14TH INTERNATIONAL *BOTRYTIS* SYMPOSIUM

ABSTRACT BOOK

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WELCOME TO THE 14TH INTERNATIONAL *BOTRYTIS* SYMPOSIUM

MESSAGE FROM THE CHAIR: BOTRYTIS2007 ORGANISING COMMITTEE

"No, *Botrytis* is not really a problem for us" were the ghastly words of rejection on what I thought was a really good research proposal. "But we only plan to use *Botrytis* as the model pathogen to study something that is most definitely a problem!" I retorted. But my anguished plea must have fallen on deaf ears, as the killer blow was delivered: "Young man, it seems to me as if you lost your keys in the dark, and are now proposing to look for it beneath the lamp where you think it might be easier to find".

And here we are gathered together from across the globe to attend the **14th** International *Botrytis* Symposium, some of us working on *Botrytis* because it is a real problem, and others 'looking for their lost keys beneath the lamp' ☺ !

On behalf of the Organising Committee, I welcome you to sunny South Africa, and more specifically to our Mother City, Cape Town. May your stay here be blessed with the wonders of this country, may your hearts be touched by the warmth of our people, and by the end of what promises to be a very exciting scientific and social programme, may we depart as friends, all hopefully a bit closer to finding our lost keys.

PAUL FOURIE

Chair: Botrytis2007
Organising Committee

MESSAGE FROM THE CHAIR: BOTRYTIS2007 SCIENTIFIC COMMITTEE

For this symposium the invited keynote speakers also served on the scientific committee and were responsible to not only help with abstract selection and the final scientific programme, but will also act as session chairs during the symposium. Special thanks to our Scientific Committee for their expert help and tireless efforts in the designing and promoting of the scientific programme.

Prof Gustav Holz (previously from the Department of Plant Pathology, Stellenbosch University, now retired) was an *ad hoc* member of the scientific committee that assisted the organisers immensely with advice, as well as evaluation of the abstracts.

The organisers strived to assemble a scientific programme that could showcase the latest body of knowledge on *Botrytis*, aiming to balance not only classical approaches and newer technologies, but also fundamental and industry-relevant research. The outcome is an exciting mix of topics and presenters that truly represent the most-prominent and emerging groups in *Botrytis* research around the globe. We look forward to each contribution and hope that not only the organisation of this symposium will be of a high standard, but that delegates will remember beautiful Cape Town and surroundings, the fine wines of South Africa, the unique ethnic heritage of South Africa and most importantly, a stimulating and thought-provoking scientific meeting.

MELANÉ VIVIER

Chair: Botrytis2007
Scientific Committee

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- Prof Paul Tudzynski (Institut für Botanik, Westfälische Wilhelms-Universität, Münster, GERMANY)
- Dr Jan van Kan (Laboratory of Phytopathology, Wageningen University, NETHERLANDS)
- *Ad hoc* member: Prof Gustav Holz (SOUTH AFRICA)

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Bayer CropScience is one of the world's leading innovative crop science companies in the areas of crop protection, non-agricultural pest control, seeds and plant biotechnology. Bayer CropScience offer an outstanding range of products and extensive service backup for modern, sustainable agriculture and for non-agricultural applications.

Abstracts

1. OPENING SESSION: *BOTRYTIS*, INDUSTRY AND THE FOOD CHAIN

Botrytis cinerea causes serious losses in many crop species worldwide. The plant protection industry has devised a multitude of active ingredients to conquer the fungus. Different modes of action were discovered but the fungus developed resistance to many botryticides. Thus a constant need for the search and development of new modes of action and new active ingredients is required. This is a constant challenge which is further extended by a changing environment. Purchasing decisions for plant protection products are increasingly influenced by a request of high value, traceability of the product and sustainability of the whole process.

Botrytis cinerea is one of the major causes of deterioration in the taste, aroma and hedonistic qualities of fruits and vegetables. However, under some specific climatic conditions, and on mature bunch of grapes, it can be implicated in a particular rot which can be the starting point for the elaboration of dessert wines. These modifications are related to a very large number of enzymatic activities produced by the fungus that transform the proteic, polyphenolic and aromatic composition of fruits. Sometimes these modifications permit growth of saprophytic fungi, belonging to various species, particularly from the *Penicillium* genus, which produce secondary metabolites with possible hygienic and organoleptic consequences.

ORAL SESSION

Keynote + Chairperson:

O1.1. Peter H. Schreier

Botrytis, industry and the food chain

O1.2. Dominique Steiger

Global economic importance of *Botrytis* protection

01.1 BOTRYTIS, INDUSTRY AND THE FOOD CHAIN

PETER H. SCHREIER

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Industry has been engaged to counteract *Botrytis* infections and their adverse effects for more than hundred years. Chemical control remains the main way to reduce grey mould on major crops, thus securing harvest value and quality.

Different modes of action of synthetic botryticides will be discussed, and combined efforts of the major industries to avoid or minimise the adverse effects of resistance will be described. Activities within Bayer Cropscience to discover new modes of action and new antifungal compounds will be presented. Furthermore, the integration of products into the food chain by a "Food Chain Partnership" gaining value and safety of food will be introduced.

01.2 GLOBAL ECONOMIC IMPORTANCE OF *BOTRYTIS* PROTECTION

DOMINIQUE STEIGER

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This analysis of the economic aspects of *Botrytis* protection is based on Bayer data, taken from the professional crop protection market at the farmer level. Price and value are just indicative and "treated hectares" means developed acreage and represents the total number of *Botrytis* treatments during the season. For practical reasons, only the "pure" *Botrytis* segment was considered, excluding side effect of products which are not registered against *Botrytis*.

In term of value, the most important investment in *Botrytis* protection is to be found in the high value crops. The grapes segment (table and wine) represents 50% value of the total *Botrytis* market, solanaceous vegetables (tomatoes, eggplants and pepper) 9%, cucurbits 7%, strawberries 6%, flowers and ornementals 5%, bulb vegetables 5%, beans and peas 5% and leafy vegetables 5%.

The top ten countries investing in *Botrytis* protection are France and Italy for wine grapes, followed by Japan and China for vegetables, Chile for table/wine grapes, Spain for vegetables and grapes, USA for grapes and fruits, Germany for grapes, The Netherlands for vegetables and Australia for grapes.

With a total value of about 225 million Euro, *Botrytis* is only a small part of the complete protection market. As an example, *Botrytis* protection in grapes represents in average only 9% of the total grapes protection investment compared to the 33% for downy mildew, 22% for powdery mildew, 16% for insecticides and 14% for herbicides.

Botrytis is however one of the major investments in term of cost per treatment at the farmer level. The average cost for *Botrytis* protection (all crops, all countries) is about 40 Euro/ha but in fact the farmer will pay less or more depending on the value of the crop, the country and the type of product he needs. For the control of the same disease the range of cost varies considerably, between 15 Euro/ha for pumpkin-wild in China, 50 Euro/ha for tomatoes protection in Spain, 80 Euro/ha for strawberry protection in USA 100 Euro/ha for grapes protection in France up to more than 130 Euro/ha for citrus protection in Japan.

The higher cost of the *Botrytis* protection, compared to other diseases is not only due to the higher value of the crops. The specificity of the *Botrytis* active ingredients should also be consider with only seven different categories of mode of action available for the farmer and a relatively narrow spectrum of activity and consequently limited possibility of crop uses. There is a limited number of multisites active ingredients used for *Botrytis* control, more than 90% of the treated hectares are protected with single-site active ingredients. This situation combined with higher development cost to answer the legitimate but increasing food chain requirements (number of crop for registration, MRLs, Import Tolerance, specific tests, etc.) leads to significantly more cost for *Botrytis* protection, but in the high value crops like table grapes, wine and small fruits, the investment for *Botrytis* protection is relatively low compared with the crop value. Returning to the example of grapes, the final cost for a good *Botrytis* protection is very marginal and varies between 0,01 and 0,05 Euro per bottle of wine depending on yield.

2. *BOTRYTIS* IDENTIFICATION AND DETECTION

Botrytis species, particularly *B. cinerea*, cause serious losses in a wide variety of crop plants and also considerable post-harvest spoilage of flowers, fruits and vegetables. To reduce these losses, methods of detecting infections (latent infections) before they become apparent are needed, as are methods to quantify the load of air-borne spores in glass houses and polythene tunnels and spores deposited on the surfaces of flowers and fruits. Modern technologies have been developed and are now being tested that can be used to quickly detect pre-symptomatic infections. These technologies, particularly monoclonal antibody-based immunoassays and real time PCR are yielding useful information. When results from these tests are combined with environmental parameters that affect the rate of disease development, they will enable growers to reduce losses by targeting fungicide applications and making informed decisions about storage and marketing. New methods of trapping and rapid quantification of air-borne spores will be addressed. Presentations will include the advantages and limitations of the new technologies in relation to older plating techniques and to their specificities. Discussions will centre on the need for comparative standards and the establishment of meaningful thresholds of fungal biomass in infected plants.

ORAL SESSION

Keynote + Chairperson:

O2.1. Frances M. Dewey (Molly)

Detection and quantification of *Botrytis* species - an overview of modern technologies

Plenary lecture:

O2.2. Daniel Kliebenstein, Heather Rowe, Erica Bakker and Katherine J. Denby

Understanding and using *Botrytis cinerea* natural variation

O2.3. J. Zhang, L. Zhang, M. D. Wu, Guoqing Li and D. H. Jiang

Molecular identification of *Botrytis* species isolated from Central China

O2.4. Shanna Bastiaan-Net, Peter Balk, Christiaan Roelofsen, Monique van Wordragen and Jurriaan Mes

Developing marker genes for the prediction of *Botrytis cinerea* infection on flower petals of *Rosa hybrida*

02.1 DETECTION AND QUANTIFICATION OF *BOTRYTIS* SPECIES- AN OVERVIEW OF MODERN TECHNOLOGIES

FRANCES M. DEWEY (MOLLY)

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Modern technologies, particularly monoclonal antibody-based immunoassays, real time PCR, and studies of microsatellite DNA are yielding important information about *Botrytis* infections that complement older dilution plating techniques. Most of these methods have been used to detect and quantify *B. cinerea*, but adapting such methods for the quantification of other *Botrytis* species should be relatively easy. Immunoassays, notably ELISA and *Botrytis*-lateral flow devices, employing a genus-specific antibody, have been used to successfully detect pre-symptomatic infections in strawberry petals and tomato fruits, and to quantify levels of infections in wine grape berries and other fruits and tissues. Tests with the *Botrytis* lateral flow devices have the advantages of being rapid (10 min), user friendly and not requiring laboratory facilities. Pre-symptomatic or latent infections have also been detected in flower petals by PCR, using rapid, simplified, methods of extracting nuclear material and species-specific probes. Identification and tracking of specific isolates is now possible using information from banding patterns of microsatellite DNA extracted from *Botrytis*-infected material. This method has been used to identify, in some plants, seed-borne, non-symptomatic, systemic infections, and to track the switch from systemic to necrotic infections. The same method has also been used to study the dynamics and host preferences of different populations of *B. cinerea* isolates. Quantification of conidia of *B. cinerea* on the surfaces of fruits has also been made possible by PCR and this method has proved to be a good predictor of decay of pears in cold storage. ELISA tests on spores, trapped and germinated in micro-titre wells, has made quantification of live, air-borne, conidia during any set time period, possible. The use of the new technologies is gradually spreading and some, such as the lateral flow devices, are now available commercially as complete kits.

02.2 UNDERSTANDING AND USING *BOTRYTIS CINEREA* NATURAL VARIATION

DANIEL KLIEBENSTEIN[1], HEATHER ROWE[1], ERICA BAKKER[2] AND KATHERINE J. DENBY[3]

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Botrytis cinerea has long been recognised as a highly diverse pathogen with natural variation modulating an extreme range of phenotypes. This natural variation has long be considered a complication in both basic science, attempting to understand how the pathogen attacks its variety of hosts, as well as in applied research, attempting to find the keys to controlling this pathogen in commercial settings. In contrast, we hypothesise that this natural variation is controlled by genetic polymorphism and that high levels of variation are central for *Botrytis cinerea* success as a broad host range pathogen. We are combining modern genomic analysis with natural variation within *Botrytis cinerea* to identify and validate the importance of unknown virulence loci within *Botrytis cinerea*. Further, we are querying how the natural variation within the pathogen interacts with standing genetic variation within the host plants such as *Arabidopsis thaliana* and tomato.

As a beginning to this analysis, we queried genetic variation in previously defined virulence genes in a collection of *Botrytis cinerea* isolates. An analysis of diversity within *Botrytis cinerea* suggests that virulence loci are under diversifying selection, potentially allowing for direct association mapping of virulence loci within the pathogen. We are extending this to complete genome resequencing of as broad a set of pathogen genotypes as possible. Preliminary analysis has identified an unknown enzyme with a deletion polymorphism that may associate with virulence. Results from this and an analysis of all secreted metabolites in this collection of pathogen isolates will be presented. As a real world test of the impact of genetic variation on farming procedures, we are comparing the genetic variation and pathogen virulence in *B. cinerea* collected from organic and non-organic vineyards.

Part of our interest is to understand how natural variation in gene expression controls differences between individuals within a species. We have been investigating the relationship between natural variation in gene expression and variation in pathogen resistance within *Arabidopsis thaliana*. We are investigating quantitative trait loci that control differential resistance to *B. cinerea* isolates. This shows that we can link global variation in plant defense gene and defense metabolite expression with altered *Botrytis* virulence. We have extended this analysis into infecting defined *Arabidopsis* signal transduction mutants with diverse *Botrytis cinerea* isolates. We will present evidence that jasmonic acid signalling is only absolutely required for response to and resistance to a subset of *Botrytis cinerea* genotypes. Collectively, these data suggest that not all *Arabidopsis thaliana* accessions utilise the same signalling networks for responding to *Botrytis cinerea* attack.

In conclusion, the combination of our observations on genetic diversity within the host and pathogen indicate that species wide conclusions about signal transduction interactions can only be made when a diverse collection of host and pathogen geneotypes are utilised.

02.3 MOLECULAR IDENTIFICATION OF *BOTRYTIS* SPECIES ISOLATED FROM CENTRAL CHINA

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The genus *Botrytis* contains 22 species and one hybrid. Traditionally, *Botrytis* species were identified on the basis of observation of cultural and morphological characteristics. Staats *et al*. (2005) showed that 22 *Botrytis* species are phylogenetically independent by analysing the DNA sequences encoding glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*), heat-shock Protein 60 (*HSP60*) and DNA-dependent RNA polymerase subunit II (*RPB2*). These DNA sequences were deposited in the GenBank public database, which can be used for identification of *Botrytis* species.

In this study, 152 isolates of *Botrytis* were collected from 26 host plants grown in central China in 2005 and 2006. Based on morphological observations, six species were identified. They are *B. cinerea*, *B. aclada*, *B. squamosa*, *B. porri*, *B. elliptica* and *B. fabae*, which accounted for 127, 10, 9, 2, 1 and 1 isolates, respectively. Two isolates were greatly different from these six species of *Botrytis* in cultural characteristics, conidial size and sclerotial production.

In order to identify the unknown species of *Botrytis*, partial DNA sequences for *G3PDH*, *HSP60* and *RPB2* of isolate OnionBc-23, and of six isolates representing the six known *Botrytis* species, were analysed by comparison of these DNA sequences with those for 22 *Botrytis* species collected from GenBank. Results showed that isolate OnionBc-23 formed an unique clade in the phylogenic trees built with the sequence data of each gene investigated, whereas isolates GarlicBC-5, GarlicBC-8, GarlicBC-2, GarlicBC-16 and LilyBC-2 representing *B. cinerea*, *B. aclada*, *B. squamosa*, *B. porri* and *B. elliptica*, respectively, were closely related to corresponding species of *Botrytis*. Interestingly, isolate BroadbeanBC-2, representing *B. fabae*, was closely related to *B. galanthina*. This study suggests that the molecular approach based on analysis of the DNA sequences of *G3PDH*, *HSP60* and *RPB2* can be used to identify *Botrytis* species. Unknown species of *Botrytis*, including isolate OnionBc-23, might be a novel species in this genus.

Occurrence of *B. galanthina* on broadbean (*Vicia faba* L.) has never been reported, therefore, further studies to clarify the identity of the BroadbeanBC-2 isolate are warranted.

REFERENCES:

Staats, M., van Baarlen, P. & van Kan, J.A.L. (2005). Molecular phylogeny of the plant pathogenic genus *Botrytis* and the evolution of host specificity. *Mol. Biol. Evol.* 22: 333-346.

02.4 DEVELOPING MARKER GENES FOR THE PREDICTION OF *BOTRYTIS CINEREA* INFECTION ON FLOWER PETALS OF *ROSA HYBRIDA*

SHANNA BASTIAAN-NET[1], PETER BALK[2], CHRISTIAAN ROELOFSEN[2], MONIQUE VAN WORDRAGEN[2] AND JURRIAAN MES[1]

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Botrytis cinerea infection in roses drastically shortens the vase-life of cut-flowers and forms a serious economic problem. Spores of *B. cinerea* are able to germinate and infect plant tissue at a humidity of 93% or higher, a condition that is frequently used during storage and transportation of cut-flowers. When flowers are sold, for example in auctions, their quality with regard to *B. cinerea* infection cannot be predicted or guaranteed. Many batches of cut-flowers therefore become spoiled, affecting both the buyer (reduction in profits) and the seller (a breeder's reputation). In order to reduce the losses because of post-harvest spoilage, we attempt to find a set of quality indicator genes that would predict the degree of *B. cinerea* infection over time.

Flowers of various batches of three different cultivars of *Rosa hybrida* were assessed for their severity of *B. cinerea* infection on the day of harvest (day 0) and after 4 and 7 days of vase-life. The degree of flower opening on day 0, day 4 and day 7 were determined as well as stem bending (bent neck), *Botrytis* development on petals and *Podosphaera pannosa* (powdery mildew) infection on leaves. In addition, the three outer petals from 10 roses per batch harvested at day 0 were sampled and frozen in liquid nitrogen for RNA isolation and subsequent cDNA amplification. From each cultivar, 12 batches, varying between 0 and 30% *Botrytis* infection on day 7, were selected for RNA isolation and gene expression profiling by real time PCR. The relative expression values, measured at day 0, are coupled to the physiological data measured at day 7. Although our data are still preliminary, we hope to develop a set of marker genes that can predict the quality after 7 days of vase-life of fresh cut roses already before the flowers appear on the world market.

2. *BOTRYTIS* IDENTIFICATION AND DETECTION

Posters

P2.1. **Rudi Aerts**, Bjorn Seels and Kathleen Heyens

Multi-purpose use of a selective *Botrytis* medium

P2.2. **Tim O'Neill**, Kim Green, Kathryn Walsh and Neil Boonham

Quantification of latent *Botrytis cinerea* in cyclamen, gerbera and poinsettia

P2.1 MULTI-PURPOSE USE OF A SELECTIVE *BOTRYTIS* MEDIUM

RUDI AERTS, BJORN SEELS AND KATHLEEN HEYENS

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Sustainable control of *Botrytis cinerea* in the production of tomato by sanitation and cultural control measures, needs a good method to determine the spore concentration in the air. A few years ago a new selective medium (SMB) for enumeration of *B. cinerea* conidia was developed. This medium gives less inhibition of spore germination and is more selective than the Kerssies medium. Germinating *Botrytis* spp. spores change the colour of SMB medium from white to atypical brown. On Kerssies medium, other fungi also change the colour of the medium but in different shades of brown. Also important, a very fast sporulation can be observed on the SMB medium. The combination of sporulation and colouring of SMB medium, makes it possible to recognise *Botrytis* spp. very fast. With SMB medium, a correlation between the concentration of spores in a tomato greenhouse and the number of infection was detected.

With the selective medium in a contact plate (Rodac plate), it is very easy to collect *Botrytis* spp. isolates from plants or plant debris. It is just necessary to touch the infected tissue with the contact plate. The selectivity of the medium is, in most cases, sufficient to get a pure culture of *Botrytis* spp. Within a few days, the typical *Botrytis* mycelium can be observed on the contact plate.

With this medium it is also possible to search for latent infections. *Botrytis* spp. can often become latent in several crops, in immature fruits but also on stems and leaves. It can be important in research to know that latent infection is present. Most researchers use a Paraquat Medium or the Overnight Freezing Incubation Technique or molecular biology techniques. It is only necessary to briefly disinfect the surface of the plant material with a possible *Botrytis* spp. infection and to incubate it on SMB. This method is also useful for searching for latent infections in seeds.

By collecting conidia of *Botrytis* spp. in the field, it is possible to identify resistant populations by using the SMB medium combined with the fungicides to which *Botrytis* spp. might be resistant. This can be carried out with an air sampler, putting Petri dishes into a field with *Botrytis* spp., or by the same method used to search for latent infections.

P2.2 QUANTIFICATION OF LATENT *BOTRYTIS CINEREA* IN CYCLAMEN, GERBERA AND POINSETTIA

TIM O'NEILL[1], KIM GREEN[1], KATHRYN WALSH[2] AND NEIL BOONHAM[2]

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Grey mould (*Botrytis cinerea*) is a major cause of post-harvest damage in cut-flowers and pot plants, resulting in losses of over £14 million annually in the UK alone. Infections can occur throughout the supply chain and may remain latent until produce is at the supermarket. Commercial crops of cyclamen and poinsettia were examined for latent *Botrytis* to determine the occurrence of symptomless infection during crop production and at dispatch. Gerbera flowers originating from plants grown overseas were tested at monthly intervals on receipt at a UK packhouse. A paraquat or freeze-thaw treatment followed by incubation and examination for *B. cinerea* sporulation, was used to assess incidence of infection; real-time PCR was used to quantify the amount of *B. cinerea* DNA associated with tissues.

B. cinerea was commonly detected in visibly healthy leaves of cyclamen and poinsettia. There was evidence over several years that both the incidence of infection and the quantity of *B. cinerea* DNA increased with crop age. In some crops, over 50% of plants were symptomlessly infected at dispatch. In cyclamen crops from three nurseries tested in 2005, *B. cinerea* was detected by a paraquat and incubation test in 2.5-5.0% of leaves at potting and 28.1-98.1% of leaves when tested 15 weeks later. There were significant differences between nurseries ($p=0.001$) and sample dates ($p<0.001$). Using the PCR test, *B. cinerea* was detected in 13.8-64.5% of leaves at potting and at a high incidence in all samples at dispatch (84.5-95.6% of leaves). The mean quantity of *B. cinerea* DNA per leaf increased between the two sample times at two nurseries (by a factor of x200 and x10) and declined slightly at the third. There were significant differences between nurseries ($p=0.023$) and sample dates ($p<0.001$).

In 2006, 20 visibly healthy cyclamen plants at the normal marketing stage were collected from each of five nurseries, tested for latent *Botrytis* in leaf samples, and subsequently assessed for visible *Botrytis* in a 5-week shelf-life test. The proportion of plants which developed visible *Botrytis* after testing positive for latent *B. cinerea* in a PCR test (14%) was significantly greater ($p=0.020$) than in plants which tested negative (3%). Although the five crops differed significantly in their mean quantity of latent *Botrytis* at dispatch, little visible *Botrytis* developed in the shelf-life test and levels did not relate to the mean quantity of latent *B. cinerea* determined earlier. One of the five crops received no fungicide active against *B. cinerea* and this was the only crop with a latent *Botrytis* level greater than 0.01 mg/g.

In gerbera flowers, symptomless *B. cinerea* was detected at a greater incidence in winter than at other times. The possibility of testing samples of flowers and pot plants to identify batches with greater levels of latent *Botrytis*, as a means of reducing the occurrence of post-harvest Botrytis leaf and flower spot and rot will be discussed.

