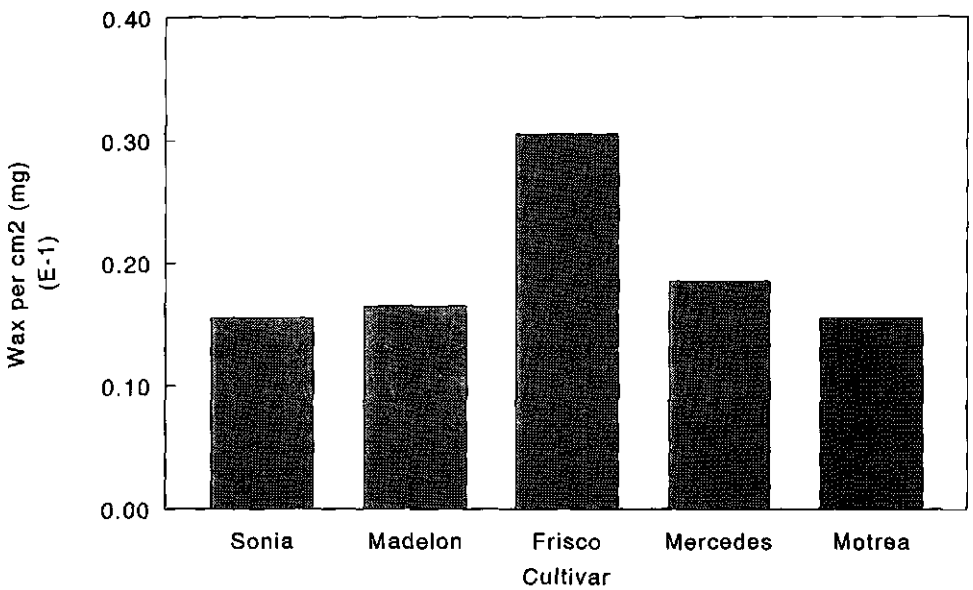

Fig. 4b Amount of wax per cm² rose petal (mg, n=6) of five cultivars.[1)]

[1)] determined according to the method described by Silva Fernandes *et al.*, 1964.

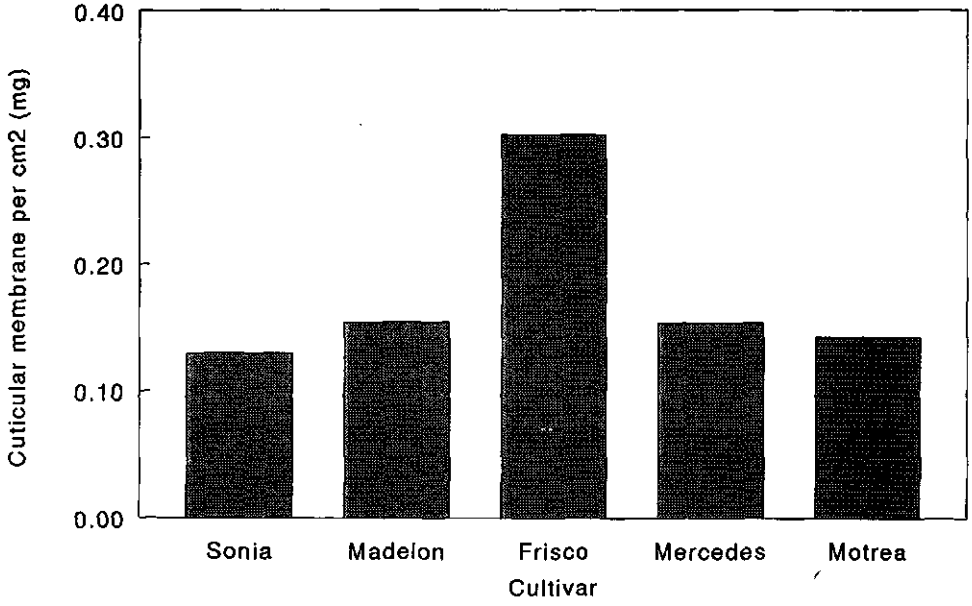

Fig. 4c Amount of cuticular membrane per cm² rose petal (mg, n=6) of five rose cultivars.[1)]

[1)] determined according to the method described by Silva Fernandes *et al.*, 1964.

(1987) suggested that spores of *B. cinerea* could be transported rapidly throughout the glasshouse. Zadoks (1967) and Frinking (1991) stated that fungi can enter and leave glasshouses very easily because of a continuous exchange of air between the glasshouse and the outside environment. These results suggest that the density of spores and the r.h. are not the most important variables regulating number of lesions, but other factors such as a combination of radiation and r.h., the structure and composition of the cuticle, or the water relations (water potential, osmotic potential) in the flower. In spring and early summer, flowers may form thicker cuticles and more wax due to high values of radiation and low r.h. (Baker, 1974; Kolattukudy, 1985). Germinated spores of *B. cinerea* may have more difficulty to penetrate the cuticle of gerbera petals in spring an summer than in autumn and winter. Additionally, conidia of *B. cinerea* on the flower surface may have been affected by radiation and relative humidity.

The role of wax and cuticular membrane in the sensivity of flowers for *B. cinerea* is still unclear. So far no correlation is found between the amount of wax and cuticular membrane per cm² and the number of lesions on gerbera- and rose flowers (Fig. 4a ,b,c).

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BIOLOGICAL CONTROL

Biological control of *Botrytis*: state-of-the-art

B. Dubos

Summary

In 1951, it was shown that fungi, bacteria and actinomycetes were able to limit the infection of *B. cinerea* on lettuce leaves though it was not until the 1970s that biological control of this pathogen on grapevine, apple and strawberry was developed using *Trichoderma* spp.

The number of target crops has now expanded greatly and the range of antagonists widened to include yeasts and bacteria. Though sometimes unsuccesful, the field results obtained have been sufficiently encouraging to have led to an examination of the modes of action of antagonistic organisms towards the pathogen and an evaluation of the effect of environmental conditions on their behaviour. These studies made it possible to select and genetically improve the antagonistic strains. Industrial production of biopesticides is now established and research has been undertaken to improve production.

Nevertheless, the application of biological control agents against *B. cinerea* is only in its infancy. To determine the principal research priorities which will lead in the future to the regular use of biological control for management of grey mould caused by *B. cinerea*, a review of present knowledge is given here.

Introduction

Agriculture will have to be more economical, but also more respectful to the environment in the next century and during this transition period biological control will probably become a recognised alternative or complement to chemical control if microbiologists and plant pathologists are sufficiently determined and convincing in dealing with those who shape agricultural policies.

As a polyphagous and ubiquitous fungus *Botrytis cinerea* Pers.: Fr. is responsible for various disease symptoms in a wide range of cultivated plants of great economic importance. It is a facultative parasite and causes serious diseases on directly consumable products, such as fruits and vegetables and it is therefore a perfect candidate for biological control.

This overview has two aims: firstly, to review the state-of-the-art knowledge and secondly to define priority research directions for establishing the practical use of biological control.

State-of-the-art knowledge

The first steps in biological control were taken by Wood (1951) who inoculated senescent lettuce leaves with antagonists (*Fusarium* sp., *Penicillium claviforme*) to prevent the primary establishment of *B. cinerea*. Positive results providing effective disease control under cold-frame conditions were obtained. Wood believed that saprophytic activity on dead lettuce tissue was, to a large extent, responsible for disease control under natural

conditions. Using the same principle, Newhook (1957) controlled grey mould on tomatoes grown in a glasshouse by spraying a spore suspension of *Cladosporium herbarum* and *Penicillium* sp. on the floral debris attached to the fruit. Bhatt and Vaughan (1962) obtained nearly 40% reduction of strawberry grey mould through the application of *C. herbarum* to senescent flowers under glasshouse conditions.

Ruinen (1961) drew attention to the lack of research on the saprophytic microflora of aerial plant surfaces. Subsequently, the role of the saprophytic microflora in the biological equilibrium was established and the dangers of its destruction, particularly through the irrational use of fungicides became known. Blakeman and Fraser (1971) and Blakeman (1972) pointed to the antagonistic function of bacteria against *B. cinerea* on chrysanthemum and beetroot leaves, but although of theoretical importance, these results were not followed by practical applications in the control of *B. cinerea.*

Paradoxically, further research on biological control of *B. cinerea* from the 1970s concerned the fungal genus *Trichoderma* whose antagonistic properties had long been known, but which was not usually a component of the microflora of the phylloplane. At the *Botrytis* symposium in Aberdeen, Scotland in 1982, Tronsmo and Dubos respectively, presented the first results concerning control of dry rot of apples and grey mould of the grapevine by strains of *Trichoderma.*

Since then, work on this antagonistic genus has progressed rapidly and the number of *Botrytis* hosts vastly increased. In parallel, the antagonistic properties of microorganisms found as part of the phyllosphere microflora have been studied. The interaction of pathogenic fungi and antagonists *in vitro*, particularly *Trichoderma* spp., have been studied extensively, but these will only briefly mentioned.

Three methods of antagonism are usually studied: 1) antibiosis – production of diffusing substances with fungistatic or fungicidal action; 2) competition – the ability to exploit environmental factors at the expense of the parasite; and 3) mycoparasitism – destruction or alteration of the hyphae of the pathogen, involving physical contact and predation, followed by enzymatic lysis.

It should be noted that reactions *in vitro* between antagonists and *B. cinerea* depend greatly on the growth conditions and especially the nutrient status of the medium, temperature and relative humidity. The important effect of temperature was shown in the interactions between *Trichoderma* and *Botrytis* (Dubos *et al.*, 1983). Most *Trichoderma* strains possess antagonistic properties between 15° and 25°C, but strains with similar properties above and below these values are relatively rare; the same occurs in antagonistic yeasts and epiphytic bacteria. Temperature therefore is a key limiting factor in the choice of strain, particularly when the latter is for use as post-harvest treatment.

Other tests are appropriate when selecting strains for a particular phase of the disease cycle. *In vitro*, Köhl and Schlösser (1989) selected strains of *Trichoderma*, capable of destroying sclerotia of *B. cinerea* at low temperature, with a view to destroying the pathogen during the survival phase. Peng and Sutton (1990) assessed the efficacy of antagonists by inoculating strawberry leaf disks first with the antagonist, then with *B. cinerea* and subsequently assessed sporulation of the pathogen on the disks.

Initial *in vitro* screening, however impressive the candidate antagonist, is often misleading; strains which may be consistently efficient *in vitro* may be unsatisfactory in the field, and the converse is also true. This observation may indicate the existence of other types of antagonistic action operating at the target than those generally investigated.

Various models studied and different modes of applying antagonists

Bio-preparations of antagonists sprayed to protect herbaceous plant parts and fruits from infection by *B. cinerea* are shown in Table 1. Postharvest treatment of fruit, vegetables and flowers by dipping or spraying with antagonists are summarised in Table 2. Soil-born inoculum of *B. cinerea* requires a different approach. For damping off diseases, the antagonist may be applied as a seed coating or incorporated directly into the soil. A compost rich in *Trichoderma* is often used to destroy the pathogen or limit its saprophytic development on crop debris or plants whose sensitive organs are in contact with the soil. For soilless crops, particularly carnation, the antagonists are supplied in the nutrient solution (Table 3). Metabolites produced by antagonistic microorganisms have also been applied for biocontrol (Table 4).

The most extensive and prolonged work on biological control of *B. cinerea* has been performed in vineyards in France, Italy, Spain, Israel and other countries. This experience will therefore be the basis for our further discussion of biological control of grey mould. Table 5 shows the results of nine years of experimentation on cv. Sauvignon in the Bordeaux region (Dubos, 1987). The protection we obtained proved excellent in some

Table 1. Biological control of B. cinerea and other Botrytis spp. by spraying antagonists on aerial plant parts

Host plants	Antagonistic microorganisms
Strawberry	*Trichoderma* spp., *T. viride*, *Gliocladium roseum*, *Penicillium* spp., *Gladosporium herbarum*, compost extract
Apple	*Trichoderma* spp., *Bacillus subtilis*
Cucumber	*T. harzianum*
Grape	*T. harzianum*, epiphytic bacteria, *Bacillus subtilis*
Cyclamen	*T. harzianum*
Beans	(*B. fabae*) *T. viride*
Onion	*Gliocladium* spp.,*Trichoderma* spp., *Aureobasidium pullulans*

Table 2. Biological control of B. cinerea during storage

Host plants	Antagonistic microorganisms
Apple	*Cryptococcus* spp., *Candida guilliermondii*, *T. viride*, *Acremonium breve*
Kiwi	*Trichoderma* spp., *Gliocladium* spp., *Paecilomyces* spp.
Pear	*Trichoderma* spp., *Gliocladium* spp., *Paecilomyces* spp.
Carrot	*T. harzianum*
Rose	*Exophiala jeanselmei*, Coryneform-type bacterium

Table 3. Biological control of B. cinerea, using antagonists applied to soil and as a seed-coat.

Host plants	Antagonistic microorganisms
Tulip bulbs	*Gliocladium roseum*, *T. hamatum*, *Streptomyces* spp.
Pinus massoniana	*Pseudomonas* spp., *Bacillus cereus*
Lettuce	*Streptomyces* spp.
Carnation	*Bacillus subtilis*, *Streptomyces* spp.
Strawberry	*Trichoderma* spp.
Miscellaneous	*Aphelenchoïdes composticola**)

*) Nematode

Table 4. Biological control of B. cinerea using metabolites produced by antagonistic microorganisms.

Host plants	Metabolite and source
Rape seedling	Pyrrolnitrin (*Pseudomonas cepacia*)
Kiwi fruit	Metabolites of *Trichoderma* spp., *Paecilomyces* spp., *Gliocladium* spp.
Pear	Metabolites of *Trichoderma* spp., *Paecilomyces* spp., *Gliocladium* spp., Pyrrolnitrin (*Pseudomonas cepacia*)
Apple	Pyrrolnitrin (*Pseudomonas cepacia*)

years, or inadequate (1983 and 1984). In all cases the efficacy was lower than that provided by dicarboximides in the absence of strains of *B. cinerea* resistant to these fungicides.

Similar results have been obtained in other regions, and for other crops (strawberry, apple, tomato) and for other antagonists.

These inconsistent results rapidly guided research to investigations about the modes of action of antagonists and the relationships between host, pathogen and antagonist.

Mode of action of antagonists in planta

Apart from the phenomena of competition, antibiosis and mycoparasitism which have been established experimentally, the induction of defence mechanisms in the host plant should be considered in this context.

Competition

Competition is probably one of the most studied modes of action and is the most important for control of grey mould. *B. cinerea* often first develops as a saprophyte on senescent leaves and flowers of strawberry, tomato and grapevines. From these nutrient bases, it produces a mycelium which invades the healthy part of the plant.

Table 5. Summary of results of biological control of B. cinerea on grape cv. Sauvignon in the Bordeaux area over 9 years.

Year	rot in treated plots (%)	rot in control plots (%)	efficacy of treatment (%)
1976	9.4	33.6	70
1977	9.5	31.4	71.7
1978	7.6	24.6	69.3
1979	4.2	22.5	81.1
1980	7	15.9	57
1981	9.7	24	59.6
1982	4	27	84
1983	25.2	34	26
1984	27	35	22.9

It has been shown in many crops that *Trichoderma* is a pioneer coloniser of senescing floral caps on developing grapes and prevents the saprophytic colonisation by the pathogen. Such competition for nutrients is thought to occur in postharvest control of apple by *Cryptococcus laurentii*, a yeast which rapidly colonises wounds using the nutrients necessary for the development of *B. cinerea* (Roberts, 1990). Competition for carbon and nitrogen sources between cells of *C. laurentii* and *B. cinerea* could be responsible for control, as conidial germination of some strains of *B. cinerea* is known to be affected by the carbohydrate and nitrogen status of their immediate environment.

Production of antifungal and fungistatic compounds

While the production of antifungal and fungistatic compounds *in vitro* is well known, this type of antagonism is rarely mentioned in biological control trials. This may be because it is difficult to demonstrate this activity *in vivo*. These metabolites were always considered to be antibiotics, and because the use of antibiotics in agriculture is prohibited in most countries, researchers tended not to select strains expressing metabolites for use on the aerial parts of plants. In the late 1980s, there was a shift in attitude, since metabolites were being used for biological control of fruit storage diseases (Janisiewicz and Roitman, 1988). Ferreira (1990) achieved a high level of protection of grape berries by applying certain strains of epiphytic bacteria and the mode of action in this case is thought to be an antibiotic which inhibits germination of *B. cinerea* conidia.

Mycoparasitism

This phenomenon has been described hardly for biological control of *B. cinerea*, except in the case of grey mould of grapevine. The incidence of infected bunches in treated and control plots usually do not differ statistically but the severity of rot in the bunches is less in the former. This probably indicates that from the time of ripening onwards, when inoculum is abundant, *Trichoderma* does not reduce the number of infection sites but prevents their development. Microscopical observations have shown that on rotting grapes the antagonist sporulates on the margin between the necrotic and healthy area, thus preventing spread of *B. cinerea* to healthy grapes in the bunch. *Trichoderma* therefore behaves as mycoparasite.

Improving the efficacy of biological control agents

When using *T. harzianum* to control grey mould of grapes, all the data suggest that a conidial concentration in the order of $10^8.ml^{-1}$ is the most suitable for bio-preparations (Dubos *et al.*, 1983, Gullino *et al.*, 1983). Below this concentration, a rapid decline in efficacy is observed, and no particular improvement seems to be achieved above this inoculum density. An identical result was obtained by McLaughlin *et al.* (1990) with *Candida* sp. to reduce lesion development in apples caused by *B. cinerea* during storage.

Adjuvants are added to bio-preparations to enhance the intrinsic properties of the antagonists, or to assist in its maintenance in the phyllosphere.

McLaughlin *et al.* (1990) noted that the addition of salt solutions improved the efficacy of *Candida* sp. on apples in storage; $CaCl_2$ was the most effective, and with certain strains it was possible to reduce the concentration of the inoculum 100-fold. This effect was unrelated to the osmotic potential of the solutions. Gullino *et al.* (1989) studied nutritional effects on the antagonistic activity of *Trichoderma* spp. on grape. Tronsmo (1986) found that *Trichoderma* was more efficient against grey mould in apple and strawberry, if the conidial suspension sprayed during blossoming contained soluble cellulose.

Strains selected for use on grape, are generally ineffective on strawberry (Gullino, 1983) and Tronsmo (1986) came to the same conclusion regarding control of diseases on apple and strawberry.

A single strain may give highly variable control from one vineyard to another, or from one year to another. Favourable results obtained with a strain of *T. harzianum* against grey mould in a vineyard in the Atlantic region will not be applicable in more northerly vineyards, such as in the Champagne region because the antagonistic potential of *Trichoderma* strains is highly temperature dependent, and operates best between 15° and 25°C; in northern vineyards, rot often develops at temperatures lower than 10°C. Similarly, the inferior results obtained in 1983 and 1984 in the Bordeaux area with the *T. harzianum* reference strains were due to very dry climatic conditions and to an explosion of grey mould before harvesting. The efficacy of biological control has been subsequently improved from 20 to 70% by use of a 'thermophilic' strain.

Where primary inoculum, which arises from within the crops, plays an essential role on the development of an epidemic, as with *B. squamosa* in onions, Fokkema *et al.* (1991) selected strains of *Trichoderma* or *Gliocladium* inhibiting sporulation of the pathogen by colonising dead leaf parts and other crop residues. Similarly Köhl and Schlösser (1989) selected *Trichoderma* strains for destruction of sclerotia of *B. cinerea* which are the primary focus of the disease in many plants.

To offer a mixture of antagonistic strains for controlling the same disease is an unattractive option and therefore attempts have been made to improve strains.

Strain enhancement

Mutagenic agents have been used by Davet *et al.* (1991) to obtain mutants of *T. harzianum* having either β-1-3 glucanase or a chitinase activity, or both. It was noted that although the antagonistic activity was improved in the laboratory, no improvement in efficacy was detected in the vineyard, probably because these strains were less competitive in the phylloplane than wild type strains. Similar results with mutants resistant to benomyl have been obtained (Mighell *et al.*, 1991).

Protoplast fusion techniques combine desirable attributes from two or more parental

strains in the progenies. By this method , Mighell *et al.* (1991) obtained several somatic hybrid strains of *T. harzianum* which combined colour and/or fungicide resistance markers. The use of these fusion products in the vineyard gave variable degrees of survival in the phylloplane, but the aptitude of wild types was always better.

Cloning and characterisation of genes involved in the general metabolism contribute to the development of expression vectors for these antagonistic species. With this objective Heidenreich and Kubicek (1991) have started to clone the *T. harzianum* gene involved in mycoparasitism of *B. cinerea* but at present no practical results have been obtained.

In view of the complexity of the problems currently associated with choice of suitable strains, the need to control *B. cinerea* more effectively, and the desire to conserve the advantages of both chemical and biological control, integrated control programmes have been devised. This was achieved for grey mould of grape by alternating biological with *T. harzianum* and chemical treatments, either by use of the four standard sprays, or by using the data derived from a predictive model; it was also achieved by mixing the antagonists with low levels of cyclic imides (Dubos *et al.*, 1983; Gullino, 1983). These strategies have been used successfully in many grape growing countries and make it possible to decrease the amount of fungicides used, and minimise the risk of the development of resistant strains. Results on strawberry, cucumber and tomato obtained with the *T. harzianum* strain T39 selected by Elad *et al.* (1991) were identical, and a formulation of this strain has been produced industrially by the Makteshim company (Israel). Limited information is available about industrial production. Liquid culture media are generally used for bacteria and yeasts but these may not be appropriate for filamentous fungi because their physiology is often highly disturbed. In the case of *T. harzianum*, chlamydospores were mainly produced (Arteconi *et al.*, 1991), but this type of propagule has not been field-tested. Jim *et al.* (1991) developed media yielding high levels of *T. harzianum* in submerged cultures, but only 1 to 10% o f the conidia survived drying. Addition of osmoticants to the medium allowed production of dessication-tolerant conidia. Usually, 40 to 50% of conidia from osmotically-enhanced media survive drying. Vial *et al.* (1991) studied the production of conidia of *T. harzianum* in a solid medium (beet pulp). They showed a relationship between decreases in respiratory activity and yield of conidia exhibiting good storage activity after 120 h. Ultrastructural studies also revealed that these conidia had reached a state of advanced maturity.

Is biological control of grey mould commercially viable?

Despite the numerous positive results, biological control of *B. cinerea* is not used extensively. Nevertheless, the company Makteshim has been developing *Trichoderma* since 1985. *T. harzianum* has been tested in Israel, Europe, South America and New Zealand on grapevine, cucumber, tomato, strawberry, bean, carrot and kiwi fruit and results were consistent in controlling *B. cinerea*. Their bio-preparation called Trichodex is registered in Israel and Yugoslavia. Another biofungicide called Mycostop (White *et al.*, 1990) based on *Streptomyces griseoviridis* should obtain registration for the control of *B. cinerea* in lettuce in Finland after toxicological trials.

By present knowledge, the use of antagonistic biocontrol agents in association with conventional fungicides seems to give satisfactory control of grey mould in a wide range of crops, and this development must be demonstrated convincingly to the policy-makers.

Concluding remarks

Chemical control is still used routinely and only rarely is consideration given to its effects on saprophytic microflora, whose vital role in the biological equilibrium is more fully appreciated. Present-day management practices for controlling diseases, particularly grey mould, is still far from the concept of integrated control!

The epidemiological approach in field conditions performed until now on grey mould, is a very conventional approach which is limited to the transient observation of phenomena.

Since *B. cinerea* attacks a wide range of host plants and is ubiquitous, epidemiological studies of grey mould should be done on a wider scale and comparisons made between different climatic regions. During their development cycle, pathogens move through various physiological states which are not receptive to the action of an antagonist. Vulnerable phases in the life cycle of the pathogen must be identified.

None of the models involving competition, mycoparasitism and antibiosis have been analysed in detail and there is a wide research area for studies of nutrient sources, the enzymes involved and the type of metabolites having a fungicidal or fungistatic action.

In the past most work on *Botrytis* spp. has been performed by mycologists who have studied the filamentous fungi, the yeasts and bacteria have received less attention. Work on these antagonists has now started and requires much more resources.

Although new methods in molecular biology do exist to improve antagonistic activities, the genes involved in antagonism are still to be identified. Studies of the precise effect of antagonists on the pathogen are therefore of great importance.

Industrial production of a biopesticide is difficult and the organism must be in a stable physiological state to survive in the formulation until it is used. Transmission electron microscopy has made it possible to assess the quality of a biopesticide (Vial *et al.*, 1992), and this method could be further developed in quality assessments.

The future perspectives for biological control in general, and especially against grey mould, lie in a wide-ranging approach, which should include research organisations, public and interprofessional bodies and industrialists from the crop protection sector, so that techniques may be standardised and models validated. It seems incomprehensible that, at a time when our governments are becoming more aware of the need for an agriculture system which is economical and more respectful to the environment, biological control is still not a player on the team!

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Biological control of *Botrytis cinerea* on grapevine by compost extracts and their microorganisms in pure culture

N. Ketterer, B. Fisher and H.C. Weltzien

Summary

Compost extracts reduced infection of grapevine by *B. cinerea*. Detached leaf bioassays demonstrated that the suppression could be enhanced to 90-95%. For proving fungicidal effects of compost extracts, cattle manure, horse manure and grape marc compost was in vestigated after a fermentation period of 8 days. These conditions promoted a number of bacteria of the genus *Pseudomonas* and spore forming genera. Previous studies have evaluated the microbial composition of the extract. The highest number of completely mesophilic aerobic bacteria was found in the cattle compost. Microorganisms were therefore isolated from extracts of that compost; a collection of 45 strains was obtained. Eight isolates reduced the disease level of *B. cinerea* to more than 98%. Subsequently a field trial was performed with the red vine cv. 'Domina' in the Ahr-valley, Germany. Due to prevailing weather conditions in 1991 there was a late infection of *B. cinerea* on berries. The treatments with compost extracts reduced the infection significantly and after addition of casein (0.5%) and pine needle oil (0.05%) before application of the extracts, the results were similar to those achieved with conventional fungicides.

Introduction

Due to the increasing environmental problems concerning the use of fungicides, alternative methods of disease control have to be studied. Biological control of plant diseases depends on antagonistic competition, antibiosis or hyperparasitism. Applications of suspensions of *Trichoderma-* or *Gliocladium* spp. have been tested with success, using their antagonistic and parasitic potential for disease suppression (Papavizas, 1985). Schönbeck and Dehne (1986) found a reduction in disease, possibly as a result of induced resistance to various obligate parasites after application of soilborne microorganisms and their culture filtrates.

For pratical use of these mechanisms, substrates with high microbe populations such as compost have been explored. Composted organic soil amendments have been used successfully to suppress soilborne pathogens. Hoitink *et al.* (1977) described the suppression of *Phytophthora cinnamoni* by composted hardwood bark. Hoitink and Fahy (1987) concluded that the microflora of composts is responsible for the suppression of soilborne plant pathogens. Soil amendments with composted manures also have depressive effects on *Erysiphe graminis* of wheat and barley if compared with plants cultivated in field soil (Budde and Weltzien, 1988). Direct application of aqueous extracts to composted manure-straw-soil mixtures to plant surfaces by dipping or spraying was first reported by Weltzien and Ketterer (1986). In these experiments the extracts reduced the downy mildew *Plasmopara viticola* on detached grapevine leaves. These promising results lead to the testing of watery extracts from composted organic

waste and the cultures of microorganisms isolated from that extracts against *B. cinerea* in laboratory and field conditions.

Material and Methods

Extraction of composts

The composts consisted of animal manure (horse and cattle) with cereal straw and amendments of top soil of fermented compost. Aqueous extracts of compost were obtained by mixing compost with tap water in ratio 1:10 (v/v). The suspension was stirred once and kept for extraction fermentation under laboratory or field conditions with temperatures ranging between 16 and 21°C. The extraction time during which the compost was covered with water was 1, 3, 7 and 14 days. Before use for disease control the extracts were filtered through cheese cloth.

Isolation of microorganisms

To isolate bacteria, the compost extracts were shaken vigorously in water (1 g compost or extract in 9 ml tap water). Dilutions to 10^{-9} were prepared and 0.05 ml of each dilution was poured into Petri dishes with the following selective media: Caso Agar, Merck No. 5458/-Bactilli; GSP Agar, Merck No. 10230/*Pseudomonades*.

Growth of microorganisms and inoculum production

The microorganisms were grown at 21°C in 100 ml Erlenmeyer flasks containing 25 ml nutrient solution. After 4 days each suspension was diluted with tap water (1:5, v/v) and the antagonistic efficiency of the isolates to *B. cinerea* was tested on detached vine leaves.

Application of compost extracts and microorganisms

A standard bio-assay was used for screening compost extracts and microorganisms. The bio-assay comprised a detached leaf test with vine leaves kept in a moist chamber. Detached leaves were inoculated with eight droplets (20 µl containing 2 x 10^6 conidia.ml^{-1}) on the adaxial side. Malt extract (1%) was added as a nutrient source for inoculation. In the screening tests, droplets contained 5 µl of the compost extract, or microorganisms suspended in a nutrient solution (15 µl malt extract). Successful infections were recorded after 2 to 3 days incubation at 21°C and a 14 h photoperiod. Grape berries, cv. Blauwer Spätburgunder, were dipped into compost extracts. After 3 days they were inoculated with a conidial suspension of *B. cinerea*. The disease severity was assessed as the percentage of diseased berries.

Field experiment

The field trial was located in a vineyard at the Ahr-valley, Germany, with the red vine cultivar Domina. The vineyard was split into 27 plots of about 30 plants each with the following treatments: horse manure compost extracts two month-old (CH2) and four month-old (CH4); CH2 + dextrose (0.5%); CH2 + casein (0.5%) + pine needle oil

Table 1. The effect of extraction times used to prepare compost extracts on the disease intensity of B. cinerea on detached grapevine leaves.

Treatment	Class of lesion size[1]			
	Extraction time (days)			
	1	3	7	14
Control (water)	4.5 a[2]	4.75 a	4.6 a	4.95 a
Horse manure	2.8 c	1.70 c	0.4 c	3.0 b
Grape marc	3.8 b	2.5 b	1.25 b	3.5 b
Cattle manure	2.5 c	1.45 c	0.3 c	2.0 c

[1]) 1 = very small lesion; 5 = large lesion
[2]) Different letters indicate statistical significance at 5% level.

(0.05%); CH2 + oil (0.05%); oil; fungicides and untreated check. Oil and casein were added immediately before application of compost extract. These eight treatments were replicated three times and assigned to plots at random. Applications were carried out with a motor backpack sprayer at a rate of 1000-1500 l per hectare at c. 12 day intervals, beginning in May until the end of August. The disease severity was assessed as percentage of diseased foilage or berries using the index of the official guidelines for pesticide evaluation (Flick *et al.*, 1989).

Results

Studies on detached vine leaves and berries

The effects of different compost extracts were studied *in vivo* on detached grape leaves and berries. In the first experiment the extraction times varied between 1 and 14 days. The results show an inhibitory effect of compost extracts on lesion sizes (Table 1) . The importance of a suitable extraction time for an effective disease suppression was apparent, the optimum time being 8 days. The suppression was enhanced by up to 60-90% by spraying compost extracts. Similar promising results were achieved in the berry test (Fig. 1).