3. BIOLOGY AND GENETICS OF *BOTRYTIS*

The genus *Botrytis* comprises two dozens species, most of which are host specific pathogens of monocot plants. In contrast, *B. cinerea* has a broad host range amongst dicotyledonous plants. The availability of full genome sequences from *B. cinerea* and related ascomycetes, and the continuous improvement of molecular genetic tools have opened the way towards a thorough understanding of the biology of *B. cinerea* and other *Botrytis* species. Recent advances include novel insights in various pathogenesis-associated processes of the grey mould fungus, including germination, penetration and host tissue destruction.

This session will discuss novel insights into the genetic factors that determine central aspects of *Botrytis* biology and their relation to its behaviour as a pathogen. Matthias Hahn will present an overview of the impact of modern techniques in molecular biology, genetics and genomics on our knowledge about the biology of the grey mould fungus, highlighting some of the major discoveries of the last years.

ORAL SESSION

Keynote + Chairperson:

O3.1. **Matthias Hahn**, Michaela Leroch, Matthias Kretschmer, Astrid Schamber, Andreas Mosbach, Oliver Rui and Gunther Döhlemann

Genetics and biology of *Botrytis cinerea*

O3.2. **Anne-Sophie Walker**, Véronique Decognet, Marc Fermaud, Alexandre Bout, Johann Confais, Pierre Leroux, Angélique Gautier, Fabian Martinez, Jean Roudet, Philippe Nicot, Marc Bardin, Philippe Robin, Mélody Potron and Elisabeth Fournier

Genetic diversity and structuring factors for *Botrytis cinerea* French populations

O3.3. Martijn Staats, Peter van Baarlen and **Jan A.L. van Kan**

Reproductive modes of *Botrytis* species in the field and in the lab

O3.4. **Annika A.M. Bokor**, Jan A.L. van Kan and Russell T.M. Poulter

Sexual mating of *Botryotinia fuckeliana* illustrates PRP8 intein HEG activity

O3.5. **Michaela Leroch**, Manti Schwarzkopf, Astrid Schamber, Gunther Döhlemann, Janine Diwo and Matthias Hahn

Functional characterisation of a cell surface sensor-like protein in *Botrytis cinerea*

Plenary lecture:

O3.6. Bettina Tudzynski

Signaling in *Botrytis cinerea* - an overview

O3.7. **Julia Plotnikov** and Frederick M. Ausubel

Difference in infection strategies of biotrophic and necrotrophic pathogens

O3.8. Shahar Ish-Shalom, Tatiana Kaplunov, Yochanan Zutchi, Susan Lurie, Mustafa Celik, Ajay K. Pandey, Maria R. Davis and **Amnon Lichter**

Monitoring the development of *Botrytis cinerea* at low temperature

O3.9. **Colin M.C. Tan**, Michael N. Pearson, Ross E. Beever and Stephanie L. Parkes

Why fungi have sex?

03.1 GENETICS AND BIOLOGY OF *BOTRYTIS CINEREA*

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The grey mould pathogen, *Botrytis cinerea*, as well as other *Botrytis* species and the closely related white mould fungus, *Sclerotinia sclerotiorum*, are major threats for crop productions in a large variety of field and greenhouse cultures. The availability of complete genome sequences for *B. cinerea* and *S. sclerotiorum*, and the application of molecular techniques have increased remarkably our knowledge of the biology of these fungi. In my lecture, I will highlight some of the major discoveries of the last years in a synoptic view, and provide also recent data from my own research group.

03.2 GENETIC DIVERSITY AND STRUCTURING FACTORS FOR *BOTRYTIS CINEREA* FRENCH POPULATIONS

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Botrytis cinerea has long been considered as a very polymorphic fungus, in terms of morphology, host range, pathogenicity, sensitivity to various xenobiotics, ecology and genetic diversity. This plasticity may explain its large geographic distribution and its ability to colonise various environments and to cause important diseases on a large variety of crops. Despite its agronomic importance, little is known about *Botrytis* population dynamics and genetics. The available studies mainly analyse one component, often adaptation to host, as a structuring factor for the populations.

First results will be presented from a multi-factorial study of *Botrytis* populations. Samples were collected in 4 distinct French areas (Champagne, Provence, French Riviera and Bordeaux area), on various host plants or decaying substrate (grapevine, tomato, brambles, litter), treated or not with fungicides, in 2 cropping systems (open fields or glasshouses) and during 2 years, in spring and autumn. This sampling plan led to the set up of at least 32 distinct populations per season of collection, and would help to understand the effect of geography and migration at small to large scale, selection by the host plant and fungicide programmes, effect of the ecological type and cropping system on the diversity and genetic structure of *Botrytis* populations. All the isolates were analysed for their diversity according to 8 neutral microsatellite markers, and part of the collection was screened for its sensitivity to various fungicides. These data are managed through the specifically-developed database POPULABOT.

The first results from autumn 2005 confirmed the occurrence of a sibling species living in sympatry with *Botrytis cinerea sensu-stricto.* In France, *B. cinerea* consists of a complex of 2 genetically isolated species: *B. cinerea* Group I (proposed as *B. pseudocinerea*) and *B. cinerea* Group II (or *B. cinerea sensu-stricto).* In our sampling, strains belonging to Group I were highly differentiated from group II strains and exhibited private alleles for 2 microsatellite loci. They were found in any region but their frequencies never exceeded 10%.

Genetic diversity was regularly high, indicating an equilibrated distribution of the various microsatellites alleles in the populations, due to migration and/or sexual reproduction. The occurrence of regular re-combination was confirmed by the low values of multi-locus linkage disequilibrium and by the few numbers of repeated multi-locus genotypes (clones) in most of the populations. However, in some Provence tomato glasshouses, genetic diversity reached very low levels, whereas the multi-locus linkage disequilibrium and the number of clones increased significantly, showing a strong effect of the cropping system on the population dynamics.

03.3 REPRODUCTIVE MODES OF *BOTRYTIS* SPECIES IN THE FIELD AND IN THE LAB

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The genus *Botrytis* comprises 22 species, for most of which a teleomorph was described, named *Botryotinia*. For virtually all the *Botryotinia* species, it is unknown how frequently sexual reproduction occurs in nature. Occurrence of the teleomorph stage of *Botrytis cinerea* (*Botryotinia fuckeliana*) in the field has only been reported five times in literature since its first description in 1865. Nevertheless, studies on genetic diversity in France have suggested that *B. cinerea* shows abundant sexual reproduction. For other *Botrytis* / *Botryotinia* species, such studies have barely been performed. One objective of our study was to assess the genetic diversity in two host-specialised species, i.e. *Botrytis elliptica* and *B. tulipae*, and to infer from the diversity data whether these species reproduce sexually or asexually in bulb fields in the Netherlands.

AFLP fingerprinting provided the most efficient method to differentiate isolates within each species and therefore this method was used for population analyses of *B. elliptica* and *B. tulipae*. Isolates of both species were sampled during successive growing seasons in experimental field plots in various locations in the Netherlands. Among 174 *B. elliptica* isolates, 105 genotypes could be discriminated and 87 genotypes were found only once, reflecting high genotypic variation. Clonal genotypes were found only within growing seasons and in the same location. We conclude that sexual recombination occurs in the *B. elliptica* population. Among the 170 B. tulipae isolates, 25 genotypes could be discriminated and 4 genotypes were found only once, reflecting a low genotypic variation. Clonal genotypes were frequently found in different growing seasons and different locations. We conclude that the *B. tulipae* population is mainly clonal with occasional recombination.

A second, rather different study that has been ongoing for more than a decade, deals with the unusal observation that certain natural *B. cinerea* isolates behave as dual maters, i.e. they are able to mate successfully with two reference strains for the mating type alleles MAT1-1 (strain SAS56) and MAT1-2 (strain SAS405). Dual maters even occur (at low frequency) in progeny originating from a cross between SAS56 and SAS405. The mechanism underlying this behaviour could not be studied because the DNA sequences of the MAT alleles were not available until recently. The genome sequences of strains B05.10 (MAT1-1) and T4 (MAT1-2) have provided insight in the configuration of both MAT alleles. Compared to other ascomycetes, the *B. cinerea* MAT locus has some unusual features. We have started to analyse the MAT locus configuration in dual mater strains by PCR and sequencing analyses. The first results suggest that dual mater strains possess a MAT1-2 locus, however, we were not able to detect any MAT1-1 like sequence as yet. These results suggest that the dual mater behaviour is not the consequence of a conversion from heterothallism to homothallism, i.e. the presence of both MAT alleles/genes in a single thallus. An alternative mechanism for the phenomenon may need to be proposed.

03.4 SEXUAL MATING OF *BOTRYOTINIA FUCKELIANA* ILLUSTRATES PRP8 INTEIN HEG ACTIVITY

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Inteins are protein elements that are removed post-translationally from a host protein. This is an autocatalytic spicing process necessary for the maturation of the host protein (exteins) and requires a set of highly critical splicing residues. In addition to the essential N and C-terminal splicing domains, most inteins (full-length inteins) encode a homing endonuclease (HEG). During meiosis, in a diploid heterozygous for the presence/absence of the full-length intein, the HEG recognises the empty ~30bp, allelic target site and introduces a double-stranded DNA break. This break triggers the host's homologous recombinational repair system, which copies the intein into the empty allelic site. Thus, the HEG enables the intein coding sequence to act as a mobile element, spreading through the gene pool of a species. Potentially, the intein might also undergo horizontal transmission to other species.

Our group has discovered a large number of inteins and several of them are located in the PRP8 gene of mycelial fungi. These include the full-length inteins in the human pathogens *Aspergillus fumigatus*, *Histoplasma capulatum* and the plant pathogen *Botrytis cinerea* (BciPRP8). For a full-length intein to replicate, a species must have a sexual cycle and there must be polymorphism for the presence/absence of the intein in the gene pool of the species. Only *B. cinerea* (telemorph *Botryotinia fuckeliana*) fulfils both these criteria.

Crosses between *B. cinerea* strains Bc18 (which carries the BciPRP8 intein) and SAS405 (which has an 'empty' PRP8 allele) were carried out to investigate whether the BciPRP8 HEG is active and can facilitate super-Mendelian inheritance of the intein and whether this gene conversion occurs during meiosis. Complete sets of individual ascospores were isolated from asci and cultured. These offspring cultures were screened for the presence/absence of the BciPRP8 intein. The BciPRP8 flanking regions were also analysed to determine the extent of any gene conversion.

All offspring screened positive for the Bc18 parental BciPRP8, illustrating a 100% super-Mendelian inheritance of the BciPRP8. In contrast, the DNA marker Daf1 was inherited in a perfect 1:1 ratio verifying the meiotic origin of the offspring cultures. Sequencing of the exteins in the parental strains revealed C-extein SNPs at the immediate flank and within two introns. The immediate flank of all offspring resembled the Bc18 donor parent while further downstream an indel and 2 SNPs were inherited in either a 4:4 ratio or a 6:2 ratio.

We have tested the validity of the HEG hypothesis and demonstrated that the HEG of BciPRP8 is active in meiosis and spreads into its empty target allele. This meiotic 'homing' process results in a super-Mendelian inheritance of BciPRP8 (100%) and the variation of C-extein polymorphisms suggests that the homing takes place at the DNA four-strand stage. This is the first natural system that demonstrates an intein acting as a meiotic driver that specifically alters the genetic makeup of a species. The only other eukaryote system showing in vivo activity was an artificial yeast system which transmitted its HEG less efficiently (89%). Only one example of a population displaying a meiotic driver has previously been described, the P element invasion in *Drosophila melanogaster*.

03.5 FUNCTIONAL CHARACTERISATION OF A CELL SURFACE SENSOR-LIKE PROTEIN IN *BOTRYTIS CINEREA*

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Germination of *Botrytis cinerea* conidia can be induced by several chemical and physical stimuli. In addition to organic carbon sources, contact to hard hydrophobic surfaces (e.g. the host cuticle) in the absence of nutrients can induce germination. While carbon source-induced germination is controlled by a Gα3 subunit of the heterotrimeric G protein, in a cAMP dependent fashion, an intact Ste11-Ste7-BMP1 MAP kinase cascade is required for germination of *B. cinerea* conidia on hydrophobic surfaces. The mechanism of surface contact perception in this pathway is still unknown.

In order to identify molecular sensors involved in surface recognition, we chose a candidate gene approach, taking advantage of the available *B. cinerea* genome sequence. A putative homolog of a yeast cell surface protein involved in filamentous growth and osmoregulation, BcMsb2 was identified. Similar to yeast Msb2, BcMsb2 contains a large extracellular domain of about 1450 amino acids, a single transmembrane domain and a short cytoplasmic tail. *B. cinerea* mutants with a msb2 deletion were constructed. When germinated on hydrophobic surfaces, Δmsb2 mutants showed increased numbers of germ tubes as well as increased elongation of the germ tubes compared to the wildtype. The increased elongation of germ tubes both on hydrophobic as well as on hydrophilic surfaces appears to be due to premature germination and a reduced surface recognition, because it was correlated with the absence of usually occuring appressorial swellings. Because of reduced penetration efficiency, the msb2 mutants showed delayed lesion formation on intact leaves and flowers. Thus, Msb2 seems to be a cell surface sensor-like protein, that is involved in the regulation of initial stages of germ tube appearance, outgrowth and surface penetration. Our further studies are aimed at the identification of interaction partners and downstream signalling components of BcMsb2, e.g. by yeast-2-hybrid experiments.

03.6 SIGNALLING IN *BOTRYTIS CINEREA* - AN OVERVIEW

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Sensing the environment and ensuring appropriate cellular responses are crucial challenges confronted by fungal pathogens and all living organisms in general. Failure at any step of signal sensing, transduction or cellular responses elicits abnormal growth, differentiation or host-pathogenic interactions. Conserved signal transduction pathways such as the cAMP-dependent and several MAP kinase pathways were shown to be important for almost all cell functions during morphogenesis, differentiation, and pathogenic interactions. In the past years, significant progress was made in characterisation of several signalling components in *B. cinerea*, allowing us to better understanding the complicated regulatory network in the pathogen. Several new genes involved in signalling processes and affecting pathogenicity, but also first indications for cross-talks between different signalling pathways have been studied and will be briefly summarised here.

After a general overview, I will concentrate on new insights in the complex role of the cAMP-dependent signalling pathway which is involved in multiple processes in plant-pathogenic fungi, including growth, conidiation and spore germination, nutrient sensing, and virulence. In *B. cinerea*, the main components of this pathway, such as Gα subunits of heterotrimeric G-proteins, the adenylate cyclase BAC, two catalytic subunits (*bcpka1* and *bcpka2*) and the regulatory subunit (*bcpkaR*) of the cAMP-dependent protein kinase (PKA) have been recently characterised. While the Δbcpka2 mutant showed wild-type-like growth, conidiation, germination and infection pattern, the Δbcpka1 and ΔbcpkaR mutants displayed the most pronounced phenotypes. They show strong growth defects on all media, and invasive growth in the plant tissue is significantly impaired. Surprisingly, and in contrast to other filamentous fungi, the *B. cinerea* Δbcpka1 and ΔbcpkaR mutants behave very similar and do not show contrasting phenotypes as it would have been expected for overexpression of PKA in the ΔbcpkaR mutant. PKA activity assays revealed a total loss of PKA activity in both the Δbcpka1 and ΔbcpkaR mutants suggesting that the deletion of *bcpkaR* results in degradation of the catalytic subunit(s).

03.7 DIFFERENCE IN INFECTION STRATEGIES OF BIOTROPHIC AND NECROTROPHIC PATHOGENS

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A diversity of plant pathogenic microorganisms can be classifed into two major groups, biotrophs and necrotrophs, although many pathogens fall into the intermediate groups of hemibiotrophs and heminecrotrophs. The strategies of biotrophic and necrotrophic fungal attacks are different. The necrotroph *Botrytis cinerea* kills plant cells in front of growing hyphae, whereas the biotroph *Erysiphe orontii* has a long symbiotic phase and even stimulates host-plant metabolism at the initial stages of the infection process.

Our experiments show major differences in the susceptibility of various *Arabidopsis* defense-related mutants to *B. cinerea* and *E. orontii.* Thus, the *Arabidopsis pad4* mutant is highly susceptible to *E. orontii* but resistant to *B. cinerea.* Similarly, *ein2* and *jar1*which are highly susceptible to *B. cinerea* are resistant to *E. orontii.*

The number of genes induced in response *to E. orontii* is much higher (2200) than in response to *B. cinerea (*1600). The effects of biotrophs and necrotrophs on *Arabidopsis* carbohydrate and amino acid metabolism also differ greatly e.g. expression level of sucrose degradative enzymes was 30-fold higher in *Botrytis*-infected than in *Erysiphe*-infected *Arabidopsis.* Similarly, the asparagine degradation pathway was 50-fold more active in the necrotrophic than in biotrophic infections. Biosynthesis of ET and JA in *B. cinerea* infected plants was 20 times higher than in *Erysiphe*-infected *Arabidopsis.* We found a striking difference in induction of some *PR* genes, in the necrotroph *B. cinerea* and the biotroph *E. orontii.* The former induced *PDF1.2* and *Thi2.1,* whereas the latter did not. On the other hand, *PR1, BGL2, PR5 etc*. were induced in both types of interactions.

The biotroph *E. orontii* infects and colonises plant cells with active metabolism. It has a long-lasting mutualistic stage when it does not destroy plant structures. During its mutualistic stage, it can even stimulate the formation of new host structures in infected cells such as new membrane complexes, dictyosomes and polarised ER cisternae so as to exploit the plant biosynthetic machinery by making infected cells compete with the growing plant apices and developing flowers for assimilates. In contrast, the necrotroph *B. cinerea* is adapted to the metabolism of older plants and/or their senescing parts with their activated catabolic pathways. With its arsenal of pectolytic and proteolytic enzymes, *B. cinerea* can induce autolysis of plant cells. This turns the host's own enzymatic machinery towards itself, churning out monosaccharides, amino acids, vitamins etc., which are utilised by a fast-growing necrotrophic pathogen.

03.8 MONITORING THE DEVELOPMENT OF *BOTRYTIS CINEREA* AT LOW TEMPERATURE

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The fungus *Botrytis cinerea* is the major cause for decay in table grapes and many other fresh produce during storage at low temperature. One objective of this study was to quantify the development of *B. cinerea* on grapes using qPCR, antibodies and the green fluorescent protein (GFP). Another objective of the study is to follow the expression of genes which may be involved in cold tolerance of the fungus.

'Superior' grapes were inoculated with a GFP strain of *B. cinerea* which was incubated at 0°C or 20°C. The results show that GFP enables qualitative imaging of disease progression at both storage regimens. Antibodies formulated as lateral flow devises were able to identify *B. cinerea* only in final stages of decay. qPCR analysis of the samples resulted in identification of the developing fungus from the first day of inoculation, and disease progression could be translated into quantitative models. Analysis of gene expression by qPCR, following transition from 20°C to 0°C in defined media, resulted in rapid expression of several genes selected from a SSH library. Some of these genes have no assigned function so far, while others have defined functions in other systems without an assigned function in cold tolerance. Several gene othologs identified in the literature to be cold-induced in other organisms, are also cold-induced in *B. cinerea* when infecting grapes. Other genes, such as fatty acid desaturase and RNA helicase, predicted to be cold-induced in yeast, were not expressed in a similar manner in *Botrytis*.

It is predicted that better understanding of the mechanisms involved in cold tolerance of *B. cinerea* will enable the improvement of means to control decay after harvest.

03.9 WHY FUNGI HAVE SEX?

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The evolution of sex is an area of much debate. "Birds do it and the bees do it", but why? We are exploring the hypothesis that sex in fungi provides a mechanism for the elimination of fungal viruses (mycoviruses) and avoidance of re-infection. The essential feature of this hypothesis is that while mycoviruses readily pass to asexual progeny, they are eliminated in the sexual process. Moreover, mycoviruses are not infectious in the 'normal' sense, but are only transmitted between strains by protoplasmic fusion. Fusion is controlled by vegetative (somatic) incompatibility genes that are recombined by sex, generating new virus-free strains, incompatible, both with their parents, and most of their sibs. Fungi provide a favourable test group because, unlike higher organisms, both asexual and sexual reproduction commonly occurs in the same species. *Botrytis cinerea* is a grey mould fungus which has both reproductive methods. This fungi also has associated viruses, specifically *Botrytis* Virus X (BVX).

To test the hypothesis that sex in fungi provides a mechanism for the elimination of viruses and prevention of re-infection, we have crossed *Botrytis* isolates containing BVX, and analysed the progeny using one step RT-PCR. So far, 84 crosses have been attempted and of those, 15 were successful. Contrary to expectations, results have shown that sexual reproduction does not totally eliminate viruses, BVX being detected in 8 out of 25 progeny from a sexual cross between the REB702-1 x REB705-1 and 15 out of 30 progeny from a REB705-1 x REB702-1 cross. Eleven single conidiospores of a BVX positive REB705-1 parent were tested for the presence of BVX and of those, 27% were negative for BVX and 72% were positive for BVX.

We have found that while many of the progeny produced by the sexual process (ascospores) have lost BVX, others retain it. Transmission rates to asexual spores are much higher, but some progeny do appear to be virus-free. These findings do not support the original hypothesis that sex acts to eliminate viruses.

Comparisons of the fitness of virus-containing isolates with isolates that lack viruses, indicate that BVX does not have a significant deleterious effect on its host, and indeed may slightly stimulate growth. Growth of BVX positive progeny and negative progeny were grown on plates of MEA and their day to day growth was recorded and analysed to see if there is a difference in growth characteristics. Again, these findings do not support the original hypothesis that viruses are deleterious to their host. In summary, we reject the 'virus elimination' model of sex in fungi.

3. BIOLOGY AND GENETICS OF *BOTRYTIS*

Posters

P3.1. **Michaela Leroch**, Andreas Mosbach, Andreas Böhm and Matthias Hahn

Hydrophobic surface contact perception and the role of hydrophobins in *Botrytis cinerea*

P3.2. Christina Huesmann, Julia Schumacher, Leonie Kokkelink and **Bettina Tudzynski**

The cAMP-dependent signalling pathway and its role in spore germination, growth and virulence of *Botrytis cinerea*

P3.3. **Anne-Sophie Walker**, Johann Confais, Pierre Leroux, Laurence Bill, Véronique Decognet, Marc Fermaud, Alexandre Bout and Elisabeth Fournier

Resistance to fungicides and genetic diversity among *Botrytis cinerea* populations

P3.4. **Marc Fermaud**, Fabian Martinez, Anne-Sophie Walker, Elisabeth Fournier, Véronique Decognet, Alexandre Bout, Jean Roudet and François Delmotte

Genetic structure and diversity of *Botrytis cinerea* populations in French vineyards at regional and national scales

P3.5. Elisabeth Fournier, Johann Confais, Véronique Decognet, Marc Fermaud, Alexandre Bout and **Anne-Sophie Walker**

Effect of host plants on sympatric genetic differentiations in French populations of *Botrytis cinerea*

P3.6. Melody Potron, **Anne-Sophie Walker**, Elisabeth Fournier and Philippe Robin

POPULABOT©: an interactive database to manage *Botrytis* populations collections

P3.7. Véronique Decognet, **Marc Bardin**, Anne-Sophie Walker, Marc Fermaud and Philippe Nicot

Genetic structure of *Botrytis cinerea* populations from vegetable greenhouses in France

P3.8. **Vivienne Gepp**, Julia Rebellato, Elisa Silvera, Pablo Gonzalez, Silvana Vero and Yohana Ferreira

Preliminary results of morphological, genetic and fungicide resistance characterisation of *Botrytis cinerea* isolates in Uruguay

P3.9. Stefania Pollastro, Rita Milvia De Miccolis Angelini, Caterina Rotolo, Wassim Habib and **Franco Faretra**

Characterisation of *vacuma* and *transposa* biotypes of *Botryotinia fuckeliana*

P3.10. Ross E. Beever, Colleen Higgins, **Matthew D. Templeton** and Mark T. Anderson

Vegetative incompatibility in *Botryotinia fuckeliana* (*Botrytis cinerea*)

P3.11. Mingde Wu, Lei Zhang, **Guoqing Li**, Daohong Jiang, Mingsheng Hou and Hung-Chang Huang
Hypovirulence and double-stranded RNA in *Botrytis cinerea*

P3.12. Maria Antonietta De Guido, Angelantonio Minafra, Agostino Santomauro and **Franco Faretra**
Molecular characterisation of mycoviruses in *Botryotinia fuckeliana*

P3.1 HYDROPHOBIC SURFACE CONTACT PERCEPTION AND THE ROLE OF HYDROPHOBINS IN *BOTRYTIS CINEREA*

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Conidial germination in *Botrytis cinerea* can be induced by several chemical and physical stimuli. While carbon sources are required for germination on hydrophilic surfaces such as glass, conidia are able to germinate in pure water on artificial hydrophobic surfaces (e.g. polypropylene) and on the plant cuticle.