When rinsing off the extracts from the leaf surface 2 days before incubation of the pathogen the effect of suppression was lost completely (Fig. 2).

In the mycelial growth test with *B. cinerea* the extract-water agar mixtures were covered with 8 ml standard malt agar and inoculated with a mycelial disk. Extracts of all three composts reduced the mycelial growth. Even the extracts obtained after eight days extraction time inhibited mycelial growth strongly (Fig. 3) and inhibition of germination of conidia was observed by light microscopy.

In other experiments we found, after sterile filtration or heat sterilisation of the horse manure compost extract that the broth remained ineffective (Fig. 4).

Fig. 5 summarises the microbial composition of the extracts. The total number of colony forming units (cfu) increased from 1 to 7 days of extraction time and decreased thereafter. The highest count was 10^8 cfu.ml^{-1} after 7 days.

To examine the antagonistic effects further we isolated microorganisms from different composts and compost extracts and tested their efficacy against *B. cinerea* on grapevine

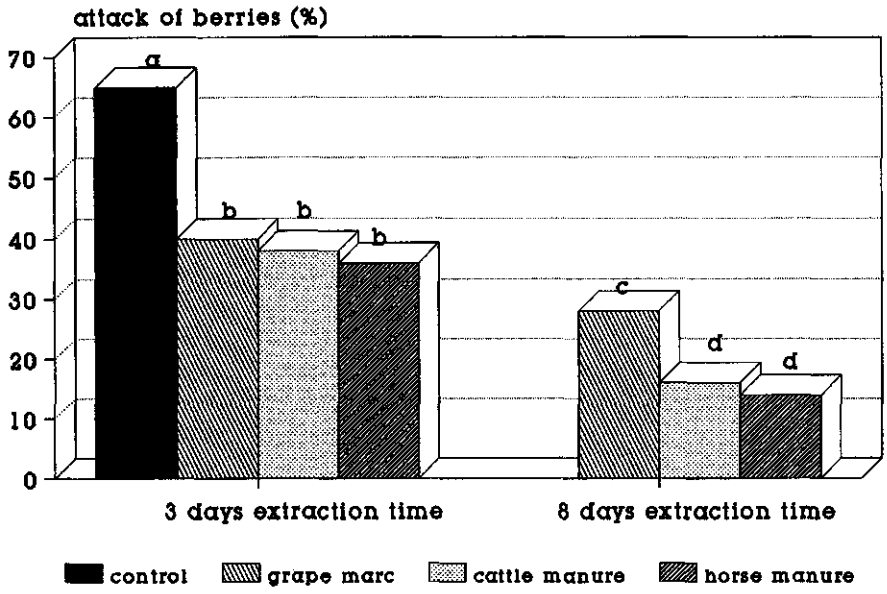

Fig. 1. Effect of three extracts produced in two extraction times on disease intensity of B. cinerea on grape berries.

Different letters indicate significant differences, p = 0.05

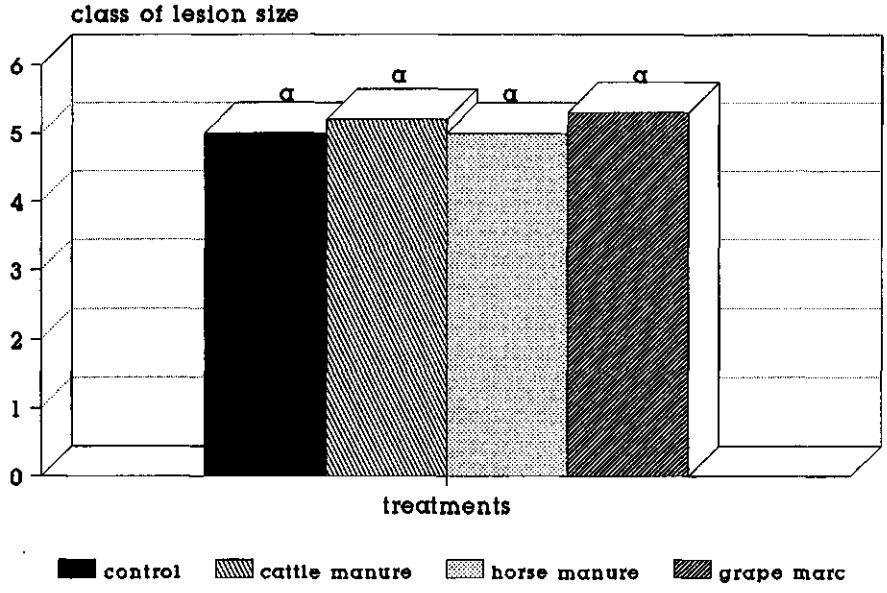

Fig. 2. Effect on disease intensity of removing the extracts before inoculation with B. cinerea.

Different letters indicate significant differences, p = 0.05

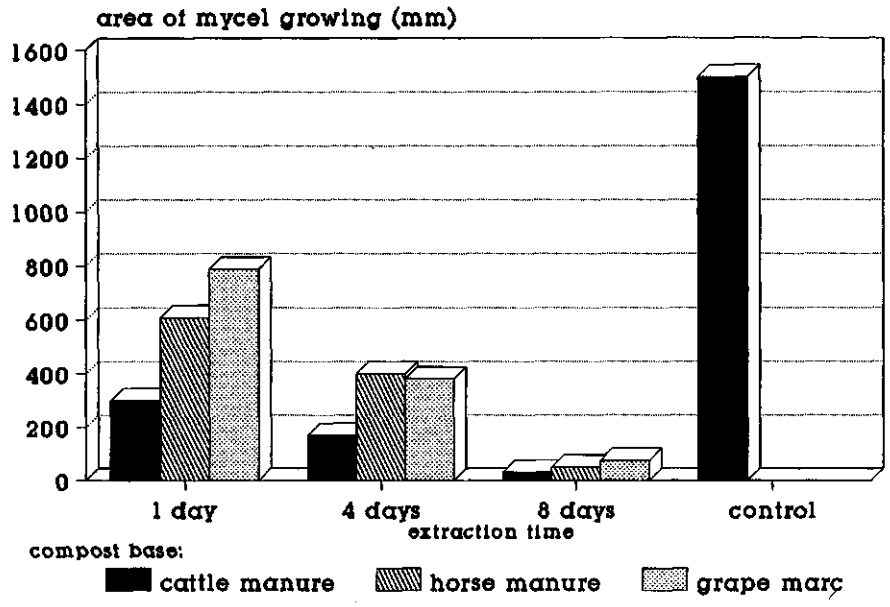

Fig. 3. Effect of different compost extract-water agar mixtures on mycelial growth of B. cinerea.

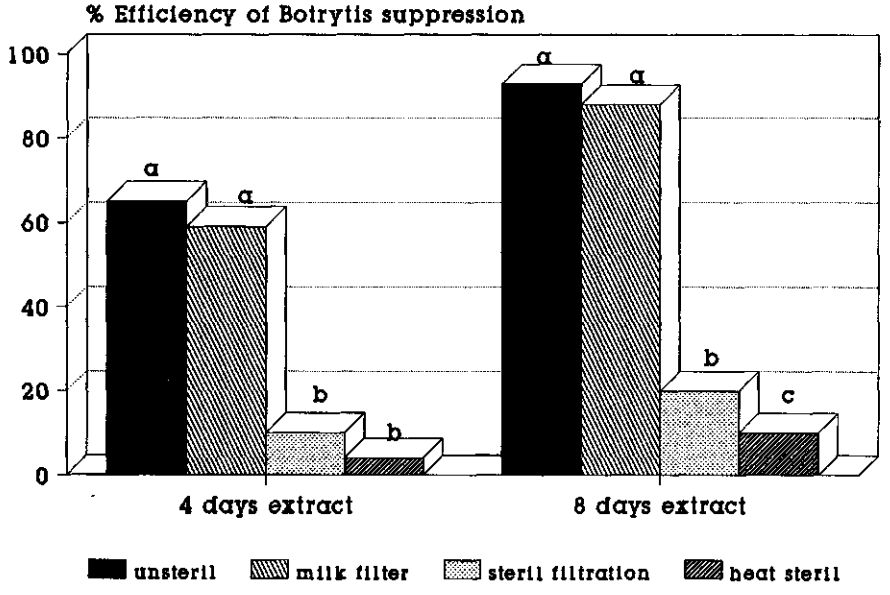

Fig. 4 Effect of sterilised horse manure compost extract on infection of grapevine leaves by B. cinerea.

Different letters indicate significant differences, p = 0.05

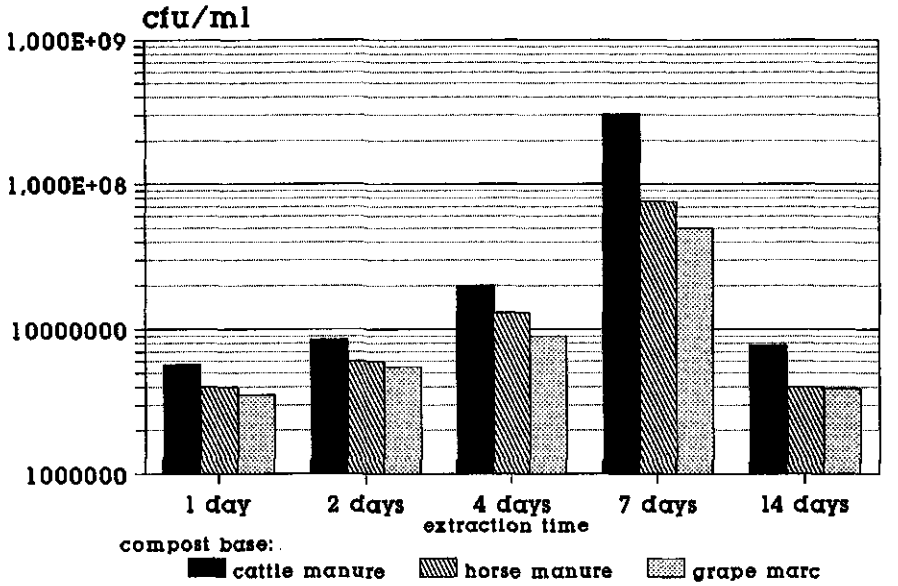

Fig. 5. Number of colony forming units obtained from three compost extracts after different extraction times.

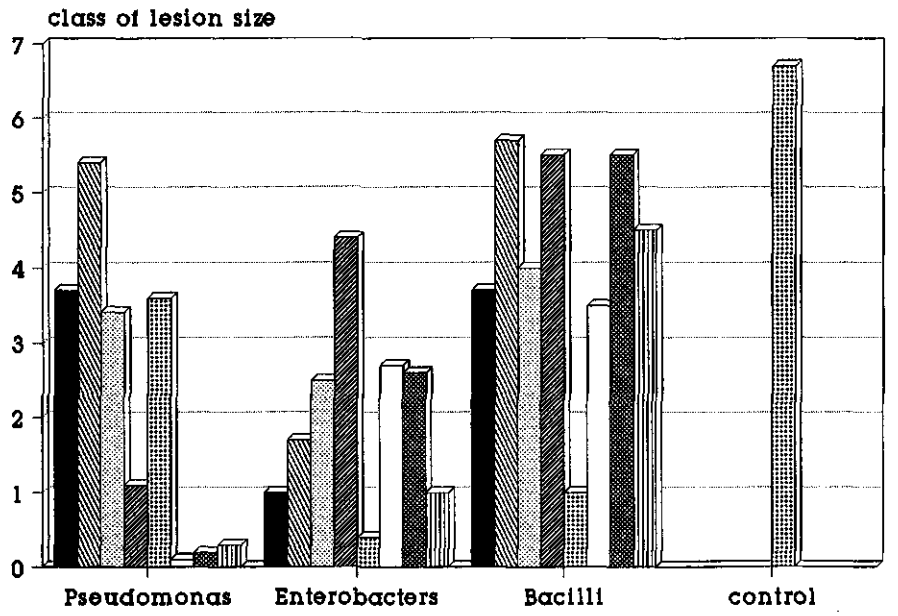

Fig. 6. Effect of microbial isolates on B. cinerea on grapevine leaves.

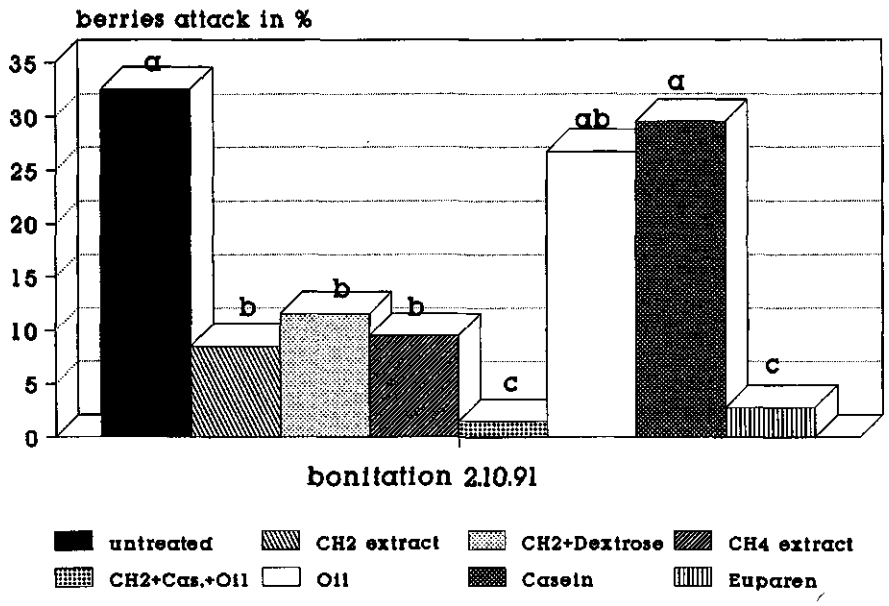

Fig. 7. Control of grey mould of grapes by compost extracts containing nutrients and pine needle oil in comparison to fungicides.

leaves (Fig. 6). Eight isolates, namely four species of *Pseudomonas*, three isolates of *Enterobacteriaceae* and one species of *Bacillus* were highly inhibitory to *B. cinerea*.

Field experiment

Due to the prevailing weather conditions in 1991 there was a late infection of *B. cinerea* on berries. Treatments with compost extracts reduced this attack significantly compared to controls (Fig. 7). When CH2-extract was added with casein (0.5%) and pine needle oil (0.05%) before spraying, the results were similar to those achieved after application of conventional fungicides. There was no significant difference in efficacy between the treatments of CH2-extract, CH4 extract and CH2 amended with dextrose. The yield increased from 1.1 kg per grapevine plant on the untreated control to 1.8 kg per plant in the plots treated with CH2 + casein + oil. CH 4, CH2 and CH2 + dextrose produced a yield increase of 1.4 - 1.5 kg per plant.

Discussion

The suppressive potential of extracts was largely based on the presence of active microorganisms. Consistent results were obtained with detached leaves and berries in laboratory experiments and under field conditions. The total number of microorganisms and the efficiency of the extracts was increased and results were more consistent after addition of nutrients. A new system for biological plant protection seems to be possible, but more information is required about the mode(s) of action and methods of producing the extracts.

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Effect of substrate, temperature and time of application on the effectiveness of three antagonistic fungi against *Botrytis cinerea*

N.E. Malathrakis and O. Kritsotaki

Summary

The influence of substrate, temperature and time of application of the antagonistic fungi *Trichoderma* sp., *Penicillium* sp. and *Acremonium alternatum* against *B. cinerea* were studied using potato dextrose agar, discs of cucumber fruits and young bean plants. The antagonists were applied as a spore suspension up to 5 days before the inoculation with *B. cinerea*. Tests were performed at four temperatures and growth of *B. cinerea* recorded after 2 to 4 days. It was found that the earlier the application of the antagonists and the higher the incubation temperature, the stronger was the inhibition of *B. cinerea*.

Antagonists needed less time for growth on PDA and young bean plants, to be effective against *B. cinerea* than on cucumber discs. On cucumber discs complete control was not achieved, even if the antagonists have been applied 5 days before the pathogen. *Penicillium* sp. was more active at low temperatures than the other two antagonists.

Introduction

We found previously that *Trichoderma harzianum*, *Penicillium* sp. and *Acremonium alternatum* were effective against *Botrytis cinerea* Pers.: Fr. *in vitro* (Malathrakis and Klironomou, 1992), but in greenhouse experiments only the first was moderately effective. This is a common phenomenon with biological control agents and several factors are probably involved (Dubos, 1987); three of these, substrate, temperature and time of application were studied to enhance the performance of the above antagonists.

Material and Methods

Tests were carried out on patoto dextrose agar (PDA), discs of cucumber fruits and young bean plants. Inoculum of *T. harzianum*, *Penicillium* sp. and *A. alternatum* were applied at different times before or after the inoculation of the substrate with *B. cinerea*. Tests on PDA were carried out simultaneously with the three antagonists at 10°, 15°, 20° and 25 °C but on cucumber discs and young bean plants the tests were carried out separately at the same temperatures. The inoculum of the antagonistic fungi was prepared as follows: stock cultures of each fungus were transferred onto PDA plates and incubated at 22 °C for 4 days. The spores produced were harvested in sterile distilled water, centrifuged at 400 rpm for 10 min, the supernatant removed and the pellet resuspended in water. The spore concentration was determined with an haemocytometer and spores were kept at 5°C before use in each experiment.

Antagonism on PDA

To examine the effect of time of establishment of the antagonist on *B. cinerea* five treatments were used in which 16 Petri dishes containing PDA were evenly seeded with 0.5 ml of spore suspension of each antagonist containing 10^6 spores.ml^{-1}, 3, 2 and 1 day before the inoculation or on the same day and 1 day after. Four dishes of each antagonist were incubated at each of the four temperatures. Four other unseeded dishes were similarly incubated as controls. All the plates were inoculated centrally with a 5 mm plug of young mycelium of *B. cinerea* on day zero. The diameter of the mycelial growth of *B. cinerea* was recorded in each Petri dish 2 days later and the mean and standard deviation for each treatment calculated.

Antagonism on cucumber discs

Fresh Dutch type cucumber fruits (*Cucumis sativus* L.) were surface-sterilised with alcohol and cut into discs c. 5 mm thick. Seven discs were placed in each 15 cm Petri dish. The discs in four dishes were then seeded evenly with the antagonist 5, 4, 3, 2 and 1 day before inoculation with *B. cinerea*, and on the same day and the day after. For each disk 40 µl of a spore suspension containing 2 x 10^6 spores.ml^{-1} were used. As for the PDA experiment, the discs were incubated at four temperatures and on day zero all dishes were inoculated. The diameter of the rotting zone on the cucumber discs was recorded 2 days later.

Antagonism on bean plants

Plants of bean (*Phaseolus vulgaris* L.) grown in 6 cm pots with two fully developed leaves were used in these experiments. Ten punctures were made with a needle in each leaf before the antagonists were applied. A spore suspension of antagonist containing 10^6 spores.ml^{-1} was sprayed onto 24 of these plants until run off on 4, 3, 2 and 1 day before inoculation with *B. cinerea* and on the same day of inoculation. Six plants for each antagonist treatment were placed in growth chambers at 10°, 15°, 20° and 25°C on a 12/12 h photoperiod and 100 r.h. Six plants untreated with antagonists were also in cluded in each chamber. All plants were spray-inoculated with *B. cinerea* on day zero with a suspension containing 10^6 spores.ml^{-1}. The infection of wounded bean leaves was recorded 3-4 days later on a 0 to 5 scale (0 = no infection, 5 = infection of c. 1 cm diameter) and the mean and standard deviation of each treatment was calculated.

Results

PDA tests

The antagonists seeded 3, 2 or 1 days before inoculation by *B. cinerea* were already well-developed and 2 days after the inoculation with *B. cinerea* the growth of *Penicillium* sp. and *Acremonium alternatum*, but not that of *Trichoderma* sp., was inhibited near the inoculum.

It was found that the higher the temperature and the earlier the application of the antagonists, the greater the inhibition of *B. cinerea* (Table 1). To obtain complete inhibition all three antagonists, with a few exceptions, needed to be applied more than 3

Table 1. Growth of B. cinerea on PDA seeded by spores of three antagonists, 2 days after incubation at four temperatures

		Penicillium sp.	*A. alternatum*	*Trichoderma sp.*
10°C	Control	5.7 + 0.5		
	–3[1)]	0.0 ± 0.00	2.8 ± 0.45	4.0 ± 0.90
	–2	1.5 ± 0.30	2.2 ± 0.25	2.3 ± 0.25
	–1	2.3 ± 0.25	6.0 ± 0.60	2.8 ± 0.50
	0	3.5 ± 0.65	5.3 ± 0.25	4.0 ± 0.0
	+1	6.3 ± 0.75	5.5 ± 0.30	5.7 ± 0.50
15°C	Control	20.0 ± 0.70		
	–2	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
	–1	0.0 ± 0.00	11.0 ± 0.40	2.3 ± 0.50
	0	16.7 ± 1.15	19.2 ± 10	15.5 ± 1.65
	+1	15.8 ± 0.75	18.0 ± 10	19.8 ± 0.25
20°C	Control	33.7 ± 0.85		
	–1	0.0 ± 0.00	1.0 ± 0.00	0.0 ± 0.00
	0	0.0 ± 0.00	26.7 ± 10	15.5 ± 1.65
	+1	30.7 ± 1.45	35.7 ± 0.50	34.7 ± 1.05
25°C	Control	41.0 ± 0.4		
	–1	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
	0	3.5 ± 0.85	19.2 ± 2.20	6.5 ± 0.30
	+1	33.7 ± 4.1	35.2 ± 0.80	35.2 ± 1.00

[1)] Mean ± S.E., n = 4. Time (days) antagonist present. Minus and plus indicates that the antagonists were applied before or after the day of inoculation by *B. cinerea* (day 0)

days before the inoculation with *B. cinerea* at 10°C, 2-3 days at 15°C and only 1 day previously at 20° and 25°C. *A. alternatum* was generally less inhibitory than the other two species and this was evident through the binocular microscope 24 h after their application, at 20° and 25°C.

Cucumber discs

The infection of cucumber discs by *B. cinerea* was inhibited by all the antagonists; the higher the temperature and the earlier the application of the antagonists, the stronger the inhibition of infection. For complete inhibition at 10° and 15°C all the antagonist needed more than 5 days establishment. *Penicillium* sp. was the most, and *Trichoderma* sp. the least effective.

Bean plants

The antagonists developed on the dead tissue around the wounds on leaves after 1 to 3 days depending on the fungus and development was faster at 25°C and than at lower temperatures (Table 2). *Trichoderma* sp. developed more rapidly than *Penicillium* sp., which developed faster than *A. alternatum*. To be completely effective at 10° and 15°C *Penicillium* and *Trichoderma* needed to be applied 2 days before inoculation with

Table 2. Effect of time of application of antagonist and temperature on infection caused by B. cinerea

		% Leaf wounds infected[1]		
		Penicillium sp.	*A. alternatum*	*Trichoderma* sp.
10°C	Control	11.6 ± 2.70	75.3 ± 9.80	16.7 ± 3.30
	–2[2]	0.0 ± 0.00	12.7 ± 4.10	0.0 ± 0.00
	–1	2.2 ± 1.00	33.5 ± 7.40	0.0 ± 0.00
	0	8.4 ± 0.70	48.7 ± 8.50	10.6 ± 3.45
15°C	Control	46.5 ± 4.50	100.0 ± 0.00	92.8 ± 3.10
	–2	1.8 ± 0.90	6.7 ± 4.60	0.5 ± 0.32
	–1	2.1 ± 2.10	20.7 ± 11.40	11.2 ± 3.80
	0	30.5 ± 4.40	84.7 ± 11.90	91.3 ± 4.98
20°C	Control	78.4 ± 12.10	88.7 ± 7.20	0.0 ± 0.00
	–2	0.4 ± 0.40	0.0 ± 0.00	0.0 ± 0.00
	–1	0.4 ± 0.40	0.0 ± 0.00	0.0 ± 0.00
	0	22.2 ± 9.70	32.0 ± 13.3	7.5 ± 3.60
25°C	Control	76.7 ± 6.00	33.0 ± 7.70	26.7 ± 5.90
	–2	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
	–1	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
	0	17.6 ± 2.70	2.7 ± 1.90	0.0 ± 0.00

[1]) Mean ± S.E., n = 6.
[2]) Time (days) antagonist present. Minus and plus indicates that the antagonists were applied before or after the day of inoculation by *B. cinerea* (day 0).

B. cinerea, but for similar efficacy at 20° and 25°C all the antagonists needed only 1 day. Antagonists applied the same day as *B. cinerea* (day 0) were still effective at 20° and 25°C, less so at lower temperatures.

Discussion

These tests suggested that substrate, temperature and time of application of antagonists relative to inoculation of the pathogen considerably influenced the performance. All were less effective on cucumber discs than on PDA or young bean plants, and, irrespective of the substrate, the earlier the application of the antagonist the greater its effectiveness at all temperatures. As grey mould is most serious in greenhouses in Greece during winter, with temperatures in the range of 12°-17°C (Malathrakis, 1989), biological control using the antagonists *Penicillium* sp., *A. alternatum* or *Trichoderma* sp. will not be effective, as they need a higher temperature to develop.

Fresh wounds without resident mycelium of *B. cinerea* may be easily protected by antagonists if they are applied soon after the wounds have been made (Fokkema, 1991). However grey mould usually develops after the pathogen has become established on dying or senescent tissues. On present evidence protection against this type of infection

may be difficult unless colonisation of these substrates has been inhibited (Newhook, 1957).

Antagonists should be selected which develop rapidly on fresh wounds or senescent tissues at low temperature and are more adaptive to conditions prevailing in protected cropping (Dubos, 1992).

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Biological control of *Botrytis* leaf blight of onions: significance of sporulation suppression

J. Köhl, W.M.L. Molhoek, C.H. van der Plas, G.J.T. Kessel and N.J. Fokkema

Summary

Saprophytic antagonists can be applied to suppress the sporulation of *Botrytis* spp. on necrotic tissue. The effect of sporulation suppression on an epidemic of *Botrytis* spp. was studied in a field experiment with onions. When 30-50% of the necrotic tissue was removed from the field, the content of *Botrytis* spores in the air above the crop was reduced by 34% and the epidemic delayed. In late August, the number of lesions on the leaves caused by *B. cinerea* and *B. squamosa* was 1.1 lesions cm^{-2} in the control and 0.6 lesions cm^{-2} after removal of the necrotic tissue.

The potential microbial antagonist *Gliocladium roseum*, was sprayed as a conidial suspension onto the plants weekly, but this did not result in colonisation of the necrotic tissue and had no effect on the *Botrytis* epidemic. Further screening of antagonists with emphasis on their ecological competence is necessary.

Introduction

Botrytis spp. sporulate primarily on dead tissue of the host plants. The necrotic tissue may also provide a suitable substrate for saprophytic antagonists. In this substrate, antagonists may interfere with the saprophytically growing mycelium of *Botrytis* spp. and suppress sporulation of the pathogen. An effect of introduced antagonists on the epidemic of *Botrytis* can only be expected when most of the infections during the epidemic are caused by spores produced within the crop and inoculum produced outside the crop is less important. A field experiment with onions was used to measure the effect of suppressing sporulation on epidemics of *B. squamosa* and *B. cinerea*, causing onion leaf blight and leaf spot respectively. *Gliocladium* spp. have suppressed sporulation of *Botrytis* in bioassays almost completely (Fokkema *et al.*, 1992), and therefore a strain of *G. roseum* was used as an antagonist in the field experiment described here.

Materials and Methods

Onions were sown in plots of 9 x 12 m in a randomised block design with six replicates per treatment. Plots were separated by 12 m wide sugar beet strips to minimise interplot interference. To obtain a homogeneous infection with *B. squamosa* at the beginning of the epidemic, sclerotia of the pathogen, grown on sterile onion leaves in wet chambers, were spread over the plots at the end of April. Sclerotia are regarded as the primary inoculum of *B. squamosa* (Ellerbrock and Lorbeer, 1977).

To stimulate the effect of an antagonist, all onion leaves which were necrotic over at least 50% of their length were cut and removed from the plots weekly such that during the experiment c. 30-50% of dead leaf tissue was removed. In a second treatment,

G. roseum was applied weekly as a conidial suspension. Conidia were produced on autoclaved wheat kernels and suspensions were prepared by adding tap water with 0.01% Tween 80 to the cultures. After filtration through a double layer of cheesecloth, the conidial concentration was adjusted to c. 1 x 10^6 conidia.ml^{-1}. No further treatments were carried out in the control plots.

Rotorods (Sampling Technologies, Los Altos Hills, Ca, USA) were used to trap airborne spores (Edmonds, 1972). Two rotorods were used simultaneously in the centre of each of two plots of each treatment at a height of 0.3 m. The concentration of airborne spores was calculated from the number of spores trapped during 15 min.

Ten plants per plot were sampled weekly from June 11 until August 20. Lesions were counted for all leaves except those with more than 50% the leaf length necrotic. The surface of the green leaves was measured and the number of lesions per cm^2 was calculated. The length of green and necrotic leaf parts was also recorded.

Samples of necrotic leaf tissue were washed thoroughly to remove adhering spores and homogenised, and dilutions of the homogenate were plated on malt agar containing 15 μg tetracycline.ml^{-1} to determine the colonisation of the tissue by the applied antagonist.

Air temperature, relative humidity and leaf wetness were continuously monitored.

Results

Due to a dry period at the beginning of May, no sporulation of *B. squamosa* was found on the introduced sclerotia. Within 21 days, many of the sclerotia were attacked by collembola (e.g. *Sminthurus* spp.). Thus, it can be assumed that most infections of *Botrytis* spp. during the field experiment were caused by natural inoculum. Comparison

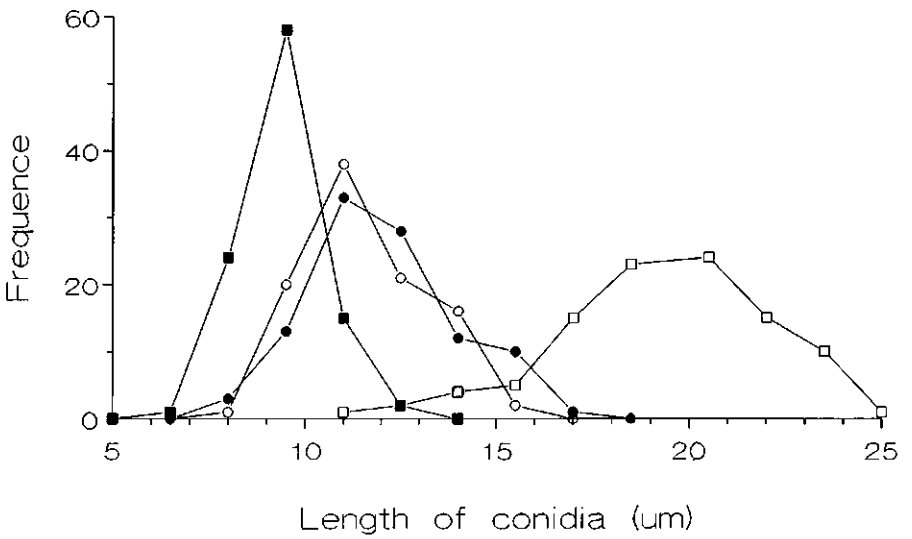

Fig. 1. Length of conidia of B. cinerea (-■-) and B. squamosa (-□-) grown on sterile onion leaves and of conidia produced on necrotic onion leaf tips (-○-) or trapped with Rotorods (-●-) in the field.

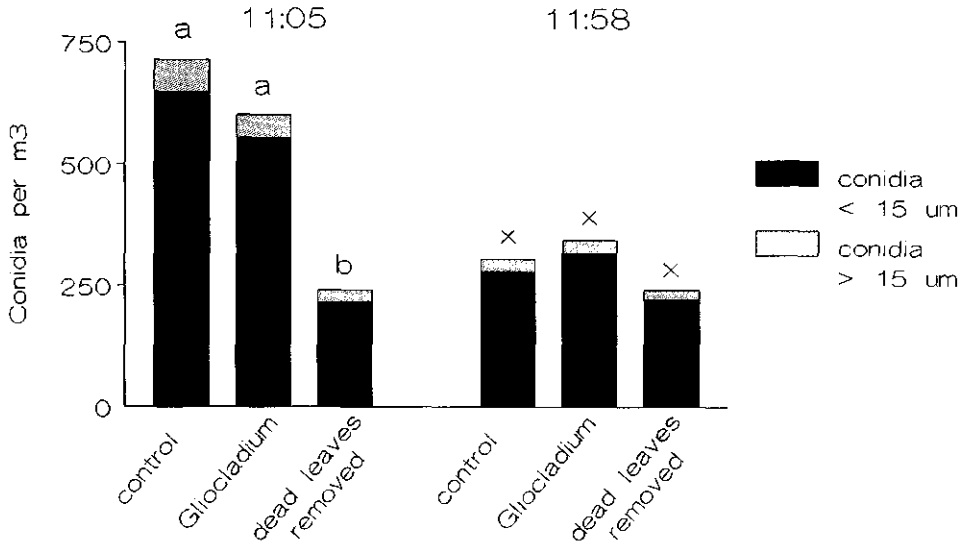

Fig. 2. Concentration of airborne conidia of Botrytis cinerea (< 15 µm) and B. squamosa (> 15 µm) on August 16 after removal of necrotic leaf tissue or application of Gliocladium roseum. Columns with the same letter do no differ significantly according to LSD-test (p = 0.05).

of the size of the *Botrytis* conidia (Ellis, 1971) collected with the Rotorods or produced on dead onion leaf tips, with those produced by isolates of *B. cinerea* and *B. squamosa* on sterile onion leaves showed that the majority of *Botrytis* conidia produced in the field belonged to *B. cinerea* (Fig. 1).

On five days in August, spores were trapped when abundant sporulation was found in the crop. Each day, two to four runs of 15 min were carried out between 10.00 a.m. and 1.00 p.m. during which period a peak of spore release could be expected (Lacy and Pontius, 1983). The spore load above plots where the necrotic tissue had been removed, was always lower than in the control plots. Reduced spore loads measured at high concentration levels of airborne conidia were significant but, not at low levels (Fig. 2). The results of all spore trappings are summarised in Fig. 3. Spores were trapped in between a sugar beet buffer crop and a plot treated with the antagonist averaged 50% of the concentration measured above the control plot at the same time. *Botrytis* spp. did not sporulate on sugar beet tissue. Most of the trapped spores had the size of conidia of *B. cinerea* (Fig. 1). Spraying with *G. roseum* had no effect on the spore load above the treated plot.

The first *Botrytis* lesions occurred on June 11 but the epidemic was interrupted by a period of hot and dry weather. In the middle of July, new lesions were found and their number increased exponentially until late August. *B. cinerea* was frequently isolated from surface sterilised lesions, whereas *B. squamosa* was never found. In plots where necrotic leaf tissue had been removed, the slope of the regression line was significantly lower ($p < 0.001$) than the control (Fig. 4). In late August, 1.1 lesions cm^{-2} were counted in the control plots, whereas only 0.6 lesion cm^{-2} were found in plots where an antagonist had been simulated by the removal of 30-50% of the necrotic tissue. The treatments in

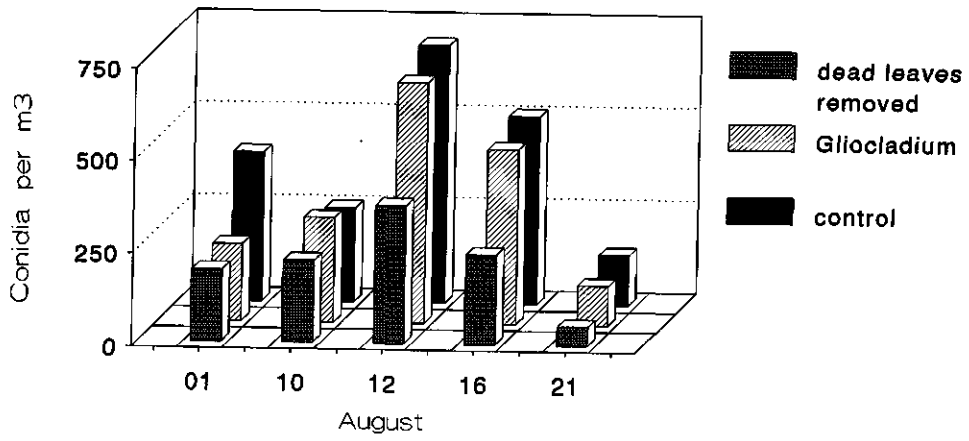

Fig. 3. Concentration of airborne Botrytis conidia during 5 days in August after removal of necrotic leaf tissue or applications of Gliocladium roseum (means of 2-4 measurements per day).

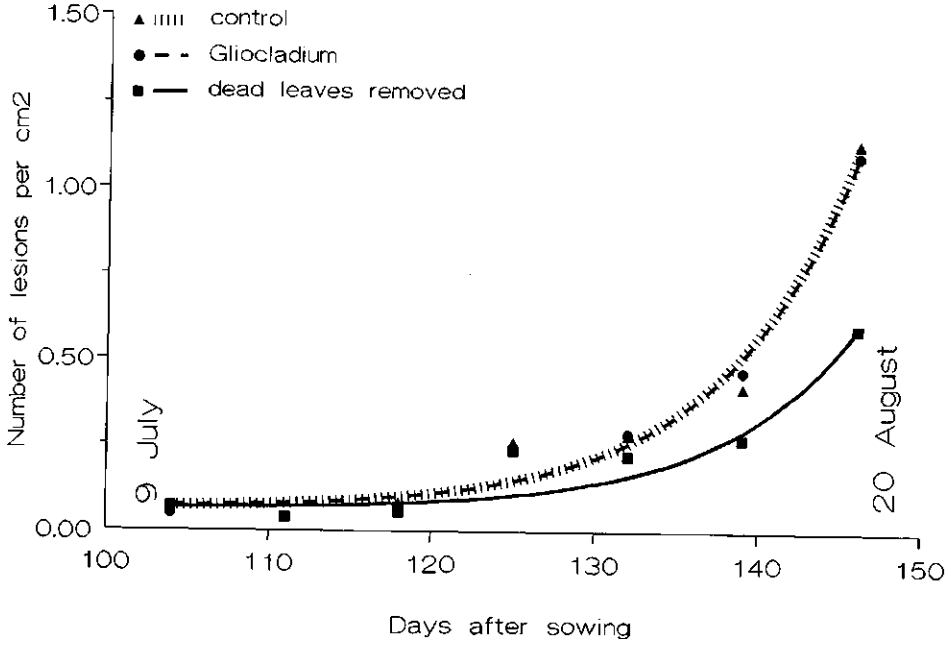

Fig. 4. Number of lesions caused by B. cinerea or B. squamosa on onion leaves after removal of necrotic tissue or applications with Gliocladium roseum.

which *G. roseum* was applied did not affect the number of lesions and it was not possible to isolate *G. roseum* from necrotic leaf tissue sampled 6 days after the antagonist had been sprayed.

Discussion

A clear relationship between the amount of suitable substrate for sporulation of *Botrytis* spp., the spore load in the air above the crop and the number of lesions on leaves was found in the field experiment. Therefore, an epidemic of *Botrytis* spp. in onions mainly depends on spores produced inside the field on host plants. This seems true for the host specific pathogen *B. squamosa* and the non-specific, ubiquitous *B. cinerea* which was dominant in our field experiment. Since conidia were also trapped between the plots, it can be assumed that some interactions between plots occurred. In a large field, the effects of sporulation suppression will be more distinct.

Necrotic tissue is a much more attractive substrate for saprophytic antagonists than the surface of an intact leaf. Consequently, control of diseases caused by *Botrytis* spp. by suppression of sporulation seems to be more feasible than the use of antagonists to prevent infections on the green leaf. Along this line, Peng and Sutton (1990) found that spraying with *G. roseum* was equally effective as fungicides in reducing sporulation of *B. cinerea* on dead leaves and the incidence of fruit rot in strawberry fields.

G. roseum did not colonise the necrotic leaf tissue under our conditions. This can be due to the spraying technique, to the quality of the conidia applied or to the ecological competence of the antagonist which is primarily a soil inhabitant. Further screening of antagonists amongst saprophytes naturally occurring on necrotic leaf tissue and of other strains of *Gliocladium* spp. and *Trichoderma* spp. in bioassays and under field conditions is necessary.

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Biological control of *Botrytis* rot of apple

M.L. Gullino, D. Benzi, C. Aloi, A. Testoni and A. Garibaldi

Summary

Two yeast isolates identified as *Trichosporon* sp. and *Candida* sp. effectively controlled rot of apple caused by *B. cinerea*. Wounded fruits (cv. Golden Delicious), treated with 10^6 cells.ml^{-1} of the biocontrol candidates and inoculated with the pathogen showed a significantly lower disease incidence as compared with control fruit at both 2-4° and 22 °C. The biological agents were effective when disease incidence was moderate and remained satisfactory even if the disease was more severe.

Introduction

Postharvest losses due to rot caused by *Botrytis cinerea* Pers.: Fr. can be serious on several perishable crops, even in production areas where the most advanced storage technologies are available. Where permitted, fungicides are a primary control measure for postharvest rots (Eckert and Ogawa, 1985). During recent years, a combination of biological and regulatory factors has increased interest and efforts in the development of agricultural production, storage and marketing systems that utilise reduced chemical inputs.

Public awareness of fungicide residues in food has increased, and government regulatory agencies are responding by reassessing the use of many pesticides. Moreover, development of fungicide resistance, common in the case of postharvest pathogens (Romano and Gullino, 1983), has stimulated efforts to develop alternative systems for disease control.

Under these perspectives, the search for biological control agents (BCA) effective against postharvest disease looks challenging (Wilson and Wisniewski, 1989). The present work summarises results, previously published (Aloi *et al.*, 1991; Gullino *et al.*, 1991, 1992), obtained in Italy by searching for BCA active against *Botrytis* rot of apple.

Material and Methods

Potential antagonists were isolated from the surface of apples harvested in unsprayed orchards (Gullino *et al.*, 1991). Their biocontrol activity was tested on apple, cv. Golden Delicious, surface sterilised in sodium hypochlorite (8% as chlorine), rinsed with tap water and punctured at the equatorial region (three punctures per apple). The antagonists were applied at 10^6 cells.ml–1 for yeast, or 10^8 cells.ml^{-1} for bacteria by using 10 µl cell suspension per wound (Tables 1 and 2) or by dipping fruit for 2 min in a cell suspension (Table 3). After treatment with the antagonists, fruits were kept at 20-22°C for 12 to 24 h, then inoculated with a conidial suspension of *B. cinerea* (5 x 10^5 conidia.ml^{-1}) using 10 µl suspension per wound or by dipping fruits for 2 min in this suspension. A mixture of four to six isolates of *B. cinerea*, sensitive (S, Tables 1 and 2) or benzimidazole-resistant

Table 1. Effectiveness of different microorganisms against Botrytis rot of apple at two temperatures.

Treatment	Rot incited by *B. cinerea* (% of the control)	
	2-4°C	22°C
2.33 Y[1)]	13 abc[2)]	21 b
4.4 Y	10 ab	9 a
1.16 B*	45 cde	41 d
1.7 B	40 abcd	13 ab
4.42 B	45 cde	62 e
5.50 B	48 de	93 f
11.31 Y	45 cde	33 c
11.33 B	72 e	40 cd
TBZ (1g.l^{-1})	13 abc	5 a
Control	100 f	100 f
	(27)[3)]	(49)[3)]

1) Y = Yeasts, used at 10^6 cells.ml^{-1}; B = Bacteria, used at 10^8 cells.ml^{-1}
2) Values of the same column, followed by the same letter do not differ significantly following Duncan's Multiple Range Test (p = 0.05)
3) Diameter (mm) of lesions in control fruit

Table 2. Effectiveness of two biocontrol candidates against Botrytis rot of apple cv. Golden Delicious, caused by isolates which were benzimidazole-sensitive (S) or -resistant (RB) (Gullino et al., 1992).

Treatment	Rot incited by *B. cinerea* (% of the control)			
	S		RB	
	2-4°C	22°C	4°C	22°C
Candida sp. 4.4	10 a[2)]	9 a	16 a	37 a
Trichosporon sp. 2.33	13 a	21 a	34 a	25 a
TBZ (1g.l^{-1})	13 a	5 a	62 b	99 b
Control	100 b	100 b	100 b	100 b
	(27)[3)]	(49)[3)]	(22)[3)]	(33)[3)]

2) and 3) see Table 1

(RB, Tables 2 and 3), were used for inoculations. After inoculation with the pathogen, apples were kept at 20-22°C or stored at 2-4°C. In the latter case, apples were transferred from 2-4° to 20-22°C for 10 days before measuring the diameter of the decayed tissue around the wounds.

Results and Discussion

The screening of hundreds of bacterial and yeast isolates yielded some microorganisms that markedly inhibited *Botrytis* rot. Most microorganisms which were active against

Table 3. Effectiveness of several treatments against rot of apple after wound inoculation with B. cinerea (fruit kept 30 days at 2°C + 8 days at 20-22°C) (Aloi et al., 1991).

Treatment	*B. cinerea*	Rot diameter (mm) in trial		
		I	II	III
1) Not wounded	–	0.1 a[1)]	0.0 a	0.0 a
2) Control[2)]	–	1.7 c	20.2 e	7.8 d
3) Control[3)]	+	24.2 f	55.6 g	34.7 g
4) TBZ (1 $g.l^{-1}$)	+	11.5 e	61.4 h	33.2 f
5) 2.33	–	0.2 a	1.6 b	2.5 b
5) 4.4	–	0.7 b	3.4 c	10.2 e
7) 2.33	+	2.1 c	19.3 d	6.7 c
8) 4.4	+	4.8 d	29.4 f	7.5 d

[1)] see table 1, but p = 0.01
[2)] not treated, not inoculated with *B. cinerea*
[3)] not treated, inoculated with *B. cinerea*

B. cinerea also showed activity against *Penicillium* expansum. Yeasts generally showed a higher activity than bacteria.

Two yeasts, coded as 2.33 and 4.4 and identified as *Trichosporon* sp. and *Candida* sp. respectively (Gullino *et al.*, 1992) showed effective and consistent biocontrol against *B. cinerea* at 2-4° and 20-22°C (Table 1). The two biocontrol candidates showed an activity comparable to that of the fungicide TBZ when rot was caused by benzimidazole-sensitive strains of *B. cinerea*. On the contrary, in the presence of benzimidazole-resistant *B. cinerea*, TBZ failed to control *Botrytis* rot (Table 2). The antagonists did not cause any significant damage to apple fruit. The best biocontrol activity was observed in the presence of attacks of medium intensity (Table 3, trial I).

In conclusion, the effectiveness of *Trichosporon* sp. 2.33 and *Candida* sp. 4.4, looks promising. The levels of the efficacy of these two BCA is similar to that reported for other biocontrol candidates (Wilson and Wisnieswki, 1989; Roberts, 1990). The good activity shown by the two yeasts against *B. cinerea* suggest that they are worth to be appraised for use on other crops commonly affected by *Botrytis* rot during storage and transit, such as kiwi, grape and strawberry. Studies on the mode of action of these two antangonists are required to develop the best method of application.

Acknowledgement

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Biological control of *Botrytis cinerea* on cold stored dutch white cabbage

C. Leifert, D.C. Sigee and H.A.S. Epton

Summary

Bacterial antagonists of *B. cinerea* were isolated from Dutch white cabbage on the basis of *in vitro* inhibition in plate assays. All species showing antagonism at 4°C were in the genera *Pseudomonas* and *Serratia*. Cabbage disks taken from internal leaves needed heat treatment (50°C for 15 min) to overcome the initial resistance of the plant tissue, which prevented spore germination and fungal spoilage for periods of at least 2 months. On heated cabbage disks spores of *B. cinerea* germinated and mycelium covered the whole disk within 2 weeks. Spoilage of heated disks could be prevented by dipping of disks in suspensions of *Pseudomonas* and *Serratia* antagonists.

Introduction

Botrytis cinerea Pers.: Fr. is one of the most important post-harvest diseases of fruit and leafy vegetables. For many plant species *B. cinerea* is an opportunistic pathogen attacking weakened, wounded or senescent tissues. Healthy leaf tissues have, however, been described as being highly resistant to attacks of *B. cinerea* (Newhook, 1951). In contrast to many other fungi, it can cause spoilage at temperatures (1 to 10°C) which are used for long term storage of fruit and vegetables. Rot of cabbage by *B. cinerea* usually starts after 2-3 months of cold storage and is often confined to the outer, dried out, senescent cabbage leaves (Wale, 1980). Post-harvest treatment of fruit and vegetables with fungicides such as benomyl (Benlate) or iprodione (Rovral) greatly reduced the losses due to rot by *B. cinerea* and allowed cabbages to be stored for up to 9 months (Leifert *et al.*, 1992a). However, the post-harvest use of fungicides is of growing concern and this is likely to result in legislation preventing the use of some or possibly all such applications in the EC. This situation and reports of increasing resistance of major post-harvest pathogens such as *B. cinerea* to fungicides (Wilson and Wisniewski, 1989) prompted a search for alternatives to fungicide control of post-harvest fungal diseases. One alternative to fungicides is the use of bacterial and yeast antagonists as potential biocontrol agents for pathogens of a variety of stored vegetables and fruit (Wilson and Wisniewski, 1989).

We have recently reported the isolation from Dutch cabbage of strains of *Serratia liquefaciens*, *Serratia plymuthica* and *Pseudomonas fluorescens* with *in vitro* activity against *B. cinerea* and *Alternaria brassicicola*, and the effect of nutrition and temperature on *in vitro* antagonisms in plate assays (Leifert *et al.*, 1992a,b). This paper describes some interactions between plant resistance and antagonistic *Serratia* and *Pseudomonas* spp. observed when studying spore germination of *B. cinerea* on heat-treated and untreated cabbage leaf tissue disks.

Material and Methods

Stock cultures of *B. cinerea*, previously isolated from diseased Dutch white cabbage, were maintained on cabbage agar 5 (50 g homogenized cabbage tissue, 10 g agar, 1 l distilled water) at 5°C in the dark. For sporulation, *B. cinerea* was grown for 5 days on malt extract agar at 20°C in the dark followed by 3 days under UV-light. Spores were harvested by pouring 10 ml of sterile Ringer's solution onto a fungal plate and suspending spores using a bacteriological loop. Spore suspensions were filtered through a double layer of muslin and adjusted to a concentration of 4 x 10^6 spores.ml^{-1} using a haemocytometer .

Leaf disks (15 mm diameter) were cut from internal leaves of cabbages (the outermost five leaves were discarded) using a cork-borer. Disks were transferred to a 25 ml universal bottle and either heated for 20 min at 50°C or used directly. Disks were left to cool and then placed in a bacterial suspension of *Pseudomonas* or *Serratia* isolates from Dutch white cabbage (Leifert *et al.*, 1992a). After inoculation with bacteria, disks were transferred to a tissue culture assay plate (8.5 x 13 cm) with 24 wells of 15 mm diameter (Fig. 1). A non-heated disk was placed under the inoculated heated disk to mimic the situation in a stored cabbage head where susceptible, senescent outer leaves overlay resistant metabolically active inner leaves. Assay plates were then placed open in a

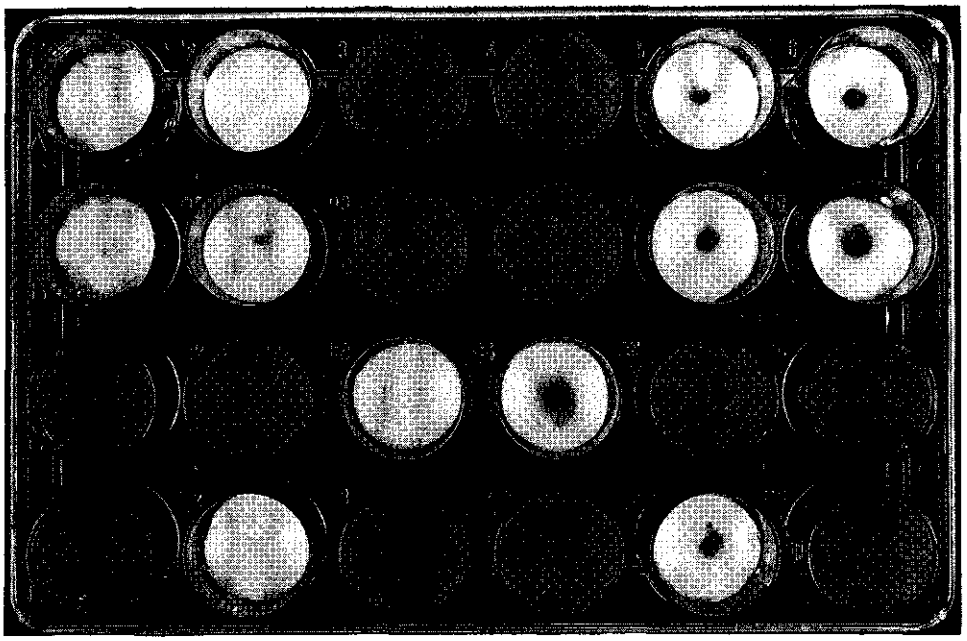

Fig. 1. Leaf disks in assay dish

Assay dish with leaf disks infected with *B. cinerea* by applying 2.5 ml of a fungal suspension containing (10^4 spores) into the centre of the disk after 8 weeks of incubation at 4°C in the dark. Disks showing visible fungal growth have been heat-treated (15 min at 50°C) prior to inoculation with *B. cinerea*. Disks without fungal growth had not been heated. Disks on the right half of the tray were wounded in the centre and fungal spores were applied to the wound; disks in the left half were not wounded.

Table 1. Suppression of B. cinerea by Pseudomonas and Serratia spp. on heated cabbage leaf disks.

No. of *B. cinerea* spores inoculated per disk	Bacterial inoculum (cfu.ml^{-1})					
	P. fluorescens CL 42,66,82		*S.plymuthica* CL43		*S. liquefaciens* CL80	
	$7x10^7$	$7x10^6$	$7x10^7$	$5x10^6$	$5x10^7$	$5x10^6$
10^4	+	V	V	–	V	–
10^3	+	+	+	-	+	V
10^2	+	+	+	+	+	+

V = variable results (inhibition of fungal growth in 1 or 2 out of 3 experiments); + = inhibition of fungus until end of experiment in all 3 experiments (6 weeks after control disks showed fungal growth).
Each experiment was repeated 3 times. – = no inhibition of fungal growth.

laminar flow cabinet until the disk surfaces were dry. Afterwards 2.5 µl of fungal suspension containing 10^2, 10^3 or 10^4 spores was placed in the centre of the disk. Assay plates were placed over water in a seed tray (15 x 20 cm) and the tray sealed with plastic film. Trays were incubated at 4°C in the dark for 10 weeks.

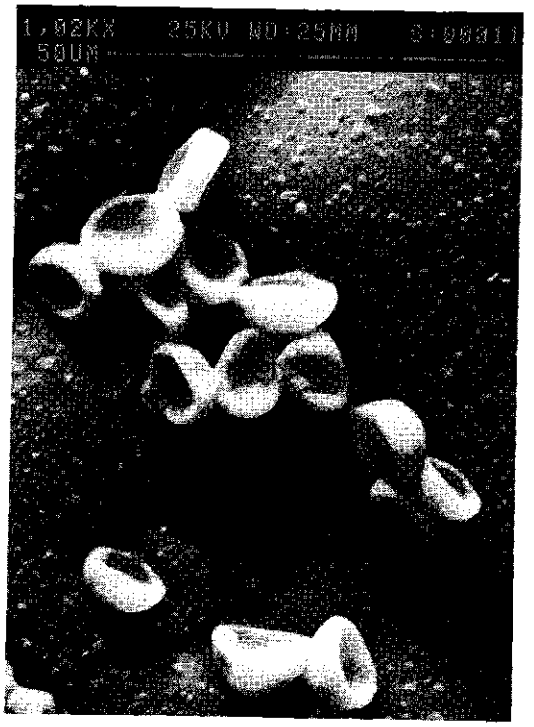

Fig. 2. Non-heated, non-wounded disks

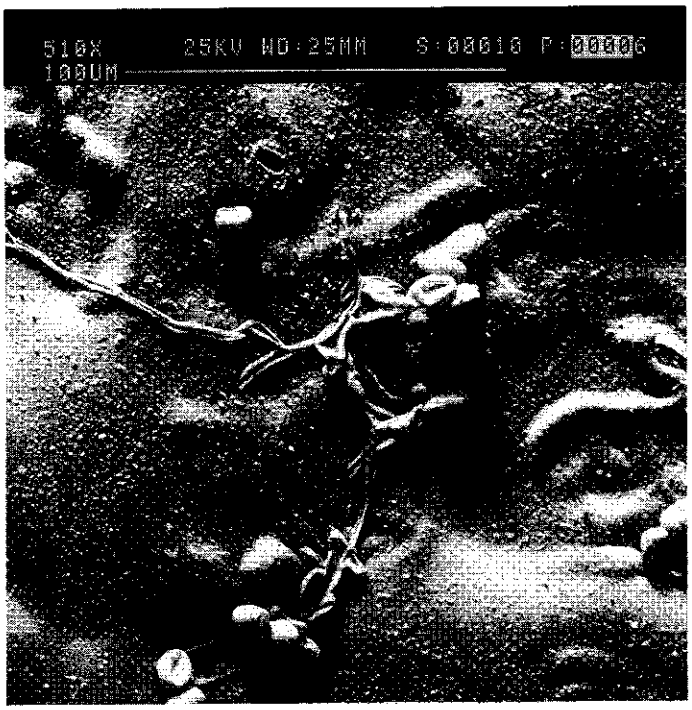

Fig. 3. Non-heated, wounded disks

Results and Discussion

When spores of *B. cinerea* were inoculated onto non-heated disks they failed to germinate and no spoilage of leaf disks occurred for periods of up to 3 months (Fig. 1 and 2). When leaf disks were wounded in the centre with a sterile pipette tip some spores germinated in the vicinity of the wound but again failed to establish and cause spoilage on the leaf disk (Fig. 1 and 3). This suggests that plant resistance mechanisms are active and prevent spore germination and fungal establishment. Heating of cabbage disks resulted in rapid fungal growth and 14 days after inoculation the fungal mycelium covered the whole disk (Fig. 1 and 4). This suggests that heating had abolished the plants resistance mechanism. Fungal establishment could be prevented on heated disks by dipping of disks into bacterial suspensions of *Serratia* and *Pseudomonas* which showed *in vitro* antagonism in plate assays. All non-identified bacteria which did not show *in vitro* antagonism could not prevent fungal development. After inoculation with some antagonistic *Serratia* and *Pseudomonas* strains (CL42, CL43, CL66, CL80 and CL82) leaf disks remained without signs of spoilage until the end of experiments, after 2 months (Table 1).

Since *Serratia* and *Pseudomonas* antagonists can persist on untreated cabbage leaves in high numbers [10^3 - 10^4 cfu/cm^2 leaf surface (Leifert *et al.*, 1992a)], and increase in numbers on damaged and heated cabbage tissue, they may be potentially useful bio-control agents against fungal pathogens when plant resistance mechanisms break down at the onset of leaf senescence. We are currently testing this hypothesis by determining

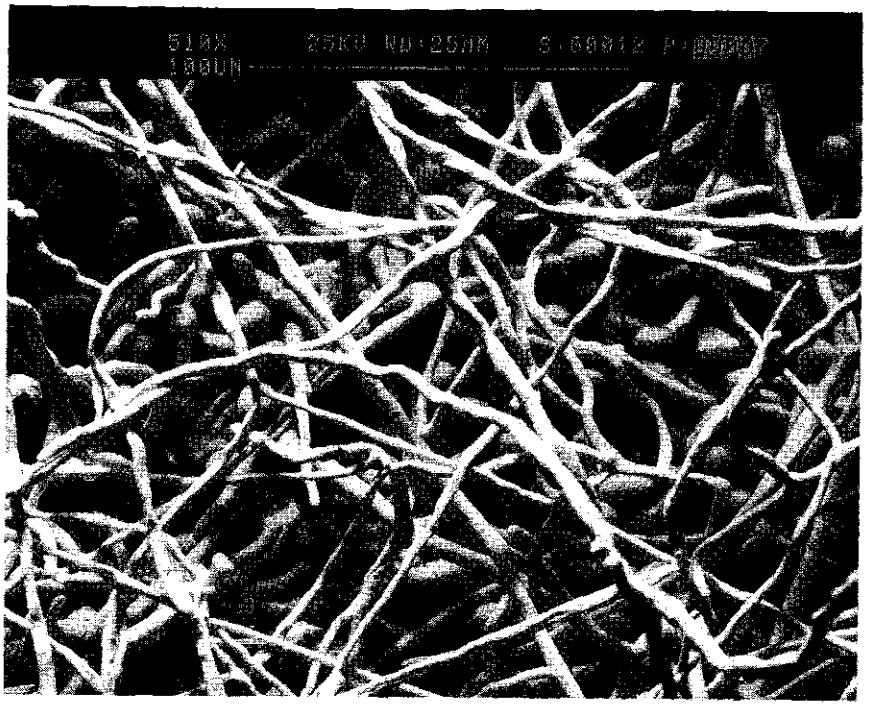

Fig. 4. Heated, non-wounded disks

Figure 2-4: Electron micrographs of cabbage leaf disks taken 10 days after inoculation with *B. cinerea* spores and incubation at 4°C in the dark (magnification is given on the photograph)

whether dipping of whole cabbages into suspensions of these antagonists can prevent fungal spoilage during cold storage of cabbages in two storage trials.

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Drought tolerance of *Botrytis squamosa*, *B. aclada* and potential antagonists

J. Köhl, M.C. Krijger and G.J.T. Kessel

Summary

Mycelial growth and conidial germination of *Botrytis squamosa*, *B. aclada* and antagonistic species of *Gliocladium* and *Trichoderma* were determined at water potentials between –1 mPa and –7 mPa on agar with KCl as osmoticum. *Gliocladium* spp. were at least as osmotolerant as *Botrytis* spp. *Trichoderma* spp. were distinctly more sensitive to low water potentials. The variation between strains within the genera was low. In a bioassay on dead onion leaves, *G. roseum* and *T. viride* suppressed sporulation of *B. aclada* at –1 mPa but at –6.6 mPa only *G. roseum* was effective.