By using atomic force microscopy to measure the contact forces between individual conidia and different surfaces, hydrophobic surfaces were found to bind conidia stronger than hydrophilic surfaces. To study whether a reduction in conidial surface hydrophobicity affects the response of conidia to the hydrophobic contact-induced germination, we sought to construct mutants devoid of hydrophobins in the spore wall. In the *B. cinerea* genome sequence, three hydrophobin genes were identified, one encoding a class I hydrophobin (BcMpg1), and two encoding class II hydrophobins (BcMhp1, BcMhp2). In a first step, deletion mutants in each single hydrophobin gene were constructed, and the resulting mutants tested for their hydrophobic surface characters, as well as for germination, growth on various media and plant infection. All the mutants were indistinguishable from the wild type, and they did not display any loss of the hydrophobic properties of conidial or mycelial walls. Using hygromycin and nourseothricin resistance markers for selection, double mutants in all three combinations were therefore constructed, but preliminary results indicate that none of them exhibit an 'easily wettable' phenotype which is often observed with fungal hydrophobin mutants. Gene expression studies by RT PCR revealed that the three hydrophobin genes are expressed in spores, germlings and both saprophytic and parasitic mycelium. Attempts to create hydrophobin null mutants are underway in order to reveal the role of hydrophobins in cell wall hydrophobicity of *B. cinerea*.

P3.2 THE CAMP-DEPENDENT SIGNALLING PATHWAY AND ITS ROLE IN SPORE GERMINATION, GROWTH AND VIRULENCE OF *BOTRYTIS CINEREA*

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The cAMP-dependent signalling pathway is involved in multiple processes in plant-pathogenic fungi, including growth, conidiation and spore germination, nutrient sensing, and virulence. In *B. cinerea*, some components of this pathway, such as Gα subunits of heterotrimeric G-proteins and the adenylate cyclase BAC are already characterised, and their impact on growth and virulence has been demonstrated. To get a complete picture on the role of the cAMP-dependent pathway and the cross-talks with other signalling cascades, we cloned and characterised the missing components of this pathway, such as genes for the two catalytic subunits (bcpka1 and bcpka2) and the regulatory subunit (bcpkaR) of the cAMP-dependent protein kinase (PKA). While the Δbcpka2 mutant showed wild-type growth, conidiation, germination and infection pattern, the Δbcpka1 and ΔbcpkaR mutants displayed the most pronounced phenotypes. They show strong growth defects on all media, and form almost identical small compact and sporulating colonies. Both mutants can penetrate plant tissue and perform invasive growth only up to a certain stage. As for the Δbac mutants, the development of spreading lesions by Δbcpka1 and ΔbcpkaR mutants was delayed, and soft rot of whole leaves never occurred.

Unlike in appressoria-forming fungi such as *M. grisea* and *Colletotrichum lagenarium*, where the adenylate cyclase and the PKA are essential for rapid degradation of lipid and glycogen reserves and formation of functional appressoria as prerequisites for penetration, the penetration is not impaired in *B. cinerea* Δbac, Δbcpka1 and ΔbcpkaR mutants. This result suggests a penetration mechanism different from that in appressoria-forming fungi and corresponds with the observation that *B. cinerea* can either directly penetrate host tissue or form swollen hyphal tips.

Surprisingly, and in contrast to other filamentous fungi, the *B. cinerea* Δbcpka1 and ΔbcpkaR mutants behave very similar and do not show contrasting phenotypes as it would have been expected for overexpression of PKA in the ΔbcpkaR mutant. PKA activity assays revealed a total loss of PKA activity in both the Δbcpka1 and ΔbcpkaR mutants suggesting that the deletion of bcpkaR results in degradation of the catalytic subunit(s).

P3.3 RESISTANCE TO FUNGICIDES AND GENETIC DIVERSITY AMONG *BOTRYTIS CINEREA* POPULATIONS

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Botrytis cinerea is responsible for grey mould and induce quantitative and qualitative losses on various crops, such as grapevine and tomato. In many cropping systems, control against *Botrytis* is mainly achieved by the use of chemical fungicides, which can enable the selection of a wide range of resistances, either specific to one fungicide (e.g. benzimidazoles, phenylcarbamates, dicarboximides, anilinopyrimidines, hydroxyanilides) or multiple to several unrelated modes of action (MDR : multidrug resistance). Global evolution of *Botrytis* populations, assessed by population genetics methods, may help to understand the evolution and distribution of resistances to fungicides in natural populations.

Isolates of *Botrytis* were collected in France in 2005 and 2006 from homogeneous treated/untreated grapevine plots in Champagne and Bordeaux areas, and from untreated plots in Provence and in the French Riviera areas. Additional samples were collected from brambles and litter in these vineyards, and from treated tomato grown in glasshouses in the four regions. These isolates were all characterised for their microsatellite profile, and resistance profiles to the main botryticides were assessed with *in-vitro* tests for part of them.

Phenotype analysis showed a large variety of resistance profiles, highly representing the fungicide selective pressures applied locally. For example, in Champagne, isolates from glasshouses showed mainly specific resistance to benzimidazoles, phenylcarbamates and dicarboximides, whereas in the vineyard, MDR strains represent at least half of the populations. Frequencies of resistances, maximal at vintage, tend to decrease after winter, but are still detectable at spring time. More generally, resistant isolates obtained from untreated grapevine plots, as well as untreated substrates (brambles, litter), maybe suggesting migrations between the various hosts.

Genic and genotypic diversity indexes within these populations showed a possible effect of recombination and migration. Finally, pairwise F_{ST}s, Factorial Analysis of Correspondances and Molecular Analysis of Variance enabled to estimate the genetic differentiation between (1) treated and untreated plots, at several geographical scales and (2) between the main resistant phenotypes. MDR for example, showed regularly significant but low levels of differentiation.

P3.4 GENETIC STRUCTURE AND DIVERSITY OF *BOTRYTIS CINEREA* POPULATIONS IN FRENCH VINEYARDS AT REGIONAL AND NATIONAL SCALES

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Botrytis cinerea is a haploid, filamentous, ascomycete fungus which can exhibit great phenotypic and genetic diversity. This necrotrophic fungus causes grey mould on numerous plants and, in grapevine, the disease can drastically reduce yield at harvest and wine quality.

The population genetic structure was investigated by using eight polymorphic microsatellite markers within Group-II in *B. cinerea*, i.e. by excluding the genetically isolated strains of Group-I (proposed as *B. pseudocinerea*). The spatial distribution of the genetic variability was assessed (i) at the regional scale, by sampling 10 vineyard sites in the Bordeaux region in September 2004 and (ii) at the national scale, on the basis of three sites in Champagne vineyards, three near Bordeaux and two in Provence (sampling periods: autumn 2005 and spring 2006). All the corresponding populations (sites x season) included from 21 to 49 monoconidial isolates. Specific fungicides were not applied at the sites during the sampling season.

At the regional scale, a high genotypic variability was found within Group II. This was illustrated by the occurrence of few repeated multi-locus genotypes within the populations tested. Low levels of linkage disequilibrium were detected within most of the populations, suggesting that recombination events occur. These results would bring indirect evidence that sexual reproduction may predominate over clonal reproduction within the populations considered. Such results will be discussed according to the population definition which is, in particular, dependent upon both the sampling strategy and plot size. A weak, but significant, genetic structure was found amongst populations ($Fst = 0.02$), but no significant pattern of isolation-by-distance was detected across the Bordeaux populations. The results from similar analyses performed at the national scale will be presented and compared with the previous ones. The hypotheses of isolation-by-distance and possible barriers to gene flow at a larger geographic scale will be discussed in relation to dispersal ability of the pathogen in the French populations.

P3.5 EFFECT OF HOST PLANTS ON SYMPATRIC GENETIC DIFFERENTIATIONS IN FRENCH POPULATIONS OF *BOTRYTIS CINEREA*

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Sympatric, ecological divergence may mainly involve parasites that differentiate via host shifts, because different host species exert strong disruptive selection and because both host and parasites are continually co-evolving. Due to their particular life styles, phytopathogenic fungi may be particularly strongly submitted to sympatric divergence, because in these organisms, adaptation alone allows the restriction of gene flow between populations developing on different hosts.

This study focuses on the generalist ascomycete fungus, *Botrytis cinerea* (*sensu-stricto*) developing on different host plants, and is part of a larger project aiming to characterise the influence of several factors on the genetic structuration and dynamics of *B. cinerea* French populations. Here we sampled populations of *B. cinerea* in 4 French regions (Champagne, Provence, French Riviera and Bordeaux area), on different hosts (grapevine, brambles, and litter) in close sympatry within each geographic area (not more than 150 m between populations from different hosts in each locality). This sampling scheme was repeated at different dates (fall 2005 and spring 2006). All isolates were genotyped with 8 microsatellite markers, and data were analysed using standard population genetics.

In addition to confirming that *B. cinerea* reproduces sexually, our results showed that the fungal populations developing on litter was not significantly differentiated from populations from grapevine and brambles. On the contrary, populations from the two plants (grapevine and brambles) were significantly differentiated, indicating restricted gene flow, even in sympatry. In contrast, only weak geographical differentiation could be detected. These results support the possibility of sympatric divergence by host adaptation in generalist parasites.

P3.6 POPULABOT©: AN INTERACTIVE DATABASE TO MANAGE *BOTRYTIS* POPULATIONS COLLECTIONS

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Population genetics studies quickly generate large collections of isolates that need to be managed through an accurate database to be fully useful.

The POPULABOT database results from a collaborative work between four INRA teams in Versailles, Bordeaux, Avignon and Antibes (France), involved in *Botrytis* population studies.

This database aims to:

- manage and share the collections present in distant laboratories and collected on various host-plants, treated or not with fungicides, in the field or in greenhouses, in various areas and in various seasons;
- collect, sort, manage and extract administrative and experimental data associated to the strains, especially phenotypic and molecular information. Molecular data handled so far by the database are RFLP, microsatellites and DNA sequences (with an available BLAST module); and
- prepare these data in appropriate formats for their analysis with various population genetics softwares.

This database is web-hosted and was written with MySql and phpMyAdmin informatic languages, with a web interface in Php. Some external modules were developed in Perl and VBA. It can be easily modified to include other types of experiments, and can be adapted to other fungal species.

POPULABOT should include, in the beginning of 2008, more than 7 000 strains of *Botrytis,* probably representing the largest organised collection for this fungus, and being a powerful tool for developing theoretical population genetics applications and for studying population dynamics and evolution within this species.

P3.7 GENETIC STRUCTURE OF *BOTRYTIS CINEREA* POPULATIONS FROM VEGETABLE GREENHOUSES IN FRANCE

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Botrytis cinerea can cause severe losses in sheltered crops. Chemical control is the main way to control grey mould, together with preventative cultural methods and climatic control. Information on the genetic structure of the pathogen's population could lead to a better understanding of epidemics and the development of better strategies of disease control. The objective of our research was to determine the level of diversity in tomato commercial greenhouses and to study the effect of three factors (geographic scale, cropping system and host) on the structuration of *B. cinerea* populations using 8 microsatellite markers.

In 2002, two greenhouses were investigated in Provence. In 2003 and 2004, isolates were collected in one of the previous greenhouses and in two other greenhouses located within a 50 km radius. In 2005 and 2006, populations were studied at the national scale, in three tomato greenhouses in each of three regions (Provence, Champagne and Bordeaux). All isolates were sampled from sporulating lesions on tomato plants.

The SSR characterisation of isolates in Provence in 2002, 2003 and 2004 revealed the presence of one or several dominant multilocus genotypes in each greenhouse, combined with an extreme diversity of the remaining isolates. A strong geographic structure of populations was suggested because (i) the sampling sites shared few common genotypes and (ii) none of those genotypes dominant on one site was dominant on the other. Unexpectedly, isolates collected in the three greenhouses in this region in 2005 and 2006 shared the same dominant genotype. In contrast, in Champagne and Bordeaux regions, none of the repeated multilocus genotypes were dominant.

In the glasshouse sampled repeatedly from 2002 to 2004, in which tomato and lettuce were consecutively produced in a yearly rotation, no dominant genotypes were found amongst the isolates collected from lettuce, and no genotypes were shared with isolates from tomato. In 2005 and 2006, *Botrytis* populations from tomato greenhouses were compared to those sampled from other substrates (grapevine, litter or blackberries) collected outside in the close vicinity of the greenhouses. High level of diversity was also observed for these populations which shared few common genotypes with tomato populations.

Our results suggest that migration and exchange of inoculum are frequent among neighbouring greenhouses. Since few genotypes were shared between the populations inside or outside the greenhouses, the results suggest a possible host specialisation of *B. cinerea*. Differences in populations sampled from lettuce and tomato in the same greenhouse supports this hypothesis. The systematic occurrence of dominant genotypes in all greenhouses suggests that the cropping system influences the genetic structure and that endogenous secondary inoculum plays an essential role in the development of grey mould epidemics in commercial tomato greenhouses.

P3.8 PRELIMINARY RESULTS OF MORPHOLOGICAL, GENETIC AND FUNGICIDE RESISTANCE CHARACTERISATION OF *BOTRYTIS CINEREA* ISOLATES IN URUGUAY

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Botrytis cinerea as a species is known to include at least two subspecies and to exhibit great morphological diversity, as well as to readily develop resistance to fungicides. In Uruguay, it causes important losses in commercial crops such as grapes, tomatoes, strawberries and Eucalyptus seedlings. Disease management is hindered by the variable climate and lack of knowledge of population dynamics and of sensitivity to the fungicides used for its control. A survey was carried out in order to obtain isolates of *B. cinerea* from different crops and determine genetic factors, colony morphology and sensitivity to fungicides.

Single-spore isolates were obtained from grapes, tomatoes, strawberries, blueberries and roses in the south of Uruguay. Growth rate of colonies was measured on malt-agar medium. The number, distribution and size of sclerotia, mycelial type and sporulation were evaluated according to Martinez *et al.* (2003). The presence of the Boty transposable element was investigated using PCR with specific primers developed by Muñoz *et al.* (2002). The isolates were analysed for double stranded RNA using the technique described by Castro *et al.* (2003).

The majority of the isolates produced sclerotia, these were generally placed irregularly and their numbers ranged from 0 to over 100 per 90 mm Petri dish. The mycelium was often sparse. Minimal inhibitory concentrations were above 128 ppm carbendazim in 52% and above 16 ppm iprodione in 30% of the isolates. The Boty transposable element was detected in 60% of the isolates analysed and no double stranded RNA was found in any of them.

The results show that there is diversity in morphological, genetic and fungicide sensitivity within the *B. cinerea* population in southern Uruguay. This research continues in order to compare characteristics of isolates obtained from different crops and other localities.

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P3.9 CHARACTERISATION OF *VACUMA* AND *TRANSPOSA* BIOTYPES OF *BOTRYOTINIA FUCKELIANA*

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Botryotinia fuckeliana (anamorph: *Botrytis cinerea*), the causal agent of grey mould on well over 200 host plants, is well-known for its broad adaptability to all adverse conditions. Different genetic sources of variation, including the presence/absence of two transposable elements (TE), Boty and Flipper, seems to be involved. *Vacuma* (Boty-Flipper-) and *transposa* (Boty+Flipper+; Boty+Flipper-; Boty-Flipper+) isolates have been reported in similar ratios on green tissues, and the latter prevalent on ripening fruits.

The distribution of *vacuma* (Boty-Flipper-) and *transposa* (Boty+Flipper+; Boty+Flipper-; Boty-Flipper+) biotypes among 840 isolates, from 23 naturally-infected host plants from 15 countries (Albania, Belgium, Chile, Crete, Croatia, Germany, Greece, Israel, Italy, Japan, Lebanon, New Zealand, Portugal, Serbia and Switzerland), was assessed through PCR with specific primers for Boty and Flipper.

On the whole, in all the countries and on all the crops, Boty+Flipper+ isolates were prevalent (69.2%); Boty+Flipper- and Boty-Flipper- isolates were found at similar frequencies (13.8% and 14.4%, respectively), while Boty-Flipper+ isolates (2.6%) were mostly detected in fungal samples collected from strawberry in Croatia. Different ratios *vacuma* : *transposa* isolates were found on shoots and leaves (1:5) and on ripening fruits (1:16).

The response of all isolates to fungicides commonly used against grey mould (benzimidazoles, dicarboximides, anilinopyrimidines, phenylpyrroles, fenhexamid and dichlofluanid) was evaluated in a colony growth test. Generally, *transposa* isolates were resistant to one or more of the tested fungicides, while *vacuma* isolates showed more frequently the "wild-type" sensitive phenotype.

Observations on additional phenotypic features were carried out on isolates from grapevine, all sensitive to fungicides, and representative of the four groups (20 Boty-Flipper-; 20 Boty+Flipper+; 5 Boty+Flipper- and 5 Boty-Flipper+). These were characterised for: (i) colony growth at different temperatures (5-40°C), (ii) production of conidia and sclerotia, and (iii) virulence on berries of different grape cultivars. Boty-Flipper- and Boty+Flipper+ isolates were also compared for their sensitivity to high osmotic pressure. Generally, no significant correlation was found between TE absence/presence and the biological parameters evaluated. The only exceptions were: (i) sclerotia production, higher in *vacuma* than in *transposa* isolates; (ii) virulence, slightly higher in *transposa* than in *vacuma* isolates. Nevertheless, such small differences do not explain exhaustively the different frequency of the two biotypes on green tissues and on ripening fruits, the reasons of which remain to be clarified in deeper details.

P3.10 VEGETATIVE INCOMPATIBILITY IN *BOTRYOTINIA FUCKELIANA* (*BOTRYTIS CINEREA*)

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In ascomycete fungi, vegetative incompatibility is typically determined by a series of vegetative incompatibility (*vic*) genes that exist in two alleles. Strains that carry identical alleles at all loci, are compatible; those that differ at one or more loci are incompatible. Previous work has established that the *Botryotinia fuckeliana* population contains at least 66 distinct vegetative compatibility groups, consistent with the presence of at least 7 *vic* genes (Beever and Weeds, 2004). A simple model of incompatibility proposes that rejection is triggered by the formation of a heterodimer of different *vic* gene products (Glass and Kaneko, 2003). Two broad hypotheses have been suggested for the nature of the downstream rejection process: first, a form of apoptosis (programmed cell death) perhaps involving autophagy as characterised in yeast (Ohsumi, 2001) and second, necrosis perhaps acting directly on plasma membrane function (Sarkar *et al.*, 2002; Marek *et al.*, 2003). In order to distinguish whether apoptosis or necrosis is the predominant process involved, we have developed an in-house series of Expressed Sequence Tag (EST) gene libraries (over 40,000 ESTs), derived from interacting compatible and incompatible isogenic strains, differing at one *vic* locus. We are using these libraries to identify which genes are up- or down-regulated, following incompatible interactions. Genes associated with incompatibility will be identified by direct comparison of compatible and incompatible EST libraries, focusing on genes potentially associated with apoptosis and necrosis, and by *in silico* subtraction.

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P3.11 HYPOVIRULENCE AND DOUBLE-STRANDED RNA IN *BOTRYTIS CINEREA*

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Twenty-one strains of *Botrytis cinerea*, isolated from 13 species of plants grown in China, were compared for pathogenicity on *Brassica napus*, mycelial growth on potato dextrose agar, and presence of double-stranded (ds) RNA. After inoculated on detached leaves of oilseed rape (*Brassica napus*) at 20℃ for 72 hours, the strain CanBc-1 isolated from oilseed rape was severely debilitated in pathogenicity and caused no lesions on leaves of oilseed rape, compared to the 20 virulent strains. The hypovirulent strain CanBc-1 grew slower than the virulent strains on PDA at 20℃, with sparse conidia and no sclerotia formed on the colonies after incubation on PDA at 20℃ for 15 days. The virulent strains grew rapidly on PDA at 20℃ with profuse conidia or sclerotia formed on the colonies. A dsRNA of approximately 3.0 kb in length was detected in CanBc-1, and four randomly selected hypovirulent single conidium (SC) isolates of CanBc-1, with low mycelial growth rates and severely-debilitated pathogenicity. A dsRNA was not detected in the 20 virulent strains and four randomly selected virulent SC isolates of CanBc-1, with high mycelial growth rates and strong pathogenicity. This finding indicated that the 3.0 kb dsRNA could transfer through asexual reproduction to conidia. Results of the horizontal transmission experiment showed that the hypovirulent trait of CanBc-1 was transmissible and the 3.0-kb dsRNA was involved in the transmission of hypovirulence. Analysis of a 920-bp cDNA sequence generated from the 3.0-kb dsRNA of CanBc-1 indicated that the dsRNA element was a mycovirus, designated as *Botrytis cinerea* debilitation-related virus (BcDRV). Further analyses showed that BcDRV is closely related to *Ophiostoma mitovirus* 3b infecting *O. novo-ulmi*, the causal agent of Dutch elm disease. Mitochondria in hyphal cells of CanBc-1 became degenerated without formation of cristae, compared to the virulent isolate CanBc-1c-66 of *B cinerea*. This is the first report on the occurrence of *Mitovirus*-associated hypovirulence in *B. cinerea*.

P3.12 MOLECULAR CHARACTERISATION OF MYCOVIRUSES IN *BOTRYOTINIA FUCKELIANA*

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Double-stranded RNA (dsRNA) and virus like particles (VLPs) are very common in *Botryotinia fuckeliana*. In many isolates of the pathogen, dsRNA molecules in variable numbers and sizes have indeed been observed. These are often associated with isometric or, less frequently, rod-shaped VLPs. So far, it seems that dsRNAs and VLPs are generally in a cryptic state in *B. fuckeliana*.

It is crucial to understand the possible biological roles of dsRNAs, as a source of variation in *B. fuckeliana*, and their use as molecular markers or as expression vectors. In the present work, molecular characterisation of the dsRNAs carried by the *B. fuckeliana* strain WS276, known for the presence of both VLPs and dsRNAs, is reported.