Introduction

Botrytis squamosa and *B. aclada* (*B. allii*) cause leaf blight and neck rot in onions, respectively. During epidemics both pathogens produce inoculum on dead onion leaf tissue. To suppress sporulation of *Botrytis* spp., saprophytic antagonists were introduced. Peng and Sutton (1990) found *Gliocladium roseum* and *Trichoderma viride* highly suppressive to sporulation of *B. cinerea* after field applications on dead leaves of strawberry. In bioassays on dead onion leaves both antagonists suppressed sporulation of *B. aclada* almost completely (Köhl *et al.*, 1990).
One of the most limiting abiotic factors for both *Botrytis* spp. and microbial antagonists is the continuous fluctuation in the water content of dead tissue due to the microclimate in the crop. In our study, the sensitivity to low water potentials of the pathogens and antagonistic *Gliocladium* spp. and *Trichoderma* spp. was compared.

Material and Methods

Mycelial growth rates

Petri dishes with malt extract agar (1 g malt extract.l^{-1}, 15 g agar.l^{-1}) were inoculated with agar disks (5 mm) from the edges of colonies of the test fungi. The water potential of the agar had been adjusted by adding KCl. A thermocouple psychrometer with multiple sample holders (Decagon Devices Inc., Pullman, WA, USA) was used to determine the water potential of the agar. Samples were left in the apparatus for 30 min before measuring to allow equilibration. Demineralised water, 0.5 M KCl, 0.5 M NaCl and saturated KCl solutions served as standards. Plates were incubated at 18°C and the daily mycelial growth rates were calculated from the colony diameters measured after 3 to 7 days. To compare the susceptibility of the several fungi to low water potentials, the mycelial growth was expressed relative to the growth at –1 mPa.

Spore germination

Water agar (15 g agar.l^{-1}) in sterile microtitre plates was inoculated with conidial suspensions applying 10 µl per 6 mm well. Conidia were obtained from cultures on malt agar after incubation at 18°C for 5 to 10 days. The colonies were flooded with water to remove the conidia. After filtration through a double layer of cheesecloth, suspensions were adjusted to 1 x 10^4 conidia.ml^{-1}. The water potentials of both the water agar and the suspensions used for inoculations were adjusted by adding KCl. After an incubation period of 24 h at 18°C, plates were placed for 60 min in a chamber containing formalin vapour to stop further fungal growth. Fifty spores per treatment were counted in five replicates to determine the percentage germination; the length of ten germtubes of germinated conidia was measured in five replicates using a Minimop image analyzer (Kontron, Enching-Munich, FRG).

Bioassays

Antagonism of *T. viride* T004 and *G. roseum* 1813 against *B. aclada* was tested in a bioassay on dead onion leaf segments. Therefore, autoclaved leaf segments were placed in Petri dishes containing water agar adjusted to –1 or –7 mPa by adding KCl. Leaves were separated from the agar by sterile plastic grids to avoid diffusion of KCl into the leaves. After 7 days of equilibration, the measured water potential of the leaves was –1 mPa or –6.6 mPa. Malt broth (10 g malt extract.l^{-1}) was inoculated with conidia and incubated for 14 days at 18°C in order to produce mycelium. The mycelium was filtered, washed with sterile water and homogenised in a blender for 60 sec. Suspensions of mycelial fragments of *B. aclada* (1 x 10^4.ml^{-1}) were sprayed on the dead onion leaf segments in five replicates per treatment. After 24 h incubation at 18°C, the antagonists (1 x 10^5 mycelial fragments.ml–1) were applied. The water potentials of the mycelial suspensions were adjusted with KCl to the water potential of the substrate to be treated. After 7 days at 18°C, the leaf surface covered with conidiophores of *B. aclada* was estimated (class 0 = 0%; 1 = 1-5%; 2 = 6-25%; 3 = 26-50%; 4 = 51-75%; 5 = 76-100% covered).

Results

Mycelial growth

B. squamosa was slightly more susceptible to low water potentials than *B. aclada* (Fig. 1a). *Gliocladium* spp. were at least as osmotolerant as *Botrytis* spp., whereas distinctly higher susceptibility was found for *Trichoderma* spp. (Fig. 1b and 1c). Almost no variation was found between strains within the genera *Trichoderma* and *Gliocladium*.

Spore germination

At -4 mPa, spore germination was moderate for *B. aclada* W, and high for *Gliocladium roseum* 1813, whereas spores of *Trichoderma viride* T004 failed to germinate (Fig. 2). Spores of all fungi failed to germinate at –5 mPa or lower water potentials. The germtube growth in all fungi tested was more affected by low water potentials than their mycelial growth (Fig. 3) and *T. viride* was the most susceptible species.

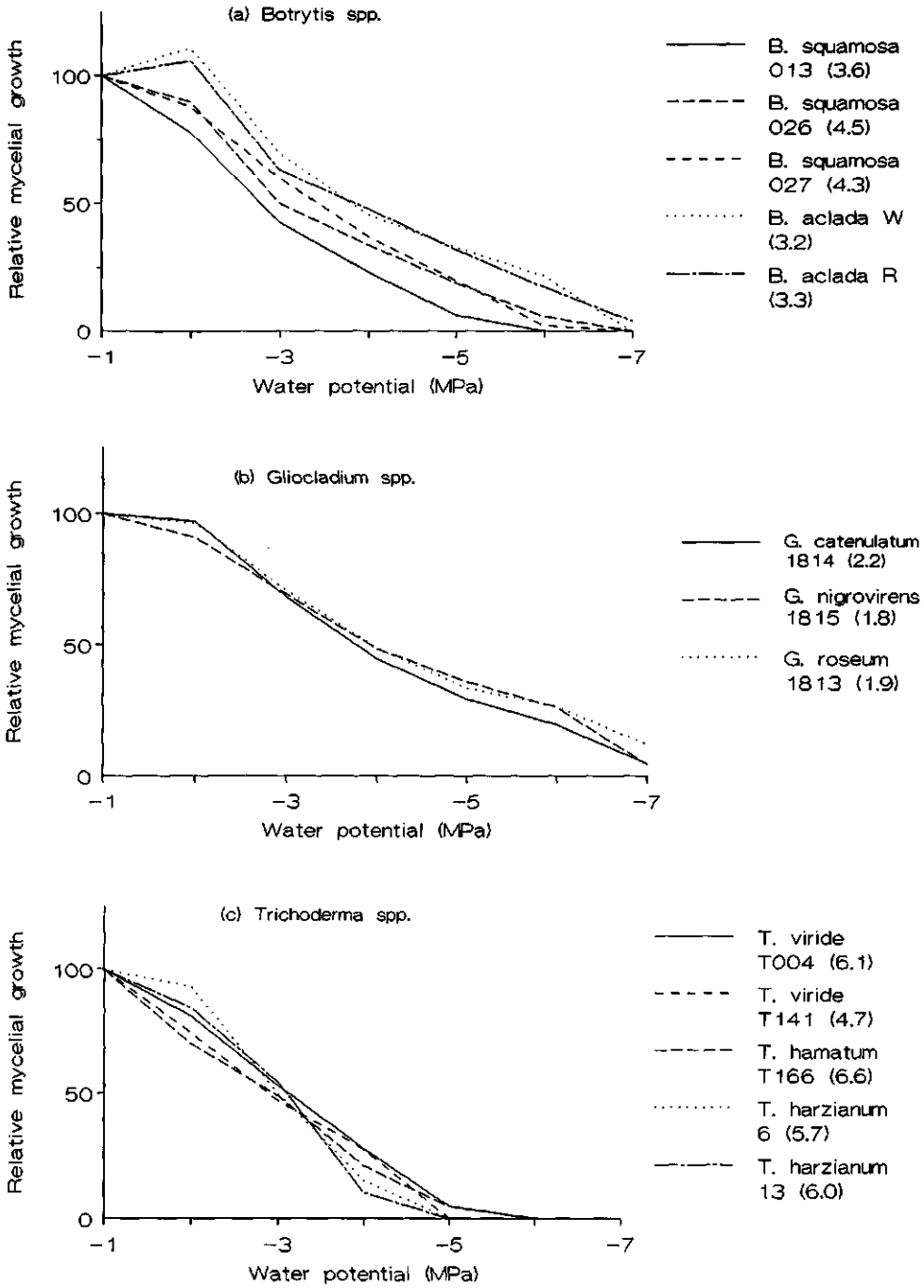

Fig. 1. Relative mycelial growth (−1 mPa = 100) of (a) Botrytis squamosa and B. aclada, (b) Gliocladium spp. and (c) Trichoderma spp. at different water potentials on malt agar. Growth rates at −1 mPa in mm 24 h^{-1} in brackets.

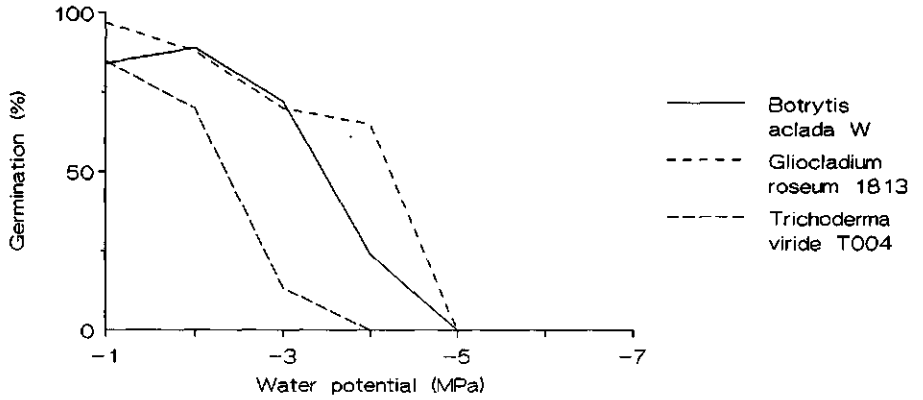

Fig. 2. Germination of conidia of Botrytis aclada W, Gliocladium roseum 1813 and Trichoderma viride T004 at different water potentials on water agar after 24 h at 18°C (means of 50 conidia in five replicates).

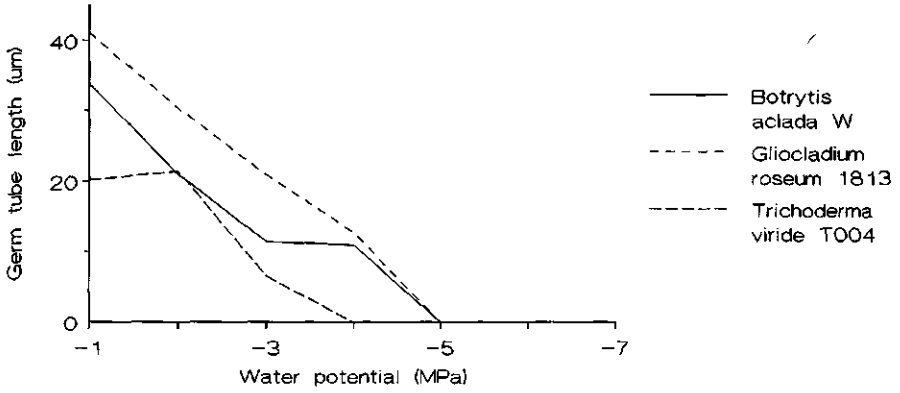

Fig. 3. Germtube lengths of conidia of Botrytis aclada W., Gliocladium roseum 1813 and Trichoderma viride T004 at different water potentials on water agar after 24 h at 18°C (means of ten germtubes in five replicates).

Bioassay

On wet leaves with –1 mPa, *G. roseum* 1813 and *T. viride* T004 suppressed sporulation of *B. aclada* (Fig. 4). At –6.6 mP a, only *G. roseum* was efficient.

Discussion

Drought tolerance is one of the key factors for the application of antagonists aimed at the suppression of *Botrytis* spp. Potential antagonists in the genera *Gliocladium* and *Trichoderma* markedly differed from each other with respect to their growth on agars with decreasing water potential. Only *Gliocladium* spp. were at least as tolerant as *Botrytis* spp. The variation between strains within the genera was unexpectedly low. Consequently, selection of drought tolerant strains of *Trichoderma* seems less promising.

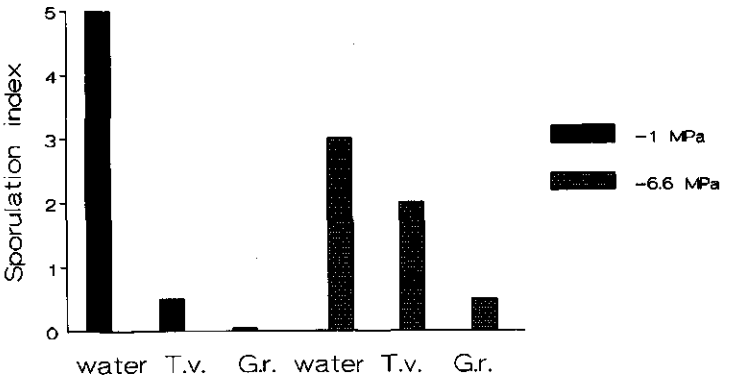

Fig. 4. Sporulation of Botrytis aclada W on dead onion leaves at -1 mPa and -6.6 mPa after application of Gliocladium roseum 1813 or Trichoderma viride T004.

Results obtained on agar with osmotica added are not necessarily representative of the conditions in the natural substrate where matric potential is more important. Alderman and Lacy (1984), found a close relationship between mycelial growth of *B. aclada* at a range of water potentials on onion leaf tissue and on agar when KCl was used as osmoticum. In our own experiments, *G. roseum* 1813 was tolerant of low water potentials on agar with low osmotic potential, as well as on dead leaf tissue with low matric potential, whereas *T. viride* T004 was less tolerant under both conditions.

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Efficacy of a grapefruit seed extract BC-1000 for control of *Botrytis cinerea* in table grape in Chile

M.A. Esterio, J.G. Auger, E. Vazquez, M. Reyes and F. Scheelje

Summary

The efficacy of BC-1000, a seed extract of grapefruit for control of *B. cinerea* in table grapes was tested in large field experiments in the 1990/1991 season. BC-1000 was as effective as benomyl or vinclozolin at concentrations of 1500 ppm. When applied in combination with fungicides the results were equivalent or superior, depending on the presence of benomyl and vinclozolin-resistant strains.

Introduction

In Chile, control of *Botrytis cinerea* Pers.: Fr. in table grapes is becoming increasingly difficult because of the development of strains of the fungus resistant to benzimidazole and dicarboximide fungicides. Therefore, we have developed a natural organic product, BC-1000, which has a different mode of action that could be useful for control of *B. cinerea*. BC-1000 is an extract of grapefruit seeds known to have antifungal properties (Anonymus, 1990a,b). Its efficacy, either alone or in combination with chemical fungicides for control of *B. cinerea* in table grapes was tested in a number of experiments. Some of the results were already published (Esterio and Auger, 1990a,b).

Material and Methods

A series of experiments in vineyards located in the Central valley of Chile were carried out during the 1990/1991 season.

In the experiments, all with 15 treatments, BC-1000 (1500 ppm), benomyl, benomyl + captan, captan and vinclozolin, (at commercially advised rates) were applied in various combinations at five growth stages during the development of berries of the cv. Thompson Seedless. The treatments and growth stages are summarised in Table 1. Each treatment had four replicates, with 20 plants per treatment.

To ensure a uniform population of *B. cinerea*, an inoculation was performed shortly before the pre-bloom stage by spraying two plants in the middle of each plot with a conidal suspension containing 9×10^4 conidia.ml^{-1}. The suspension contained conidia of isolates of *B. cinerea* sensitive and resistant to 50 ppm benomyl, in a 1:1 ratio.

The following parameters were evaluated: 1) field infection level throughout the five growth stages; 2) final postharvest decay level (after storage of 21 days at 0°C, and 11 days at 12°C); and 3) development of benomyl (50 ppm) and vinclozolin (20 ppm) resistant isolates using the method of Leroux and Gredt (1979). Concurrently, a test was carried out to evaluate any possible phytotoxic effects of BC-1000, on the vines and berries of cvs Flame Seedless, Thompson Seedless and Ribier at 1500 and 1800 p pm in comparison with standard captan sprays.

The infection level was determined from pre-bloom after harvest. Before the fungicide treatments were applied, 100 flowers or berries from each treatment were collected from the two central plants in each plot. These samples were incubated in Petri dishes containing water agar at 20°C for 20 days, and periodically assessed for infection.

To determine post-harvest decay, two 8.2 kg grape boxes were harvested from every plot; one box was subjected to the standard *Botrytis* postharvest control treatment (fumigation with SO_2), while the other remained untreated. Later, the boxes were kept at 0°C for a 21-day-period and thereafter left at room temperature for 2 to 13 days. The decay level per fruit box was determined after this period through visual inspection. Decay was also determined by weighing the total berries which showed symptoms of the disease. Data were subjected to analysis of variance and Duncan's multiple range test.

The laboratory test were carried out at the Plant Pathology Laboratory of the Plant Health Department of the Faculty of Agricultural and Forest Sciences, University of Chile.

Results and Discussion

The data from one of the experiments are given in Table 2. Treatments with BC-1000 alone (T1 and T2) gave similar results to those with benomyl or vinclozolin alone (T12

Table 1. Overview of the different treatments in experiments to control B. cinerea in table grapes, cv. Thompson Seedless in the 1990/1991 season.

Treatment	Application				
	Pre-bloom	Full bloom	Fruit set	Véraison	Preharvest (10 days before harvest)
T1	BC1[1)]	BC1	BC1	BC1	BC1
T2	BC2[2)]	BC2	BC2	BC2	BC2
T3	BC2	BC2	C	C	V
T4	V[3)]	B+C	C	C	V
T5	V	B+C	C	C	BC2
T6	V	B+C	BC2	BC2	V
T7	B[4)]+C[5)]	B+C	C	C	V
T8	B+C	BC2	C	C	V
T9	BC2	B+C	C	C	V
T10	B+C	B+C	C	C	BC2
T11	V	BC2	C	C	BC2
T12	B	B	B	B	B
T13	V	V	V	V	V
T14	Untreated control				
T15	Standard vineyard treatment				

1) BC1 = BC-1000 1.200 ppm
2) BC2 = BC-1000 1.500 ppm
3) B = benomyl
4) C = captan
5) V = vinclozolin

Table 2. Infection of flowers or berries of cv. Thompson Seedless by B. cinerea determined at the different growth stages as treatments applied. (Each figure is the average of four replicates. Infections are given as percentage of the untreated control).

Treatment	Pre-bloom	Full bloom	Fruit set	Véraison	Pre-harvest	Post-harvest
T1	11.75	2.75	1.0	4.25	5.75	1.45
T2	14.75	4.75	2.5	3.0	2.75	1.95
T3	18.5	6.5	1.25	3.75	3.5	1.05
T4	10.5	3.5	2.25	3.25	4.0	1.55
T5	16.5	6.0	2.0	4.25	4.3	0.80
T6	14.0	5.25	2.0	5.75	4.0	2.25
T7	14.0	5.25	2.25	3.25	5.25	0.50
T8	12.5	1.25	1.5	3.3	4.5	0.60
T9	14.75	5.25	1.25	2.05	3.5	1.25
T10	12.5	5.0	0.5	4.0	3.0	0.80
T11	12.5	6.75	1.75	5.0	3.0	1.35
T12	14.5	3.25	1.75	5.0	5.0	1.00
T13	11.25	5.75	2.0	5.8	4.5	2.75
T14	13.5	5.5	2.8	3.3	5.0	4.8
T15	16.5	5.25	1.5	4.0	5.0	1.25

Table 3. Percentage of isolates of B. cinerea resistant to 50 ppm benomyl obtained at various growth stages.

Treatment[1]	Growth stages						
	Pre-bloom	Full bloom	Fruit set	Véraison	Pre-harvest	Harvest	Post-harvest
T1	63.1[2]	54.5	77.7	72.7	71.8	72.3	77.8
T2	42.4	74.7	72.2	71.5	73.9	66.5	70.8
T3	76.0	74.5	72.8	72.6	82.9	87.7	81.3
T4	87.6	67.6	89.1	84.8	77.1	85.6	88.0
T5	82.8	52.4	78.4	74.3	83.7	83.9	87.2
T6	63.2	73.9	80.6	80.8	89.4	91.3	88.3
T7	85.1	68.0	81.2	75.6	89.6	86.8	82.4
T8	46.4	37.2	77.9	84.2	75.0	88.4	74.7
T9	72.2	24.0	81.7	76.3	83.5	68.1	79.2
T10	48.6	42.3	86.8	77.5	72.9	72.0	70.7
T11	58.3	81.5	80.1	76.4	75.8	76.2	72.8
T12	38.1	55.1	91.7	84.3	83.2	84.2	88.8
T13	87.0	48.5	81.9	75.7	78.4	81.4	82.4
T14	53.4	13.6	82.6	83.4	82.5	79.4	79.0
T15	81.2	68.0	81.2	75.6	89.6	86.8	82.4

[1]) See Table 1

[2]) Average of four replicates, isolating three colonies per treatment

and T13). Some combinations, such as T5, T7 and T8 resulted in a lower percentage of postharvest decay. The results of the other field experiments were not so successful, probably due to the presence of a larger number of fungicide-resistant strains in the population of *B. cinerea*. A high number of benomyl-resistant isolates were obtained in all field experiments, the number being highest in the benomyl-treated plots and the lowest in the BC-1000 treated plots (Table 3).

There was little difference in the incidence of benomyl resistant isolates between the treatments at the end of the season, but at earlier dates, there was a larger variation which could not be correlated with type of treatment. Most of the benomyl-resistant isolates also showed some limited resistance to vinclozolin.

No visible damage was seen on berries or on the rachis in the phytotoxicity tests.

The efficacy of BC-1000 in concentrations of 1500 ppm was similar to that of benomyl and vinclozolin and combinations of the three compounds increased their effectiveness, depending on the presence of resistant isolates of *B. cinerea*. These results were obtained in experiments carried out in one season.

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CHEMICAL CONTROL

Chemical control of *Botrytis* spp.

M.L. Gullino

Summary

The main factors involved in designing effective chemical control strategies against *Botrytis* spp. on different crops are reviewed. Some of the problems related to the use of fungicides are summarised and the ways to counteract them are discussed. The need to integrate the use of chemicals with other control measures is stressed.

Introduction

A few *Botrytis* spp. cause severe losses on many economically important crops. Although alternative control measures are often proposed and attempted, especially in the case of protected crops, control of these fungi still relies mainly on extensive use of synthetic chemicals. However, in spite of the availability of several effective fungicides and improved application techniques, control of *Botrytis* spp. is often difficult and incomplete.

This review focusses on some aspects of chemical control of *Botrytis* spp., with special emphasis on the problems that can be encountered under different situations and on the factors which can cause these problems.

Factors affecting chemical control of *Botrytis* spp.

When considering chemical control of diseases caused by *Botrytis* spp., it is important to take into account the biology of the pathogen and crop diversity, cultural conditions, availability of fungicides and possible development of resistance towards some of these fungicides.

Pathogen biology and crop diversity

The control of *Botrytis* spp. is extremely difficult because of the ability of this pathogen to attack crops at almost any stage of growth, or during storage and transit, and to affect all the plant parts, including cotyledons, leaves, stems, flowers and fruits.

Different species of *Botrytis* attack different hosts. Among them, *Botrytis cinerea* Pers.: Fr. has the widest host spectrum and causes severe damage on several economically important fruit, vegetable and ornamental crops. Other species for example *B. aclada* Fresen., *B.fabae* Sard., *B. squamosa* Walker, and *B. tulipae* Lind show a restricted host range and as a consequence generally cause less severe economic losses.

The use of fungicides to control the various species of *Botrytis* encounters different problems. For instance, treatments devised for protection of certain parts of a plant may not be sufficient to protect other tissues. Fungicide resistance spreads and develops at a different rate in various species. Among these species, *B. cinerea* has shown a particular aptitude to develop fungicide resistance.

Crop diversity influences *Botrytis* control in terms of practical approaches, availability of chemicals and implementation of possible strategies. In fruit crops most of the available fungicides active against this pathogen are registered for use in field sprays. A limited number of chemicals is registered for use as postharvest treatment with disparities between different countries in which chemicals may be applied. Toxicological and environmental considerations make the situation most complicated and rather diversified in the case of vegetables like tomato, strawberry, lettuce, cucumber, onion and garlic, especially when these are grown in glasshouses or in polyethylene tunnels. In ornamentals, like cyclamen, rose, gerbera, pelargonium, tulip and lily, aesthetics strongly influences the choice of the fungicide because fungicide residues may be visible on the blossoms. Moreover, possible toxic effects on humans causes restrictions in the use of some chemicals (Garibaldi and Gullino, 1990).

Cultural conditions

Different approaches are used in designing chemical control strategies in open field or in protected crops. Grey mould caused by *B. cinerea* is generally more serious in greenhouse crops, where conditions favour development and spread of the pathogen. However, more innovative control strategies can be designed in greenhouse crops (Jarvis, 1989) and fungicides can be applied by means of environmentally safer techniques. For instance, formulations of dicarboximides which are active by sublimation have been developed, with the aim of reducing the amount of residues on fruit and at minimising farmer's exposure to chemicals during spraying. Fumigation with sulphur dioxide has been, and still is, widely used for table grape (Eckert and Ogawa, 1988). Postharvest treatments can be carried out by applying chemicals in aqueous washes and dips, sprays or as dust. Recently, sprays have been largely used in order to reduce environmental problems caused by discharge of large quantities of fungicide.

Available fungicides

Two groups of chemicals, the benzimidazoles and dicarboximides which are highly active against *Botrytis* spp. are currently available. Diethofencarb, a N-phenylcarbamate, has been developed over the past decade to control *B. cinerea* strains resistant to benzimidazoles (Kato, 1988). Multisite fungicides such as chlorothalonil, dichlofluanid and thiram are also available, but severe restrictions often limit their use on some crops. Ethylene bisdithiocarbamate (EBDC) fungicides are still largely employed against *B. squamosa*, alone or in mixtures with dicarboximides (Lorbeer and Vincelli, 1990). Some ergosterol biosynthesis inhibiting fungicides (EBIs) have also shown useful activity against *Botrytis* spp. (Reinecke *et al.*, 1986) and recently developed fungicides with good anti-*Botrytis* activity are expected to appear on the market in the next few years.

Benzimidazoles, originally highly active, lost part of their importance during the last decade on most crops due to the appearance and persistence of resistant strains (see later) and to toxicological problems. At present, they have limited use on grapevine and vegetable crops (Gullino and Garibaldi, 1986a). In the case of vegetable crops, beside problems of resistance, the use of benzimidazoles has been severely restricted in many areas. In Italy they can no longer be used on greenhouse-grown vegetables during the cultivation, although they can be used as postharvest treatment. In the latter case there is

now a trend to replace them with more effective chemicals due to the presence of fungicide resistant strains (Eckert and Ogawa, 1988).

Dicarboximides replaced benzimidazoles in most situations in the late 1970s and early 1980s. Their initial high activity has been, at least in part, lost due to development of resistance on some crops (Lorenz, 1988), but their use remains crucial on most crops.

Diethofencarb, which showed remarkable activity against benzimidazole resistant *B. cinerea*, has been registered and used in some countries such as France and Israel; however, its use again rapidly led to the occurrence of resistant strains of *B. cinerea* (see later).

Among ergosterol biosynthesis inhibiting fungicides (EBI), some compounds show satisfactory activity against *Botrytis* spp. Their fungicidal activity has been particularly exploited against *B. cinerea*. For instance, tebuconazole, alone and mixed with tolyfluanid, shows a good activity against grey mould on several crops. Prochloraz is applied on tulip against *B. tulipae* (A. Koster, personal communication). However, the use of EBIs against *Botrytis* spp. is uncommon and the selectivity of EBIs must be carefully evaluated. Repeated application in greenhouses can cause phytotoxicity in some crops.

Among **new fungicides**, the phenylpyrrole CGA 173506, the pyrimidine mepanipyrim (Maeno *et al.*, 1990) and the triazole SSF-109 (Murabayashi *et al.*, 1990) have been recently developed. CGA 173506 has a broad spectrum of activity, including *Botrytis*, *Monilinia*, *Sclerotinia*, *Rhizoctonia*, *Alternaria*, *Fusarium*, *Septoria*, *Tilletia*, *Helinthosporium* and *Gibberella* (Ghemann *et al.*, 1990). The interest attracted by this chemical is related to the fact that it belongs to a new chemical group of fungicides with activity against *B. cinerea*, *Venturia* spp. and *Monilinia fructicola* (Maeno *et al.*, 1990). The triazole SSF-109 seems to be specifically and highly active against *Botrytis* spp., and its use on bulb crops has been reported (Anema *et al.*, 1988). It is highly desirable that fungicides like these with modes of action different from that of currently available chemicals, soon become available to growers.

Multisite fungicides, although less effective than specific ones, are interesting because of their wider spectrum of activity which permits control of secondary pathogens. They are also good partners for specific fungicides in anti-resistance strategies. As a consequence, their use is still highly recommended, particularly on crops where the number of sprays in a season is high.

Fungicide resistance

Resistance to benzimidazoles, dicarboximides and diethofencarb complicates chemical control of *Botrytis* spp. on several crops. The relative importance of fungicide resistance varies on different crops and in different areas, according to the selection pressure exerted by the use of these groups of chemicals (Staub, 1991).

At the time of introduction of the benzimidazoles, there were no other highly effective fungicides that could have been used as companion materials. Accordingly, the benzimidazoles were overused, and resistance developed quickly and limited their usefulness within 2 to 4 years in most areas, with very few exceptions (Delp, 1988).

Dicarboximide resistance developed in the 1980s, although not so rapidly and in a less dramatic way. This type of resistance now affects *B. cinerea* in many crops (Lorenz, 1988). Resistance to both benzimidazoles and dicarboximides is now widespread in *B. cinerea* on grapevine, vegetable and ornamental crops (Gullino and Garibaldi, 1986b; Leroux and Valentin, 1991). In the case of protected crops, resistance spreads quite rapidly through the entire greenhouse. A similar rapid spread of resistance has been

observed in commodities affected by postharvest attacks (Eckert and Ogawa, 1988). Resistance to benzimidazoles is also present in *B. squamosa* (Presley and Maude, 1980), *B. tulipae* (Duineveld and Beijersbergen, 1975) and *B. elliptica*. In the case of this latter pathogen, dicarboximide resistance has also been reported (Migheli *et al.*, 1989). In countries where diethofencarb is already registered, resistance to this chemical also developed rapidly (Leroux and Valentin, 1991; Elad *et al.*, 1992).

Multisite fungicides, on the contrary, are still effective after years of use. Only in the case of dichlofluanid, some loss of efficacy has been reported, probably due to the development of resistance (Malathrakis, 1989; Rewal *et al.*, 1991), but in these cases the wide range in sensitivity of isolates within the wild populations of *B. cinerea* must be considered (Garibaldi *et al.*, unpublished results).

In crops which are frequently sprayed, monitoring for fungicide resistance is important because it permits the implementation of control strategies using chemicals which are still effective (Gullino *et al.*, 1989). Different monitoring techniques have been described: some are simple and rapid and can be directly used by growers or technicians working in the extension service (Lorenz, 1988).

Mixtures of fungicides or rotation of their use helps to reduce the spread of resistance. The mixture of thiram with a dicarboximide showed good efficacy in the presence of fungicide resistance in *B. cinerea* on several crops (Boureau, 1988; Gullino et *al.*, 1989; Creemers *et al.*, 1990). It should be stressed, however, that the use of thiram is not recommended due to its activity against some benificial organisms.

Diethofencarb, a fungicide showing negative cross resistance to benzimidazoles, when used in mixtures with carbendazim controls benzimidazole-resistant (RB) as well as benzimidazole and dicarboximide-resistant (RBD) populations of *B. cinerea*. This mixture represents a short term anti-resistance strategy, able to solve problems of loss of efficacy due to fungicide resistance, but as described above, where its use has been intensive, double resistance to benzimidazoles and diethofencarb has developed already. The mixture of carbendazim with diethofencarb cannot be used where benzimidazoles are not permitted (i.e. greenhouse-grown vegetables in Italy); this is one more example of how the severe restrictions recently imposed in many countries complicate the implementation of anti-resistance strategies.

Strategies in chemical control of *Botrytis* spp.

Although chemicals still play a major role in control of *Botrytis* spp. on most crops, it is clear that their use leads to practical problems of various types in many cases. For this reason, the exclusive use of fungicides can no longer be considered to be a workable approach to the control of *Botrytis* spp. on most crops. The choice of the chemical still remains crucial: new fungicides having different modes of action are urgently needed.

The integration of chemical treatments with cultural management systems, epidemiological models and biological control agents, is now becoming a realistic and rational strategy for management of diseases caused by *Botrytis* spp.

For *B. cinerea*, good results have been achieved on the greenhouse-grown crops tomato, cucumber and strawberry, by integrating the use of chemicals with careful control of environmental parameters. Reducing the persistence of water films on the plant by increased ventilation and heating strongly reduced grey mould incidence under severe disease pressure on several crops (Elad and Zimand, 1991; Gullino *et al.*, 1991; Nicot and Allex, 1991).

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Action of sterol biosynthesis inhibitors against *Botrytis cinerea*

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Summary

Fungicides which inhibit sterol 14α-demethylation (DMIs) are registered to a limited extent for control of *B. cinerea*. Additional sterol biosynthesis inhibitors (SBIs) for control of *B. cinerea* are required. This led us to study the selective toxicity of DMIs to *B. cinerea*. A cell-free sterol 14α-demethylase assay of the fungus able to synthesise C4-desmethyl sterols was developed. This opens the possibility to study the correlation between fungitoxicity of DMIs and the potency to inhibit the demethylase in an important target fungus. The assay also provides the possibility to study quantitatively the structure-activity relationships of candidate fungicides and to screen for other potential target sites in sterol biosynthesis. Methods to study other parameters which may influence toxicity of DMIs against *B. cinerea*, such as accumulation in mycelium and resistance development, were also developed. Accumulation of fenarimol in mycelium was transient and was mediated by energy-dependent efflux as described previously for other filamentous pathogens. Laboratory resistance was detected, but it was unstable upon subculturing of resistant isolates in the absence of selection pressure. This is probably caused by the poly- and heterokaryotic nature of the fungus.

Introduction

In the last two decades chemical control of grey mould caused by *Botrytis cinerea* Pers.: Fr. has been severely restricted by the development of resistance to benzimidazole and dicarboximide fungicides. Fungicides which inhibit sterol biosynthesis (SBIs) were developed during this period but only a few of them were registered for grey mould control. In general, SBIs did not replace the benzimidazoles and dicarboximides. This is of serious concern since SBIs have a number of advantageous features, such as a relatively low resistance risk. The unavailability of modern systemic fungicides for control of *B. cinerea* may result in an increased re-use of conventional fungicides which lack curative action. Growers will also be urged to rely again much more on alternative methods of disease control. In itself, this is a good development, but alternatives are not always available or are difficult to achieve . In the context of this situation a study on the activity of SBIs against *B. cinerea* was started. The study will be restricted to the largest group of SBIs, the triazoles which inhibit sterol biosythesis by inhibition of cytochrome P450-dependent sterol 14α-demethylase activity ($P450_{14DM}$). These are generally described as demethylation inhibitors (DMIs).

The goal of the study was to investigate parameters which may influence the selective toxicity of triazole DMIs to *B. cinerea*. It is expected that fundamental knowledge gained about this topic may contribute to the development of DMIs which show a good field performance against grey mould.

Results and Discussion

Biological activity

The biological activity of triazole DMIs was investigated in radial growth tests on PDA. EC_{50} values for radial growth varied significantly among 17 compounds tested. Among the compounds with the highest fungitoxicity found were itraconazole, an antifungal agent for control of mammalian pathogens (EC_{50} 10^{-8} M), and tebuconazole (EC_{50} 10^{-8} M), the only triazole registered for control of *B. cinerea*. Triazoles with a low fungitoxicity were triadimenol (EC_{50} 10^{-6} M), triadimefon (EC_{50} 5.10^{-6} M) and flutriafol (EC_{50} 9.10^{-6} M). These results indicate a significant range in toxicity (900 fold) of triazoles. The question is whether these figures correlate with differences in sensitivity of $P450_{14DM}$ or whether other factors are involved in selective toxicity, such as accumulation in fungal mycelium.

Development of a cell-free bioassay for sterol 14α-demethylase

The affinity of DMIs to $P450_{14DM}$ can be assessed by measuring spectrophotometrically the interaction between the DMIs and microsomal cytochrome P450 isozymes or by measuring the inhibitory potency on $P450_{14DM}$ activity. In studies with *Penicillium italicum* measurement of the inhibitory potency on $P450_{14DM}$ activity gave the most relevant infor mation (Guan *et al.*, 1992a,b). Therefore, attempts were made to develop a similar cell-free bioassay for the sterol 14α-demethylase of *B. cinerea*. The protocol for preparation of the *P. italicum* assay was not successful for *B. cinerea* and it therefore, had to be modified. Modifications were made in the mechanical disruption method of the mycelium and the nature of the incubation mixture. Details of the protocol will be published elsewhere. The final protocol yielded cell-free extracts which were able to incorporate 20-30% of [^{14}C] mevalonic acid in non-saponifiable lipids of which 35-45% consisted C4-desmethyl sterols. Other sterols formed were lanosterol and eburicol. Incubation of the mixture with the imidazole DMI imazalil (10^{-7} M) fully prevented the synthesis of desmethyl sterols and induced the accumulation of eburicol, the substrate of sterol 14α-demethylase. The IC_{50} value of imazalil (concentration which inhibits desmethyl sterol synthesis by 50%) was about 10^{-8} M. Results of tests with various triazole DMIs will be published later. The results should elucidate whether compounds with a low toxicity towards *B. cinerea* have a relatively low inhibitory potency on $P450_{14DM}$ activity. If not, other factors influence negatively their toxicity such as low accumulation or fast metabolic inactivation.

Significance of the sterol 14α-demethylase assay

The development of the cell-free bioassay could provide a biochemical tool for agrochemical companies to do quantitative studies of structure-activity relationships at the enzyme level and hence to optimise activity of DMIs specifically against $P450_{14DM}$ of *B. cinerea*. This approach might be profitable since *B. cinerea* is regarded as a world-wide target fungus in agriculture. Such an optimalisation has not been done before because of the lack of a cell-free bioassay for any filamentous plant pathogen, except for the recently developed assay of *P. italicum*. As a substitute, companies sometimes use a

cell-free $P450_{14DM}$ assay of *Saccharomyces cerevisiae*. In general, analogs of particular DMIs may show a correlation between toxicity to whole organisms such as *Pyricularia oryzae* and *Drechslera sorokiniane* and the activity in the $P450_{14DM}$ assay of yeast (Fujimoto *et al.*, 1988). However, this may not be true for *B. cinerea* since $P450_{14DM}$ of different organisms may possess a differential sensitivity to DMIs. Hence, optimalisation using the *S. cerevisiae* assay does not necessarily lead to compounds with maximum intrinsic activity to *B. cinerea*.

The cell-free bioassay developed here also opens the possibility to determine the inhibitory potency of different stereo-isomers (diastereomers, enantiomers) of the same compound on $P450_{14DM}$ activity. Such results are more conclusive for the identification of biologically active isomers than *in vivo* toxicity bioassays since in this instance metabolism may interfere with a proper interpretation of the results, such as in the case of triadimefon (Gasztonyi, 1981; Deas *et al.*, 1984). A knowledge of the toxicity of the various isomers of one DMI is important since it may be that only one isomer is responsible for most of the activity. The effects of the remaining isomers should also be taken into acount since they may have beneficial or deleterious effects on non-target sites in the plant. Hence, it may be relevant to alter production of these agrochemicals in such a way that the content of the most active isomer in the product is as high as possible (Burden *et al.*, 1987).

Another spin off from using the cell-free bioassay is that the test can also be used to identify compounds which interfere in sterol biosynthesis at other sites. This statement is confirmed by results of preliminary experiments with allylamines and morpholine fungicides. In addition, we identified an experimental sterol 14α-demethylase inhibitor which induced a sterol accumulation pattern different from those found with commercial SBIs. This particular chemical inhibited both C4-desmethyl and C4,4-dimethyl synthesis. Such a chemical may lack usual cross-resistance patterns and be of interest to develop new candidate fungicides.

Accumulation of SBIs in fungal mycelium

The mechanism of laboratory resistance in a number of filamentous fungi against DMIs is based on increased energy-dependent efflux of the compounds from mycelium. Such an increased secretion probably prevents accumulation of the compounds at the target sites to concentrations inhibitory to sterol 14α-demethylase (De Waard, 1980, 1988; Kalamarakis *et al.*, 1991). Accumulation in wild-type isolates was always higher than in resistant mutants and was characterised by a transient accumulation of the fungicides. The efflux mechanism involved has never been elucidated.

We demonstrated that energy-dependent efflux of fenarimol also operates in mycelium of *B. cinerea*. Incubation of mycelium of a wild-type isolate with fenarimol (90 nM) gave the typical transient accumulation patterns. Accumulation after 1 h of incubation became stable and constant. Addition of inhibitors under such equilibrium conditions could induce an instantaneous increase in accumulation. The most active inhibitors found were ionophoric antibiotics. These compounds interfere with the proton motive force over membranes and hence may inhibit transport processes which are driven by this force. This suggests that energy-dependent efflux is also driven by membrane-bound enzymes. Further research should identify these enzymes.

Resistance development

Selection for laboratory-resistance to DMIs in various monokaryotic fungi has been described frequently. It can be achieved easily and was shown to be polygenically based (Van Tuyl, 1977). Resistance levels were relatively low. Recombination of different minor genes in the same strain or stepwise selection yielded isolates with relatively high levels of resistance (De Waard and Van Nistelrooy, 1990; Kalamarakis *et al.*, 1991).

In the present studies we tried to select for resistance to DMIs in *B. cinerea* by mass selection of conidia, which had been treated or untreated with mutagenic agents on agar with a minimal inhibitory concentration of different fungicides. Developing colonies were continuously subcultured on agar with sublethal concentrations of the fungicides. Resistance levels were determined in radial growth tests with inoculum taken from DMI-containing agar and from the first subcultures on agar without fungicides.

Resistance levels of isolates tested with inoculum from fungicide-containing agar ranged from 6-19; resistance levels of the same isolates tested with inoculum from fungicide-free plates ranged from 2-4. This indicates that the decreased sensitivity observed is not a stable genetic character. This may relate to the poly- and heterokaryotic nature of *B. cinerea*. Attempts will be undertaken to acquire isolates with stable levels of resistance to DMIs. Only such isolates can be used to test whether the mechanism of resistance is based on increased efflux from mycelium or decreased affinity of $P450_{14DM}$ to DMIs.

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Investigations on the dicarboximide resistance of *Botrytis cinerea* II. Incomplete cross resistance in the Rheingau

J.F.R. Borge, N. Abolgassani and E. Schlösser

Summary

All isolates of *B. cinerea*, representative for 2.600 ha of vineyards in the Rheingau, exhibited incomplete cross resistance to dicarboximide fungicides. As compared to sensitive isolates (ED_{50} 5.0 $\mu g.ml^{-1}$) they had a low level of resistance to iprodione and metomeclan (ED_{50} 25 $\mu g.ml^{-1}$) and a high level of resistance to vinclozolin and procymidone (ED_{50} 500 $\mu g.ml^{-1}$).

Introduction

Resistance of *Botrytis cinerea* Pers.: Fr. to dicarboximide fungicides is widespread in fungal populations on various crop plants, including grapevine (Pommer and Lorenz, 1987). The resistance situation in the vineyards of the Rheingau has never been assessed before. Thus, *B. cinerea* was isolated in 1985 and 1986 from representative vineyards of the region.

Material and Methods

B. cinerea was isolated from infected plants, as described by Borge (1988). Conidia produced were kept without further subculturing on agar media by storage of conidia at 4°C.

Agar plate assay

The sensitivity to various fungicides was tested as described by Borge and Schlösser (1990).

Detached leaf assay

Detached leaves of *Vitis vinifera* cv. Müller-Thurgau were placed on moist filter paper disks in Petri dishes, with the abaxial surface uppermost. The fungicides were applied to leaves with a hand-sprayer to give a fine film. For inoculation, 50 µl droplets of a 1% malt extract solution, containing 750-1100 conidia were placed on intercostal areas. After 10 days at 18-22°C and a 12/12 photoperiod with artificial illumination of 10.000 lux, the size of the necrotic lesions was measured and attributed to the following classes: 0 = lesions absent; 1 = few minute lesions; 2 = lesions of 1 x drop diameter; 3 = 2 x drop diameter; 4 = 3 x drop diameter; 5 = lesions > 3 x drop diameter. From the class distribution a disease index was calculated:

$$I = \frac{n_1 + 2n_2 + 3n_3 + 4n_4 + 5n_5}{n_0 + n_1 + n_2 + n_3 + n_4 + n_5}$$

The values for each variant of the leaf assay are the average from three replicates with six inoculation points each.

Results

Agar plate assay

The 20 isolates of *B. cinerea* collected in 1985 with 50 µg a.i. vinclozolin.ml^{-1} as threshold level, differed in their reaction to the four fungicides with regard to production of sclerotia (Table 1).

Iprodione and metomeclan proved to be more effective than vinclozolin and procymidone.

In 1986 resistant isolates were selected with vinclozolin resistance (V-R) and iprodione resistance (I-R), at 50 and 5 µg a.i. as discriminatory levels, respectively. A similar reaction (Table 2) was observed with mycelial growth from fungal suspensions with six I-R and V-R isolates as shown in Table 1, regardless of which fungicide had been used to select the resistant isolate.

Production of sclerotia on agar plates (Table 3) showed the same general pattern.

Table 1. Effect of dicarboximide fungicides on sclerotia production in agar plates of 20 isolates of B. cinerea collected in 1985 in the Rheingau.

Fungicide	number sclerotia/agar plate[1)]				
	µg a.i.ml^{-1}				
	0	5	10	25	50
Iprodione	41	44	42	0	0
Metomeclan	41	12	25	0	0
Vinclozolin	41	15	10	8	18
Procymidone	41	20	11	6	16

[1)] average of five replicates for each of the 20 isolates.

Table 2. Effect of dicarboximide fungicides on colony diameter from conidial suspensions on agar plates of six iprodione-resistant (I-R) and six vinclozolin-resistant (V-R) isolates of B. cinerea collected in 1986 in the Rheingau.

Fungicide	colony diameter, as % of untreated check[1)]											
	µg a.i.ml^{-1}											
	0		5		10		25		50		100	
	I-R	V-R	I-R	V-R	I-R	V-R	I-R	V-R	I-R	V-R	I-R	V-R
Iprodione	100	100	78	87	85	90	0	0	0	0	0	0
Vinclozolin	100	100	77	83	83	87	81	82	81	83	74	77

[1)] average of five replicates for each isolate.

Table 3. Effect of dicarboximide fungicides on sclerotia production from conidial suspensions on agar plates of six iprodione-resistant (I-R) and six vinclozolin-resistant (V-R) isolates of B. cinerea collected in 1986 in the Rheingau.

Fungicide	number of sclerotia/agar plate[1)]											
	µg a.i.ml^{-1}											
	0		5		10		25		50		100	
	I-R	V-R	I-R	V-R	I-R	V-R	I-R	V-R	I-R	V-R	I-R	V-R
Iprodione	48	72	119	25	59	23	0	0	0	0	0	0
Vinclozolin	68	39	106	19	21	21	12	49	10	16	9	14

[1)] average of five replicates for every isolate.

Table 4. Reaction of three dicarboximide-sensitive isolates of B. cinerea to iprodione and vinclozolin on detached grapevine leaves.

Fungicide	µg a.i.ml^{-1} [1)]				
	0	2.5	5.0	10.0	25.0
Iprodione	4.7[1)]	3.7	2.7	0.4	0
Vinclozolin	4.8	3.9	2.5	0.9	0

[1)] lesion index is the average of three replicates of six inoculation sites for every isolate.

Table 5. Reaction of eight dicarboximide-resistant isolates of B. cinerea to iprodione and vinclozolin on detached grapevine leaves.

Year of isolation	µg a.i.ml^{-1}	lesion index[1)]	
		Iprodione	
	0	50	100
1985	4.8	1.3	0.4
1986	4.7	0.8	0.2
		Vinclozolin	
	0	500	1000
1985	4.9	2.7	1.8
1986	4.8	2.5	1.7

[1)] average of three replicates of six inoculation sites for every isolate.

Detached leaf assay

Agar plate assays can be informative, but often disagree with assays *in planta*. For this reason the reaction of resistant isolates was tested on detached grapevine leaves. Dicarboximide-sensitive isolates proved equally susceptible to vinclozolin and iprodione (Table 4), with an ED_{50} of about 5.0 µg a.i.ml^{-1}.

With resistant isolates there was the same diverse reaction, as observed with the agarplate essays (Table 5). They were more sensitive to iprodione than to vinclozolin, with ED_{50}-values of about 25 µg and 500 µg a.i.ml^{-1}, respectively.

Discussion

On agar plates as well as in detached leaf assays, resistant isolates of *B. cinerea* showed a range of reactions to the four dicarboximides tested, being more sensitive to iprodione and metomeclan than to vinclozolin and procymidone. In the leaf assay, sensitive isolates reacted equally to iprodione and vinclozolin, with an ED_{50} of about 5.0 μg a.i.ml^{-1}, while resistant isolates had ED_{50}-values of 25 μg a.i.ml^{-1} for iprodione and 500 mg a.i.ml^{-1} for vinclozolin, respectively. These differences were apparent, regardless whether the isolates had been selected for resistance towards iprodione or vinclozolin. This incomplete cross-resistance was described by Vulic *et al.* (1984), but these authors also indicated that some isolates behaved this way, while others showed complete cross-resistance.

The vineyards in the Rheingau consist of c. 2600 ha in a single 35 km stretch along the Rhine, extending only 0.5 to 3.0 km from the river. As a result of the continuous grapevine cultivation for centuries a regional population of *B. cinerea* has probably evolved. This may be the reason why all isolates from this region reacted similarly and without exception to the dicarboximide fungicides. Whether populations in other regions behave similarly remains to be established.

The described phenomenon has two implications. First, complete cross resistance cannot automatically be assumed to occur in all areas in which resistance to a given fungicide has been detected. Second, in the case of incomplete cross resistance, some fungicides may still be useful despite the presence of resistant isolates, provided they are applied with prudence.

Acknowledgment

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Stability of benzimidazole resistance and diethofencarb sensitivity of *Botrytis cinerea*

H. Ishii, M. Fukaya, F. Faretra, H. Takeda and Y. Tomita

Summary

Monospore or monomycelial isolates of *B. cinerea* having the phenotypes 'carbendazim-sensitive, diethofencarb-resistant' (S,R), 'highly carbendazim-resistant, diethofencarb-sensitive' (HR,S), 'intermediately carbendazim-resistant, diethofencarb-resistant' (IR,R), and 'highly carbendazim-resistant, diethofencarb-resistant' (HR,R) were obtained. They were phenotypically stable during subculture on media in the presence and absence of carbendazim. A mixture of diethofencarb and thiophanate-methyl was highly effective in reducing growth of the HR,S and S,R isolates, but not against the IR,R and HR,R isolates.

Introduction

In Japan, benzimidazole-resistant strains of *Botrytis cinerea* Pers.: Fr. appeared in the mid-70's and caused severe loss of disease control by benzimidazole fungicides (Takeuchi, 1987). Benzimidazole resistance persisted in the field, prompting the use of the N-phenylcarbamate fungicide diethofencarb for combatting the resistant strains. This fungicide, which is specifically active against benzimidazole-resistant strains, was synthesised in Japan (Nakamura *et al.*, 1986) and commercially released in France as a mixture with the benzimidazole fungicide carbendazim. This was the first example of a practical application of the phenomenon of negatively correlated cross-resistance. In Japan, subsequently, diethofencarb was registered as a mixture with either thiophanate-methyl or procymidone, although registration of these mixtures has not yet been obtained for grapes. The objectives of this work were: 1) to monitor field isolates for their resistance to benzimidazoles and sensitivity to diethofencarb in vineyards; 2) to examine the stability of resistance or sensitivity to carbendazim and 3) diethofencarb *in vitro*; and to test the effects of a mixture of diethofencarb with thiophanate-methyl on *B. cinerea* isolates in the laboratory.

Material and Methods

Monitoring of benzimidazole resistance and diethofencarb sensitivity

In 1990, isolates of *B. cinerea* were obtained from five commercial fields and 1 experimental field in Akita Prefecture. In 1991, monitoring was repeated for the same experimental field. In that field, a mixture of diethofencarb with thiophanate-methyl was applied five times in total during the 2 years. Mycelial disks of each isolate previously cultured on potato dextrose agar (PDA) plates at 24°C for 3 days were transferred to PDA plates containing thiophanate-methyl (70% WP) or diethofencarb (25% WP). After incubation at 24°C for 2 days, the minimum inhibitory concentrations (MIC) of each fungicide were determined.

Stability of resistance or sensitivity to carbendazim and diethofencarb

Eight Italian and two Japanese isolates were used. Each isolate was subcultured on PDA plates supplemented with carbendazim at 0,0.1, 10, and 100 μg.ml^{-1}at 20°C to test for their sensitivity to carbendazim and diethofencarb. After incubation at 20°C for 2 days, the inhibition of mycelial growth (%) was calculated for each fungicide. This test was repeated every 3 days over a period of 18 days, and then four times every month.

Efficacy of a mixture of diethofencarb with thiophanate-methyl

Activity of the fungicide was determined using the 'Cucumber Fruit Methods' reported by Tezuka and Kiso (1976). In experiment 1, four isolates were cultured separately on PDA plates. In experiment 2, conidial suspensions (ca. 1 x 10^5 conidia.ml^{-1}) prepared from four isolates were mixed in various ratios and dropped onto PDA plates. The cultures were incubated at 20°C for 4-5 days. Pieces of cucumber fruit dipped into a suspension of thiophanate-methyl (a.i. 50 mg.ml^{-1}), diethofencarb (a.i. 83 mg.ml^{-1}) or a mixture of thiophanate-methyl and diethofencarb at the same concentrations were placed on the colonies grown on PDA plates. The cultures were kept at 20°C in a moist atmosphere. The length of decay caused by *B. cinerea* was measured 4-5 days after the start of incubation, and the protective value for the decay development by the fungicide was calculated.

Results and Discussion

Fukaya *et al.* (1979) reported that benzimidazole-resistant populations of *B. cinerea* declined in vineyards over the following 2 years when spray applications of benzimidazole fungicides were discontinued. In this work, the distribution of isolates

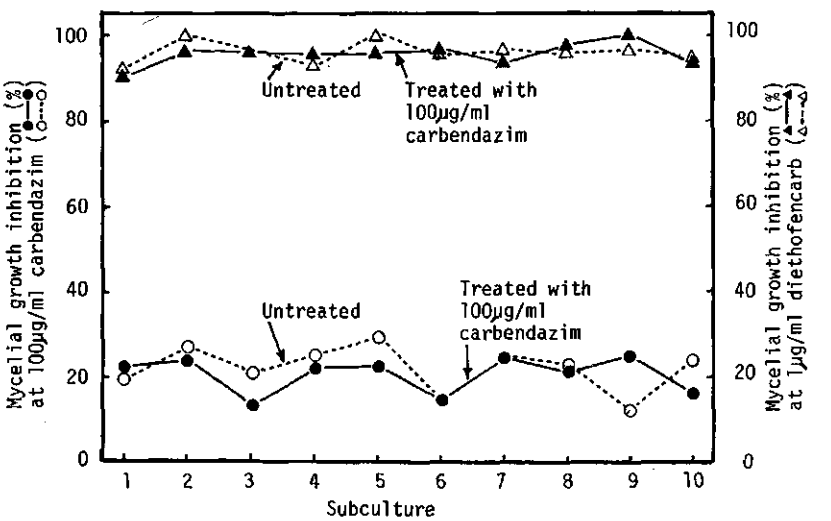

Fig. 1. Isolate SAS405 (HR,S). Stability of carbendazim resistance and diethofencarb sensitivity in an isolate of Botrytis cinerea.

showing different degrees of sensitivity to thiophanate-methyl and diethofencarb in fungal populations was monitored in the same vineyards where selection pressure from benzimidazole was removed after withdrawal of the chemical in 1975. Of 94 isolates tested in 1990, seven (7.4%) were highly resistant to thiophanate-methyl but most of the remaining isolates were sensitive. In an experimental vineyard, on the other hand, 31 out of 36 isolates (86.1%) were highly resistant to thiophanate-methyl and sensitive to diethofencarb (HR,S). In the following year, 125 isolates obtained from the experimental vineyard contained 101 isolates (80.8%) which were highly resistant to thiophanate-methyl; one of them was resistant to diethofencarb. Isolates with the phenotype HR,R (high resistance to benzimidazoles and resistance to diethofencarb) have been described previously (Faretra *et al.*, 1989; Pallastro and Faretra, 1992). It was not clear however whether appearance of these new variants was related to the application of diethofencarb in the vineyard. For *Venturia naschicola*, causing scab on Japanese pear, the recombinant phenotype with high resistance to benzimidazoles and resistance to *N*-phenylcarbamates was found as a result of crosses of the isolates (Ishii and Van Raak, 1988).

Instability of dicarboximide resistance in *B. cinerea* has been explained by resensitisation in the resistant strains (Lorenz and Eichhorn, 1982). It has also been suggested that heterokaryosis provides a mechanism for maintaining nuclei carrying genes for dicarboximide resistance (Summers *et al.*, 1984). For benzimidazole resistance in *B. cinerea*, however, circumstances might be different. Meyer *et al.* (1977) reported that a high level of resistance was retained for a long time and through many generations when the fungus was grown in the absence of the fungicide. No resistant strains appeared through the 'training culture'. In the present study, when monoascospore or monomycelial isolates were tested, the level of resistance or sensitivity to carbendazim or diethofencarb did not change markedly, irrespective of the existence of carbendazim in the culture media (Fig. 1). The changes of phenotype from IR,R (intermediate resistance to benzimidazole with resistance to diethofencarb) to HR,R, and from HR,R to IR, R

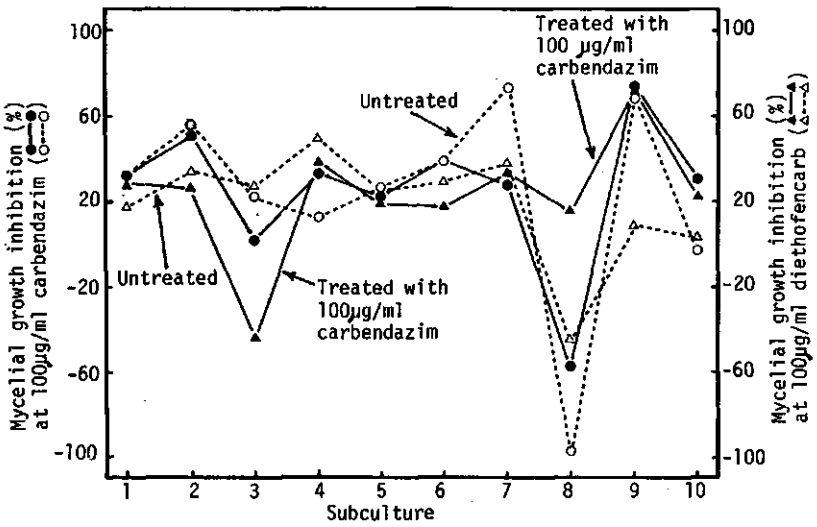

Fig. 2. Isolate VB 73 (HR,R). Stability of carbendazim resistance and diethofencarb sensitivity in an isolate of Botrytis cinerea.

Table 1. Effects of a mixture of diethofencarb and thiophanate-methyl on grey mould development on cucumber fruit

Plot	Ratio (%) of isolates in inoculum source[a)]				Protective value
	S,R	IR,R	HR,S	HR,R	
A	100	0	0	0	77.8
B	0	100	0	0	5.3
C	0	0	100	0	78.5
D	0	0	0	100	6.0
E	72	25	0	0	23.8
F	50	50	0	0	25.1
G	25	75	0	0	–56.5
H	75	0	25	0	75.0
I	50	0	50	0	81.2
J	25	0	75	0	100.0
K	0	75	25	0	–3.9
L	0	50	50	0	8.0
M	0	25	75	0	10.1
N	0	0	75	25	12.7
O	0	0	50	50	1.5
P	0	0	25	75	1.4
Q	50	25	25	0	–72.3
R	25	50	25	0	0.9
S	25	25	50	0	11.7

[a)] S,R: sensitive to carbendazim and resistant to diethofencarb;
IR,R: intermediately resistant to carbendazim and resistant to diethofencarb;
HR,S: highly resistant to carbendazim and sensitive to diethofencarb;
HR,R: highly resistant to carbendazim and resistant to diethofencarb.

were not observed. The HR,R phenotype of isolates derived from single ascospores was stable. On the other hand, the HR,R phenotype of isolates obtained by mass isolation from infected tissues was unstable in successive subcultures (Fig. 2). The mass-isolation cultures seemed to be heterokaryons or mixtures of different isolates.

Recently, reduced performance of a mixture of diethofencarb and carbendazim has become apparent in French vineyards due to the increase in IR,R strains (Leroux, personal communication). The effect of such double resistant strains on the decrease of effectiveness of the fungicide mixture has also been reported in vegetables (Elad *et al.*, 1992). Diethofencarb alone or in a mixture with thiophanate-methyl was effective against the HR,S isolate in experiment 1 using the 'Cucumber Fruit Methods' (data not presented). However, these fungicides have no effect against the IR,R and the HR,R isolates. In experiment 2 involving mixed conidial suspensions of the isolates, the mixture of diethofencarb and thiophanate-methyl provided strong protection when the inoculum source comprised only the S,R (benzimidazole-sensitive and diethofencarb-resistant) and/or the HR,S isolates. When the IR,R or HR,R isolates were included in the inoculum, the fungicide efficacy decreased markedly (Table 1). Even when the double resistant isolates comprised 25 % of the inoculum, the fungicide did not provide good

control, suggesting that the IR,R and/or the HR,R strains can cause a practical problem under severe disease pressure.

It has been demonstrated that the binding of carbendazim to cell-free protein is lower in highly carbendazim-resistant isolates and intermediately resistant isolates than in sensitive isolates (Ishii and Takeda, 1989). On the other hand, biochemical data on the binding of diethofencarb have not yet been reported. Methods for the double resistant strains are urgently required.