Four out of the six dsRNAs, the most abundant ones, were cloned by different techniques (DOP-PCR, RACE, RT-PCR, Single Primer Amplification) and sequenced. Four sequences were derived with the lengths of 5,261 bp (dsRNA0; AM491608), 1,793 bp (dsRNA1; AM491609), 1,566 bp (dsRNA2; AM491610) and 1,383 bp (dsRNA3; AM491611). Molecular characterisation, sequence analysis and phylogenetic analysis of the four dsRNAs proved the co-presence of two different viruses, belonging to Totivirus and Partitivirus genera, co-infecting the fungal strain.

Genome analysis of dsRNA0 revealed two coding regions: ORF1, close to 5' end, coding for a coat protein of 765 amino acids with a putative mass of 81 kDa; ORF2 coding for a putative RdRp of 837 amino acids containing eight conserved replicase motif domains reported for viruses infecting lower eukaryotes; ORF1 and ORF2 overlapped for four nucleotides (ATGA) in a hypothetical coupled termination-re-initiation mechanism also reported for other Totivirus. DsRNA0 sequence could be attributed to the genome of a new putative Totivirus species, for which the name *Botryotinia fuckeliana* Totivirus 1 (BfTV1) is proposed.

The three dsRNA fragments 1 to 3, having similarity with Partitivirus species, represent the genome of a second mycovirus co-infecting the WS276 strain, for which the name *Botryotinia fuckeliana* Partitivirus 1 (BfPV1) is proposed; they are associated to viral isometric particles (40 nm). dsRNA1 contains a single ORF coding for a sequence of 540 amino acids showing high identity with RdRps of Partitivirus. dsRNA2 is also a monocistronic sequence, coding for a putative coat protein of 436 residues, with a presumptive weight of 47 kDa. dsRNA3 has a sequence quite identical to dsRNA2, with an internal deletion of 210 bp in the coding region. Virus species in the genus Partitivirus have generally a bipartite genome, but in few cases a third dsRNA molecole is present, probably a satellite, whose sequence does not show similarities with the two genomic coding sequences or with other known sequences in databases. Although the presence of defective dsRNA for Partitiviridae is reported, the deleted dsRNA detected in *B. fuckeliana* is the first report of defective dsRNA for mycoviruses of Partitivirus genus whose origin and function need to be clarified.

4. ECOLOGY AND EPIDEMIOLOGY

Botrytis cinerea and other *Botrytis* species are important pathogens of nursery plants, vegetable, ornamental, field and orchard crops. Bulbs, seeds and other propagation material also suffer infection. One intriguing phenomenon associated with *Botrytis* infection is the ability of this pathogen to be quiescent in the host tissue for varying periods either during the crop growing season or post-harvest. The rapid conidia germination, infection, mycelium growth and conidiation occur under a wide range of microclimate conditions and pose severe disease management problems all around the world. Moreover, the ability of *B. cinerea* to be active at temperature as low as 0°C makes it also an important and challenging pathogen during storage and shipment. Nevertheless, the limiting factor for epidemic outbreaks is usually the occurrence of the appropriate microclimatic conditions, rather than the amount of inoculum. The production of high numbers of conidia poses a long lasting threat to susceptible hosts. It was claimed that infection can occur only in high relative humidity conditions. Indeed, the role of water films and nutrients in germination and infection has been long recognised. However, it is interesting that the pathogen adapted itself to infect plants when no visible water film occurs. Penetration of leaves and stems, floral organs and wounds of various ages and consequences of ecological niche change in host crops will be demonstrated. Detailed studies on the precise conditions that promote infection, disease development and survival of inoculum have provided the essential epidemiological information required for the design of control strategies. For example, cultural methods have been developed that increase aeration and drying of the plant canopy to reduce the risk of *Botrytis* epidemics. Rational warning systems based on conditions highly conducive to conidia germination and host penetration for disease development have been developed for some crop systems.

In the Ecology and Epidemiology session, presentations will address, among others, different aspects in grapes and other crops, related to eenvironmental and nutritional factors influencing the activation and spread of quiescent infections; interdependent effects of climate and vegetative growth on grey mould incidence; infection of flowers and fruits in relation to weather conditions and fruit age; Thrips as a vector of *Botrytis* to flowers, systemic infection; Inoculum ecology; Predicting the seasonal risk and forecast of *Botrytis* infection risk; competitive colonisation of *B. cinerea* and other species.

Oral Session

Keynote + Chairperson:

O4.1. Yigal Elad

Epidemiology and ecology of *Botrytis* spp.

O4.2. Stella M. Zitter and **Wayne F. Wilcox**

Environmental and nutritional factors influencing the activation and spread of quiescent infections of *Botrytis cinerea* in grapes

O4.3. **Marc Fermaud**, Héctor Valdés-Gómez, A. Calonnec, Jean Roudet and Christian Gary

Interdependent effects of (micro) climate and vegetative growth on grey mould incidence in grapevine

O4.4. **Xiangming Xu** and Angela Berrie

Infection of blackcurrant flowers and fruits in relation to weather conditions and fruit age

O4.5. K. Schmidt, D.A.J. Teulon, S.D. Wratten and **Marlene V. Jaspers**

***Thrips obscuratus*, as a vector of *Botrytis cinerea*, to cause grape flower infection**

O4.6. A.P. Rajaguru, E.N.K.Sowley, A.E.vandenBon, F.M.Dewey and **Michael W. Shaw**

Location and persistence of systemic *Botrytis cinerea* in lettuce, Primula and other species

O4.7. **Chris Spies**, Adele Mcleod and Sandra Lamprecht

Inoculum ecology of *Botrytis cinerea* in rooibos (*Aspalathus linearis*) nurseries in the Western Cape province of South Africa

O4.8. Robert M. Beresford, **Katherine J. Evans** and Jacqueline Edwards

Predicting the seasonal risk of Botrytis bunch rot in wine grapes

O4.9. R.W. Emmett, M. Welsh, O. Villalta, **Jacqueline Edwards**, R. Holmes, J. Lopresti, B. Tomkins and D. Partington

Evaluation of the Broome *Botrytis* model for the forecast of *Botrytis* infection risk in table grapes

O4.10. Ruth Walter, **Marco Harms** and Heinrich Buchenauer

Investigations on the competitive colonisation of *Botrytis cinerea* and *Penicillium expansum* on grapes

04.1 EPIDEMIOLOGY AND ECOLOGY OF *BOTRYTIS* SPP.

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Botrytis cinerea and other *Botrytis* species are important pathogens of vegetable, ornamental, field and orchard crops, as well as nursery plants. Bulbs, seeds and other propagation material can all become infected. One intriguing phenomenon associated with *Botrytis* infection, is the ability of the pathogen to be quiescent in the host tissue for varying periods during the crop's growing season or in the harvested produce. Rapid conidiation, conidium germination, infection and mycelium growth occur under a wide range of microclimatic conditions, and pose severe disease management problems in crops all over the world. Moreover, the ability of *B. cinerea* to be active at temperatures as low as 0ºC makes it an important and challenging pathogen during storage and shipment. The limiting factor for epidemic outbreaks is usually the occurrence of the appropriate microclimatic conditions rather than the amount of inoculum. The comparable importance of different sorts of inoculum varies with the crop and cropping system. Conidia of *Botrytis* are generally regarded as short-lived in the field, and their season-to-season survival is limited. Sclerotia can survive adverse environmental conditions and, alongside mycelium, are regarded as important in winter survival. Interestingly, sclerotia of *B. squamosa* serve as the source of conidia, as well as apothecia-producing ascospores, in onion production. Mycelium that is protected by plant debris can survive adverse conditions, including warm, dry summers. In some cases, mycelium can also be carried on seeds.

The production of high numbers of conidia poses a long-lasting threat to susceptible hosts. The production, liberation and dispersal of conidia are ongoing processes that depend on microclimatic conditions. *Botrytis* releases its conidia mainly in dry air currents. It can be assumed that *Botrytis* inoculum is always present in the field, awaiting the appearance of suitable conditions for infection. It has been claimed that infection can occur only under conditions of high relative humidity. Indeed, the role of water films (and nutrients) in germination and infection has long been recognised. However, it is interesting that the pathogen has developed the ability to infect plants in the absence of visible water films. Penetration of leaves, stems, floral organs and wounds of various ages, and the consequences of ecological niche change in host crops, will be demonstrated. Detailed studies of the specific conditions that promote infection, disease development and the survival of inoculum, have provided the essential epidemiological information required for the design of control strategies. For example, cultural methods have been developed that increase aeration and drying of the plant canopy to reduce the risk of *Botrytis* epidemics. Rational warning systems, based on conditions highly conducive to conidia germination and host penetration, have been developed for some crop systems.

A combination of measures can be used to reduce the occurrence of *Botrytis*-induced problems in crops. Increased emphasis on alternative, non-chemical control, requires improved knowledge of *Botrytis* ecology and epidemiology in affected crops. Significant knowledge gaps exist concerning the microclimatic conditions required for infection during different phases of the host's life cycle. Furthermore, there is a need for fast and efficient methods for the detection of latent infections. Similarly, biotic and abiotic factors predisposing host tissue to infection by *Botrytis*, also merit further study.

04.2 ENVIRONMENTAL AND NUTRITIONAL FACTORS INFLUENCING THE ACTIVATION AND SPREAD OF QUIESCENT INFECTIONS OF *BOTRYTIS CINEREA* IN GRAPES

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Grape berries can become infected with *B. cinerea* at or shortly after flowering. These infections typically remain quiescent through harvest, although a proportion of them sometimes become active as berries approach maturity. Should this occur, affected berries serve as primary disease foci, from which secondary spread can occur during the pre-harvest period. Our objective was to investigate environmental and nutritional factors that might influence both quiescent infection activation and post-veraison secondary spread.

To examine the effect of atmospheric humidity, potted Chardonnay vines were inoculated with conidia during flowering and maintained outdoors, protected from rain; then, they were moved to a glasshouse or a humid growth chamber (>92% RH, 20oC) for various durations either at (i) veraison, or (ii) 10 days before harvest. Periods in the humid chamber at veraison never had an effect on disease activation. However, the incidence of clusters diseased at harvest increased linearly as the humid duration increased during the immediate pre-harvest period, i.e., from 27% following the 0-day treatment to 61% following the 4-day treatment ($r2 = 0.90$) in the first experiment, and from 11% after 0 days to 81% after 9 days ($r2 = 0.60$) in the second. High plant water status also increased both the activation and spread of infections. Potted Chardonnay vines were inoculated at bloom, grown in an outdoor shelter and irrigated as necessary until veraison, then assigned to one of two post-veraison irrigation treatments: (i) Wet (irrigated almost daily), and (ii) Dry (irrigated only upon signs of water stress). At harvest, the incidence of diseased clusters in these treatments was 56 and 17%, respectively ($P = 0.02$). However, following post-harvest incubation of clusters under high RH, there was no longer a significant difference between the two treatments (61 and 42% incidence, respectively; $P = 0.30$). In a related potted-plant experiment to examine soil water effects on secondary spread, 3 Pinot noir berries per cluster were injected with *B. cinerea* conidia at veraison, then vines were irrigated to maintain one of three different regimes: High, medium, or low soil water content (33.7, 23.2, and 13.9% SWC, respectively). At harvest, the disease had spread to an average of 15, 11, and 6 non-inoculated berries per cluster in these respective treatments. Finally, to examine the effects of berry nitrogen content on disease spread, field-grown Chardonnay vines were provided four weekly sprays of 0.8% urea beginning at veraison, and either 1 or 3 berries per cluster were injected at veraison with *B. cinerea* conidia. In both cases, disease spread was three times greater in urea-treated clusters than in those of control vines. Data from similar experiments designed to test the hypothesis that increased berry N levels promote the activation of quiescent infections, were statistically inconclusive but provided a consistent trend in support of the hypothesis.

Our results suggest that pre-harvest rains can promote bunch rot epidemics through a variety of mechanisms beyond de novo infections related to surface wetness, viz. activation of quiescent infections via increased atmospheric humidity and availability of water to the plant through the soil, and increased secondary spread due to these same higher soil moisture levels. Secondary spread is also favoured by increased berry N, as may be quiescent infection activation.

04.3 INTERDEPENDENT EFFECTS OF (MICRO) CLIMATE AND VEGETATIVE GROWTH ON GREY MOULD INCIDENCE IN GRAPEVINE

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In an experimental vineyard near Montpellier, France, various levels of grapevine growth and canopy development were generated over a period of three years (2004-06) by implementing different management policies, including irrigation and cover cropping. This experiment was implemented to further investigate the relationships between grey mould expression at harvest, and some of the major factors affecting the disease development, *i.e.* (micro) climate, fruit composition, vine vegetative and reproductive growth.

B. cinerea developed in all experimental plots in 2004 because climatic and microclimatic conditions were conducive to grey mould. Three main (micro) climatic variables were confirmed as favourable, particularly during the last twenty days before harvest, *i.e.* precipitation, duration of R.H. $>$ 90% in the canopy, and low potential evapo-transpiration. The cropping system influenced also significantly final expression of the disease in 2004. The perennial cover crop decreased the incidence by a factor of 4, compared with chemical weed control. However, in 2005 and 2006, under dry summer conditions, disease developed only in the most vigorous vines which were both irrigated and fertilised. Therefore, grey mould expression was influenced positively by a greater canopy development. Ten variables of shoot vigour or vine capacity were highly correlated with grey mould incidence ($P < 0.01$) and selected among 17 tested (e.g. total leaf no., leaf dry matter, total dry matter, leaf area per m of row and pruning weight). Lastly, three main variables were highlighted among those ($n = 15$) representative of yield components, cluster architecture and fruit composition, *i.e.* bunch weight, fruit dry matter, and titrated acidity.

These relationships were established in a context of interaction between (micro) climate and grapevine vegetative growth. Differences observed between 2004, 2005 and 2006, evidenced that unfavourable climatic conditions for disease development can be counterbalanced by conditions of high vine growth and associated canopy and cluster features.

04.4 INFECTION OF BLACKCURRANT FLOWERS AND FRUITS IN RELATION TO WEATHER CONDITIONS AND FRUIT AGE

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Most of the UK and European blackcurrant crop is processed for juice, and as with other fruit crops, there is pressure from consumers to reduce pesticide inputs to ensure residue free juice. Recent research has focused on developing integrated pest management systems. Little or no new research has been conducted on diseases, other than evaluation of new fungicides, for some 20 years. Effective integrated disease control is dependent on a good understanding of the biology and epidemiology of the diseases. *Botrytis* and leaf spot (*Drepanopezizza ribis*) are important diseases of blackcurrant that reduce yield and quality, and require frequent fungicide applications to achieve control. New varieties have been introduced with resistance to mildew, but not to *Botrytis* and leaf spot. *Botrytis* is often difficult to control in blackcurrants and results in considerable losses in yield and fruit quality. There are several gaps in knowledge, such as principal source of inoculum, spray timing, effect of fruit age on susceptibility to *Botrytis*, and importance of pre-harvest fungicide sprays. We have carried out research studies to investigate the effects of environmental conditions and fruit age on infection of fruit by *Botrytis*.

Plants of the varieties Baldwin and Ben Hope were inoculated with conidia of *Botrytis cinerea* at different stages: (1) full bloom, (2) late flowering, (3) early fruiting, (4) mid-fruiting and (5) near harvest. Inoculation of flowers was done under 15°C in controlled environmental cabinets; inoculated plants were subjected to a 24-hour wet period. Flower infection was assessed one week later. For inoculation on fruit, there were 16 treatments (four temperatures 10, 15, 20 and 25°C with four wet periods 4, 8, 12 and 24 h). Number of infected flowers, aborted young fruit, and fruit with disease symptoms, were assessed on each inoculated plant. Number of latent infection on fruit were assessed post-harvest after 10-14 days incubation inside Petri dishes at ambient temperature.

Nearly 75% of flowers were infected or dropped, following inoculation at the full bloom and late flowering stages. The two varieties differed very little in their susceptibility to *Botrytis* during the flowering stage, but differed significantly in their responses to *Botrytis* infection during the fruiting stage. On Baldwin, ca 50% of fruit were infected (aborted or dropped), compared to ca 10% on Ben Hope. For inoculation on green fruit, almost all fruit at harvest were latently infected, i.e. no visual symptoms (symptoms were only shown after post-harvest storage). Near the harvest, the incidence of latent infection on Ben Hope was significantly less than on Baldwin. Finally, temperature (10-25°C) and duration of wetness (4-24 h) appeared not to have systematic effects on the incidence of flower and fruit infection.

These results suggest that Baldwin needs to be protected from *Botrytis* from flowering to near harvest, whilst Ben Hope needs to be protected during the flowering period. Further validation is needed.

04.5 *THRIPS OBSCURATUS*, AS A VECTOR OF *BOTRYTIS CINEREA*, TO CAUSE GRAPE FLOWER INFECTION

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The epidemiology of Botrytis fruit rot in grapevines has been studied intensively, but there are still many uncertainties about the infection pathways, including the mechanisms of latent infection. Latent *B. cinerea* is common around berry receptacles, where there are deep crevices and small wounds that were created by senesce of stamens. In New Zealand, the endemic *Thrips obscuratus* are very numerous on grape flowers, and this study investigated their potential role as vectors of *B. cinerea* conidia.

A scanning electron microscope study found that *T. obscuratus* adults placed in a vial with a sporulating culture plug of *B. cinerea* for 24 h, had many conidia distributed over their cuticles. The conidia were most numerous on the wings, trapped between cilia, and attached to setae of the body, both near the mouthparts and the ovipositor. Light microscope studies of grape floral tissues that had been exposed to the contaminated thrips also found *B. cinerea* conidia within the receptacle areas of berry tissues and thrips eggs within the pedicel tissues.

The capacity of *T. obscuratus* to transfer *B. cinerea* conidia from infected to uninfected grape flowers was further demonstrated in an apparatus that had the 'source' and 'destination' inflorescences placed in two insect rearing cages, separated by a connecting tunnel, whose seals could be opened to provide access between cages. A healthy, thrips-free Riesling inflorescence from greenhouse grapevines was placed within the source insect cage. Five 1.5 cm sporulating *B. cinerea* culture plugs were then placed within the inflorescence, followed by 20 adult thrips. After 24 h, the sealed tunnel was opened to give the thrips access to the destination cage, which contained a healthy thrips-free grape inflorescence. After 3 days, the thrips were removed from the destination inflorescence and it was subjected to 7 days moist incubation. After six repetitions of the experiment, Botrytis rot covered 88% of the inflorescences. In a further nine repetitions of the experiment, the transfer of conidia was directly assessed after 3 days by washing each inflorescence and plating out the wash liquid onto a selective medium. The mean number of *B. cinerea* CFU was $8x10^2$ per inflorescence.

The feeding behaviour of adult *T. obscuratus* was also observed in the laboratory by continuous video recording. Five adult thrips were introduced to a small cluster of 3-5 grape flowers and their behaviour recorded for 10 minutes. Results showed that *T. obscuratus* adults spent most time walking (38.8%) and feeding (24.6%), with different time proportions on nectar (6.3%), pollen (5.7%) and stigma tissues (12.6%), as well as grooming (16.9%). The thrips' grooming activities were clearly likely to dislodge any attached conidia, and their behaviour on the different flower positions was considered likely to wound the tissues or to deposit conidia in the naturally susceptible areas.

This study provided evidence of the interaction between *T. obscuratus* and Botrytis rot. It also supports the many reports of grape latent infection and where it was found in berries.

04.6 LOCATION AND PERSISTENCE OF SYSTEMIC *BOTRYTIS CINEREA* IN LETTUCE, PRIMULA AND OTHER SPECIES

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Botrytis cinerea is known to grow extensively inside some hosts, without causing symptoms. By isolation of surface sterilised tissues on MEA or *Botrytis* selective medium, we have found *B. cinerea* in roots, stems or leaves of lettuce, wild and cultivated Primula, cyclamen, Poinsettia and dandelion. In Poinsettia, cyclamen and Primula, genetically identical isolates were found in the same plant, more often than expected from the frequencies of microsatellite alleles in the general populations within the crops sampled. More detailed study has been done in lettuce and Primula.

Three isolates of *B. cinerea* were transformed with the Green Fluorescent Protein gene by Agrobacterium mediated transformation. Hybrid Primula seedlings (six leaf stage) and flowers were inoculated with the transformed pathogen. GFP expressing *B. cinerea* was isolated from symptomless, non-inoculated leaves and stems of plants which had been inoculated as seedlings. It was also isolated from ovaries of flower inoculated plants, the mature seeds from these flowers, and from roots and leaves of seedlings grown from these. Infection of leaves was 80% and 70% for the two isolates tested after 4 weeks, and seed infection was 32% and 19%. Visualisation was difficult because the host autofluoresced brightly.

In lettuce, seed grown in a spore free airflow, gave rise to infected plants. Surface sterilisation of seed before planting reduced but did not eliminate infection. Infection after one month was mainly restricted to the roots, but spread to stems and leaves later. Hyphae in non-symptomatically infected lettuce were visualised by immunostaining with monoclonal antibody BC12. They formed a sparse network in the cortex of roots and stems and throughout leaves.

According to these findings, internal infection of several species can occur over long periods without causing symptoms, but with regular colonisation of newly developing tissues. Hyphae are internal, and not associated with vascular tissue. Infection of seed can occur from infected flowers in lettuce and Primula, and the resulting internally infected seed give rise to long-term infected plants.

04.7 INOCULUM ECOLOGY OF *BOTRYTIS CINEREA* IN ROOIBOS (*ASPALATHUS LINEARIS*) NURSERIES IN THE WESTERN CAPE PROVINCE OF SOUTH AFRICA

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Grey mould, caused by *Botrytis cinerea*, is the most important foliar disease of rooibos seedlings in nurseries. Although the disease is primarily controlled through fungicide applications, improvement of cultural control measures is required to reduce fungicide usage and lessen dependence on chemical control measures. The effective use of cultural control measures require knowledge of the inoculum sources and dispersal of the pathogen. Therefore, the aim of the study was to identify the primary inoculum sources of the pathogen by investigating the inoculum ecology of *B. cinerea* in rooibos nurseries, which included monitoring of dicarboximide resistance in *B. cinerea* populations. A survey was conducted in four nurseries in the Clanwilliam region across two growing seasons (March to July 2003 and 2004). Levels of air-borne inoculum of *B. cinerea* were monitored on a monthly basis within and around (up to 20 m from the edge of the nursery) the nurseries using spore traps. The incidence of plant material infected by the pathogen within and around the nurseries was also determined by sampling and analysing plant material (rooibos seedlings, weeds and natural vegetation) and organic debris within and around nurseries. Low numbers of *B. cinerea* colonies were obtained from spore traps both within and outside the nurseries, suggesting that air-borne inoculum was low. The incidence of plant material and organic debris yielding *B. cinerea* was higher outside the nurseries than within the nurseries, indicating the importance of these materials as potential sources of inoculum. *Botrytis cinerea* isolates that were obtained from air and infected plant material were assessed for resistance to a dicarboximide fungicide (iprodione) at 1 and 3 μg/ml active ingredient. The incidence of dicarboximide-resistant isolates at the start of the growing season within and outside the nurseries was 21.94%. However, as the season progressed, the incidence of dicarboximide-resistant isolates decreased to 14.37% during May, but increased again to 23.20% during July. A relatively high percentage (17.53%) of isolates collected outside the nurseries was found to be dicarboximide-resistant. Two of the nurseries had a significant higher incidence of resistant isolates on plant material collected inside, than on plant material collected outside the nursery. The incidence of dicarboximide resistant *B. cinerea* isolates collected from air samples within and outside of the nurseries were similar, suggesting migration of spores accross nursery borders. Since patterns of air-borne inoculum and dicarboximide resistance levels observed in this study confirmed reports of the local dispersal of *B. cinerea*, the removal of possible hosts outside the nurseries could aid in the management of grey mould in rooibos nurseries. The study highlighted the importance of organic debris, weeds and natural vegetation in the survival and dispersal of dicarboximide-resistant *Botrytis* isolates. The study yielded valuable information for improving cultural control measures of grey mould in rooibos nurseries, which is of utmost importance due to the large emphasis that is currently placed on organic production in all agricultural crops.