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Effectiveness of some fungicides and fungicide combinations on *Botrytis cinerea* isolates

N. Delen and T. Ozbek

Summary

B. cinerea is one of the important pathogens of greenhouse crops in Turkey. Because of the continuous applications of fungicides, a high proportion of the pathogen population is resistant to benzimida zoles, dicarboximides and captan. *In vitro* tests and glasshouse experiments suggest that mixed formulations of tebucanazole + tolylfluanid, tebucanazole + dichlofluanid, diethofencarb + carbendazim and dichlofluanid alone are the most effective fungicides. Phosetyl-Al stimulated the resistance response in the host.

Introduction

Botrytis cinerea Pers.: Fr. is one of the most important pathogens of greenhouse vegetables and ornamental crops in Turkey, but because of the continuous fungicide application, *B. cinerea* has become resistant to the benzimidazoles (Delen *et al.*, 1984). Now several isolates of the pathogen have been isolated from greenhouse plants, which are moderately resistant to dicarboximides and multisite fungicides (Delen *et al.*, 1984, 1985). Because chemical control of the pathogen is becoming more difficult in Turkey, the sensitivity of *B. cinerea* to the recommended chemicals was studied. Some of the more recent fungicides and fungicide combinations were also evaluated for control of *B. cinerea* in Turkey.

Material and Methods

Infected tissues were collected in Turkey from the vegetable and ornamental greenhouses located in the coastal regions of the Aegean and the Mediterranean in 1991. The sensitivity of 37 isolates to fungicides was tested on MM medium, consisting of 20 g glucose, 1. 5 g asparagine 1.0 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.1 g $FeCl_3$, 1 g yeast extract, 20 g difco bacto agar per 1000 ml distilled water (Delen *et al.*, 1984). Five selected isolates were studied *in vitro*. Isolates 90/5-CAP and 91/12-K were sensitive and isolates 91/13-K and 91/23-I were less sensitive to benzimidazoles and dicarboximide, whereas isolate 91/26-I was sensitive to dicarboximides but less sensitive to benzimidazoles. The effectiveness of the chemicals listed in Table 4 on the isolates was determined using seedlings of broadbean (*Vicia fabae*) (Meunier *et al.*, 1985). Isolates 90/5-Cap, 91/13-K and 91/23-I were used in two steps; in the first newer chemicals were tested; the second step was used triggering a resistance response in the host, combinations of phosetyl-Al with iprodione or carbendazim were used (Cohen and Coffey, 1986).

Results

The sensitivity of collected isolates to chemicals recommended in Turkey for control of *B. cinerea* are given in Table 1.

The data in Table 1 show, that several isolates were relatively insensitive to the recommended fungicides.

Some of the isolates were especially insensitive to copper oxychloride + iprodione + phosetyl-Al (Table 2). Tebucanazole + tolylfluanid, tebucanazole + dichlofluanid, dichlofluanid alone and diethofencarb + carbendazim were the most effective for the control of isolates resistant or sensitive to benzimidazole and iprodione.

The efficacy of phosetyl-Al combinations with carbendazim or iprodione are summarised in Table 4. The iprodione + phosetyl-Al combination was the most effective treatment for control of grey mould. Carbendazim + phosetyl-Al was more effective than carbendazim alone.

Table 1. Sensitivity of B. cinerea isolates to recommended fungicides in Turkey in 1991.

Fungicide	Number of isolates	distribution of ED_{50} values in isolate population							
		<0.3	0.3-1	1-3	3-10	10-30	30-100	100-300	>300
Iprodione	37	37.1	18.9	24.3	21.6	0.0	0.0	0.0	0.0
Benomyl	37	29.7	2.7	0.0	0.0	0.0	0.0	0.0	67.6
Carbendazim	17	29.4	0.0	0.0	0.0	0.0	5.9	5.9	58.8
Dichlofluanid	23	26.1	17.4	13.0	30.4	13.0	0.0	0.0	0.0
Thiram	23	8.1	0.0	8.7	52.2	30.4	0.0	0.0	0.0
Mancozeb	24	8.3	0.0	0.0	0.0	4.2	54.2	12.5	20.8
Captan	23	4.3	4.3	4.3	34.8	30.4	13.0	8.7	0.0

Table 2. Effect of fungicides on germination of conidia of five isolates of B. cinerea.

Fungicides or combinations[1)]	doses ($\mu g.ml^{-1}$)	Germination of conidia (% of control)				
		Isolates				
		90/5-Cap	91/12-K	91/13-K	91/23-I	91/26-I
Anilazine	0.3	90.6	100.0	100.0	96.3	66.2
	1.0	46.3	81.2	100.0	71.5	37.5
	3.0	32.2	100.0	100.0	65.2	47.7
	10.0	14.8	27.8	38.0	22.7	11.1
Copper Oxychloride + Iprodione + Phosetyl-Al	0.3	66.5	100.0	100.0	100.0	88.5
	1.0	–	31.0	–	–	–
	3.0	82.7	59.8	42.0	100.0	76.7
	10.0	94.5	100.0	82.0	50.2	72.6

[1)] Imazalil, prochloraz, flusilazole, myclobutanil, tebucanazole, tebucanazole + tolylfluanid, tebucanazole + dichlofluanid, diethofencarb + carbendazim completely inhibited conidial germination.

Table 3. Statistical grouping of the characters according to their effectiveness, as tested on broad beans.

Fungicide	disease severity (%)
Tebucanazole + tolylfluanid	0.00 a[1)]
Tebucanazole + dichlofluanid	0.00 a
Dichlofluanid	4.31 ab
Diethofencarb + Carbendazim	4.31 ab
Anilazine	22.79 bc
Tebucanazole	25.31 bc
Imazalil	26.45 bc
Prochloraz	26.57 bc
Myclobutanil	27.71 c
Flusilazole	35.22 cd
TBZ	36.14 cd
Iprodione	43.24 cd
Carbendazim	51.14 de
Control	67.71 e

[1)] Figures followed by the same letter do not differ significantly, p = 0.05.

Table 4. Statistical grouping of the characters according to their effectiveness, as tested on broad beans.

Fungicide	disease severity (%)
Phosetyl-Al + iprodione	15.61 a[1)]
Iprodione	28.46 ab
Phosetyl-Al + cabendazim	33.25 ab
Carbendazim	38.85 bc
Phosetyl- Al	45.50 bc
Control	55.57 c

[1)] Figures followed by the same letter do not differ significantly, p = 0.05.

Discussion

Reduced sensitivity of *B. cinerea* to the recommended fungicides is seriously influencing the chemical control of the pathogen in Turkey. The most important aspect of the problem is, that the ratio of the isolates with reduced sensitivity is increasing each year. Because of heterokaryosis (Caten and Jinks, 1966), fungicide resistance is expected in *B. cinerea*. For this reason new and effective products will be a continuing need. This work showed that mixtures of tebucanazole + tolylfluanid, tebucanazole + dichlofluanid, diethofencarb + carbendazim and dichlofluanid alone were effective against grey mould. These chemicals may have some resistance risks (Katan *et al.*, 1989; Malathrakis, 1989), and this should be taken into consideration in designing control strategies. The results obtained with phosetyl-Al perhaps suggest that this chemical may act indirectly by stimulating a resistance response in the host (Cohen and Coffey, 1986).

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The chemical control of *Botrytis cinerea* in fruit culture

P. Creemers

Summary

B. cinerea is the most important fruit rot disease on pome and small fruits in Belgium. The appearance in 1979 of strains of *B. cinerea* resistant to dicarboximides in strawberry fields led to different control strategies. Combinations of dicarboximides with the fungicides thiram and tolylfluanid were the most effective, even in strawberry fields with a relative high population of resistant strains. The development of new fungicides belonging to different chemical groups are in progress with positive perspectives for control of *B. cinerea* in the near future.

Thermonebulisation is a new development in the control of postharvest diseases of pome fruits. This method has important advantages in comparison with preharvest treatments or other postharvest applications such as dipping or drenching.

Introduction

In Belgium, *Botrytis cinerea* Pers.: Fr. is an important pathogen responsible for severe yield losses of ripe and near-ripe strawberries. To control *B. cinerea*, repeated applications of different fungicides are applied during the blossom period. The development of resistant strains to benzimidazoles and dicarboximides has necessitated the search for new strategies to prevent or to delay the build-up of resistance that diminish the efficacy of these compounds.

B. cinerea also causes postharvest grey mould of pome fruits. In Belgium, grey mould is the major disease of pear, and on apple the second most severe after *Gloeosporium* (Creemers, 1989).

The same fungicides used in strawberry culture, can be used to control postharvest fruit rot of pome fruits. In Belgium, two to three treatments are usually applied before harvest. Recently a new method called thermonebulisation has been tested (Chapon *et al.*, 1988), which is based on the thermal fogging of fungicides in the storage room. The method is based on thermal fogging of chemicals to produce droplets of c. 1 μ. Compared with dipping or drenching, this method offers a number of advantages; the application is done after fruits are stored without further handling; 200 ton fruits can be treated per hour; no problems with residual chemical solutions occur as with dipping or drenching; no spread of spores to healthy fruits occurs and the treatment can be repeated for products with a short life during storage.

Results and Discussion

Resistance of *B. cinerea* to benzimidazoles and dicarboximides in strawberries

In 1969, the Research Station at Gorsem did the first trials with benzimidazoles (MBC)

for the control of *B. cinerea* on strawberries (Bal *et al.*, 1989). In 1973, after ten weekly spray applications to the everbearing cultivar Ostara, the efficacy of the benzimidazoles decreased sharply. In June-bearing strawberries where only four to five treatments against *B. cinerea* were applied, the efficacy of benzimidazoles also decreased, albeit slower. Since 1983 the MBC fungicides have no longer controlled this pathogen.

At present, even on plots which had not been treated with MBC fungicides during the last few years, applications of these compounds had no effect on *B. cinerea*. This shows that resistance to MBC is no longer limited to plots regularly subjected to MBC treatments.

Dicarboximide fungicides, such as vinclozolin, iprodione and procymidone were introduced later. Here also, dicarboximide-resistant strains were soon isolated from everbearing strawberries. The resistance level was much lower and resistant strains showed also a lower fitness compared to wild strains. This led to a decrease in frequency of dicarboximide-resistant strains when no dicarboximides had been applied.

The selection pressure for resistance is higher on everbearing than on June-bearing strawberries as less than half of the number of treatments are applied within one season to the latter. The number of dicarboximide-resistant strains increased during the last years on June-bearing strawberries.

With better control strategies it was possible to increase the efficacy of the dicarboximides in strawberry fields where dicarboximide-resistant strains were present. The best results were obtained when the dicarboximides were combined with thiram or tolylfluanid (Table 1 and 2).

The efficacy of the dicarboximides on June-bearing strawberries was better than on everbearing strawberries, the most pronounced difference being found with procymidone (84,1% against 8,2%).

Dicarboximide resistance occurred more often on the everbearing strawberries as was expected.

Recently we also observed a tendency for a decrease in the activity of dicarboximides in June-bearing types; the efficacy was comparable to the older fungicides thiram and tolylfluanid. The combination of dicarboximides with these compounds considerably improved control of *B. cinerea* (Creemers *et al.*, 1990; Gocha and Creemers, 1991).

Efficacy of diethofencarb against *B. cinerea* in fruit culture

Diethofencarb, a fungicide with negative cross resistance to benzimidazoles, offers more possibilities for alternating fungicides of different chemical groups in the control scheme (Gullino *et al.*, 1989; Knights *et al.*, 1991).

For the control of natural populations of *B. cinerea* it is necessary to combine diethofencarb with benzimidazoles. Sumico, a coformulation of diethofencarb with carbendazim has been registered in Belgium for commercial use on strawberries since 1990. It is possible to apply Sumico until 3 days before harvest. In 1992 a request for registration of Sumico as an agent for control of *B. cinerea* and *Gloeosporium* sp. on stored apples and pears has been submitted.

Sumico showed a good activity against *B. cinerea* in June-bearing and everbearing strawberries. The addition of thiram to Sumico gave an important increase in the level of control (Table 3). On everbearing strawberries where three sprays of Sumico were followed with three sprays of tolylfluanid there was also an improvement in control compared with the two compounds alone (Table 4).

Table 1. Control of B. cinerea on June-bearing strawberry, cv. Elsanta in four trials in 1990 and 1991.

Fungicide	Dose rate g a.i.are^{-1})	Efficacy (%)	
Iprodione	10	80.3	c[1)]
Procymidone	5	84.1	c
Thiram	16	79.5	c
Tolylfluanid	12.5	78.6	c
Procymidone + thiram	5 + 16	94.3	ab
Procymidone + tolylfluanid	5 + 12.5	95.2	ab
Vinclozolin + thiram	5 + 16	97.4	a
Iprodione + thiram	10 + 16	90.7	b

Average number of treatments: 6
Average degree of infection in the untreated plots: 13,8%
[1]) Values followed by the same letter are not significantly different at p = 0.05

Table 2. Control of B. cinerea on everbearing strawberry, cv. Selva in three trials in 1990 and 1991.

Fungicide	Dose rate g a.i.are^{-1})	Efficacy (%)	
Procymidone	5	8.2	e[1)]
Thiram	16	62.7	d
Tolylfluanid	12.5	76.0	c
Procymidone + thiram	5 + 16	88.7	ab
Procymidone + tolylfluanid	5 + 12.5	80.5	bc
Vinclozolin + thiram	5 + 16	90.2	a
Iprodione + thiram	10 + 16	79.2	c

Average number of treatments: 14
Average degree of infection in the untreated plots: 13.6%
[1]) See foot note in Table 1.

Because of the mode of action of Sumico, we recommended that the number of applications within one season should be limited to avoid the selection of strains of *B. cinerea* which are resistant to both components i.e. diethofencarb and carbendazim. We have isolated such strains from everbearing strawberry fields which were treated with only Sumico. Besides the possibility of reducing resistance build-up against MBC fungicides, alternation or combination with thiram or tolylfluanid has a positive effect on the control of other fruit rot fungi, like leather rot (*Phytophthora cactorum*) and anthracnose (*Colletotrichum gloeosporioides*) (Tables 3 and 4).

Sumico was also effective against *B. cinerea* in pome fruits where benzimidazoles are no longer effective. Normally three sprays are applied in the last 5 to 6 weeks before harvest, the last applied 14 days before harvest. The control of *B. cinerea* with Sumico was superior to spray programmes based on vinclozolin (Table 5).

Postharvest dipping treatments were more effective for control of *B. cinerea* than preharvest treatments. In Belgium, some fungicides (imazalil, thiabendazole) are

Table 3. Control of B. cinerea on June-bearing strawberry cvs Gorella, Berluta, Sivetta, Vicoda and Elsanta in nine trials during 1987-1991.

Fungicide Fungicide	Dose rate g a.i.are^{-1}	Efficacy (%)	
Diethofencarb + carbendazim	2.5 + 2.5	88.3	b[1)]
Diethofencarb + carbendazim + thiram	2.5 + 2.5 + 16.0	97.4	a
Tolylfluanid	12.5	83.3	b
Vinclozolin	5	77.0	bc
Vinclozolin + thiram	5 + 16	95.9	a

Average number of treatments: 5.7
Average degree of infection in the untreated plots: 18.8%
[1)] See foot note in Table 1.

Table 4. Control of B. cinerea in everbearing strawberry cvs Rapella and Selva in eight trials during 1987-1991.

Fungicide	Dose rate g a.i.are^{-1}	Efficacy (%)	
Diethofencarb + carbendazim	2.5 + 2.5	73.8	b[1)]
Diethofencarb + carbendazim alternated with tolylfluanid	2.5 + 2.5 12.5	88.0	a
Tolylfluanid	12.5	67.8	b
Vinclozolin	5	39.6	c
Vinclozolin + thiram	5 + 16	89.2	a

Average number of treatments: 15
Average degree of infection in the untreated plots: 16.8%
[1)] See foot note in Table 1.

permitted for postharvest use, but in practice are rarely applied. These treatments have some advantages biologically and ecologically compared to preharvest applications, such as reduced use of chemicals per unit of fruits, no effect on benificial organisms in the orchard, better control of fruit rot by a better coverage of the fruits by the protection of wounds made by picking, separation between fruits for direct sale or for long storage and a reduced risk for the selection of resistant strains of which the infection source is in the orchard.

Thermonebulisation

Experiments were done to compare thermonebulisation and dipping. The best control of *B. cinerea* was obtained by dipping (Table 6). At the first assessment in February, there was a difference in efficacy between the preharvest treatments and the thermonebulisation, but by the second assessment in June no difference was detected.

Table 5. Control of B. cinerea on pear cv. Conférence in three trials during 1988-1990.

Fungicide	Dose rate	(a.i) Efficacy (%)
Pre-harvest treatments (3 treatments)		
Diethofencarb + carbendazim	375 + 375 g.ha^{-1}	86.5
Vinclozolin	750 g.ha^{-1}	73.0
Post-harvest treatment by dipping		
Diethofencarb + carbendazim	250 + 250 mg.l^{-1}	98.2
Vinclozolin	500 mg.l^{-1}	92.8

Average degree of infection in the untreated plots: 14.8%

Table 6. Control of B. cinerea on pear, cv. Conférence during storage using different methods.

Method	Dose rate (a.i.)	Efficacy (%)	
		February '91	June '91
Preharvest			
Vinclozolin	3 x 750 g.ha^{-1}	86.5 ab[1)]	82.2 a
+ Carbendazim	3 x 375 g.ha^{-1}		
Dipping			
Vinclozolin	500 mg.l^{-1}	94.7 a	86.4 a
+ Thiabendazole	500 mg.l^{-1}		
+ Imazalil 250 mg.l^{-1}			
Thermonebulisation			
Vinclozolin	550 gr/50 ton	72.9 b	80.0 a
Thiabendazole	480 gr/50 ton	26.7 c	0.0 b
Untreated % infection		22.4	18.0

[1)] See foot note in Table 1.

The period of 14 days between harvest and thermonebulisation may have affected this results and better control may have been achieved if this treatment was carried out rapidly after harvest. Because of MBC resistant strains the efficacy of thiabendazole was inadequate. Although the efficacy of thermonebulisation is lower than that of dipping, with modification it may replace the need for pre-harvest sprays.

New fungicides against *Botrytis cinerea*

In 1991 some new fungicides from different chemical groups were evaluated for the control of *B. cinerea* on strawberries. One of these compounds, CGA 173506, is based on the natural substance pyrrolnitrin, a metabolite of *Pseudomonas cepacia* and belongs to the chemical class of the phenylpyrroles. The chemical structure for the other compounds cannot yet be given. They are presented by the RSG numbers 404 and 184.

Table 7. Fungicidal control of B. cinerea on three strawberry cultivars in 1991.

Fungicide	Dose rate g a.i.are^{-1}	June-bearing Elsanta Trial 1	Elsanta Trial 2	Vicoda 2 years	Everbearing Selva Trial 1	Selva Trial 2
CGA 173506	5.0	94.6 a[1)]	99.0 a	91.2 a	95.9 a	89.9 a
RSG 404	8.0	96.9 a	98.1 a	62.3 b	91.7 ab	66.4 b
RSG 184	5.0	87.6 abc	93.3 ab	89.0 a	90.0 ab	71.0 ab
RGS 184 + thiram	3.75 + 16.0	95.3 a	96.2 ab	78.8 a	90.9 ab	81.3 ab
Tolyfluanid	12.5	68.2 de	89.4 ab	64.1 b	75.2 b	73.4 ab
Thiram	16.0	80.6 bcd	86.5 b	55.7 b	44.6 c	66.4 b
Procymidone	5.0	62.0 e	69.2 c	11.8 c	42.1 d	6.3 c
Vinclozolin + Thiram	5.0 16.0	93.0 ab	99.0 a	62.7 b	87.6 ab	85.2 ab
Untreated % infection		12.8	13.0	33.3	15.1	16.0

[1)] See foot note in Table 1

These new fungicides showed a good activity against *B. cinerea* on June- and everbearing strawberries (Table 7). The efficacy was comparable, or better than the standard vinclozolin + thiram combination.

The release of several fungicides with a different mode of action offers more possibilities to alternate the fungicides and to prevent or to delay the selection of resistant strains for a specific fungicide group in the near future.

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Effect of fungicide application techniques on the control of *Botrytis cinerea* and development of fungal resistance

M. Besri and F. Diatta

Summary

The fungicides used to control tomato grey mould caused by *B. cinerea* are usually sprayed, but some farmers apply them also as a paste to the stem lesions. Application of benomyl and thiophanate-methyl to plants previously inoculated, did not control the fungus. Procymidone, applied as spray or paste, reduced lesion length by 25.6% and 42.5% respectively and reduced the percentage of wilted plants, particularly when applied as a paste.

Painting the *B. cinerea* lesions with benomyl, thiophanate-methyl and procymidone increased the frequency of resistant spores. No resistant spores were detected on sprayed or untreated lesions.

Introduction

Chemical control of tomato pathogens, particularly *Botrytis cinerea* Pers.: Fr., is intensive along the Moroccan atlantic coast. Despite the large number of fungicide applications (20-25 per crop), grey mould remains the most important disease of this crop (Diatta, 1984; Hormatallah, 1984). Diatta (1984) reported that *B. cinerea* occurs in 96% of the plastic greenhouses surveyed. Farmers use several fungicides to control the disease, but particularly thiophanate-methyl (Pelt 44), benomyl (Benlate) and procymidone (Sumisclex). These are either sprayed or applied as a paste on the stem lesions. Abundant sporulation of *B. cinerea* is often observed either on the sprayed plants or on the painted lesions.

Under Moroccan conditions (Besri and Diatta, 1985) and elsewere (Dekker, 1976, 1982; Delp, 1980; Leroux *et al.*, 1982; Lorenz and Eichhorn, 1982), fungicide resistance to benomyl, procymidone and thiophanate-methyl is widely distributed. However, data related to methods of fungicide application on resistant strain development in *B. cinerea* and on efficiency of control are not available.

Material and Methods

Frequency of sporulation of *B. cinerea* on treated stem lesions

Plastic tunnels in the important tomato growing areas of Dar Bouazza, Chtouka, Oualidia and Abda where benomyl, procymidone and thiophanate-methyl are used to treat the diseased plants were chosen for our study. In each region, one hundred lesions per treatment for each fungicide were observed 4 months after planting to examine sporulation of the fungus.

Efficacy of the fungicide application techniques

Stems of tomato plants (cv. Darus 149) were wound-inoculated with *B. cinerea*, using a tomato leaf disc previously steam-heated and inoculated with the fungus (Diatta, 1984). The fungicides were applied one week after inoculation when lesions developed. Application was done either as paste (0.5 $g.ml^{-1}$ of water) by painting the lesions, or as sprays at 250, 750 and 300 ppm for benomyl, thiophanate-methyl and procymidone respectively. Five replicates of ten plants were used for each fungicide and treatment. The lesion lengths were measured 14 days after fungicide application and the efficiency of the fungicide determined by the formu la:

$$\text{Efficiency } (\%) = \frac{\text{A-B}}{\text{A}} \times 100$$

Where A = length increase of the control lesion and B = length increase of the treated lesion.

The percentage of wilted plants was calculated 40 days after inoculation of plants.

Frequency of resistant spores of *B. cinerea* produced on treated tomato plants

Spores from stem lesions sprayed or painted with the three fungicides were suspended in water and the concentration adjusted to 10^3 spores.ml^{-1}. PDA Petri dishes, amended with benomyl (Benlate 50%), thiophanate-methyl (Pelt 44), procymidone (Sumisclex) at concentrations 20, 20 and 2 ppm respectively were inoculated with 0.5 ml spore suspension and the dishes were incub ated at 25°C for 6 days. The frequency of the resistant spores (F %) was then calculated by the same formula as above where A represents the number of colonies on PDA and B the number of colonies on fungicide-amended PDA. Fifteen isolates per treatment in five replicates were tested for each fungicide.

Results

Frequency of sporulating lesions

B. cinerea sporulated on tomato plants sprayed or painted by the three fungicides (Table 1), but the percentages of the sporulating lesions treated with benomyl and thiophanate-methyl were higher than those treated with procymidone. The sporulation frequency on the sprayed plants is also higher than on the painted ones for the three fungicides used, but neither spraying nor painting the lesions completely inhibited sporulation.

Effect of application technique on lesion length and percentage of wilted plants.

The application of benomyl and thiophanate-methyl as sprays or as paste did not inhibit the growth of lesions. The treatment efficiency varied from 1.2% to 5.4% 14 days after the inoculation. Procymidone applied as a spray or as paste reduced lesion length by 25.6 % and 42.5% respectively (Table 2).

Table 1. Frequency of sporulating B. cinerea lesions along the Moroccan atlantic coast.

Region	% sporulating lesions					
	Sprayed			Painted		
	B[1)]	TM	P	B	TM	P
Dar Bouazza	80	92	4	48	55	0
Chtouka	86	85	3	38	62	2
Oualidia	73	95	6	49	52	5
Abda	59	88	7	53	49	3
Mean	74.5	90	5	39.5	54.5	2.5

[1)] B = benomyl, TM = thiophanate-methyl, P = procymidone. The fungicides were applied by the growers as sprays or as paste.

Table 2. Effect of fungicide application technique on B. cinerea stem lesions after 14 days.

Fungicide	Sprayed		Painted	
	lesion length (mm)	% control[1)]	lesion length (mm)	% control
Benomyl	95.0	5.4	89.2	3.2
Thiophanate-methyl	91.5	1.2	99.0	2.2
Procymidone	81.5	25.6	53.2	42.4
Control	96.8	–	96.8	–

[1)] control, compared to lesion length before fungicide applied.

There were no significant differences between plant parts treated with benomyl and thiophanate-methyl, either sprayed or painted, and the control plants. In procymidone-treated plants sprays and painting reduced wilting from 95% in controls to 49 and 28% respectively.

Frequency of resistant spores of *B. cinerea*

Table 3 shows that painting the stem lesions with the fungicides increases the frequency of the resistant spores of *B. cinerea* to benomyl, thiophanate-methyl and procymidone. No resistant spores to procymidone were detected on sprayed or on control lesions.

Discussion

To our knowledge, no research on the effect of the fungicide application technique has been done with *B. cinerea* control or on the development of resistance. Sporulation was higher on tomato plants sprayed with benomyl and thiophanate-methyl than on those painted with the same fungicides. Similar results were found with procymidone, but the percentage of sporulating lesions was lower.

The curative treatments through spraying or painting the lesions did not stop their development, but procymidone applied as spray or paste decreased lesion length and the

Table 3. Frequency (%) of resistant spores of B. cinerea growing in the agar medium amended with the fungicide.

Origin of isolates	Benomyl (20 ppm)	Thiophanate-methyl (20 ppm)	Procymidone (2 ppm)
Painted lesion	80 a[1)]	93 a	2 a
Sprayed lesion	30 b	42 b	0 b
Control	15 c	18 c	0 b

[1)] Figures followed by different letters are significantly different, p = 0.01

number of wilted plants. Therefore fungicides must be applied preventively.

The fungicide application techniques used by the growers have limited effect on grey mould, but increase the population of resistant conidia, particularly when the stem lesions were painted. The results indicate that the latter practice is probably responsible for an important increase of the resistant *B. cinerea* isolates compared to the spraying technique. We therefore advise painting of lesions should be avoided and an integrated disease management strategy to control the pathogen introduced (Ogawa *et al*, 1977, Dekker, 1982).

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Effect on grey mould of presence of *Botrytis cinerea* strains showing reduced sensitivity to dicholofluanid

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Summary

In the winter of 1987-88 during monitoring of tomato and rose crops infected by *B. cinerea* in protected cropping, it was found that a high percentage of isolates showed reduced sensitivity to dichlofluanid *in vitro*. The field efficacy of this fungicide was not decreased throughout a 3-year trial on tomato. Sprays containing dichlofluanid provided the best protection against *B. cinerea* causing petal spotting on roses.

Introduction

The widespread distribution of strains of *Botrytis cinerea* Pers.: Fr. showing resistance to benzimidazoles and dicarboximides has restricted the number of compounds available for grey mould control in greenhouse crops. The recently introduced fungicide diethofencarb offered a possible alternative for use in places where high levels of carbendazim-resistant strains predominate (Pappas and Elena, 1988). Nevertheless, insensitive biotypes to both carbendazim and diethofencarb have been already detected in Israel (Katan *et al.*, 1989).

The long-standing protectant fungicide dichlofluanid is still used extensively against grey mould, but doubts about its efficacy were claimed following the isolation of strains exhibiting a wide range of sensitivity to this fungicide when tested *in vitro* (Malathrakis, 1989). Moreover, such dichlofluanid 'resistant' isolates also showed variable sensitivity to two other multisite fungicides, chlorothalonil and captan both used against grey mould (Elena and Pappas, 1989). A re-evaluation of the performance of dichlofluanid in the field in places where strains of *B. cinerea* with reduced sensitivity were detected, was therefore necessary.

Material and Methods

To determine the frequency of infections caused by strains of *B. cinerea* with reduced sensitivity to dichlofluanid, the 'point inoculation method' was applied. Infected plant tissues were collected in polyethylene bags and incubated overnight at room temperature. Conidia from freshly sporulating lesions of each sample were then aseptically transferred with a needle onto malt extract agar (Oxoid) containing 0.1 or 3 μg a.i.ml^{-1} of the fungicide dissolved in acetone. The final concentration of the solvent in the medium was always 1%. When necessary the sensitivity to dicarboximides and benzimidazoles was detected on media amended with 3 and 10 μg.ml^{-1} iprodione or 100 μg.ml^{-1} benomyl, respectively.

In some cases, the sensitivity to N-phenylcarbamates was also tested in media containing 100 μg.ml^{-1} diethofencarb. Solvents in these tests were acetone for iprodione

and dimethyl sulfoxide for benomyl and diethofencarb. Following 24 h incubation at 21°C in darkness, spore germination of each isolate was examined.

In three successive years (1985-88), dichlofluanid, alone or in alternating sprays and combined programmes with specific fungicides, was evaluated against grey mould in a protected commercial tomato crop in Tymbaki. In all trials plants of tomato cv. Tombo were grown as single stems on vertical strings and planted 50 cm apart in middle October as six rows spaced at 1 m. Each treatment was applied to a 50-plant randomised plot surrounded by unsprayed guard plants and replicated three times.

The fungicides used were: dichlofluanid; iprodione; metomeclan; chlorothalonil; carbendazim + diethofencarb (Sumico). These fungicides were applied until run-off (1120 l.ha^{-1}) using a hand-operated knapsack sprayer at a pressure of 6 kg/cm^2. Fungicide applications were made on 8-11 occasions each growing season at 15-day intervals commencing the first fortnight of December and terminating in April. Prior to each application ripe fruit was picked and weighed and the number of infected tomatoes in each plot recorded.

Fungicide treatments during the 1987-88 period were also compared on roses for the control of petal spotting on flower buds caused by *B. cinerea*. In this experiment, 4-month old plants of rose cv. Sonia were planted in July 1987 20 cm apart in seven rows. The experiment was done in a commercial glasshouse in the Marathon region. Each treatment was applied on three 9 m long randomised plots. Fungicides were sprayed on six occasions at 10-day intervals between October-December 1987. Before each application five flowers per treatment/replicate were collected at random and examined for petal spotting after storage for 3 days at 5°C.

Results

The monitoring of tomato fruit rots on two occasions during the winter of 1987-88 in Tymbaki region revealed that 80 out of 150 infections were due to *B. cinerea* strains showing reduced sensitivity to dichlofluanid. The spores of these isolates germinated on media containing 1 μg.ml^{-1} of the fungicide. However, germtube length was less than double the spore diameter, and the germination was completely arrested at concentrations of 3 μg.ml^{-1}. Isolations of such strains were few in previous years where spore germination of most isolates was inhibited by dichlofluanid at concentrations of 0.2 μg.ml^{-1}.

A high incidence of strains with reduced sensitivity to dichlofluanid was detected in 4-month-old commercial roses in the region of Marathon. In a survey made in October 1987, the spores of 23 out of 70 isolates with originated from infected roses germinated on media containing 1 μg.ml^{-1} dichlofluanid.

The predominance of *B. cinerea* strains, showing moderate resistance to dicarboximides and high resistance to benzimidazoles, was confirmed in Tymbaki and Marathon. All benzimidazole-resistant isolates tested however, were found to be sensitive to diethofencarb.

In the 1985-1986 disease control trial, all fungicide applications, except iprodione, significantly reduced fruit rot and in creased the yield of tomato. Better control was provided by the tank mixture of dichlofluanid plus half strength metomeclan (Table 1). Iprodione alone failed to control the disease, presumably due to infection by strains moderately insensitive to dicarboximides.

In the 1986-87 trial, dichlofluanid alone and in combined spray programmes gave

Table 1. Evaluation of fungicides for control of fruit rot in tomato caused by B. cinerea in the 1985-86 season.

Treatment/concentration (a.i.)	Yield (kg)	No. rotted fruits
Dichlofluanid (0.1%)	85	10.7 a
Iprodione (0.05%)	84	18.0 b
Metomeclan (0.05%)	95	8.0 a
Dichlofluanid (0.1% + iprodione (0.025%)	96	9.3 a
Dichlofluanid (0.1%) + metomeclan (0.025%)	92	6.7 a
Untreated	81	18.0 b
S.E.D.	–	1.44

Data followed by the same letter do not differ significantly at p = 0.05.

Table 2. Evaluation of various spray-programmes for control of B. cinerea fruit rot in tomato in the 1986-87 season.

Treatment/concentration (a.i.)	Yield (kg)	No. rotted fruits
Dichlofluanid (0.1%)	104	53.7 bc
Dichlofluanid (0.1%) + iprodione (0.025%)	113	36.7 a
Dichlofluanid (0.1%)*) followed by dichlofluanid (0.1%) + iprodione (0.025%)	97	50.0 abc
Dichlofluanid (0.1%)*) followed by iprodione (0.05%)	97	57.0 c
Chlorothalonil (0.1%)*) followed by chlorothalonil (0.1%) + iprodione (0.025%)	109	39.7 ab
Untreated	80	95.7 d
S.E.D.	–	4.63

Data followed by the same letter do not differ significantly at p = 0.05.
*) This fungicide was applied in the the first four sprays only.

satisfactory control and increased the yield of tomato. The best control was achieved by repeated applications of the tank mixture of dichlofluanid with half-strength iprodione (Table 2).

During 1987-88 trials, applications based on dichlofluanid significantly controlled grey mould in both crops tested. In tomato, the use of carbendazim + diethofencarb (Sumico) either alone or in half-strength tank mixes with dichlofluanid further decreased the number of infected fruit and increased yield. By contrast, Sumico alone was less effective in rose for control of the number of flowers affected by lesions caused by *B. cinerea*. For this type of infection, applications containing dichlofluanid were more effective (Table 3). In both crops, sporulating lesions were strongly suppressed on plots which received Sumico sprays.

No apparent differences in sensitivity to dichlofluanid were found among isolates originating from plants which received various treatments. On rose, after the completion of six sprays in the overall treatment with dichlofluanid, inoculum with reduced sensitivity to this chemical was increased by 5%.

Table 3. Evaluation of various fungicide applications against B. cinerea in tomato and rose culture in the 1987-1988 season.

Treatment/concentration (a.i.)	Tomato		Rose	
	Yield (kg)	No. rotted fruits	petal $\sin^{-1}p^{\circ}$	spotting %
Dichlofluanid (0.1%)	170	40.7 b[1)]	43.2	47.0 a
Dichlofluanid (0.1%)+iprodione (0.025%)	171	47.3 b	47.1	53.7 ab
'Sumico' (0.025% + 0.025%)	178	16.3 a	54.8	66.7 bc
Dichlofluanid (0.1%) + 'Sumico' (0.0125% + 0.0125%)	190	23.3 a	45.6	51.0 ab
Iprodione (0.025%) + 'Sumico' (0.0125% + 0.0125%)	171	22.0 a	62.4	78.0 cd
Iprodione (0.05%) alternated with 'Sumico' (0.025% + 0.025%)	NT[2)]	NT	58.3	72.3 cd
Untreated	160	89.0 c	66.4	83.3 d
S.E.D.		4.12	3.5	

[1)] Data followed by the same letter do not differ significantly at p = 0.05.
[2)] NT = not tested

Discussion

A wide variation in sensitivity to dichlofluanid, chlorothalonil and captan had been demonstrated among field isolates of *B. cinerea* in the early years of their commercial introduction. All these wide spectrum compounds mainly inhibit spore germination while mycelial growth is much less affected (Pappas, 1978). The present findings show that *B. cinerea* populations shift to become les ssensitive to dichlofluanid in crops grown under cover. This was probably due to high selection pressure among the wild strains followed by the continuous use of this fungicide. The increased frequency of less sensitive strains, however, should not be considered as resistance development in the field.

Dichlofluanid efficacy in localities of high inoculum pressure was always inferior to that provided by the site specific compounds (Hunter *et al.*, 1979). The results of the present work show that dichlofluanid maintains its moderate effectiveness against grey mould in the field, although the frequency of strains exhibiting reduced sensitivity in vitro was much increased. These findings are in agreement with those obtained by other workers. Inoculation experiments have shown that conidia of *B. cinerea* from isolates with reduced sensitivity failed to infect plants sprayed with field-strength dichlofluanid (Elena and Pappas, 19 89; Rewal *et al.*, 1991).

A further improvement in dichlofluanid efficacy was achieved during this work, when it was applied in mixes with compounds having specific modes of action such as iprodione or Sumico used at half recommended strength. Moreover, in cases of non-aggressive infections, the continuation of inclusion of multisite inhibitors in control programmes appeared more justifiable. Thus, dichlofluanid and its mixtures effectively restricted the spotting on rose petals when the more specific compounds failed to do this.

However, the possibility of a gradual decline in the effectiveness of conventional broad-spectrum fungicides must be considered in planning their more rational use in protected crops.

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Control of *Botrytis cinerea* in cut rose flowers by gibberellic acid, ethylene inhibitors and calcium

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Summary

Botrytis blight, caused by *B. cinerea* is a widespread problem in rose (*Rosa hybrida*) flower production in greenhouses. Despite efforts to reduce damage caused by the disease by modification of environmental conditions and use of fungicides, many flowers carry latent infections and these become visible during shipment or storage. The physiological status of the flower probably inf luences the change from latency to development of spreading lesions. The effect of ethylene inhibitors and calcium nutrition on susceptibility of rose flowers to the disease was studied and both factors were effective in controlling the disease. Exogenously applied gibberellic acid also showed potential for disease control.

Introduction

Botrytis flower blight, caused by *Botrytis cinerea* Pers.: Fr., is a widespread problem of roses (*Rosa hybrida*) grown in greenhouses. The pathogen attacks mainly flowers, but also causes necrotic lesions on young canes. Latent infection occurs and lesions are invisible at harvest, but develop under humid conditions or as flowers mature (Elad, 1988b). The most severe economic damage occurs when disease develops on flowers in storage or in transit (Elad, 1988a; Hammer and Marois, 1988). Fungicides are recommended for control of the disease on roses (Hammer and Marois, 1988), but their efficacy is limited. Roses are harvested generally in the bud stage, but infections occur between sprays on flowers that have opened slightly. Populations of the pathogen resistant to dicarboximide, benzimidazole and diethofencarb have developed (Katan, 1982; Katan *et al.*, 1989). One method of partially controlling the disease on roses is to lower greenhouse humidity by increased ventilation and heating (Marois *et al.*, 1988).

Development of flower blight on petals occurs in two stages. Initially, infection is followed by restricted colonisation, which is not visible to the naked eye, and then cessation of development. In the second stage small or spreading necrotic lesions develop when environmental conditions and host tissues are conducive (Elad, 1988b). The factors affecting the transition of tissues from resistant to susceptible are not understood, but senescence of petal tissues is probably involved.

Ethylene and Calcium

The role of ethylene in the development of *Botrytis* blight has been demonstrated (Elad, 1988a) and this is supported by the fact that inhibitors of ethylene production or activity can reduce disease severity (Elad, 1988a; Elad and Volpin, 1988). Cut rose flowers fertilised with nutrient solutions containing elevated concentrations of calcium ions,

produced less ethylene and were less affected by *Botrytis* blight than those with lower levels of Ca^{2+} (Elad and Volpin, 1988; Volpin and Elad, 1991).

Physiological stresses that increase the permeability of plant cell membranes enhance disease development, with leaked exudates serving as a nutrient source of the pathogen (Sol, 1965, 1967) and it is known that addition of Ca^{2+} ions to plant tissue retards senescence (Ferguson, 1984; Pooviah and Leopold, 1973) and limits membrane permeability (Simon, 1978). Several other plant diseases are inhibited by the addition of Ca^{2+} ions, the inhibition being associated with the ability of the ion to strengthen the cell wall and inhibit degradation by pectinolytic enzymes (Corden, 1965; Liptay and Van Dierendock, 1987).

Volpin and Elad (1991), found that the addition of calcium nitrate to the nutrient solutions used to fertilise rose plants of cvs Golden Times and Mercedes reduced the severity of postharvest *Botrytis* blight. The susceptibility of cut flowers of cv. Golden Times was increased by treatment at 4° and 10°C for 2 days, but this effect was suppressed by addition of calcium, more so at 10° than at 4°C. Cold storage of roses at 8°C shortens vase life, and storage at 3°C results in severe damage to the plasma-membrane. The induced susceptibility of flowers to *Botrytis* blight caused by cold storage could be due to increased leakage from plasmamembranes (Faragher and Mayak, 1984). At 10°C, Ca^{2+} may limit the membrane damage caused by low temperature exposure, and thereby inhibit leaching. At 4°C, Ca^{2+} was probably unable to overcome the damage caused to the membrane.

In a medium containing Na-pectate as the carbon source and 3 mM calcium, growth of *B. cinerea* and polygalacturonase activity were inhibited as compared with a Na-pectate medium without calcium. Pectate is an important compound in plant cell walls, and more than 60% of the calcium in the plant is found in cell walls (Rossignal *et al.*, 1977). *B. cinerea* produces at least four endo-polygalacturonases (Marcus and Schejter, 1983). The inability of *B. cinerea* to utilise pectate in the presence of calcium, therefore, is suggested as a mode of action of the Ca-ions.

Gibberellic acid

On the basis of these reports the physiological status of the cut flowers appears to play a major role in the change from latent infection to the appearance of spreading lesions. Goszczynska *et al.* (1990) showed that the vase-life of rose flowers was improved and senescence of petals delayed after pre-treatment with gibberellic acid (GA_3).

The objective of our study was to examine the effects of the exogenously applied GA_3 on grey mould in rose flowers, and the growth of *B. cinerea in vitro*.

Material and Methods

Rose flowers (*Rosa* x *hybrida* cv. Mercedes) grown in an experimental greenhouse or supplied by commercial growers were used in the experiments. The flowers were harvested when sepals and petals started to unfold.

Detached petals, or flowers buds left intact on the stem, were sprayed to run-off with 1 mM solutions of gibberellic acid (GA_3 Sigma, USA). Following the various treatments, the intact flowers were placed with their stem base in water and the buds enclosed in plastic bags. The detached petals were inserted through a slit in a plastic net with their base in water, and covered with a transparant polyethylene sheet. Both intact flowers and

detached petals were kept in an illuminated growth chamber at 20±2°C and 90-95% r.h. (Elad and Volpin, 1988). Experiments were repeated 3 to 6 times and each treatment was replicated 10 times.

Disease severity was assessed on a scale 0 to 5, where 0=healthy petals and 5=completely affected petals. In all the experiments (more than 20), ten flower buds or petals were used as replicates in each treatment. The results were analysed statistically and means were compared by Duncan's multiple range test.

Disks from the growing margins of mycelium from cultures or drops of conidial suspension of *B. cinerea* were inoculated onto plates of PDA containing different concentrations of GA_3 and incubated at 20±2°C. The linear growth of mycelium was measured daily and the germination of the conidia was examined under a light microscope after 24 h.

Results and Discussion

The development of grey mould on detached petals and intact buds, either with or without inoculation with conidia, was inhibited by application of a 1 mM solution of GA_3. This

Table 1. The effect of gibberellic acid on grey mould caused by B. cinerea in rose flowers.

Cultivar	Days of incubation	Disease index (0-5)	
		Control	GA_3[1)]
Detached petals			
Mercedes	16	2.8a	0.2b
Ilseta	9	2.8a	0.9b
Celica	9	3.8a	0.2b
Madelon	9	1.4a	0.8a
Madam Delbar	9	2.5a	0.5b
Cadillac	5	4.6a	2.0b
Sonia	6	2.4a	0.5b
Gabriela	11	3.1a	0.6b
Golden Times	8	3.1a	4.6b
Whole flowers			
Mercedes	14	4.7a	3.2b
Fresco	7	3.1a	3.8a
Golden Times	6	2.7a	2.8a
Sonia	6	1.9a	0.9a
Gabriela	10	4.7a	2.2b
Pasadena	9	2.1a	0.3b
Jaguar	8	1.3a	1.7a

[1)] Whole flowers were sprayed with 1 mM gibberellic acid; detached petals were dipped in 0.057 mM gibberellic acid.
Statistical significance ($p = 0.05$) between control and treatment is indicated by a different letter.

effect of GA_3 was observed in several rose cultivars (Table 1). The extent of inhibition of grey mould varied in flowers obtained from different production regions in these experiments.

Neither growth of the mycelium nor germination of the conidia was affected by addition of GA_3. Only at 10 mM GA_3, the linear growth of the mycelium was inhibited by 20%.

Since the growth and germination of *B. cinerea* were unaffected by addition of GA_3 to the growth medium, the suppression of grey mould development in the rose flowers sprayed with GA_3 may result from a decreased sensitivity of tissues to the pathogen due to the inhibition of senescence of petals by GA_3 (Goszczynska *et al.*, 1990). These authors also showed that senescence of the GA_3-treated rose flowers was inhibited, even in the presence of higher levels of ethylene evolved by the GA_3-treated than the untreated flowers. Aged rose flowers are more sensitive to ethylene than the younger flowers (Lukaszewska *et al.*, 1990). Therefore, we cannot exclude the possibility that GA_3-induced inhibition of grey mould in our work was the result of a GA-induced decrease in petal sensitivity to ethylene.

All the flowers from the numerous sources investigated in this study were infected to varying degrees by *B. cinerea*. Large quantities of fungicides are used to control this disease, with a concomitant increase in the resistance of this fungus to fungicides (Katan *et al.*, 1989). Replacement of fungicides with gibberellin and/or other growth regulators may therefore be advantageous for control of plant diseases and reduce the ecological hazards caused by excessive pesticide use.

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Mannans from *Botrytis cinerea* strains resistant to dicarboximide fungicides

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Summary

Isolates of *B. cinerea* resistant to dicarboximide fungicides are usually more sensitive to high osmotic strength than non-resistant isolates. Osmo-sensitivity of dicarboximide-resistant isolates was correlated with higher concentrations of rhamno-galacto-mannans, but not with soluble glucans, in the culture medium.

Introduction

Dicarboximide-sensitive isolates of *Botrytis cinerea* Pers.: Fr. (Bc-S) are more tolerant of high osmotic strengths than dicarboximide-resistant ones (Bc-R) (Albert, 1979; Leroux and Gredt, 1979).

The higher sensitivity of Bc-R is probably due to defective cell wall synthesis with the result that the wall may give insufficient support to the plasmalemma. Here we tested the hypothesis that more soluble fungal cell wall fragments could be released in liquid cultures of Bc-R isolates.

Material and Methods

Three Bc-R and three Bc-S isolates (Table 1) were grown in 300 ml Erlenmeyer flasks with 150 ml Czapek-Dox medium. The medium was inoculated with a 1 ml spore

Table 1. Genetic status and origin of isolates of B. cinerea, used in the experiments

Isolates and host plants	Status and source
Dicarboximide-sensitive (Bc-S)	
Bc-L69 (apple)	homokaryotic, Dr. Laubert, 1971, Switzerland (Schönenberg TG)
Bc-L5	(raspberry) homokaryotic, Dr. Laubert, 1971, Switzerland (Meerlischachen SZ)
Bc-S1 (from grape)	field isolate from Angers (F) - Coteau du Layon (1988)
Dicarboximide-resistant (Bc-R)	
Bc-R1 VG1f (strawberry)	field isolate from Meerle (Belgium) selected on vinclozolin-containing medium
Bc-R2 CG1a (strawberry)	field isolate from Meerle (Belgium) selected on vinclozolin-containing medium
Bc-RF16 (from grape)	homokaryotic, Dr. Faretra, 1989, Bari, Italy

Table 2. Precipitation of polysaccharides from culture media of isolates of B. cinerea resistant and non-resistant to dicarboximides.

Isolate	ED50	Mycelial dry weight (g)	$P_{0.5}$ mg/g myc.	P_1 to P_4 mg/g myc.
Bc-L69	<1	1.31	150.7	7.7
Bc-L5	<1	2.87	19.8	6.7
Bc-S1	<1	12.28	30.9	31.2
Bc-R1V G1f	5	2.97	147.7	307.2
Bc-R2V G1a	>10	5.88	61.2	92.8
Bc-FR16	5	0.63	14.8	55.9

Table 3. Sugar analysis of the culture medium of B. cinerea, after DEAE column separation.

Fractions Sugar (%)	8-13	14-19	20-22	50-60	62-72
glucose	66	38	15	21	12
mannose	21	21	67	64	66
galactose	12	39	16	12	16
rhamnose	1	2	2	2	6

suspsension containing 10^6 spores and incubated on a rotary shaker (100 rpm) for 6 days. Culture filtrates were obtained by vacuum filtration through glassfiber filters (SS GF 92, 50 mm in a millipore filtration system XX 10.047) and mycelial weights were determined after drying under vacuum at 90°C. To obtain polysaccharides, the medium was acidified (pH +3) and a half volume of ethanol added to give immediate precipitation of glucans (MW between 10^6 and 0.5×10^6 Da). This precipitate, called $P_{0.5}$ appeared as a slurry that was removed immediately. Further additions of ethanol, one till four times the volume of the medium, precipitated polysaccharides of lower molecular weight (between 50 and 5×10^3 Da). These precipitates called P_1, P_2 and P_4 consisted of rhamno-galacto-mannans (Dubourdieu, 1978; Kamoen and Dubourdieu, 1990). The different precipitates were washed with ethanol, redissolved in distilled water, dialysed for 24 h, lyophilysed and the dry weights determined.

The amount of polysaccharides in the precipitates was determined with anthrone-sulphuric acid reagent and the proteins were measured according to Bradford (1976). The sugar composition of several precipitates was examined by gas chromatography (Zanetta *et al.*, 1972). From some precipitates the polysaccharides were separated from proteins by anion exchange chromatography on DEAE-Sepharose CL-6B (Pharmacia), firstly eluted with 10 mM tris buffer at pH 9 and then with 0 to 0.5 M NaCl in the same buffer. The best separation was obtained by pre-treatment of the sample by ultrasonication and by elution with a stepwise increase using NaCl.

Results and Discussion

The growth of the isolates differed substantially, but growth rates had no relationship

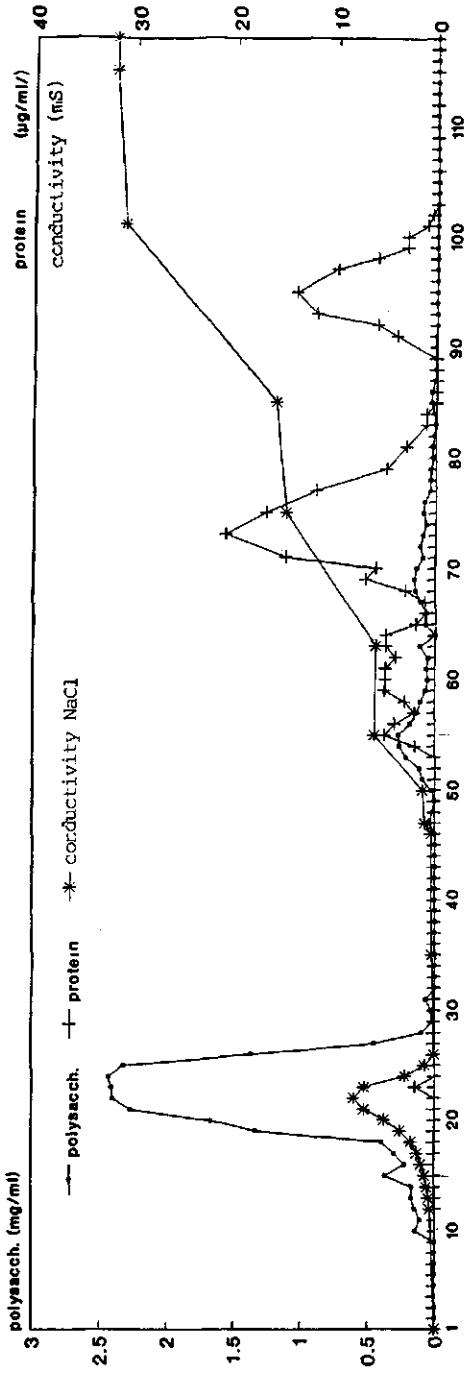

Fig. 1. DEAE-column for analysis of a rhamno-galacto-mannan from B. cinerea isolate R1V G1f resistant to dicarboximides.

with resistance of isolates to dicarboximides (Table 2). The glucan obtained in the $P_{0.5}$ fraction also showed great variation but these differences were unrelated to resistance of the isolates. The amount of rhamno-galacto-mannans obtained in the P_1 to P_4 fractions was higher for the Bc-R isolates than for the Bc-S isolates.

Further analysis showed that the sugar content of the P_2 ethanol precipitate of the sensitive isolate Bc-L69 and the resistant isolate Bc-R1V G1f contained mainly mannose, some galactose and only traces of rhamnose. Glucose was always present and it was not clear if this was a result of glucan contamination or if glucose was an essential part of the rhamno-galacto-mannan. Small amounts of proteins were also co-precipitated with the polysaccharides (Fig. 1). Table 3 shows the sugar composition of several fractions from the same separation. Most polysaccharides eluted from the unbound part of the DEAE column. Fractions 18 to 28 contained the rhamno-galacto-mannans, with some glucose. Proteins present in mg amounts were almost completely separated from the polysaccharides; only a trace of residual protein co-eluted in the fractions 55 to 80.

A higher amount of rhamno-galacto-mannans was found in shake cultures from isolates of *B. cinerea* resistant to dicarboximides than from susceptible isolates. The higher release of rhamno-galacto-mannans may have an effect on the behaviour and survival of Bc-R isolates in nature. In general Bc-R isolates are less competitive in nature than Bc-S isolates and they disappear in the field in the absence of the fungicides (Beever *et al.*, 1991). Maraite *et al.* (1980) examined the competitivity of Bc-S isolates and Bc-R isolates *in vitro*; two Bc-R isolates of six were competitive with the Bc-S isolates but competitivity *in vitro* was probably due to sporulation and growth rate and cannot explain completely the decrease of Bc-R isolates in the field in the absence of fungicides.

Dicarboximide-resistant isolates are highly sensitive to high osmotic pressure. This feature too can only partly explain the low fitnes of Bc-R isolates in nature for in many infected tissues the fungus does not grow at high osmotic pressure.

The high secretion of rhamno-galacto-mannans by Bc-R isolates in vitro may provide an additional explanation for these polysaccharides are known as elicitors of plant defence (Dixon and Fuller, 1977; Kamoen *et al.*, 1980) and might give elicitation of defence reactions. As a consequence they may be less able to survive as a parasite.

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Bacillus brevis as a biocontrol agent against *Botrytis cinerea* on protected chinese cabbage

S.G. Edwards and B. Seddon

Summary

Bacillus brevis produces the antibiotic, gramicidin S which is fungicidal to *B. cinerea in vitro*. Tests *in vitro* using *B. brevis* spores showed that gramicidin S associated with these spores was also active against *B. cinerea*. Tests using leaf disks and seedlings failed to confirm these observations *in planta* and it was found that gramicidin S binds to the leaf surface and is inactive in this form. Field trials using Chinese cabbage as a protected crop showed that biocontrol of *B. cinerea* with *B. brevis* was achievable with protection as good as the standard fungicide treatment. *B. cinerea* is dependent upon surface water for germination and infection and it is thought that *B. brevis* controlled *B. cinerea* infection by reducing the period of leaf wetness.

Introduction

Bacillus brevis Nagano is one of three strains of *B. brevis* which produces a single antibiotic, gramicidin S which since its discovery (Gause and Brazhnikove, 1944) has been known to be bactericidal; only recently has it been shown to have fungicidal activity also (Murray *et al.*, 1986). Gramicidin S is active towards *Botrytis cinerea* Pers.: Fr. (Davis *et al.*, 1987) which causes grey mould and is a major pathogen of numerous important crops (Jarvis, 1977). The disease is difficult to control because of its ability to attack crops at almost any growth stage and develop fungicide resistance (Maude, 1980). The research described in this paper aimed to test the potential of *B. brevis* to control *B. cinerea*. This work was facilitated by the availability of purified gramicidin S and the acquisition of an antibiotic-negative mutant, *B. brevis* E-1 (Iwaki *et al.*, 1972).

Results

Antagonism *in vitro*

Tests of antagonism of *B. brevis* to *B. cinerea* were carried out in liquid culture. Flasks (100 ml) containing 20 ml glucose peptone broth (Blakeman, 1975) were inoculated with conidia or mycelal plugs of *B. cinerea* and incubated for 5 days in an orbital shaker at 25°C, 100 rpm in the presence of gramicidin S (Sigma) or spores of *B. brevis* (containing 4.1 mM gram icidin S/10^5 spores) or the mutant E-1. After incubation the mycelium was dried, weighed and the increase in biomass determined. Gramicidin S was shown to be fungicidal to *B. cinerea* (Fig. 1). The antibiotic-negative mutant E-1 failed to inhibit *B. cinerea*, suggesting that the antagonistic activity of *B. cinerea* was due to the presence of gramicidin S. Germination of *B. cinerea* was completely inhibited by gramicidin S at 5 μM.

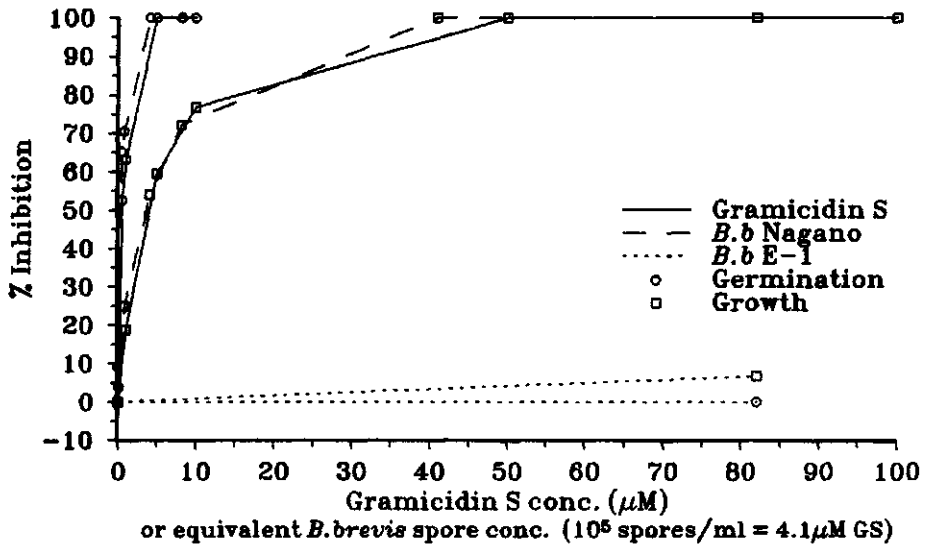

Fig. 1. Inhibition of germination and mycelial growth of B. cinerea by gramicidin S and B. brevis in liquid culture.

Antagonism *in planta*

Infection droplets containing conidia of *B. cinerea* labelled with Calcofluor M2 were placed on Chinese cabbage (*Brassica campestris* subsp. *pekinensis*) leaf sections (5 x 3 cm) from leaves previously sprayed until run-off with gramicidin S, or spores of *B. brevis* or the mutant E-1. The sections were incubated for 5 days at 15°C and 100% r.h. in the dark. After such time the infection droplets were dried at 60°C and observed by fluoroscence microscopy (Polyvar, Reichert-Jung; Excitation filter Band Pass 330-380 nm, Emission filter Long Pass 418 nm). The concentrations of gramicidin S and spores required to achieve inhibition of *B. cinerea* germination (500 μM gramicidin S and 2 x 10^7 *B. brevis* spores.ml^{-1}) were higher on the leaf sections than *in vitro*, with only 65 and 48% inhibition of *B. cinerea* germination achieved, respectively.

Chinese cabbage seedlings were also sprayed with gramicidin S, or spores of *B. brevis* or the mutant E-1, dried and sprayed the following day with *B. cinerea* (10^3 conidia.ml^{-1}). The seedlings were incubated for 20 days and assessed. The gramicidin S and *B. brevis* did not control damping-off on Chinese cabbage seedlings caused by *B. cinerea*. The highest concentrations, 500 μM gramicidin S and 2.1 x 10^7spores.ml^{-1}, were phytotoxic to the seedlings.

The *B. brevis* E-1 treatment was not phytotoxic, implying that phytotoxicity was due to gramicidin S.

Gramicidin S was strongly adsorbed to the leaf surface and in this form was inactive.

Antagonism *in situ*

A polythene tunnel planted with Chinese cabbage in 24 plots of 24 plants was used in trials during 1990 and 1991. There were six replicates of three treatments positioned in two 3x3 Latin squares plus a row of border plots at either end as guard plants. The

Table 1. Control of grey mould on Chinese cabbage in polythene tunnels by B. brevis and iprodione.

Treatment	Disease index	
	Trial no. 2	Trial no. 3
Untreated	40.3	28.4
Iprodione	13.6 (66)	6.7 (76)
B. brevis	14.2 (65	8.2 (71)
LSD	9.8	14.7

The percentage control of grey mould with respect to the untreated control plots is given in parentheses.

treatments were as follows: 1) *B. brevis*; 2) iprodione (1.5 kg/1000 l/ha Rovral WP, Embetec) and 3) untreated control. The plants were sprayed in treatments 2 and 3 until run-off at 2-3 week intervals using a hand-held applicator with a hollow cone nozzle with an output of 1.1 $l.min^{-1}$ at 3 bar pressure. During the first trial in 1990 there was a prolonged period of warm, dry weather which resulted in a very low incidence of grey mould infection and no differences in disease levels between treatments. However, no phytotoxicity with the *B. brevis* treatment occurred. In the second and third trials in 1991, grey mould developed and at harvest each plant was assessed using a disease scale 0 - 5, which denoted 0 = healty and 5 = severe infection of the cabbage heart. The total disease index for each plot was calculated and used in an analysis of variance. Differences between the treatments were highly significant ($p = 0.001$). Table 1 shows the disease index for treatments for the two field trials. The *B. brevis* treatment reduced the incidence of grey mould to a level equivalent to that of Iprodione.