04.8 PREDICTING THE SEASONAL RISK OF BOTRYTIS BUNCH ROT IN WINE GRAPES

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This presentation is a preliminary report of a new collaboration between Australian and New Zealand researchers for developing a robust and accurately calibrated model to predict the seasonal risk of Botrytis bunch rot (BBR) in wine grapes. The work is supported by the Australian Grape and Wine Research and Development Corporation and New Zealand Wine Growers Inc.

Botrytis cinerea is the major cause of bunch rot (grey mould) of wine grapes in cool climates. Berries begin to rot when latent infections at flowering become active during ripening and/or as a result of direct infection of fruit by inoculum from decaying trash within the grapevine canopy or surrounding area. Berry to berry spread of the pathogen within the grape cluster can be a major determinant of bunch rot severity, along with a prolonged ripening period that allows further expression of the disease. In Australia and New Zealand, vineyard managers are at the mercy of weather leading up to harvest, because there are severe limitations on fungicides available for late-season management. Evaluation of new chemicals or integrated measures across sites and seasons, such as manipulation of canopy microclimate and harvest date, has been hampered by the tendency to assess disease severity only at harvest. We report the use of standardised epidemic descriptors that allow such comparisons, and a process for identifying key crop and environmental factors that drive *Botrytis* epidemics.

In 2006/07, randomised complete block experiments were established at three sites in the Yarra Valley of Victoria (Vic.), two sites in the Coal River Valley of Tasmania (Tas.), one site in Hawke's Bay, two sites in Marlborough and one site in south Auckland in New Zealand. Treatments were applied variously in trials to generate *Botrytis* epidemics that differed in the time of onset and/or rate of progression. Each trial included protective fungicides applied in 'windows of time' such as early, mid or late season, and integrated control measures, including removal of floral trash at fruit set, shoot or bunch thinning, leaf plucking, vine irrigation or nutrition. The incidence of latent infection by *B. cinerea* was assessed at pre-bunch closure. BBR incidence and severity were assessed at 1-2 week intervals from veraison to harvest for development of disease progress curves.

Analyses of disease progression in relation to weather and crop variables will be reported. The use of a large number of site-years of data collected in a standardised way will enable development of a reliable model to predict the seasonal risk of BBR. This project is expected to lead to a prototype decision support system that can be developed into software for web-based access by vineyard managers or some other means of information delivery. Ready access to such information will enable integrated management and harvest date to be optimised.

04.9 EVALUATION OF THE BROOME *BOTRYTIS* MODEL FOR THE FORECAST OF *BOTRYTIS* INFECTION RISK IN TABLE GRAPES

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Botrytis rot (*Botrytis cinerea*) is the most important field and post-harvest disease of table grapes in Victoria, Australia. Relationships between the risk of *Botrytis* infection in different vineyards, and the incidence of *Botrytis* in grapes just before harvest and after cool storage, were investigated.

Trials on the production and post-harvest storage of Thompson Seedless grapes (Vitis vinifera cv. Sultana) were conducted in six commercial vineyards in the Sunraysia district of north west Victoria, Australia over two seasons, 2005/2006 and 2006/2007. Each vineyard had different management and site specifications. At each trial site, weather data (including temperature, relative humidity, leaf wetness within vine canopies) were recorded by a networked automated weather station. The severity and duration of *Botrytis* infection periods (risk of *Botrytis* infection) at each site, based on leaf wetness duration and average temperature during wetness events, were determined using the Broome *Botrytis* model (Broome *et al.*, 1995). The incidence of *Botrytis* latent infection in berries was assessed just before harvest, and the incidence of berries with Botrytis rot was assessed after cool storage for 6, 8 and 10 weeks, in the absence of sulphur fumigation.

The real time weather data collected in each vineyard were successfully processed through the Broome model to develop daily estimates of the risk of *Botrytis* infection. The total number of days when the risk of infection was moderate to high for all trial sites was substantially higher in 2005/2006 than in 2006/2007. *Botrytis* incidence in berries (i.e. latent infections just before harvest and/or berry rots after 6-10 weeks of cool storage) also varied substantially between sites each season, especially in 2005/2006. In the latter season, weather conditions in the vineyards also favoured berry infection by other fungi such as *Aspergillus*, *Penicillium* and *Rhizopus spp.*, that also produced rots in the stored grapes.

Differences in vineyard management (e.g. vine trellising, pruning, irrigation, fungicide spray programmes and/or prevalence of trash on the vineyard floor that produced *Botrytis* inoculum) contributed to differences in *Botrytis* incidence between sites each season. Differences in *Botrytis* incidence were more strongly related to differences between sites than to differences between numbers of days, with a moderate to high risk of *Botrytis* infection or numbers of days with berry protection against *Botrytis* infection provided by fungicide treatments each season. Further field and storage studies are needed to clarify the effects of different site-related factors on the incidence of *Botrytis* in grapes at harvest and after cool storage.

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Broome, J.C., English, J.T., Marois, J.J., Latorre, B.A. & Aviles, J.C. (1995). Development of an infection model for *Botrytis* bunch rot of grapes based on wetness duration and temperature. *Phytopathology* 85: 97-102.

04.10 INVESTIGATIONS ON THE COMPETITIVE COLONISATION OF *BOTRYTIS CINEREA* AND *PENICILLIUM EXPANSUM* ON GRAPES

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Botrytis cinerea is the most important bunch rot pathogen on grapes in German vine-growing areas, however, in the last ten years, increasing problems with secondary bunch rot invaders like Pencillium expansum or sour rot occurred in many regions. The reasons for the increasing problems are not completely understood so far.

In laboratory and field trials, the competitive colonisation of *B. cinerea* and *P. expansum* has been investigated under different conditions. At temperatures between 15° C and 30° C, *B. cinerea* and *P. expansum* were able to colonise wounded berries of the variety Riesling at the same rate, if they have been placed separately on the wounds. Optimal temperatures for the disease severity of the two fungi ranged between 20° C and 25° C. The development of disease severity of both fungi in variants with mixed inoculum run nearly in parallel at most temperatures until the surface of the berries was completely colonised. Then *P. expansum* developed stronger and was able to overgrow *B. cinerea* at any temperature.

P. expansum was unable to colonise grape berries with intact berry skin. In trials with sequential inoculations of the two fungi on intact and wounded berries, *P. expansum* was able to profit in its development, after *B. cinerea* had affected the substrate. *B. cinerea* seems to create the entrance for *P. expansum* through the berry skin. In trials with berries of the variety Pinot Blanc, *P. expansum* developed even stronger disease severities in mixtures with *B. cinerea* compared to the disease severity on berries where the *Penicillium* inoculum was placed on the wounds individually.

In the laboratory, the treatment of wounded berries with different botryticides (Fenhexamid, Boscalid, Cyprodinil + Fludioxonil) led in most cases to a reduced disease severity of *B. cinerea*. This could be observed both on berries that had been inoculated only with *B. cinerea*, as well as that had been inoculated with a mixture of *B. cinerea* and *P. expansum*. In contrast, the development of *P. expansum* was hardly reduced by the fungicides. Simply four days after the inoculation for some of the compounds, a small inhibitory effect on the growth of *Penicillium* could be detected. Even in the variants with mixed inoculum, *P. expansum* was able to take profit from the inhibition of *B. cinerea* and reached stronger disease severities at the same time than in the untreated check. In the vineyard, the results of the laboratory could only partly be confirmed. While *B. cinerea* was reduced in the field by the fungicide treatments, *P. expansum* was not able to take profit from the treatments compared to the laboratory study.

The current data indicate that a consequent protection strategy against *B. cinerea* should also reduce the development of *P. expansum* in the field. More attention should be payed to all options that are helpful to reduce the number of wounds.

4. ECOLOGY AND EPIDEMIOLOGY

Posters

P4.1. A.P. Rajaguru, A.E.vandenBon and **Michael W. Shaw**

Population differentiation of *Botrytis cinerea* by host and locality

P4.2. A.E. Vanden Bon, E. Karapatzak, T.M. O'Neill, K. Walsh and **Michael W. Shaw**

Types of infection of ornamental hosts by *Botrytis cinerea*

P4.3. **Aminath Shafia** and Michael W. Shaw

Behaviour of latent infection of *Botrytis cinerea* in lettuce (*Lactuca sativa*)

P4.4. Véronique Decognet, **Marc Bardin**, Yannie Trottin-Caudal and Philippe Nicot

Genetic analysis reveals a rapid evolution of population structures of *Botrytis cinerea* after the introduction of isolates in tomato glasshouses

P4.5. **Vivienne Gepp**, Julia Rebellato, Angelo Marveggio, Elisa Silvera, Elsa Perdomo, Oscar Bentancur, Luis Curbelo and Santiago Contarin

Botrytis bunch rot epidemiology in Tannat grapevines in southern Uruguay

P4.6. **Marc Fermaud**, C. Deytieux, J. Roudet, V. Veyssière, B. Donèche and L. Geny

Assessment of ontogenic resistance to *Botrytis cinerea* in grape berries and effect of water availability

P4.1 POPULATION DIFFERENTIATION OF *BOTRYTIS CINEREA* BY HOST AND LOCALITY

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Botrytis cinerea is known to grow extensively inside some hosts without causing symptoms; in others, such as grapes and strawberry, restricted quiescent growth occurs. Growth throughout a symptomless plant implies a very intimate evasion of host defences, which makes the wide host range of the pathogen surprising.

We have characterised approximately 300 isolates of *B. cinerea*, using the nine microsatellite loci published by Fournier *et al.* (2002). Systematic collections of isolates were made from non-symptomatic *Rubus fruticosus* (blackberry) and *Fragaria* x *ananassae* (strawberry) fruits, and from roots or leaves of wild *Primula vulgaris* (primrose) and *Taraxacum agg.* (dandelion) in Reading, Brighton and Bath, separated by 90 km or more. Isolates recovered from a *Primula* x *polyantha* crop at Reading were also examined.

Isolates were usually haploid at all loci. No identical haplotypes were found in the populations from field/wild hosts. Identical haplotypes were rare in the protected crop.

In the wild non-fruit species, incidence of non-symptomatic infection was very variable, both geographically and between years; in dandelion it was much more common in the roots than the leaves. In 2005, incidence in Primrose was 21% at Brighton, but zero at Bath; incidence in dandelion in the same year was 40% at Reading, about 14% in Brighton and Bath.

In the genetic data, three clear host groupings were significantly ($P < 0.001$) separated by CVA: strawberry, blackberry and primrose/dandelion isolates. The separation between strawberry and blackberry is not confounded with location because equal sized samples were taken from each locality. Location differences, looking either at data pooled over species or within a species, were also significant. All three sites differed in allele frequencies at several loci.

According to these findings, the *B. cinerea* population is very variable, with individual isolates apparently best adapted to growth on particular host species. This presumably reflects a need for particular alleles at functional loci in disequilibrium with the microsatellite loci in order to best attack a particular host. It also means that management needs to consider species individually, according to the main sources of inoculum for that host. It seems extremely surprising that isolates from blackberry and strawberry fruits should be so distinct, but this may reflect the need for distinctive adaptations during the early stages of flower infection, leading to fruit infection. The similarity between the dandelion and primrose isolates suggests that differentiation must be functional rather than based on a co-evolutionary relationship.

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Fournier, E., Giraud, T., Loiseau, A., Vautrin, D., Estoup, A., Solignac, M., Cornuet, J.M. & Brygoo, Y. (2002). Characterization of nine polymorphic microsatellite loci in the fungus *Botrytis cinerea* (Ascomycota). *Molecular Ecology Notes* 2: 253-255.

P4.2 TYPES OF INFECTION OF ORNAMENTAL HOSTS BY *BOTRYTIS CINEREA*

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Botrytis cinerea is known to grow extensively inside some hosts without causing symptoms; restricted quiescent growth occurs in others. This may influence management strategies, such as fungicide timing in commercial crops.

Material for this study came from two sources. Collections were made at several times during the growing cycle from roots, stems and leaves of non-symptomatic poinsettia (*Euphorbia pulcherrima*) grown from imported cuttings at two commercial nurseries in the UK; from roots, corms, leaves and flowers of seed-grown crops of *Cyclamen persicum* (cyclamen) at Reading University and one of the commercial nurseries; and from leaves of a *Primula* x *polyantha* crop at Reading. Approximately 450 isolates of *B. cinerea* were genotyped at using nine microsatellite loci (Fournier *et al.*, 2002).

Isolates were usually haploid at all loci. Identical haplotypes were rare except when multiple isolations were made from one host plant. Results were analysed by canonical variate analysis with randomisation tests, Kolmogorov-Smirnoff tests and AMOVA. Populations isolated from different hosts grown on the same nursery were significantly distinguishable. Populations on the same host species but from different nurseries were also distinguishable.

Systemic infection was common in cyclamen, evidenced both by recovery of *B. cinerea* from the corm interior and multiple isolations of the same haplotype from the same plant. Seed of 14 heads were examined after symptomatic disease had been apparent on the flowers. Two had a very high proportion of infected seed; the others had little. In each head all seed harboured the same haplotype of *B. cinerea*. Primula isolates from crops of cvs. Quantum and Forza grown side by side in the same greenhouses were significantly differentiated, suggesting either a continuing importance of seedborne infection or very specific host-pathogen matching. Poinsettia cuttings had a moderate level of symptomless infection when received from overseas suppliers. As infection accumulated through the season, the population composition changed substantially. The air-borne spores trapped around a poinsettia crop were similar to, but still significantly distinguishable from the population of *B. cinerea* in the crop. No *B. cinerea* could be found in non-symptomatic parts of gerbera plants, even after sporulating infection had appeared on leaves and been removed.

We hypothesise tentatively that various types of *Botrytis*/host species relationships exist. Some, like gerbera, are affected mostly by spatially limited infections from air-borne spores. Others, including poinsettia, harbour spatially extended non-symptomatic infections, either from the mother plant or from air-borne spores. A final group, including lettuce, cyclamen and primula, are infected systemically by air-borne spores, but also may have significant levels of seed-borne infection at maturity.

REFERENCES:

Fournier, E., Giraud, T., Loiseau, A., Vautrin, D., Estoup, A., Solignac, M., Cornuet, J.M. & Brygoo, Y. (2002). Characterization of nine polymorphic microsatellite loci in the fungus *Botrytis cinerea* (Ascomycota). *Molecular Ecology Notes* 2: 253-255.

P4.3 BEHAVIOUR OF LATENT INFECTION OF *BOTRYTIS CINEREA* IN LETTUCE (*LACTUCA SATIVA*)

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The behaviour of the fungal pathogen *Botrytis cinerea*, which causes grey mould disease of lettuce, was studied. It has been previously shown that *B. cinerea* can grow throughout roots, stems and leaves of lettuce plants, starting from seed or seedling infection, without symptoms ever appearing on the growing plants, and that the infection is commonest in roots or stems, not leaves.

Lettuce seedlings (at 6 leaves stage) were inoculated with *B. cinerea* ES13 (previously isolated from lettuce) as a spore suspension (106 spores/ml) sprayed or as dry spores brushed on the leaves. *Trichoderma harzianum T-*39 was inoculated by placing plugs of an agar culture in the growing medium at transplant (at 4 leaves stage). Leaf, stem and root samples selected randomly from the plants at harvest were incubated on *Botrytis* Selective Media to investigate the occurrence of the pathogen within the tissues as latent infection.

Inoculation of plants with *B. cinerea* alone or in combination with the biocontrol agent *T. harzianum* T-39 did not produce aggressive symptomatic infection on the inoculated leaves or subsequently. At harvest, no plants showed any visual symptoms of *B. cinerea*. There was no correlation between dry weight and the number of successful isolations of *B. cinerea* made from a plant. However, the dry weight at harvest of shoots of plants inoculated with *B. cinerea* was reduced by about 20% ($P < 0.001$). Inoculation of the plants with *T. harizianum* T-39 partially reversed this effect of the *B. cinerea* inoculation, but inoculation with *T. harzianum* alone did not increase dry weight.

Despite this substantial physiological effect, *B. cinerea* was isolated from all organs of plants at equal frequency, whether inoculated or not with the pathogen as seedlings. We are genotyping isolates to determine whether this was the inoculated isolate. The frequency of isolation of *B. cinerea* was also unaffected by inoculation with *T. harzianum* T-39. However, *Trichoderma* spp. were isolated more frequently from plants inoculated with *T. harzianum* T-39.

According to these findings, latent *B. cinerea* within lettuce plant tissues, whether in leaf, stem and roots, does not affect plant growth. However, unsuccessful infection by spores triggers changes in the plants which reduce growth, without preventing the continued growth of latent infection within the plant. This latent quiescent or systemic infection may be a source of inoculum at post-harvest stages of the crop.

P4.4 GENETIC ANALYSIS REVEALS A RAPID EVOLUTION OF POPULATION STRUCTURES OF *BOTRYTIS CINEREA* AFTER THE INTRODUCTION OF ISOLATES IN TOMATO GLASSHOUSES

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Botrytis cinerea can rapidly produce massive amounts of inoculum on diseased plants. In addition to the endogenous inoculum, the air-borne spora of *Botrytis* over a canopy may also include exogenous inoculum carried from a variety of hosts in open fields or even greenhouses. The relative proportion of both types of inoculum may influence the efficacy of control methods and the impact of selective pressures resulting from their implementation.

A trial was set up to quantify the impact of endogenous inoculum on disease development and on the population structure of *B. cinerea* in four compartments of an experimental tomato glasshouse of CTIFL (Balandran, France). Plants were grown in quasi-commercial conditions. Isolates were collected from the air spora 4 days before and 14 days after inoculation, of 6 plants per compartment, with one of two reference strains (differing in their microsatellite profile and aggressiveness on tomato). Disease development was monitored and isolates were collected on stem lesions 60 days after inoculation. All the collected isolates were analysed for their diversity according to 9 neutral microsatellite markers.

Among 80 isolates collected in the air spora prior to inoculation (while all plants were healthy), none had microsatellite profiles similar to either of the reference strains. Following inoculation, lesions developed and sporulation was observed on all inoculation points. Within 14 days, microsatellite profiles identical to those of either introduced strain represented 66% of the 353 samples characterised from the air spora. The other multilocus genotypes detected from air-borne spora were very diverse. This extremely high level of diversity confirms earlier data obtained with *B. cinerea* populations collected on different hosts and suggests that the entry of spores into the glasshouse (although these structures are usually considered as confined) is probably an important phenomenon which occurred regularly during the growing season.

The disease spread steadily to non-inoculated plants and incidence reached an average of 3-7 lesions per plant by 90 days after inoculation. Among 240 isolates collected from stem lesions at 60 days after inoculation, 58% and 33% had microsatellite profiles similar to the aggressive and to the less aggressive reference strains, respectively. This suggests that the displacement of the initially dominant air-borne population of *Botrytis* was concomitant with its negligible contribution to the epidemic on tomatoes.

These results are compatible with the hypothesis of a polycyclic development of *Botrytis* epidemics in tomato greenhouses and illustrate the importance of endogenous inoculum in this growing system. Furthermore, they raise the question of possible host specificity within *Botrytis cinerea* in naturally occurring epidemics, as few multilocus genotypes were shared between air-borne and plant populations.

P4.5 BOTRYTIS BUNCH ROT EPIDEMIOLOGY IN TANNAT GRAPEVINES IN SOUTHERN URUGUAY

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In spite of being a very difficult disease to manage in vineyards in the variable Uruguayan climate, there has been little research in Botrytis bunch rot epidemiology, especially in the Tannat variety, characteristic of this country.

In order to determine the factors affecting the development of bunch rot in the Tannat variety grapes in Southern Uruguay, latent infections and rot intensity were monitored in commercial vineyards during the 2005-06 and 2006-07 seasons. Possible primary inoculum sources and midrow vegetation at flowering were evaluated in 2005, and grape berry volume was estimated from veraison to harvest in the second season.

In spring, *B. cinerea* was detected in remains of grape bunches and very occasionally on branches or dry leaves, when these were held 10 days at approximately 100% relative humidity. It was also found sporulating, after incubation in humid chambers, on senescent white clover (*Trifolium repens*) flowers and on *Lamium amplexicaule* and *Conyza bonaeriensis* plants growing under the rows. The percentage of symptomless berries that yielded *B. cinerea* when incubated in humid chambers increased from flowering to the end of December, then decreased to near zero in mid January (pre-veraison) and later increased steadily to 18% (0 - 71%) near harvest in 2006 and 12.4% (0.8 - 31%) in 2007. Bunch rot increased exponentially from a month after veraison to harvest. At harvest, incidence was 24% (0 - 55%) of bunches with rot in 2006 and 17% (0 - 39%) in 2007. Severity was 3.6% (0 - 9.6%) and 1% (0 - 3.2%), respectively. There was slightly more rot in 2005-06 than in 2006-07, although rainfall, relative humidity and cloudiness were higher during grape ripening in the second season. Vineyards with greater fresh weight of foliage of understory vegetation at flowering also had more pre-harvest bunch rot. Berry volume differed most between vineyards at the beginning of the evaluation period, around veraison, but the relationship with bunch rot at harvest was not clear.

P4.6 ASSESSMENT OF ONTOGENIC RESISTANCE TO *BOTRYTIS CINEREA* IN GRAPE BERRIES AND EFFECT OF WATER AVAILABILITY

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Management of grapevine Botrytis bunch rot and optimised use of fungicides should be based on the experimental knowledge of the duration and dynamics of fruit susceptibility to infection by the pathogen. The first objective is to show, under controlled conditions, quantitative effects of the berry stage of development, from bunch closure to maturity and over-maturity, upon the development of the pathogen. Veraison is usually considered as the development stage at which fruit become susceptible. The existence and characterisation of such a threshold effect have been examined thoroughly. The second objective is to further investigate the effect of water availability, measured by water activity (a_w) on *in vitro* growth of *B. cinerea*. Different *transposa* strains (within Group II) were tested. Furthermore, because exosmosis increases during ripening and modifies the availability of water on the grape skin, free water was assessed at the berry surface by measuring the a_w value at different stages of berry development. In all the experiments, the susceptible cultivar Sauvignon Blanc was used.

A laboratory bioassay was developed to assess ontogenic resistance in relation to the development of the pathogen. Artificial inoculations of undamaged berries were performed using different *transposa* strains (Group II). The following variables were measured as representative of some of the major elementary steps during the infectious process: incidence of infected berries, severity of rot expansion at the berry surface, and intensity of sporulation. For the first two epidemiological variables, significant differences in the pathogen development were demonstrated according to the berry stage. When testing the *Botrytis* strains on different solid PDA media modified for water activity, the highest radial growth was obtained for a_w of *ca*. 0.99. In contrast, a significantly reduced and very low growth was noted for a low value of a_w (*ca*. 0.93). Some differences on PDA media were also observed in morphology (mycelial growth and sporulation) between the different strains tested. These differences will be analysed in relation to the strain aggressiveness as tested on grape berry.

The potential of these results for better understanding the epidemic development of Botrytis bunch rot in vineyards and for use in disease management programmes, will be discussed.