Leaf wetness studies

During the trials it was noted that *B. brevis*-treated plots dried quicker after irrigation than untreated ones. This phenomenon was further investigated in greenhouse studies. The periods of leaf wetness on Chinese cabbage leaves sprayed with *B. brevis* and untreated leaves were recorded using automated sensors (Envirolog, Envirolog Measurement Ltd.). The electrical resistance (Ohm) across the leaf surface was measured and correlated to the water present on the leaf surface; the more water on the leaf surface, the lower the electrical resistance. Fig. 2 shows the duration of leaf wetness of *B. brevis*-treated and untreated plants, as indicated by the electrical resistance across the leaf surface. The *B. brevis* treatment reduced the period of leaf wetness by 7 9%.

Discussion

Initial data from *in vitro* work suggested that antibiosis was the mode of antagonism by *B. brevis* against *B. cinerea*. However, the antibiotic was less effective *in planta* because it was adsorbed to the leaf surface and was inactive in this state. Nevertheless, biocontrol of *B. cinerea* with *B. brevis* was achieved in two trials with protected Chinese cabbage indicating another mode of antagonism.

B. cinerea needs high humidity for germination and infection, and epidemics are related to periods of wet, humid weather (Yunis *et al.*, 1990). Practical methods of

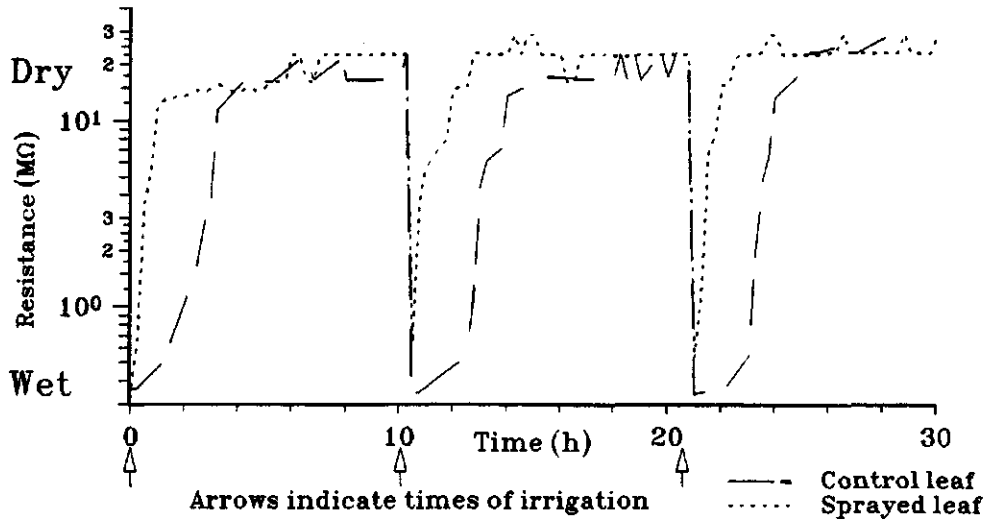

Fig. 2. Leaf wetness of control and Bacillus brevis sprayed leaves.

controlling *B. cinerea* include avoidance of high humidity and minimising periods of leaf wetness. *B. cinerea* has been controlled successfully in environmentally controlled greenhouses (Jarvis, 1989).

It is postulated that *B. brevis* controlled *B. cinerea* infection of the protected Chinese cabbage by reducing the period of leaf wetness. If confirmed, this treatment may be effective when combined with an integrated control system, but would not be effective if prolonged periods of high humidity prevail.

Acknowledgment

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Integration of biological and chemical control for grey mould

Y. Elad and G. Zimand

Summary

The biocontrol agent *Trichoderma harzianum* (T39) prepared as a wettable powder (Trichodex) was sprayed in field experiments for the control of grey mould (*Botrytis cinerea*) was compared with the conventional fungicides vinclozolin, iprodione, diethofencarb + carbendazim and tebuconazole + dichlofluanid in greenhouse-grown cucumbers, tomatoes and strawberry and in vineyards. Effective control was usually achieved by the biocontrol preparation when applied alone but in strawberry, there were often failures in control. Tank mixes of the biocontrol agent with dicarboximide fungicides were effective for controlling grey mould in all crops. Alternation of Trichodex with dicarboximide fungicides or with diethofencarb + carbendazim generally resulted in a level of control similar to that obtained with the standard fungicides. The alternating programme achieved a reduction in fungicides residues in the fruits and the surrounding ecosystem, whilst the population of *B. cinerea* was exposed less to the fungicides and therefore less likely to develop resistance to them.

Introduction

More development and commercial exploitation of biological control systems for insect pests has occurred than for plant diseases. One of the reasons for this is the availability of cheap and efficient chemical fungicides and their ease of application to foliage. Another problem is that the biotic and abiotic factors in the phylloplane vary and are generally unfavourable for an introduced microflora (Elad, 1990).

However, a number of foliar diseases, including several caused by *Botrytis cinerea* Pers.: Fr., can be reduced by pre-inoculation of the phylloplane with epiphytic filamentous fungi, yeasts or bacteria (Blakeman and Fokkema, 1982) and several bacteria and actinomycetes controlled grey mould of lettuce (Wood, 1950). Biological control of grey mould was suggested by Newhook in 1957. He sprayed tomato plants with *Cladosporium herbarium* and *Penicillium* spp. and reduced the incidence of grey mould of fruits. Later, bacteria and *Streptomyces* spp. were reported to be effective in suppressing *B. cinerea* on leaves of beetroot, onion and lettuce (Clark and Lorbeer, 1977; Pennock-Vos *et al.*, 1990; White *et al.*, 1990). *C. herbarium* effectively controlled blossom blight and fruit rot of underripe strawberries (Bhatt and Vaughan, 1962). Redmond *et al.* (1987) controlled rose grey mould with *Exophiala jeanselmei*.

Satisfactory control of *B. cinerea* using isolates of *Trichoderma* and *Gliocladium* spp. on grapes (Dubos and Bulit, 1981; Bisiach *et al.*, 1985; Gullino and Garibaldi, 1988), strawberry (Tronsmo and Dennis, 1974; Peng and Sutton, 1990) and apple (Tronsmo and Raa, 1977) has been achieved. Combinations of *Trichoderma harzianum* with

vinclozolin at reduced rates were tested for the control of the disease on cyclamen (McCain et al., 1985).

Material and Methods

We have tested a preparation of *T. harzianum* in greenhouses on tomato (*Lycopersicon esculentum*), cucumber (*Cucumis sativus*) and strawberry (*Fragaria ananasa*) and in vineyards (*Vitis viniferea*) for control of grey mould. The experiments were performed at several sites in Israel. The greenhouses were covered with 0.15 mm-thick polyethylene (Infrasol 266, ultraviolet absorbing - UVA - + infrared repellent - IR -; Ginnegar, Israel) and the soil (sandy loam) was fumigated with methyl bromide (Bromine Compounds, Be'er Sheva, Israel) prior to planting. Each plot contained 10 to 15 plants and guard rows were used between treated rows. The experiments were laid out in randomised block design with five replicates. Treatments were given according to commercial practice and the disease was evaluated on the fruits or stem nodes of the greenhouse crops and on grape berries.

An isolate of *T. harzianum* (T-39) previously isolated from a cucumber fruit was grown and formulated by Makhteshim Chemical Works Ltd. (Be'er Sheva, Israel). The preparation - Trichodex 25 WP - was applied at a rate of 0.2-0.4% (0.5 $g.l^{-1}$ a.i.) unless otherwise specified. The following fungicides were used in the experiments: vinclozolin (Ronilan 50 WP, BASF AG, Ludwigshafen, Germany) 0.5$g.l^{-1}$; iprodione (Rovral 50 WP, Rhone-Poulenc, Lyon, France) 0.5 $g.l^{-1}$; a factory-prepared mixture of diethofencarb (250 $g.kg^{-1}$) and carbendazim (250 $g.kg^{-1}$) (Resec WP, Agan Chemicals Co., Ashdod, Israel), 0.25 $g.l^{-1}$each; and a factory-prepared mixture of tebuconazole (100 $g.kg^{-1}$) and dichlofluanid (400 $g.kg^{-1}$) (Silvacur WP, Bayer Ltd., Ludwigshafen, Germany), 0.15 + 0.6 $g.l^{-1}$, respectively.

The fungicides and the biocontrol agent were sprayed to run off with a knapsack motor sprayer (Echo SHR 200E, Kiorizo Corp., Japan) at a volume of 1000 $l.ha^{-1}$ using a Conjet DX6 spray nozzle. Populations of *T. harzianum* were present at a level of 3×10^5 to 8×10^5 CFU per leaf, and 10% of that on fruits, but remained high after the second and third sprays.

Calculations were made for average disease reduction compared with non-treated controls. The percentage of cases giving positive results was calculated three times: in the first, second and third calculation a reduction of 40, 50 and 60% repesctively was regarded as positive.

Results and Discussion

Table 1 summarises the results of 70 experiments.

T. harzianum isolate T-39 proved to be a reliable biocontrol agent of cucumber grey mould. In most of the experiments the preparation significantly decreased the severity of the disease. In some experiments, when the biocontrol preparation was applied along with the dicarboximide fungicides iprodione or vinclozolin, a slightly better efficacy was observed, compared with either agent alone. This may be due to the presence of resistance to these fungicides in populations of *B. cinerea* in the greenhouse.

The ultimate aim of our work was to reduce the usage of fungicides during the growing season without loss of control; for this reason we alternated *T. harzianum* application with fungicides. A level of control similar to that obtained with the standard

Table 1. Efficacy of Trichoderma harzianum T39 (formulated as Trichodex) for control of grey mould in seventy experiments.

Method of control	Minimum % for seccessful disease reduction	Grape	Cucumber	Tomato	Strawberry
		35[1)]	13[1)]	11[1)]	11[1)]
Average grey mould severity (%)	–	37.0	35.3	26.7	41.8
Standard fungicide: % control	–	69	35	69	80
% Expts. with effective control	40[2)]	94	62	100	100
	50	84	54	90	91
	60	66	38	70	91
T. harzianum: % control	–	50	47	61	42
% Expts. with effective control	40[2)]	67	69	100	45
	50	54	69	82	36
	60	36	31	45	27
T. harzianum + fungicide tank mix, % control	–	68	45	X[3)]	73
% Expts. with effective control	40[2)]	88	50	X	100
	50	87	50	X	100
	60	62	50	X	100
Alternate sprays of *T. harzianum*/fungicide: (%) control	–	86	52	70	74
% Expts. with effective control	40[2)]	100	100	100	100
	50	78	100	100	100
	60	67	33	100	100

[1)] Number of experiments
[2)] See Material and Methods for calculation of wthresholds considered as positive control. Thresholds were chosen at the rang e of 40 to 60% disease reduction, according to common control achievements obtained in greenhouses under commercial conditions
[3)] X = not tested

fungicide treatments recommended for grey mould control was achieved, with no failures in control as occurred with *Trichoderma* alone. The alternating use of biocontrol agent and fungicides had two beneficial effects; first, there was a reduction in fungicide residues in fruits and the surrounding agro-system. Second, the population of the pathogen was exposed less to the fungicides and were then less likely to develop resistance. Resistance to common fungicides has become widespread in greenhouse populations of *B. cinerea* (Elad *et al.*, 1992) and consequently the range of chemicals

available for control of grey mould is limited. Similar results have been obtained in Italy by spraying dicarboximide + thiram alternately with *Trichoderma* to control grey mould in tomatoes and strawberries (Gullino *et al.*, 1991).

McKenzie *et al.* (1991) concluded that poor activity often shown by *Trichoderma* against *B. cinerea* under severe disease pressure can be explained in part by its poor survival on the phylloplane in the field. The preparation tested in the present study ensured satisfactory survival of an efficient population of the antagonist for 1 month under commercial greenhouse conditions and a moderate population also became established in the non-treated control plots. This was probably due to secondary distribution of the fungal propagules in the air of the greenhouses. Secondary distribution may be important for establishment of the biocontrol agent, but in these test conditions it was insufficiently effective to reduce grey mould in the controls.

An examination of the relationships between microclimatic conditions and disease control achieved by *Trichoderma* revealed a negative effect of surface wetness and a positive effect of temperature above 20°C. This may explain the failure of *T. harzianum* to control grey mould in Experiment 3. These results showed that it is possible to incorporate biocontrol of plant pathogens into integrated pest management programmes. More isolates of *Trichoderma* which effectively control the disease under a wider range of conditions are needed, as the pressure to develop biological control strategies is increasing (Elad, 1990). Meanwhile, control can be achieved by use of combinations of the biocontrol agent and fungicides.

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Control of *Botrytis* spp. in tulip with reduced input of chemical crop protection

A.Th.J. Koster and L.J. van der Meer

Summary

Foliage and flowers of many flowerbulb species can be infected by *Botrytis* spp. causing the so-called 'fire' disease. At present, these diseases are controlled mainly by weekly sprays of the crops with dithiocarbamate compounds, supplemented with a dicarboximide or benzimidazole compound in three sprays around flowering time.

To reduce the input of chemical crop protectants and to avoid the use of ethylene thiourea (ETU)-forming dithiocarbamate compounds (e.g. zineb, maneb, mancozeb), which are expected to be banned in the future, field experiments were carried out in among others tulips with a number of potentially effective alternative chemicals. We tested chlorothalonil, chlorothalonil plus prochloraz, fluazinam, and CGA 173506 sprayed in low dosages once every fortnight. These compounds were applied alone or in a combination with dicarboximide or benzimidazole fungicides.

Data obtained indicate that low-dosage applications of chlorothalonil, chlorothalonil plus prochloraz, or fluazinam in combination with dicarboximide or benzimidazole fungicides at fortnight intervals, resulted in a 70-82% reduction of inputs and in control of *Botrytis* spp. at least equal to that of the presently applied spraying scheme.

Introduction

Many flowerbulb crops, including tulips, lily and gladiolus, grown in the field can be infected by *Botrytis* causing a disease called 'fire'. The specialised *Botrytis* spp., *B. tulipae* in tulip (Beaumont *et al.*, 1936; Price, 1970), *B. elliptica* in lilies (Ward, 1888; Doss *et al.*, 1988) and *B. gladiolorum* in gladiolus (Timmermans, 1941) are mainly responsible for the disease. Depending on the conditions, *B. cinerea* may also infect bulbous plants (Schönbeck, 1967).

In tulip, infected leaves and stems initially show characteristic small, round, dark green lesions; these turn whitish with or without peripheral water-soaking or 'aggressive' spreading lesions develop which cause early senescence with subsequent loss of bulb yield. Petals are extremely susceptible to *Botrytis* infection and massive sporulation can generally be observed on necrotic tissues.

At present, 'fire' disease is controlled by cultivation techniques, such as removal of flowers and other plant debris, by crop rotation and by spraying with fungicides. Over several years, weekly sprays with dithiocarbamates supplemented with a dicarboximide or a benzimidazole compound in three sprays around flowering time have been effective (Anonymous, 1980). Dithiocarbamate compounds are expected to be banned in the future, particularly because of the persistent and mobile properties of the metabolite ethylene thiourea (ETU) in sandy soils used for flowerbulb cultivation (Lagas *et al.*,

1990), but for the more general need to reduce the input of agrochemicals in current agrosystems.

A research programme was started to find alternative chemicals, to reduce the input of chemical crop protectants, to improve spraying techniques and to develop non-chemical control methods and a prediction system for timing of fungicide application. This paper describes experiments designed to test alternative chemicals that can be applied effectively in low quantities.

Material and methods

Plant material and spray schedules

Bulbs of tulip cv. Renown (circumference 9-10 cm) were harvested, stored at 17°C, dipped before planting in a water suspension of captan and carbendazim (a.i. 0.5% and 0.2% v/v, respectively) and planted in the field in November.

Crops were sprayed with a water suspension 500 $l.ha^{-1}$ of mancozeb, chlorothalonil, chlorothalonil/prochloraz, fluazinam or CGA 173506 (Ciba-Geigy AG) with or without procymidone and/or carbendazim in amounts given in Table 1. Crops were sprayed with a portable field-plot sprayer (AZO, Ede, The Netherlands; 1.2 F nozzle) weekly or at fortnightly intervals from the time that leaves of neighbouring plants reached each other until plants began to senesce.

Experimental design

The experiments were performed in 1989, 1990 and 1991 on various locations in The Netherlands using plots of 1.5 m^2 with 150 planted bulbs each in three replicates, and an adjacent sprayed guard row, such that the spraying area for each plot was 2.5 m^2. The test plots were not inoculated. The percentage of diseased foliage was recorded c. 1 month before harvesting the bulbs, and the weight of harvested bulbs was determined. Bulb yields were expressed relative to yields from untreated control plots which were set at 100. Data were analysed using analysis of variance ($p = 0.05$).

Results

The effects of sprays with various combinations of chemical crop protectants on the infection of tulip plants by *B. tulipae* in 1990 and 1991 are presented in Table 2. The results obtained in 1989 were similar (data now shown). All treatments were effective against *B. tulipae*. Compared with current weekly sprays of dithiocarbamates around flowering time, applications at fortnightly intervals of chlorothalonil alone were significantly less effective. All other treatments were as, or more, effective. As expected, data on bulb yield and on percentage of diseased foliage showed a strong inverse relationship.

No harmful effects of the various combinations of chemicals was observed in tulips.

Discussion

Combinations of chemicals with different modes of action were more effective than chemicals applied alone. In practice, apart from the efficacy of the fungicides, the risk of

Table 1. The various treatments carried out to control B. tulipae in field experiments in 1989, 1990 and 1991.

Treatment	Code used in Table 2
Untreated	A
Mancozeb 1.88 kg a.i.ha^{-1}, with the addition of twice 0.25 kg a.i.ha^{-1} procymidone and once 0.15 kg a.i.ha^{-1} carbendazim, at weekly intervals	B
Chlorothalonil, 0.75 kg a.i.ha^{-1}, at fortnightly intervals	C
Chlorothalonil, 0.75 kg a.i.ha^{-1}, with the addition of 0.125 kg a.i.ha^{-1} procyimidone and 0.075 kg a.i.ha^{-1} carbendazim at fortnightly intervals	D
Chlorothalonil/prochloraz, 0.75 and 0.23 kg a.i.ha^{-1} respectively	E
Chlorothalonil/prochloraz, 0.75 and 0.23 kg a.i.ha^{-1}respectively, with the same additions as mentioned under D	F
Fluazinam, 0.5 kg a.i.ha^{-1}	G
Fluazinam, 0.5 kg a.i.ha^{-1}, with the same additions as mentioned under D	H
CGA 173506, 0.5 kg a.i.ha^{-1}	J

Botrytis species becoming resistant and the implications for the environment and safety of workers, are also important.

Unlike other *Botrytis* species (Pommer and Lorenz, 1983, Presly and Maude, 1986; Lörcher *et al.*, 1987), strains of *B. tulipae* resistant to dicarboximide and/or benomyl compounds have not been found in the field. To avoid the development of specific fungicide resistance, applications of compounds with various modes of action in alternation or in combination are generally preferred to consecutive applications of a single compound.

Application of fluazinam or CGA 173506 alone also would be unwise, since recent results obtained with CGA 173506 show that *Botrytis* spp. can acquire resistance to this phenylpyrrole compound under laboratory conditions (Hilber, personal communications); relevant data on fluazinam are lacking at presence. Consequently, chlorothalonil or fluazinam with additions of chlorothalonil/prochloraz with or without additions may

Table 2. Effects of combinations of fungicides on the control of B. tulipae in tulip cv. Renown.

Treatment	1990		1991	
	% diseased foliage	bulb yield	% diseased foliage	bulb yield
A	80	100	70	100
B	33	118	23	142
C	40	108	33	134
D	27	114	13	150
E	–	–	23	137
F	–	–	13	151
G	37	111	20	153
H	23	120	13	164
J	23	123	10	150
LSD	15.60	10.96	7.60	7.41

be used effectively and without serious risks of resistance in *B. tulipae*. A combination of CGA 173506 and additions has not yet been included in our experiments.

In comparison with the presently applied standard treatment with dithiocarbamate, considerable reduction in the total volume of sprayed chemicals each year were realised using the alternative combinations of fungicides. This reduction amounted to 70 and 82% for chlorothalonil/prochloraz plus additions and for fluazinam plus additions, respectively. Moreover, further release of ETU from tulip crop sprays was prevented.

The reduction of the amount of fungicides used and particularly the reduction of the frequency of application help to reduce exposure of workers to fungicides in the field.

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Control of grey mould of tomatoes in greenhouses with fungicides and antagonists

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Summary

Tomatoes grown in greenhouses in Greece suffer severe damage caused by *B. cinerea.* Control of this fungus is based entirely on fungicide application and during recent years strains of the pathogen resistant to fungicides have predominated. To find alternative control methods, the fungi *Trichoderma harzianum, Penicillium* sp. and *Acremonium alternatum* have been assessed as potential biocontrol agents in tomatoes. In one experiment where strains of the pathogen resistant to fungicides predominated, the antagonists were applied as suspensions containing 10^6 spores.ml^{-1} at 4 and 7 day intervals, and compared with three fungicides currently used against *B. cinerea.* Neither the antagonists nor the fungicides were effective. In the second, the antagonists were applied at concentrations of 10^6 and 5 x 10^6 spores.ml^{-1} at 7 day intervals, or at 5 x 10^6 spores.ml^{-1} alternated with iprodione every third week. In this experiment, the antagonists alternated with iprodione or iprodione alone, were equally effective. The *Penicillium* isolate established more readibly on the petals of tomato flowers than *T. harzianum,* whereas *A. alternatum* was not recovered from flowers. *B. cinerea* was isolated more frequently from tomato flowers treated with *Penicillium* sp. and *A. alternatum* at concentrations concerning 10^6 spores.ml^{-1}, than in controls. *B. cinerea* also was recovered in lower frequency from flowers treated with antagonists plus iprodione, or iprodione alone.

Introduction

Due to fungicide resistance in *Botrytis cinerea* Pers.: Fr. control of grey mould in several greenhouse-grown crops has been difficult. To overcome this several workers have studied the effectiveness of microbial antagonists, combinations of conventional fungicides, and the integration of antagonists with fungicides (Gullino and Garibaldi, 1988; Creemers, 1992). Preliminary experiments which evaluated *Penicillium* sp., *Trichoderma harzianum* and *Acremonium alternatum* as potential biocontrol agents for *B. cinerea in vitro* or on young bean plants have given promising results (Malathrakis and Kritsotaki, 1992). This paper examines the effectiveness of three antagonists, several fungicides and antagonists in mixed programmes with iprodione.

Material and Methods

The methods and materials are described elsewhere (Malathrakis and Kritsotaki, 1992). Two experiments were performed in a plastic greenhouse from early September until late April. The treatments were arranged in a randomized block design with four replicates. The treatments were applied commencing in mid November when the temperature and

Table 1. Effectiveness of Acremonium alternatum and Trichoderma sp. against tomato grey mould.

Treatments	No. infection sites/plot	
	15 Feb.	13 March
T. harzianum 10^6/4d	47 a	75 a
T. harzianum 10^6/7d	42 a	60 ab
A. alternatum 10^6/4d	46 a	62 ab
A. alternatum 10^6/7d	34 a	48 b
Iprodione 0.05% + dichlofluanid 0.12%	48 a	62 ab
Iprodione 0.05% + thiram 0.20%	52 a	60 ab
Iprodione 0.05%	51a	64 ab
Dichlofluanid 0.12 %	47 a	70 ab
Sumico 0.05%	40 a	62 ab
Control	41 a	51 b

Figures followed by the same letter do not differ significantly according to Duncan's Multiple Range Test (p = 0.05).

Table 2. Effectiveness of antagonists integrated with iprodione against grey mould

	% Infected fruits per plant		No. Infected sites per plot
	26 March	18 April	18 April
Acremonium sp. 10^6	8.5	8.0 a	44 a
Acremonium sp. 5 x 10^6	9.4	5.2 ab	32 bc
Acremonium sp. 5 x 10^6 + iprodione 0.05%	1.9	1.6 c	11 d
Penicillium sp. 10^6	10.2	5.9 ab	35 abc
Penicillium sp. 5 x 10^6	9.5	5.9 ab	33 bc
Penicillium sp. 5 x 10^6 + iprodione 0.05%	2.3	1.6 c	11 d
Trichodex 0.2%	8.6	3.1 bc	27 c
Trichodex 0.2% + iprodione 0.05%	2.9	1.7 c	11 d
Iprodione 0.05% 0.8	1.0 c	6 d	
Control 10.0	6.2 a	41 ab	

Figures followed by the same letter do not differ significantly according to Duncan's Multiple Range Test (p = 0.05).

humidity became conducive to grey mould. In the first experiment *A. alternatum* and *T. harzianum* were applied as a suspension containing 10^6 spores.ml^{-1} at 4 and 7 day intervals and compared to several fungicides (Table 1). In the second experiment *A. alternatum*, *Penicillium* sp. and Trichodex (a commercial formulation of *T. harzianum*; Makthesim Co, Israel), alone or integrated with iprodione, were evaluated. The first two fungal antagonists were applied as suspensions containing 10^6 and 5 x 10^6 spores. ml^{-1}. Trichodex was applied at the rate of 2 g.l^{-1} of water. The antagonists and iprodione were applied at 7 and 15 day intervals respectively. Treatments in which *A. alternatum*, *Penicillium* sp. and *T. harzianum* were applied at the same rates and

alternated every third week with iprodione, were also compared. Iprodione applied alone at 15 day intervals was used as a standard treatment.

The number of infection sites on plants in each plot and percentage infected fruits were recorded. In the first experiment, the severity of the ghost spot symptoms on the fruits was also recorded on a 0 to 5 scale. In the second experiment, the fungal flora of tomato flowers was also monitored. Senescing flowers were collected from all the plots and ten small pieces of each were plated separately on patoto dextrose agar (PDA), in the presence of antibiotics and incubated at 22°C for 3 days. The fungal colonies were counted and the percentage colonies of each antagonist recovered was calculated. The sensitivity of the population of *B. cinerea* to iprodione and dichlofluanid was also monitored.

Results

In the first experiment neither the fungicides nor the antagonists were effective (Table 1). The ED_{50} values for iprodione for spore germination in *B. cinerea* ranged from 5 to 11 $\mu g.ml^{-1}$ throughout the work. No significant differences occurred between treatments for percentage infected fruits or ghost spot, but some differences were initially found for the number of infection sites/plot treated with iprodione and plots treated otherwise.

In the second experiment iprodione alone at 15 day intervals, or integrated with any of the antagonists every third week controlled grey mould (Table 2) effectively. When antagonists were used alone, *T. harzianum* was slightly effective, but *Penicillium* sp. and *A. alternatum*, irrespective of the inoculum concentration, were ineffective (Table 2). Isolates showed that *T. harzianum* and *Penicillium* sp. were established on senescent tomato flowers but *A. alternatum* was not (Table 3). *B. cinerea* was isolated regularly from senescent tomato flowers, but less from plots treated with iprodione than from other plots. The ED_{50} value for iprodione for *B. cinerea* throughout the cropping season was < 2 $\mu g.ml^{-1}$ and for dichlofluanid < 0.1 $\mu g.ml^{-1}$.

Discussion

Neither the fungicides nor the antagonists applied alone were consistently effective against grey mould in this study. The inadequate performance of the fungicides can be explained on the basis of the data obtained by resistance monitoring. In the first experiment, the ED_{50} values of iprodione to *B. cinerea* ranged from 5 to 11 $mg.ml^{-1}$ with no significant difference between the treatments and showed clearly that *B. cinerea* was resistant to iprodione, as described by Katan (1982), and no effective control could be expected. Similarly, the failure of dichlofluanid in these experiments may be that in the pathogen population strains with reduced sensitivity (ED_{50} values ranging from 0.7 to 2.7 $\mu g.ml^{-1}$) prevailed throughout the growing season as described by Malathrakis (1989). This work provides further evidence that dichlofluanid under greenhouse conditions may fail to control grey mould when strains with reduced sensitivity are present from the beginning of the season. Pappas (1992) reported effective control of grey mould under the same conditions, but in this case the resistant isolates represent c. 50% of the population at about the middle of the growing season.

In the second experiment, a population of *B. cinerea* sensitive to iprodione prevailed throughout the growing season (ED_{50} < 0.1 $\mu g.ml^{-1}$) and it controlled grey mould.

Iprodione integrated with *Penicillium* sp., *T. harzianum* and *A. alternatum* applied

Table 3. Fungal flora of tomato flowers treated by fungicides and antagonists

	I	II	III	IV	V	VI[1)]
A. alternatum 10^6	29	0	19	3	11	1
A. alternatum 5 x 10^6	14	0	63	1	4	3
A. alternatum 5 x 10^6 + iprodione 0.05%	1	0	59	5	37	3
Penicillium sp. 10^6	27	0	97	0	0	1
Penicillium sp. 5 x 10^6	1	0	97	0	0	6
Penicillium sp. 5 x 10^6 + iprodione 0.05%	3	0	98	3	14	1
Trichodex 0.2%	12	0	10	43	0	7
Trichodex 0.2% + iprodione 0.05%	16	0	55	35	3	1
Iprodione 0.05% 3	0	12	7	1	1	
Control (water) 12	0	16	0	41	4	

[1)] I = *Botrytis*
II = *Acremonium* sp.
III = *Penicillium* sp.
IV = Trichodex
V = *Cladosporum*
VI = Others

every third week was equally effective as iprodione alone at 15 day intervals. The contribution to this result due to the antagonists, is difficult to assess because there was no treatment with iprodione alone applied at 3 week intervals. In our experience iprodione applied at 3 week intervals is not usually so effective.

Except *T. harzianum* which was slightly effective, the antagonists alone were ineffective, and this contrasts markedly with results from the laboratory. At least in the second experiment this could be partly explained by the data obtained from the monitoring of the fungal flora of the flowers. *A. alternatum* was not established on flowers and this tissue initiates the infection of the fruits; hence the failure of this fungus to control grey mould. In the laboratory *A. alternatum* established satisfactorely on young bean plants and competed against *B. cinerea* at temperatures as low as 10°C. Its failure to establish on tomato flowers may be due to its low competitiveness to the microbial flora on this tissue.

T. harzianum and *Penicillium* sp. well established at both spore concentrations, but only the former species was effective. *T. harzianum* applied as Trichodex has been specifically selected and commercially formulated for control of *B. cinerea*. It has been tested and found to be effective against grey mould by others. *Penicillium* species are part of the natural flora of tomato flowers and have been monitored on the plots of all the treatments and regularly from several greenhouses (Malathrakis, unpublished). It was probably present in sufficient densities as part of the natural flora in all the treatments, including the controls. On this basis there was no check without *Penicillium* sp. for comparison. In these cases, the role of the natural flora against grey mould can be evaluated only if fungides effective against them, but not against *B. cinerea*, are used in the experiment.

Whatever the protection of the natural population of *Penicillium* sp. is, however, it is probable, as Fokkema (1991) claimed, that the application of large amounts of highly effective and competitive strains would deprive the remains of flowers of nutrients, which are considered to be the main food base of *B. cinerea* and might reduce fruit infection. Hence more research is needed to select such strains from the natural population.

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