5. DISEASE MANAGEMENT

A wide range of strategies are available for control of diseases caused by *Botrytis* spp. These include chemical applications, cultural management, biological control and host resistance. Chemical control is a major strategy in management of many *Botrytis* diseases. In time, the arsenal of fungicides available is constantly subject to changes. Therefore, the search for new and better fungicides proceeds continuously. The keynote lecture on chemical control by Maarten de Waard will review fungal drug transporters known to play an important role in sensitivity and resistance to fungicides and on inhibitors of these drug transporters, described in literature as modulators. Some modulators display disease control while they are almost not fungitoxic *in vitro.* This may be ascribed to inhibition of drug transporters that act as pathogenicity factors. Hence, modulators may act as leads in the discovery of new disease control products. In terms of biological control, there are many publications reporting successful control of *Botrytis* spp. in the laboratory and in controlled environments, but very few are commercialised. Where effective biological controls are available, industry uptake has often been slow and we examine some of the barriers to greater use by producers. Despite these challenges, most recent research is very encouraging as scientists identify unique combinations of biological controls and natural products that provide *Botrytis* disease control equal to that of conventional fungicides. This session will focus on new developments in chemical and biological disease control.

Oral Session 1

Keynote + Chairperson:

O5.1. **Maarten de Waard**, Aurelie Huser Ramin Roohparvar, Henk-jan Schoonbeek and Lute-Harm Zwiers

Developments in indirect disease control of plant pathogens

O5.2. **Matthias Kretschmer**, Michaela Leroch, Melanie Wiwiorra, Anne-Sophie Walker and Matthias Hahn

Multiple fungicide resistance in *B. cinerea* field strains is correlated with over-expression of efflux transport proteins

O5.3. **Rita Milvia De Miccolis Angelini**, Caterina Rotolo, Wassim Habib, Stefania Pollastro and Franco Faretra

Single nucleotide polymorphisms (SNPs) in *Botryotinia fuckeliana* genes involved in fungicide resistance

O5.4. Chang-Lin Xiao

Resistance of *Botrytis cinerea* to thiabendazole and alternatives for management of post-harvest grey mould in apple in Washington State of USA

O5.5. **Sandra C. Lamprecht**, Johan A. Brand, Chris F.J. Spies and Paul H. Fourie

Integrated management of Botrytis grey mould of rooibos (*Aspalathus linearis*) seedlings in nurseries

O5.6. Maria Lodovica Gullino

***Botrytis* management in vegetable crops: challenges and perspectives**

Oral Session 2

Keynote + Chairperson:

O9.1. **Philip Elmer**, Tony Reglinski, Peter Wood, Kirstin Wurms, Frank Parry, Jonathan Saunders, Stephen Hoyte, Annette Ah Chee, Mike Spiers, Joseph Taylor, Ron Marsden, Tracy Taylor and Danyang Ying

Practical integration of biological control strategies for Botrytis bunch rot management in vineyards

O9.2. Luciano V. Cota, **Luiz A. Maffia** and Eduardo S. G. Mizubuti

Clonostachys rosea* in strawberry leaves: autoecology and antagonism to *Botrytis cinerea

O9.3. **Ilaria Pertot**, Kanak Bala, Denis Bassetti and Yigal Elad

Efficacy of medicinal plant extracts for the control of *Botrytis cinerea*

O9.4. Richard Finkers, Adriaan W. van Heusden, **Jan A.L. van Kan**, Paul Maris and Pim Lindhout

Resistance to *Botrytis cinerea* in a population of *Solanum habrochaites* introgression lines

O9.5. **Rudi Aerts**, Liesbeth Vogels, Sara Gielen, Bjorn Seels and Kathleen Heyens

Sustainable control of *Botrytis* by sanitation and cultural measures

O9.6. Lena Shpialter, **Yigal Elad**, Dalia Rav David, Irit Dori, Liana Ganot, David Shmuel, Eli Matan and Yoel Messika

Cultural methods for grey mould (*Botrytis cinerea*) management in lisianthus

05.1 DEVELOPMENTS IN INDIRECT DISEASE CONTROL OF PLANT PATHOGENS

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Control of *Botrytis cinerea* in a wide range of crops by fungicides is a major disease management strategy. Search for new fungicides remains urgent since many of the older chemicals loose their efficacy because of resistance development or loss of registration. The main part of the presentation will focus on compounds that provide indirect disease control activity by inhibition of fungal ATP-binding cassette (ABC) transporters, important for virulence of plant pathogens.

The secretion of plant defence compounds from fungal plasma membranes, mediated by ABC transporters, can play an important role in virulence of plant pathogenic fungi, by preventing the intercellular accumulation of plant defence compounds to potentially toxic concentrations. As a result, plant pathogens may survive plant tissue containing plant defence compounds. Such a protection mechanism implies that compounds, capable of inhibition of the activity of ABC transporters (modulators), may increase fungal sensitivity to plant defence compounds, and that these inhibitors may display indirect disease control based on increased efficacy of natural defence reactions in host plants against plant pathogens. We have tested a number of such compounds from different chemical classes for indirect disease control of *Mycosphaerella graminicola* on wheat seedlings, and of *B. cinerea* on grapevine leaves.

Most of the tested alkaloids, flavanones, opiods, phenazines, polyphenols and tricyclic polyaromatic compounds are known as medical drugs or secondary metabolites of plants. The vast majority of these compounds do not possess *in vitro* toxicity to growth of *M. graminicola*. However, in preventative and curative foliar spray experiments, many of the compounds reduced disease development on wheat seedlings. Compounds with the highest activity were amitriptyline (a tricyclic polyaromatic compound), loperamide (an opiod), and promazine (a phenazine). These compounds also increased the ABC transporter mediated accumulation of the azole fungicide, cyproconazole, in biomass of *M. graminicola*. Hence, the results suggest that the observed disease control activity of the modulators can be based on indirect disease control.

The same set of modulators was also tested for their potency to control *B. cinerea* on grapevine leaves. Comparable to the situation in *M. graminicola*, modulators such as amitriptyline, loperamide and phenazines, were able to control grey mould. The ABC transporter, BcatrB, is known to provide protection against the grapevine phytoalexin resveratrol. Preliminary results indicate that the modulators and resveratrol display synergistic toxicity to *in vitro* growth of *B. cinerea*, and that resveratrol can inhibit ABC transporter activity of the fungicide fludioxonil, mediated by BcatrB. These results suggest that indirect disease control of grey mould on grapevine leaves by modulators, can be the results of increased accumulation of resveratrol in fungal biomass during pathogenesis.

We propose that modulators of ABC transporter can be used as lead compounds for further development of compounds with optimised indirect disease control activity.

05.2 MULTIPLE FUNGICIDE RESISTANCE IN *B. CINEREA* FIELD STRAINS IS CORRELATED WITH OVEREXPRESSION OF EFFLUX TRANSPORT PROTEINS

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In French and German vinegrowing areas, increasing populations of *B. cinerea* strains with a low-level resistance to chemically different fungicides (refered to as multidrug resistance, MDR) have been observed (see accompanying poster by Kretschmer *et al.*). Phenotypic analyses and preliminary genetic studies indicated that three types of MDR strains occur, which seem to be determined by mutations in only two genes. Uptake experiments with ^{14}C-labeled fungicides have provided evidence for a correlation of the MDR phenotype with increased activity of efflux transport. In order to proof a causal link between MDR phenotype and increased efflux transport, and to understand its molecular basis, different experimental approaches were performed.

1. From the genome sequence of *B. cinerea*, PCR fragments representing candidate genes encoding putative MDR-type ABC- and MFS-transporters were amplified and used for the generation of macroarray filters. The filters were hybridised to ^{32}P-labeled cDNAs from susceptible as well as MDR field isolates. In all MDR strains tested, one or few genes encoding ABC- and MFS-transporters were significantly overexpressed relative to susceptible strains. In MDR1 strains, the gene encoding ABC transporter AtrKI2 was most strongly expressed, along with increased expression of four other efflux transporter genes. In MDR2 strains, the gene encoding MFS transporter Mfs19 was induced. MDR3 strains, in most cases, showed increased expression of both sets of genes.
2. Sequencing of the promoter regions of *atrKI2* and *mfs19* did not reveal significant differences between MDR1, MDR2 and susceptible strains. This indicates that overexpression of *atrKI2* and *mfs19* in MDR strains is not due to promoter mutations, but rather to mutations in genes encoding regulatory proteins, e.g. transcription factors.
3. Two MDR1 strains were used for knock-out mutagenesis of the *atrKI2* genes. Mutant strains were analysed for their resistance to various fungicides. Indeed, the *atrKI2* knock-out mutants had lost part of the MDR1-specific resistance to some fungicides. Detailed phenotypic characterisation of the mutants, together with the expression studies described below, will reveal the specificity and the role in MDR of the AtrKI2 ABC transporter.
4. *atrkI2* and *mfs19* cDNAs have been cloned into an expression vector and transformed into a yeast mutant that lacks multiple ABC-transporter genes, and which is hypersusceptible to many antifungal drugs. The response of the transgenic yeast cells expressing the AtrKI2 and Mfs19 transporters to various fungicides is currently tested. Because we have found that MDR1 strains show increased resistance to plant-derived defense compounds (e.g. eugenol), we will also test the transporters for their activity on various non-fungicide compounds.

05.3 SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS) IN *BOTRYOTINIA FUCKELIANA* GENES INVOLVED IN FUNGICIDE RESISTANCE

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The high risk of acquired resistance to fungicides in *Botryotinia fuckeliana* (*Botrytis cinerea* anamorph), poses a serious threat to the chemical control of grey mould, although several fungicides with different modes of action are available against the disease. DNA sequence variations of the genes coding for the target proteins of three groups of fungicides (dicarboximides, hydroxyanilides and carboxamides), were analysed in order to explore the genetic bases of resistance.

Partial sequences of the histidine kinase gene (Daf1) from field isolates and laboratory mutants with different response to dicarboximides, were compared. Low-resistant mutants showed two alternative thymine substitutions (T-to-G or T-to-A) in the codon 365, leading to the change of an isoleucine residue of sensitive strains to serine or asparagine (I365S or I365B) within the second unit of the six tandem repeats. Two point mutations in the gene sequence, A-to-C in the codon 369, changing glutamine to proline (Z369P), and A-to-G in the codon 373, changing asparagine to serine (B373S), were associated with a moderate level of resistance. Guanine to adenine (G-to-A) transitions, causing the substitutions of glycine with asparagine at position 357 (G357B), or glycine with serine at position 446 (G446S) in the protein sequence, were exhibited by high-resistant mutants displaying different sensitivity to high osmotic pressure, a pleiotropic effect. An allele-specific primer extension method for PCR detection of Daf1LR alleles in field isolates of *B. fuckeliana*, was then developed.

Sequence analysis of the putative 3-keto reductase gene (ERG27) in laboratory mutants of *B. fuckeliana*, resistant to the hydroxyanilide fenhexamid, identified a single point mutation (G-to-T) in spontaneous mutants, leading to a change from glycine to valine (G5V) at the position 5, according to the amino acidic sequence of the transcript BC1T_00806 available on the *Botrytis cinerea* Sequencing Project database (Broad Institute of Harvard and MIT). In UV-induced mutants, the G-to-A transition, causing the substitution of the same amino-acid residue with serine (G5S), or the cytosine to thymine (C-to-T) transition, leading to a proline to leucine replacement at position 409 (P409L), was associated with high resistance. The C-to-T transition, causing the substitution of threonine with isoleucine at the position 45 (T45I), was instead associated with a low level of resistance.

Gene sequences coding for the succinate dehydrogenase complex from laboratory boscalid-resistant mutants and their parental sensitive strains, were compared. In all mutants, C-to-T transitions, causing amino-acid substitutions in the highly conserved cysteine-rich region of the iron-sulfur protein, were observed. High-resistant mutants showed single or two-nucleotide replacements at the codon 225 in the second Cys-rich cluster (S2), leading to replacement of proline with leucine (P225L) or phenylalanine (P225F). Low-resistant mutants showed a single mutation at the codon 272, resulting in a histidine to tyrosine replacement (H272Y) in S3. The gene sequences of the flavoprotein and the two transmembrane subunits of the succinate dehydrogenase were not affected.

05.4 RESISTANCE OF *BOTRYTIS CINEREA* TO THIABENDAZOLE AND ALTERNATIVES FOR MANAGEMENT OF POST-HARVEST GREY MOULD IN APPLE IN WASHINGTON STATE OF USA

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Washington State produces approximately 50% of the apples in the United States. Post-harvest diseases are a limiting factor for the long-term storage of apples. Grey mould caused by *Botrytis cinerea* is a major post-harvest disease of apple in the region. Thiabendazole (TBZ) is commonly used as a post-harvest drench treatment for control of post-harvest diseases. In 2004 and 2005, fludioxonil and pyrimethanil as post-harvest fungicides, and pyraclostrobin+boscalid as a pre-harvest fungicide, were labeled for use on apple in the U.S. In this study, resistance of *B. cinerea* to TBZ and use of newly registered fungicides as alternatives to TBZ for control of grey mould, were investigated.

A total of 139 single-spore isolates of *B. cinerea* (69 recovered from non-drenched fruit and 70 from TBZ-drenched fruit collected from commercial fruit packinghouses) were tested for TBZ resistance on PDA, amended with 10 μg/ml of TBZ. In 2004-2006, Red Delicious apples were harvested from commercial orchards and drenched with fludioxonil, pyrimethanil and thiabendazole. Fruit were then stored at 0°C in controlled atmosphere for 7 months, after which decay was evaluated. In a separate experiment conducted in research orchards in 2004-2006, a premixed formulation of pyraclostrobin+boscalid (Pristine) was applied at 7 days before harvest on Fuji and at 7 or 14 days before harvest on Red Delicious. Fruit were harvested, wounded and inoculated with the conidial suspension of *B. cinerea*. Fruit were stored at 0°C in air for 8-12 weeks, after which decay was evaluated.

Of the 139 isolates of *B. cinerea* tested, two were resistant to TBZ. The two TBZ-resistant isolates were recovered from decayed apple fruit that had not been drenched after harvest with TBZ. No TBZ-resistant isolates of *B. cinerea* were observed among the 70 isolates that were recovered from TBZ-drenched apple fruit. In 2004 and 2006, incidence of grey mould resulting from natural infections, was 11% and 4%, respectively. Fludioxonil and pyrimethanil were equally effective and reduced grey mould by 89-94% and 95-97%, respectively. TBZ was significantly less effective than fludioxonil and pyrimethanil in 2004, but not in 2006. In 2005, incidence of grey mould was low (<1% in the non-treated control), and no decay was observed in pyrimethanil-drenched fruit and no difference observed in grey mould incidence among other treatments. In comparison with the non-treated control, Pristine applied at 7 days before harvest reduced grey mould on Fuji by 93-96%. On Red Delicious, Pristine applied at 7 or 14 days before harvest provided a similar level of control and reduced grey mould by 68-78% and 83-85% in 2005 and 2006, respectively.

The results indicate that TBZ-resistant strains of *B. cinerea* were present at a low frequency in apple orchards in the region, and suggest that a post-harvest drench treatment with either fludioxonil or pyrimethanil, and a pre-harvest treatment with Pristine applied within two weeks before harvest, are effective alternatives for control of grey mould in apple.

05.5 INTEGRATED MANAGEMENT OF BOTRYTIS GREY MOULD OF ROOIBOS (*ASPALATHUS LINEARIS*) SEEDLINGS IN NURSERIES

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Rooibos (*Aspalathus linearis*) is a perennial indigenous legume that is grown in the Cedarberg area of the Western Cape province of South Africa. The plant is used for making rooibos tea, a herbal tea that is high in anti-oxidants, contains no caffeine, and is low in tannins. Grey mould, caused by *Botrytis cinerea*, is the most important foliage disease of rooibos seedlings in nurseries. The disease occurs on older (2 to 4-mo-old) seedlings, and is characterised by a rotting of the stem which extends into the leaves. The lesions are usually on the lower stems, but can also occur higher on the stems and even on the stem tips. Grey mould is favoured by cool, wet conditions, and has been recorded in rooibos nurseries throughout the rooibos production area. A study conducted to determine the incidence of the pathogen on different plant parts and in different rainfall areas, showed that the pathogen was significantly more frequently isolated from the lower parts of seedlings compared to the upper parts of the stems, and significantly more frequently from the high than the intermediate and low rainfall areas. Management of the disease relies heavily on chemical control, but incorrect application of the spray programme can cause a significant increase in resistance of *B. cinerea* against iprodione. Although chemical control is the most important component of the management strategy, a number of cultural practices are included to ensure effective management of grey mould. These practices focus on reducing the relative humidity and improving air circulation in seedbeds to create conditions unfavourable for infection by the pathogen and development of the disease. The recommended practices include optimum seedling density, correct orientation of seedbeds and seedling rows, correct irrigation scheduling, weed control and sanitation. Surveys conducted annually at 30+ nurseries from 2003 to 2007, showed that compliance with this management strategy significantly decreased the incidences of *B. cinerea* on rooibos seedlings in nurseries, and contributes to better establishment of seedlings after transplantation.

05.6 *BOTRYTIS* MANAGEMENT IN VEGETABLE CROPS: CHALLENGES AND PERSPECTIVES

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Several species of *Botrytis* (mainly *B. cinerea*, *B. squamosa*, *B. allii*) cause severe losses on economycally important vegetable crops. Among them, *B. cinerea* is particularly widespread on crops such as tomato, strawberry, lettuce, basil, etc. *Botrytis* management still relies on the usage of chemicals. A number of fungicides, belonging to different chemical groups, with diversified modes of action, are registered for use at least on the most important crops. The use of chemical control finds, however, some constraints due to the loss of registered fungicides, during the process of re-registration, as well as to the development of resistance towards the most applied fungicides.

The present status of fungicide availability as well as of fungicide resistance, will be critically discussed, together with measures adoptable to counteract such problems.

Biocontrol agents (mostly *Trichoderma* spp.) have been largely tested and are in some cases commercially available. Although less effective than chemicals, they can play a role in *Botrytis* management, particularly when applied under integrated plant disease management strategies.

Some examples of integrated disease management will be provided, with special reference to the Mediterranean conditions.

09.1 PRACTICAL INTEGRATION OF BIOLOGICAL CONTROL STRATEGIES FOR BOTRYTIS BUNCH ROT MANAGEMENT IN VINEYARDS

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Botrytis bunch rot in grapes, caused by *Botrytis cinerea*, has been responsible for significant crop losses in vineyards worldwide. Infected grapes also present a number of challenges to wine making, including fermentation problems, undesirable off-flavours and increased phenolics. Conventional management of *Botrytis* relies heavily upon the use of synthetic fungicides applied at key growth stages. However, this approach is no longer regarded as sustainable because of the relative ease with which fungicide-resistant strains of *B. cinerea* emerge within vineyard populations and increasing public concerns about the effects of pesticides on human and environmental health. As a consequence, more stringent regulations governing fungicide residues have been adopted and this has severely restricted chemical control options in conventionally managed vineyards, particularly during the pre-harvest period.

In a recent review, alternatives to synthetic botryticides were described and included plant defence stimulants to increase host resistance ("activators"), naturally occurring antagonistic microorganisms and natural products (Elmer and Reglinski, 2006). The authors concluded that the implementation of biologically-based control methods represented an enormous challenge, given the complex interactions between the vines, their responses to applied activators and *Botrytis* antagonists in the field, the pathogen, and the vineyard environment.

A joint partnership between NZ Winegrowers, HortResearch and Technology NZ was initiated in 1998 to develop biologically-based *Botrytis* control strategies for New Zealand (NZ) winegrowers. An isolate of the saprophytic fungus, Ulocladium oudemansii, was identified as an effective antagonist of *B. cinerea* (Reglinski *et al.*, 2005) and commercialised as BOTRY-Zen® in 2001. Vineyard studies (2002-2007) in commercial and research blocks have confirmed that early season fungicides such as dichlorfluanid, cyprodinil/fludioxionil and fenhexamid can be replaced with BOTRY-Zen® with no significant loss of *Botrytis* control at vintage. More recently, laboratory and field studies have identified five biologically-based treatments to complement early season BOTRY-Zen®. One of these, a chitosan-based formulation (ARMOUR-Zen®), will be commercially available for the 2007-08 vintage in NZ. The practical integration of complementary biologically-based treatments for Botrytis bunch rot control in New Zealand will be described.

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09.2 *CLONOSTACHYS ROSEA* IN STRAWBERRY LEAVES: AUTOECOLOGY AND ANTAGONISM TO *BOTRYTIS CINEREA*

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A successful programme of biological control of *Botrytis cinerea* (Bc) with isolate PG 88-710 of *Clonostachys rosea* (Cr) was established in Canada by J.C. Sutton's group. In a similar programme we are conducting to manage diseases caused by Bc, four Cr isolates were selected from Brazilian conditions. Their autoecology and antagonism to Bc in strawberry leaves were studied. Isolate PG 88-710 was used as a standard.

Each isolate (including a Bc isolate aggressive to strawberry) was grown on potato dextrose agar (PDA). Conidial suspensions (10^6 for Cr or 10^5conidia/mL for Bc), were sprayed on strawberry leaves. Establishment of either fungus was indirectly assessed by quantifying sporulation on leaf tissues: 1cm-diameter leaf discs were transferred to paraquat-chloramphenicol-agar medium (PCA) in Petri dishes, incubating at 20ºC. After 10-12 days, disc area with sporulation of either Cr or Bc was assessed with appropriate rating scales.

At the autoecological studies, each Cr isolate was applied on leaves, which were kept from 0 to 48h intervals in a moisture chamber. All isolates colonised the leaves, varying from 7 to 16%, disregarding moisture chamber interval. Leaves remained up to 36 h without wetness. Regardless of the delay in the time to establish wetness, the isolates colonised the leaves, varying from 11 to 14%. Each isolate was applied on leaves that were kept under 10 to 30ºC. Temperature strongly affected colonisation: at 10°C, no isolate sporulated on leaf discs, and 25.6°C was estimated as the optimum for colonisation. Temporal dynamics of Cr isolates on whole plants was studied. All Brazilian isolates survived in green leaves but leaf colonisation decreased from 16-18% to 4-6% from 1 to 49 days after application.

Similar procedures as above were followed at the antagonism studies, but Bc inoculation was also performed. Leaves were inoculated with Bc, sprayed with each Cr isolate, and kept from 0 to 36 h without wetness: all Cr isolates reduced Bc sporulation by more than 93%. On inoculated leaves, kept in moisture chamber on intervals varying from 0 to 48h after Cr application, Bc sporulation was reduced by more than 90%. At 10°C, no isolate reduced Bc sporulation; at 15°C, reduction on sporulation varied from 37 to 72%; at 20°C, the reduction was more than 85%; at 25 and 30°C, Bc did not sporulate. Each Cr isolate was applied on leaves at intervals ranging from 0 to 12 days, either before or after Bc inoculation. At all application times before inoculation, Bc sporulation was reduced from 47 to 97%. At most application times after inoculation, Bc sporulation was reduced by more than 95%. In whole plants, each isolate was applied on leaves. After 1 to 49 days, Bc was weekly inoculated, and its sporulation was assessed. Reduction of Bc sporulation was 52-62% and 10-19%, 1 and 7 days after Cr application, respectively.

In conclusion, the four Brazilian isolates of Cr were similar to PG 88-710 regarding leaf colonisation and antagonism to Bc. Temperature is the key environmental factor for their success.

09.3 EFFICACY OF MEDICINAL PLANT EXTRACTS FOR THE CONTROL OF *BOTRYTIS CINEREA*

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The ancient tradition of finding healing powers in plants, was confirmed by several clinical studies that found that some plant extracts possess antimicrobial activity and can be used to control several viral, bacterial and fungal pathogens of humans. Plant extracts of *Calendula officinalis*, *Baptisia tinctoria*, *Thuja occidentalis*, *Rubus nigrum*, *Rosmarinus officinalis*, *Juniperus communis*, *Citrus grandis*, *Eugenia caryophyllata*, *Juglans nigra*, *Thymus vulgaris*, *Lavandula angustifolia*, *Leptospermum scoparium*, *Cupressus sempervirens* and *Meleleuca alternifolia* are currently used in human medicine for their antimicrobial properties. Propolis, the resinous substance collected from various plant sources and transformed and used by bees, is not a plant extract, but can be considered a plant derivate material. Propolis, too, has antimicrobial properties. Plants are interesting sources for the discovery of new antimicrobial molecules that degrade quickly and have minimal environmental impact. The toxicological effects of these medicinal plants on humans are already well-known. We investigated the potential use of the above plant extracts against *Botrytis cinerea*. Concentration of plant extracts resulting in 50% reduction of *B. cinerea* conidia germination was calculated (LC_{50}). The effects of the test materials on conidial germination and germ tube elongation were tested on glass slides and detached tomato leaves. Plant extracts that were found to be effective under these conditions were then tested under controlled conditions on detached tomato and bean leaves and grape berries, and on whole tomato and grapevine plants.

Several extracts negatively affected germination and germ tube elongation, but only *T. vulgaris*, *E. caryophyllata*, *L. angustifolia* and propolis provided good and consistent control of *B. cinerea* in the detached tissue and whole plant experiments. The composition and specific active molecules of these effective extracts can be further characterised for the possible identification of new tools for plant protection.

09.4 RESISTANCE TO *BOTRYTIS CINEREA* IN A POPULATION OF *SOLANUM HABROCHAITES* INTROGRESSION LINES

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Quantitative resistance to *Botrytis cinerea* has been reported in wild relatives of tomato (*Solanum lycopersicum)*. The resistance levels reported for these genotypes are high, however, breeding for resistance has been hampered by (presumed) polygenic inheritance. We identified resistance in *S. habrochaites* LYC4 and studied its inheritance. First, a QTL mapping study was performed in a segregating F_2 population (n=174) of a cross between the susceptible *S. lycopersicum* cv. Moneymaker and *S. habrochaites* LYC4. The genotypes of the progeny were determined by molecular marker methodology, including AFLP. Initially, two QTLs were identified that confer resistance to *B. cinerea* (QTL1 and QTL2). During confirmation of these QTLS in segregating BC_2S_1 families, a third QTL was discovered, of which the effect could only be seen in the absence of QTL2.

We considered it likely that the relatively small F_2 population and the difficult quantitative bioassay hampered detection of the full spectrum of QTLs for resistance. Therefore, we developed an introgression line (IL) population of 31 genotypes, each containing a separate *S. habrochaites* LYC4 introgression in the *S. lycopersicum* genetic background. On average, each IL contained 5.2% of the *S. habrochaites* genome and the total population provided an estimated coverage of 95% of the *S. habrochaites* genome. The resistance to *B. cinerea* of each IL was assessed in a quantitative greenhouse trial. The effect of the three previously identified loci could be confirmed and seven additional loci were detected. Some ILs contained multiple QTLs and the increased resistance to *B. cinerea* in these lines was indicative of a completely additive effect.

We conclude that this set of QTLs offers good perspectives for breeding of *B. cinerea* resistant cultivars. Screening an IL population was more sensitive for detection of QTLs conferring resistance to *B. cinerea* than the analysis in an F2 population. Analysis of separate ILs, each containing a distinct QTL, might in the future provide more insight in the underlying physiological and molecular mechanisms that contribute to resistance.

09.5 SUSTAINABLE CONTROL OF *BOTRYTIS* BY SANITATION AND CULTURAL MEASURES

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Chemical control of *Botrytis cinerea* in the production of tomato is not always very efficient. A greenhouse trial with artificial inoculations on stem wounds, shows that the efficacy of fungicides is only 24 hours sufficient while infections become visible after 2 weeks. So growers cannot treat infections adequately - when they see an infection, it is already too late.

There is a need for another control management system. In the search for a more sustainable method for controlling *B. cinerea* in the production of tomatoes in greenhouses, a method based on sanitation and cultural control measures was developed. The main aims of this method are lowering the spore production of *B. cinerea* in the greenhouse and lowering the chance of infection.

Most of the spores of *B. cinerea* present in the neighbourhood of the tomato plants are formed in the greenhouse, especially on infected stems and stems of bunches. In heated greenhouses, leaves are rarely infected (sometimes in spring). Treating and healing infected stem wounds, prevent the plant from dying and will also decrease the infection pressure.

The chance of an infection can be lowered by making a very smooth wound on the stem that will dry as soon as possible. More than 90 % of the infections on stem wounds appear at irregular wounds. Taking measures to decrease the period of wound wetness, give a decreased number of infections as well.

Old stems of bunches must be removed to prevent infections on these stems. It is important that they are totally removed.

In a few greenhouses, the combination of these sanitation and cultural control measures was implemented in 2004 and compared with the natural infections at the same greenhouses in 2003, a year with a lot of sunshine and fewer infections than average. In the greenhouse of the research centre, Proefcentrum Hoogstraten, the number of infections decreased in 2004 by 61% when implementing the control measures. The 2004 season was more humid with more infections than average. In 2005, the number of infections decreased again by 95% in comparison with 2003. In the greenhouse of a professional grower, the combination of the proposed measures was only partially applied in 2005. The number of infections in 2005 decreased by 74% in comparison with 2004.

In two other greenhouses, the combination of the measures was strictly applied, with a good result. In one greenhouse, two infected and dying plants were not treated nor removed, therefore *B. cinerea* produced a lot of spores. The concentration of spores in the air in the neighbourhood of these dying plants increased till 116 spores/m^3, while the concentration in the control greenhouse remained 3.3 spores/m^3. At this time, 0.55% of the stems in the control greenhouse became infected, while in the greenhouse with the two sporulating plants, 2.3% of the stems became infected.

09.6 CULTURAL METHODS FOR GREY MOULD (*BOTRYTIS CINEREA*) MANAGEMENT IN LISIANTHUS

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Grey mould (*Botrytis cinerea*) severely affects the cut-flower crop of lisianthus (*Eustoma grandiflorum*). Lisianthus is grown mainly in unheated greenhouses during the winter. Following planting in September, the plant grows to one flower-bearing stem that is harvested beginning in December until January (first flowering wave). Cut plants go on to produce several branches that are harvested during the second flowering wave in the spring. The fungus infects the bases of the stems of whole plants, and plant stubs that are left after flowers have been harvested. The crop's typically dense canopy and the fact that leaf rosettes are close to the ground, prevent adequate air movement, encouraging the development of disease. Chemical fungicide treatments frequently fail to suppress this disease. The aim of this research was the study and implementation of integrated management strategies for the control of grey mould in lisianthus.

Under controlled conditions, infections were favoured at temperatures of 18-22ºC, RH levels lower than the saturation point, and high light intensity. We found that *B. cinerea* infection of lower leaves and the spread of rot towards the stem, are important factors in plant mortality, in addition to the direct infection of stem wounds. Leaf infection often occurs as leaves come into contact with moist soil. Therefore, relatively high levels of soil moisture tend to encourage the spread of the disease. Methods for separating the lower leaves from the moist soil, were investigated and found to successfully decrease plant mortality. Microclimate management through the use of polyethylene soil covers, deep burial of the drip irrigation system, reduced planting densities, fertilisation with increased levels of calcium, and the use of chemical fungicides, were examined in growth chambers and under commercial conditions in unheated, polyethylene greenhouses.

The use of polyethylene soil coverings and the deep burial of drip irrigation lines significantly decreased the humidity in the greenhouse and the amount of moisture around the stem bases. These methods suppressed grey mold on stem bases and plant stubs. Decreased planting densities resulted in significantly lower disease levels. However, reduced planting density did not result in high frequency of healthy plants, as counted in the higher density treatment, because of the net outcome of reduction in diseased plants on one hand and the initial total number of plants in the plots on the other hand. Nevertheless, the combination of reduced plant density or forcing air into the lower canopy with chemical control methods, resulted in significantly improved disease control, as compared with each treatment alone. An integrated grey mould control system will be described. It was developed for farmers based on the results of our research.

5. DISEASE MANAGEMENT (1)

POSTERS

P5.1. **Marcela Esterio**, Héctor García, Jaime Auger and Cecilia Ramos

Analysis of the population composition of *Botrytis cinerea* in *Vitis vinifera* cv. Thompson Seedless, exposed to different fungicide programmes: *in vivo* study

P5.2. **Marcela Esterio**, Jaime Auger, Cecilia Ramos and Héctor García

Actual status of *Botrytis cinerea* sensitivity to fenhexamid in Chile

P5.3. **Marco Harms**, Katja Erzgräber, Eva Alexander and Roland Ipach

Long lasting activity of different botryticides on grapes

P5.4. M. Berrios, R. Saini, R. Santamaría, V. Navia and **Benjamin Valiente**

Control of *Botrytis cinerea* and sour rot in export table grapes by the post-harvest electrostatic fungicide application system (Typhoon® Service) in Chile

P5.5. **Sabine Fillinger**, P. Leroux, J. Bach, C. Al Hajj, M. Gredt and D. Debieu

Fenhexamid: mode of action and resistance in the phytopathogenic fungus, *Botrytis cinerea*

P5.6. **Karien Lubbe** and G. van den Berg

Evaluation of selected fungicides for the control of *Botrytis cinerea* on *Leucospermum* flowers

P5.7. **Nadia Korolev**, M. Mamiev, T. Zahavi and Y. Elad

Resistance to fungicides among *Botrytis cinerea* isolates from different plant hosts in Israel

9. DISEASE MANAGEMENT (2)

Posters

P9.1. **Sakhr Ajouz**, Marc Bardin and Philippe Nicot

Evolution of *Botrytis cinerea* to pyrrolnitrin, an antibiotic produced by biological control agents

P9.2. Saravanakumar Duraisamy, Sandro Frati, Davide Spadaro, Angelo Garibaldi and **Maria Lodovica Gullino**

Metschnikowia pulcherrima mediated iron depletion reduces *Botrytis cinerea* infection in apples

P9.3. **Rudi Aerts**, Alexandra Denayer, Bjorn Seels and Kathleen Heyens

Modelling infection of *Botrytis cinerea* in tomato as a tool for better control

P9.4. Marco A. Jacometti, Steve D. Wratten and **Monika Walter**

Managing *Botrytis cinerea* on grapes through the use of organic mulches

P9.5. **Arnaud Cousin**, Vincent Jacus, Aude Toulouse and Pierre-Antoine Lardier

Pyrimethanil and boscalid used to fight the aromatic deviations in French wines due to the presence of geosmin, a volatile compoud produced by *Penicillium expansum*

P9.6. **Rudi Aerts**, Kathleen Heyens, Miguel F.C. De Bolle and Bruno P.A. Cammue

Bio-control of *Botrytis cinerea* in tomatoes

P9.7. **Béatrice Charnay** and Martine Cazin

Estimating the field *Botrytis* risk to minimise the disease

P9.8. **Kristy Morris**, Andrew MacNish, Annemarie de Theije and Michael Reid

Strategies for control of post-harvest *Botrytis* infection in roses

P5.1 ANALYSIS OF THE POPULATION COMPOSITION OF *BOTRYTIS CINEREA* IN *VITIS VINIFERA CV*. THOMPSON SEEDLESS, EXPOSED TO DIFFERENT FUNGICIDE PROGRAMMES: *IN VIVO* STUDY

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A field trial was carried out during the 2002/03 season in order to genetically and phenotypically characterise *B. cinerea* populations recovered from Thompson Seedless flowers and berries, subjected to a high fungicide selection pressure. The trial consisted of control programmes, based on four iprodione (P1), fenhexamid (P2) and fenhexamid & tebuconazole (Tiebreak™) (P3) applications. These applications were made during flowering, cluster closure, veraison and pre-harvest, for each one of the programmes. Plants that were not subjected to botrycides during the season (P4), were used as control. The study included the following: 1) monitoring of genotypic frequencies in the different programmes through the season in all the *B. cinerea* isolates recovered (n=337), using the *Boty* and *Flipper* transposable elements as population markers; 2) genetic variability analysis through RAPDs trials in isolates recovered post-harvest (n=115); 3) classification of *B. cinerea* isolates in Group I or II through PCR-RFLP according to the Bc-*hch* gene restriction pattern; and 4) analysis of the level of sensitivity to iprodione and fenhexamid in isolates recovered in post-harvest. The genotypic frequency was characterised by a small number of isolates corresponding to *vacuma* and *boty* genotypes, with predominance of the *transposa* genotype, except for the population that was exposed to fenhexamid, where higher proportions of the *vacuma* genotype were detected (22.2 and 41.4%, in harvest and post-harvest periods, respectively). The populations were genetically grouped by control programmes, with the exception of the ones coming from programme P3. In addition, groupings were detected at genotype level. All individuals analysed belonged to Group II of *B. cinerea* (B*c-hch*2). Only the population exposed to fenhexamid presented significant differences from the unsprayed control population, with average levels of EC_{50} that fluctuated between 0.083 and 0.021 $\mu g \cdot mL^{-1}$(P2 and P4, respectively). In addition, 50% of the isolates of the *vacuma* genotype recovered from P2 (n=6) showed EC_{50} values associated to resistance ($EC_{50} \geq 0{,}1\ \mu g \cdot mL^{-1}$). The results obtained indicate that a high fenhexamid selection pressure in one season can generate changes in the *B. cinerea* genotypic frequency, thus decreasing the exposed population's sensitivity, mainly in the *vacuma* genotype, which probably has a higher adaptation capacity to this fungicide.

P5.2 ACTUAL STATUS OF *BOTRYTIS CINEREA* SENSITIVITY TO FENHEXAMID IN CHILE

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The sensitivity to fenhexamid was evaluated in 472 isolates of *Botrytis cinerea*, recovered during the last two seasons from symptomatic berries from different cultivars of export table grape (*Vitis vinifera* L.), collected from different geographical zones in Chile (Regions III to VI). The EC_{50} values calculated, based on their myceliar growth, were used as criterium. According to previous studies carried out in Switzerland and France, the resistant isolates would show $EC_{50} \geq 0{,}1$ $\mu g \cdot mL^{-1}$ values. The sensitivity threshold calculated for the *B. cinerea* population analysed in this study corresponded to 0.084 $\mu g \cdot mL^{-1}$ (EC_{50}). According to this threshold, 450 isolates (95.3%) presented high sensitivity to fenhexamid; 9 isolates (1.9%) had lower sensitivity, and 13 isolates (2.8%) behaved as resistant isolates with $EC_{50} \geq 0{,}1$ $\mu g \cdot mL^{-1}$ values. Among these last isolates, 3 presented high EC_{50} values (0.7, 3.9 and 8.4 $\mu g \cdot mL^{-1}$). Through PCR, it was possible to determine that 8 of the 13 resistant isolates corresponded to the *transposa* and 5 to the *vacuma* genotype. With the PCR-RFLP technique and the H*ha*I enzyme, it was established that all the resistant isolates belong to Group 2 of *B. cinerea* (B*c-hch*2), so the loss of sensitivity would correspond to resistance acquired as time elapsed. Finally, in order to determine if the resistant isolates belonged to the HydR2 or HydR3 phenotypes, the conidial germination behaviour was analysed for these isolates at 0.4 and 4 $\mu g \cdot mL^{-1}$ of fenhexamid. The three isolates that showed high EC_{50} values corresponded to phenotype HydR3, with an elongation of the germ tube similar to the one of the control at both concentrations. The 10 remaining isolates corresponded to phenotype HydR2, without or with a restricted germ tube elongation both at 0.4 and 4 $\mu g \cdot mL^{-1}$ of fenhexamid. Up to now, the presence of *in vitro B. cinerea* resistant isolates in Chile has not been associated to fungicide efficacy losses in the field.

P5.3 LONG LASTING ACTIVITY OF DIFFERENT BOTRYTICIDES ON GRAPES

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The long lasting activity of a fungicide is an important attribute for the treatments against *Botrytis cinerea* in grapes. Especially for the fungicide applications at veraison (BBCH growth stage 81), the long lasting activity is of special interest because in most cases it is the last treatment during the growing season.

Between 2003 and 2005, the long lasting activity of different fungicides against *Botrytis cinerea* (Boscalid, Fenhexamid, Pyrimethanil, Tolylfluanid, Cyprodinil + Fludioxonil) on grapes has been investigated in the field and the laboratory. Grapes of the variety Müller-Thurgau have been treated in the field with the recommended field rate of each fungicide at veraison. The development of grey mould was recorded in weekly intervals. In parallel, bunches have been taken to the laboratory and inoculated artificially with conidia of *B. cinerea*.

In 2003, up to 41 days after the last treatment, a sufficient effect against *B. cinerea* could be observed in the field. After that, the degree of efficiency decreased rapidly in all variants. In 2004 and 2005, no mentionable infections could be recorded because of unsuitable wheather conditions for the fungus. With artificial inoculations under standardised conditions, the long lasting activity of the compounds ranged between 19 days in 2003 and 44 days in 2005. It was surprising that in all years the differences between the fungicides have been low. A remarkable loss in efficiency could be observed for all fungicides nearly at the same time.

These observations indicate that besides the wheather conditions and plot specific factors, mainly physiological parameters of the berry and the berry skin seem to influence the long lasting effect of the fungicides. This has to be considered for the assessment of the efficiency of very late treatments against *Botrytis* (e.g. two weeks after veraison). Trials with additional late treatments at BBCH growth stage 85 (berry softening) between 2001 and 2005 ($n = 15$) revealed a wide range of effectiveness (80 % to -11%) against *B. cinerea* compared to the standard spraying schedule (treatment at BBCH GS 77 and GS 81). The influence of physiological parameters on the effectiveness of the fungicides might be an additional reason for the strong variability of the results for the treatments with late fungicide application in the field.

Further investigations are necessary to understand the factors that influence the effectiveness of fungicide treatments in the field against *B. cinerea* on grapes. This may lead to an improved protection strategy and help to develop better tools for recommendations in viticultural practise.

P5.4 CONTROL OF *BOTRYTIS CINEREA* AND SOUR ROT IN EXPORT TABLE GRAPES BY THE POST-HARVEST ELECTROSTATIC FUNGICIDE APPLICATION SYSTEM (TYPHOON® SERVICE) IN CHILE

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Fourty trials were conducted during the 2004-05 season with demonstrative applications on table grapes, in order to confirm the effectiveness of the post-harvest fungicide application by an electrostatic system (Typhoon® Service) against grey mould (*Botrytis cinerea)* and sour rot (complex of fungi, bacteria and yeasts). Trials were done in 14 export companies, 16 of them with Thompson Seedless variety and 24 of them with Red Globe variety. Each treatment included 1,920 boxes (8,2 kg).

During the 2006-07 season, Typhoon® was evaluated at commercial level. An inspection of the fruit treated in Chile was made at destiny location in Philadelphia, USA (April 2007). The variety Crimson Seedless was kept in cold storage at 0°C for 30 days (in that time is included: packing house, ship travel, ports at origin and destination, fumigation at destiny and beginning of sale).

All treatments were complemented with SO_2 generators. Fungicides assessed (fenhexamid and iprodione) were applied in mixture. The evaluations were immediately made after the cold storage period, determining the levels of grey mould (both berry and pedicel rot and Botrytis slip skin on berries) and sour rot (both based weight).

Botrytis and sour rot levels detected in all varieties treated by Typhoon® were statistically lower than the ones obtained with the traditional pre-harvest treatment. One of the most remarkable results was the effect of the mixture of fenhexamid with iprodione which resulted in highly reduced rotting levels.

P5.5 FENHEXAMID: MODE OF ACTION AND RESISTANCE IN THE PHYTOPATHOGENIC FUNGUS, *BOTRYTIS CINEREA*

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The use of fungicides remains the major method to control *Botrytis cinerea*, although resistance development threatens the efficiency of these products. Among the available active compounds, one of the most recent is fenhexamid. We have shown that this hydroxyanilide affects sterol biosynthesis by inhibiting the 3-ketoreductase, one of the enzymes involved in sterol C4 demethylations. This mode of action is different from other sterol biosynthesis inhibitors (SBIs) already known. Moreover, fenhexamid exhibits a rather narrow activity spectrum (mainly *B. cinerea* and related species such as *Sclerotinia* spp. and *Monilinia* spp.) compared to the other SBIs. We have isolated fenhexamid resistant strains from Champagne vineyards, which can be classified into three resistance categories. Enzymatic studies have shown for all of them a reduced 3-ketoreductase sensitivity towards fenhexamid, suggesting target modification as resistance mechanism. The sequence of the *erg27* gene encoding the 3-ketoreductase, has revealed a strong polymorphism for one resistance category (HydR1), which corresponds to the naturally resistant cryptic species, *B. pseudocinerea*. The second resistance category (HydR2) exhibits a wild-type Erg27 protein sequence. Finally, for the third resistance class (HydR3) showing medium to high resistance to fenhexamid, several point mutations have been identified in the *erg27* sequence from these isolates. The major mutation is a replacement of the phenylalanine, at position 412 in the putative transmembrane domain, by a serine (16 isolates), or an isoleucine (3 isolates), or a valine (1 isolate). These replacements confer high resistance levels to fenhexamid, as we could show by introducing one of these *erg27(HydR3)* alleles into the sensitive B05.10 strain. Other point mutations identified between the positions 195-400 confer different resistant levels towards fenhexamid. The correlation between the mutations and sensitivity to fenhexamid will be discussed.

P5.6 EVALUATION OF SELECTED FUNGICIDES FOR THE CONTROL OF *BOTRYTIS CINEREA* ON *LEUCOSPERMUM* FLOWERS

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Leucospermum is indigenous to South Africa and is produced commercially as a cut-flower. The largest proportion of flowers produced in South Africa is exported *via* airfreight to the Netherlands, but also to other European countries. *Botrytis cinerea* has always been a damaging post-harvest pathogen on *Leucospermum,* but the recent introduction of shipment as a way of transport has lead to an increase in post-harvest losses. This research project aimed to identify fungicides for the effective control of this pathogen, since the fungicides commonly used show resistance and is not accepted by the EU markets. A list of fungicides with the potential for control of *Botrytis* were first screened *in vitro* and included benomyl, carbendazim + flusilazole, chlorothalonil, cyprodinil + fludioxonil, fenhexamid, iprodione and pyrimethanil. The results of the *in vitro* test were used to identify fungicides to be evaluated under field conditions. The following fungicides were evaluated under field conditions: cyprodinil + fludioxonil, fenhexamid and pyrimethanil. All three fungicides showed significant control of the pathogen. Although fenhexamid was the only fungicide that ensured less than 10% infection by *B. cinerea*, it is recommended that all three fungicides be tested at other localities.

P5.7 RESISTANCE TO FUNGICIDES AMONG *BOTRYTIS CINEREA* ISOLATES FROM DIFFERENT PLANT HOSTS IN ISRAEL

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Grey mould (*Botrytis cinerea*) is a damaging disease of numerous greenhouse- and field-grown crops in Israel. Chemical control remains an important means of grey mould control, although it is impeded by the development of pathogen resistance. This research was undertaken in order to monitor the resistance to the newly introduced anilinopyrimidine, hydroxyanilide, phenylpyiridinamine and phenylpyrrole fungicides. The monitoring of resistance to the old benzimidazole and dicarboximide fungicides was also carried out. More than 500 *B. cinerea* isolates from eight hosts in 22 locations throughout Israel were examined, using mycelial growth test (Hilber and Schüepp, 1996). Resistance to benzimidazoles and dicarboximides was widely spread among open field and especially greenhouse isolates. Isolates resistant or less sensitive to fluazinam and pyrimethanil were found in greenhouses with a frequency of about 8%. A few isolates were recovered also from open fields treated with the target fungicides. Resistance to fludioxonil and phenhexamid was very rare (about 0.5%). Among isolates found to be resistant, only about 30% were resistant to one class of fungicides, whereas the remaining isolates exhibited resistance to two or more fungicides simultaneously. In greenhouse experiments, resistance to benzimidazoles was accompanied by almost complete failure of control, and resistance to fludioxonil caused significant (up to 50%) loss of control efficacy. Resistance to iprodione, fluazinam and pyrimethanil was associated with 5 to 10% reduction in control efficacy. Low-level resistance to fenhexamid had no practical importance. In comparison to the sensitive (wild-type) isolates, isolates resistant to fluazinam formed smaller lesions on bean leaves and slower growing colonies on PDA. Isolates resistant and sensitive to other fungicides, appear to be equally fit. *Botrytis* selective media, based on BSTM (Edwards and Seddon, 2001) amended with discriminatory doses of fungicides, were developed for specific isolation and enumeration of resistant phenotypes from the air of greenhouses or open fields.

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P9.1 EVOLUTION OF *BOTRYTIS CINEREA* TO PYRROLNITRIN, AN ANTIBIOTIC PRODUCED BY BIOLOGICAL CONTROL AGENTS

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Botrytis cinerea is known to develop resistance to many chemical fungicides. In order to control the disease, biological control could be a good alternative and many biological control agents (BCA), possessing different modes of action (including antibiosis, hyperparasitism, nutrient competition and induction of host resistance), have been described in the past years. But to date, field use of BCAs has been limited and scientific information is lacking regarding the durability of biological control efficiency. Consequently, it is often considered that resistance of plant pathogens to BCAs will develop less frequently, and there are clearly few documents dealing with the capacity of plant pathogens to overcome the effect of BCAs. But possible loss of efficacy of a BCA could result from the pre-existence of strains of the plant pathogens with low susceptibility in natural populations. It could also arise if plant pathogens have the ability to produce natural mutants with reduced susceptibility under the selection pressure of BCAs used by farmers. The objective of the present study is to estimate the risk of losing efficacy of biocontrol due to selection pressure exerted by BCAs on *B. cinerea* populations.

In this study, efforts have been focused on antibiosis and particularly on the antibiotic pyrrolnitrin which has been identified in diverse BCAs known to have a significant effect on *B. cinerea* (*Serratia plymuthica*, *Pseudomonas* sp.).

To evaluate a possible decrease in sensitivity to pyrronitrin under selection pressure, ten successive generations (G1 to G10) of 5 isolates of *B. cinerea* were produced *in vitro* in the presence of a sub-lethal dose of the antibiotic. For each isolate, three independent repetitions were carried out. As a control, ten successive generations were also produced for each isolate in absence of pyrrolnitrin. We observed a significant reduction in the sensitivity to pyrrolnitrin in the 10^{th} generation produced in the presence of the antibiotic (G10P). The resistance factor (RF) was closed to 10 for some G10P variants, and different evolution patterns were observed among the 5 isolates. Finally, the production of 10 additional generations with increasing doses of pyrrolnitrin resulted in the development of variants of *B. cinerea* with high levels of resistance ($RF > 800$). These results suggest that continuous exposure to pyrrolnitrin may lead to rapid adaptation of the fungus.

Works in progress include analysis of the fitness of pyrrolnitrin-resistant strains and bioassays on plants with pyrrolnitrin-producing biocontrol bacteria to evaluate a possible loss of efficacy against the resistant variants. Furthermore, we will test the possibility of "reverse adaptation" of resistant variants in absence of selection pressure.

P9.2 *METSCHNIKOWIA PULCHERRIMA* MEDIATED IRON DEPLETION REDUCES *BOTRYTIS CINEREA* INFECTION IN APPLES

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Metschnikowia pulcherrima has been studied as a biocontrol agent against post-harvest fungal diseases. This yeast produces a red pigment called pulcherrimin, formed non-enzymatically from pulcherriminic acid and ferric ions. The study was carried out to investigate the effect of pulcherrimin production, utilising ferric ions by *M. pulcherrima* strain BIO126 isolated from apple fruits, against *Botrytis cinerea in vitro* and on apples *in vivo*. *Metschnikowia* strain BIO126 produced white colonies without iron, and the same strain produced pale to dark red colonies with increased supplementation of iron in PDA medium. Conspicuously, the wider halos were observed around colonies with lower concentrations of iron, indicating the movement of precursors from *Metschnikowia* to find sufficient amount of iron to form the pigmentation. Similarly, increased supplementation of iron resulted in reduced halos by strain BIO126.

In addition, a *Botrytis* conidial suspension was prepared and flooded on a PDA medium amended with different concentrations of iron. *Metschnikowia* strain BIO126, streaked on the centre of the plate, caused an inhibition zone. A higher inhibition was measured with low iron amendment, while a lower inhibition was noticed with increased concentrations of iron in the medium. Moreover, the microscopic observations revealed the non-germination and degeneration of *Botrytis* conidia and mycelia, respectively in the pigmented inhibition zones *in vitro*. Besides this, an *in vivo* experiment was conducted on apples cv. Golden Delicious treated with *Metschnikowia* strain BIO126, grown in YPD broth, amended with different concentrations of iron, and artificially inoculated with *B. cinerea.* A high level of pathogen infection was recorded from increased concentrations of iron. Lower concentrations of iron supplementation caused a reduction in percent infection. Apples inoculated with *B. cinerea* alone recorded the highest infection compared to all other treatments.

In conclusion, our findings elucidated the iron utilisation by *Metschnikowia* strain BIO126 for the production of pulcherrimin that depletes the iron either in the substrate, or in the environment, which ultimately affected the growth of *B. cinerea in vitro* and on apples *in vivo*. This could be a useful mechanism in improving the control of post-harvest fungal pathogens by *M. pulcherrima* strains.

P9.3 MODELLING INFECTION OF *BOTRYTIS CINEREA* IN TOMATO AS A TOOL FOR BETTER CONTROL

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Control of *Botrytis cinerea* in tomato on stem wounds by fungicides, is only effective if a treatment is applied within 24 hours after infection, while infections only become visible after 2 weeks. So growers cannot do a treatment when they see an infection because it is much too late. Therefore, it is important to calculate the risk for new infections so growers can decide if a treatment would be necessary after plant maintenance. This treatment can be an application of fungicides or a biological control agent. Knowing the infection risk is also interesting to manage this plant maintenance. Making stem wounds can be delayed when the infection risk is too high.

The three most important parameters for calculating the infection risk, are the probability of spore deposition on stem wounds, the duration of wound wetness, and the time that a conidiospore needs to infect the plant.

The probability of spore deposition on stem wounds is correlated with the number of spores in the air of a greenhouse. This can be measured by taking air samples together with modelling spore deposition. Spore deposition can be measured by putting small dishes with a selective *Botrytis* medium in a greenhouse. With a spore concentration of 50 spores/m^3 air, 2.5 % of the wounds will be hit by a *Botrytis* spore after 5 hours.

The duration of wound wetness depends on the greenhouse climate and the transport of moisture through the vessels to the wound. The wound wetness can be measured with an IR- camera. This transportation of moisture to the wounds takes a few days, so the duration of wound wetness is a matter of days and not of hours. This means that the time that wounds can be infected by spores of *B. cinerea*, is rather long.

Germination and penetration of *Botrytis* spores into the plant is influenced by temperature, nutrition, age of the spores, and the defence mechanism of the plant. The optimum temperature for spore germination is 20°C, with a small difference between 15° and 25°C. At 20°C, 50% of the spores are germinated in optimum conditions after 230 min. At 15° and 25°C, 50% of the spores is germinated after 285 min. If plant juice is added to the medium, germination is delayed by 120 min.

Penetration in onion epidermis, previously treated with chloroform and rinsed with water, was studied by placing *Botrytis* spores suspended in a Gamborg's medium on the hydrophobic side of the epidermis. After incubation, the fungal cells were stained using lactophenol cotton blue. This penetration process takes about 7 hours (including germination), or 10 hours if chloroform was not used. On tomato epidermis, this process takes approximately 26 hours.

Bringing these three parameters into account, it is possible to calculate the infection risk for stem wounds in the production of tomato.

P9.4 MANAGING *BOTRYTIS CINEREA* ON GRAPES THROUGH THE USE OF ORGANIC MULCHES

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In New Zealand vineyards, *Botrytis cinerea* has typically been managed through the prophylactic use of agrichemicals at a cost of NZ$ 500-700/ha/year. Four organic mulches (anaerobically and aerobically fermented *marc* (grape pressings), inter-row grass clippings and shredded office paper) were tested to enhance the activity of soil flora and fauna below vines, to disrupt the disease lifecycle and improve the vines' resistance to the pathogen in a commercial vineyard in Blenheim (2003-2006). Plastic mesh bags, containing naturally infected vine debris, were placed under vines on bare ground (control) and at the soil-mulch interface in July. Moisture and temperature were monitored at the soil-mulch interface. *Botrytis* sporulation and debris degradation rate were assessed at flowering (December) and at leaf plucking (February). Bait lamina probes and Biolog Ecoplates were placed at the soil-mulch interface to measure soil biological diversity and microbial activity. At harvest, yield and *B. cinerea* bunch severity (% berries per bunch with sporulating *B. cinerea* in the field) were determined. Canopy density, leaf nutrients, berry skin strength, and berry nutrients, including sugars and phenolics, were measured. Resistance of berries to *B. cinerea* was assessed for ripe, surface sterilised and artificial inoculated fruit.

All mulches led to a reduction in *B. cinerea* sporulation. This reduction was significantly correlated with elevated rates of vine debris decomposition and increased soil biological activity. Over both years, compared with the controls, all treatments gave a 3 to 20-fold reduction in *B. cinerea* sporulation, a 1.6 to 2.6-fold increase in vine debris degradation, and in the two *marc* and the paper treatments, a 1.8 to 4-fold increase in activity of soil organisms. The mulches also altered vine characteristics and elevated their resistance to *B. cinerea* probably through changes to the soil environment. Functional soil biological activity, as measured by Biolog Ecoplates and bait lamina probes, was increased 2-4 times in the two *marc* and paper treatments, compared with the control, an effect relating to the elevated soil moisture and reduced temperature fluctuations under these mulches. Nutrient levels and carbon : nitrogen ratios were also affected in these treatments. The mulched paper lowered vine canopy density by up to 1.4 times that of the other treatments, an effect which probably led to elevated light penetration into the canopy and consequently increased canopy temperature, photosynthesis and lower canopy humidity. These changes to soil and vine characteristics increased grape skin strength by up to 10% in the paper treatment and sugar concentrations by 1.2-1.4 ^{o}Brix in the two *marc* and paper treatments. The severity of *B. cinerea* bunch infections in the anaerobic *marc*, aerobic *marc* and paper treatments, was reduced to 12%, 3% and 2.2% of the control, respectively, averaged over both harvests. These reductions led to *B. cinerea* bunch infection being below the economic threshold (5%) for fungicide intervention.

P9.5 PYRIMETHANIL AND BOSCALID USED TO FIGHT THE AROMATIC DEVIATIONS IN FRENCH WINES DUE TO THE PRESENCE OF GEOSMIN, A VOLATILE COMPOUND PRODUCED BY *PENICILLIUM EXPANSUM*

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For several years, wines from a certain number of French wine-producing regions have been marked by aromatic deviations evoking fresh mushroom, humus or wet ground. These defects are prejudicial on organoleptic quality of musts, grape juices and wines, and were always found correlated to the presence of different microorganisms on the grapes. The major molecule responsible for these defects is trans-1, 10-dimethyl-trans-9-decalol, more known under the name of geosmin, a powerful aromatic compound with an earthy smell, produced very early in grapes in the vineyards. Geosmin has been identified from pure cultures of fungi belonging to the *Penicillium* genus and *Streptomyces* sp. (actinomycete bacteria), isolated from rotten grapes. Even if other species of *Penicillium* are able to produce geosmin, it has been considered that *P. expansum* is the main producer and produces geosmin only in the presence of *Botrytis cinerea*, leading to the earthy odour of wine.

Because aromatic deviations are not always correlated with the presence of geosmin-producing microorganisms, several factors are probably influencing the geosmin production, for example quantity of fungal species, their development stage, their physiological state or the potential presence of some precursor compounds. For these reasons, the principal methods of control are orientated towards prophylaxis (strength and yield control, bunch and vegetation ventilation, harvesting at optimum ripeness and removal of affected bunches). Moreover, considering that no specific oenological treatment is allowed to remove geosmin, the use of fungicide remains the most effective tool to prevent the development of *B. cinerea* so far.

Here we show the efficacy of two fungicides from BASF, pyrimethanil and boscalid, towards to *P. expansum* and *B. cinerea*. This approach implies two levels of observation: the first one using laboratory conditions and the second one collecting the results of several years (2003-2007) of studies in vineyards. These results underline the downward trend of the geosmin production when grapevines are treated with BASF products and will be disccussed.

P9.6 BIO-CONTROL OF *BOTRYTIS CINEREA* IN TOMATOES

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Grey mould, caused by *Botrytis cinerea*, is a major problem in tomato production in greenhouses, thereby causing the biggest economic losses of all known tomato diseases. *B. cinerea* infects the tomato plant mainly through stem wounds caused by breaking or cutting off leaves, a general procedure in tomato plant maintenance. Such an infection can cause an entire plant to die and results in high yield losses if no adequate measures are taken. At this moment there are no known resistant tomato cultivars. Furthermore, most fungicides currently registered for use in tomato production in Flanders, only have a preventative effect and as such need to be applied on a regular basis independently of disease pressure. Such frequent use of fungicides has negative environmental impacts and can, in case of certain fungicides such as pyrimethanil, result in the development of resistant *B. cinerea* strains. As a consequence, there is a growing demand for biological treatment of grey mould diseases, including the use of specific biocontrol agents (BCAs).

We previously isolated a fungal BCA from *B. cinerea* infected tomato plants. This fungus, designated as BCA1, exhibited a strong *in vitro* antagonistic effect against different *B. cinerea* isolates and was further explored for its potential biocontrol effect against grey mould disease. Therefore, *in planta* inoculation experiments with BCA1 were performed on *B. cinerea*-infected plants of tomato as well as of the model *Arabidopsis thaliana* (which is also known to be a host for *B. cinerea*). On both plant species, application of spores of BCA1 prior to *B. cinerea* (strain B05-10) inoculation showed a significant preventative protection against grey mould, as no or very little disease symptoms were observed. Moreover, these results were confirmed by qPCR analysis indicating no *B. cinerea* proliferation. Currently, the mode of inhibitory action of BCA1 is being investigated. An update of the latest results will be presented.

P9.7 ESTIMATING THE FIELD *BOTRYTIS* RISK TO MINIMISE THE DISEASE

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"Cinerea" is a software tool for vine growers, designed for the evaluation of the effects of farmers practices and the agronomics field characteristics on the development of grey mould in vineyards. With the estimated impact of each factors weighted within each vineyard, a global grey mould risk in the field can be evaluated with this tool.

Since 2003, Bayer CropScience and the InVivo Opticoop Network cooperatives have worked together to create "Cinerea". Within each field, the vine-grower is able to design and justify the different solutions to control the development of grey mould. 1300 plots have been surveyed to elaborate "Cinerea" - they are representative of the 10 most important french vineyards.

The 13 main factors influencing the development of grey mould have been selected and their local impact estimated. These factors are connected to the plot characteristics (topography, soil depth, susceptibility to drought and water excess), vineyard types (cultivars, pruning type, plant vigour) and crop practices (fertilisation, grape berry moth and powdery mildew protection, grass cover and ventilation of the bunch zone practice).

P9.8 STRATEGIES FOR CONTROL OF POST-HARVEST *BOTRYTIS* INFECTION IN ROSES

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Botrytis infection greatly reduces the vase life of cut-flowers, especially roses. Current post-harvest strategies to reduce its impact are inadequate, especially given the expansion of the international trade in roses and resulting increased transit times. We are investigating improved means of post-harvest chemical control. Several oxidising agents show promise, although some proved phytotoxic. The fungicide Palladium (Switch – Fludioxinil and Cyprodinil), though not effective as a systemic vase solution additive, shows promise as a post-harvest drench. Treatment efficacy is dependent on variety as well as a time/temperature incubation window presumably related to spore development.

6. *BOTRYTIS*"-OMICS"

Botrytis cinerea is one of the major pathogens on grapevine responsible for serious impacts on quality and quantity of harvest. Community efforts allowed the sequencing of the Vitis vinifera genome and those of two *B. cinerea* strains. Along with classical molecular genetic studies, the complete genome sequence of this patho-system opens the way to new possibilities for gene-function analyses in both organisms and their interaction. From the microbiological point of view, comparing *B. cinerea* genes to other fungi helps to identify a signature in *Botrytis* genes and gene content, but also to follow up genome evolution, in particular in the *Botrytis* species complex.

ORAL SESSION

Keynote + Chairperson:

O6.1. Sabine Fillinger and the *Botrytis-Sclerotinia* genome consortium

The *Botrytis cinerea* genome

O6.2. Guillaume Robin, Isidro G. Collado, Mathias Choquer, Jean-Marc Pradier, Guillaume Morgant, Pascal Le Pêcheur, **Muriel Viaud** and the *Botrytis-Sclerotinia* genome consortium

Secondary metabolism genes clusters in *Botrytis cinerea*: a high potential for sesquiterpenes biosynthesis

O6.3. **Punit Shah**, Hind El Mubarek, Gopi K. Podila, James Atwood III, Gerardo Gutierrez-Sanchez, Ron Orlando, Maria R. Davis and Carl Bergmann

Secreted proteome from *Botrytis cinerea*

O6.4. John V.W. Becker, Andreas G.J. Tredoux, Katherine J. Denby and **Melané A. Vivier**

Transciptomic analysis of PGIP-specific resistance phenotypes against *Botrytis cinerea* reveals novel roles of PGIPs in plant defense responses

O6.5. **Katrien Curvers**, Bob Asselbergh, Soraya França, Monica Höfte and Frank Van Breusegem

Transcript profiling of the tomato (*Solanum lycopersicum*) response to the necrotrophic fungus *Botrytis cinerea*

O6.6. Priya Madhou, Joseph Mulema, Oliver Windram, Nicolette Adams, Zennia Paniwnyk and **Katherine Denby**

Unravelling gene regulatory networks governing the *Arabidopsis* response to *Botrytis cinerea* infection

06.1 THE *BOTRYTIS CINEREA* GENOME

SABINE FILLINGER AND THE *BOTRYTIS-SCLEROTINIA* GENOME CONSORTIUM

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Botrytis cinerea and *Sclerotinia sclerotiorum* are destructive polyphageous pathogens belonging to the Leotiomycetes class provoking grey and white mould, respectively. *S. sclerotiorum* is so far the most polyphageous phytopathogenic fungus attacking more than 400 plant species (*vs.* >200 for *B. cinerea*). Both have the same infection strategy, but they strongly differ in their life cycle. The asexual cycle, with the production of macroconidia, is the principal way of propagation and inoculation for the heterothallic *B. cinerea*, whereas the homothallic *S. sclerotiorum* that does not produce asexual spores, undergoes a sexual cycle. In that case, the released ascospores are the predominant inoculants. These important biological differences between such closely related species deserve interest for biological but also genomic analyses.

In 2005, the French national sequencing center (Genoscope) released the genome sequencing *B. cinerea* strain T4 (http://urgi.versailles.inra.fr/projects/Botrytis/private/; free access to consortium members or upon request). At the same time, the Broad Institute released the assembly of the genomic sequence from *B. cinerea* strain B05-10 (TMRI/Syngenta), as well as the genomic sequence of *Sclerotinia sclerotiorum* (Broad Institute; http://www.broad.mit.edu/annotation/fgi/). Automatic gene prediction of the three genomes suggests 14.000 to 16.000 potential genes for both species. Comparative analyses of both *B. cinerea* strains on one hand, and *B. cinerea vs. S. sclerotiorum* on the other hand, revealed a common set of 8400 genes, 1200 of which are absent from other fungal genomes. In addition, both species present a high degree of sequence similarity (76 % average sequence identity in orthologous proteins), but also a good conservation of gene order or even gene structure (exons and introns).

Functional annotation of *B. cinerea* and *S. sclerotiorum* genomes, involving an international consortium of 20 research groups, has been initiated with special regard on the biological differences between both species and in comparison to other pathogenic (or not) fungi. The analysis of the mating type *loci* confirms hetero- *vs.* homothallism in both species, but also reveals some interesting features with respect to its evolution. Concerning secondary metabolism, the absence of sesquiterpen-cyclase genes in the *S. sclerotiorum* genome may explain why this type of toxins is not found in this species. Other aspects that have been analysed, are primary metabolism, stress-related genes, lytic enzymes, secreted proteins, transcription-factors, photo-receptors, membrane transporters, etc. A compilation of the most interesting results will be presented. One of the first functional outputs of the whole genome sequences, is the transcriptome analysis of *B. cinerea* and *S. sclerotiorum* genes during early plant infection on newly designed, genome wide micro-arrays.

06.2 SECONDARY METABOLISM GENES CLUSTERS IN *BOTRYTIS CINEREA*: A HIGH POTENTIAL FOR SESQUITERPENES BIOSYNTHESIS

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Sesquiterpenes are secondary metabolites that are known in fungi as toxins (e.g. trichothecens) and plant hormones (e.g. abscissic acid). As a necrotrophic and polyphageous pathogen, *B. cinerea* secretes unspecific phytotoxins to kill cells from a large spectrum of plants. Amongst the phytotoxic metabolites isolated from fermentation broths, the most well-known is the sesquiterpene botrydial. Inactivation of one of the biosynthetic genes (*CND5/Bcbot1*) in three different strains previously demonstrated that botrydial is a strain-dependant virulence factor (Siewers *et al.,* 2005).

The recent availibility of the *B. cinerea* genome at the Genoscope (http://www.genoscope.cns.fr/) and at the Broad Institute (http://www.broad.mit.edu/annotation/fgi/) provided the opportunity to investigate secondary metabolism gene clusters including those putatively involved in sesquiterpenes synthesis. The "key" enzymes of these pathways are the sesquiterpene cyclases that catalyse the cyclisation of FPP (C15 farnesyl pyrophosphate). Six putative sesquiterpene cyclase encoding genes (S1 to S6) were identified in the *B. cinerea* genome. They are all surrounding by other secondary metabolism genes. The resulting clusters seems to be absent from the genome of the close genus *Sclerotinia*, suggesting recent gene gains or losses.

Functional analysis have been initiated by gene inactivation of cyclase genes in order to identify the produced metabolites and to characterise their role in *B. cinerea* virulence. S1/CND15 cyclase gene is part of the previously described "botrydial genes cluster". S1 null mutant does not produce any botrydial which confirmed that S1/CND15 corresponds to the botrydial cyclase. S2 cyclase is similar to the trichothecenes cyclase (TRI5). S2 null mutant show a reduced colonisation and a delayed conidiation on bean and tomato leaves. Identification of the corresponding metabolite is in progress.

The evolutive story of these sesquiterpene cyclase genes was initiated by looking for their presence in all *Botrytis* species that, apart from *B. cinerea*, have narrow host ranges. All together, these functional and evolutionary approaches aim to understand how *B. cinerea* has gained its sesquiterpenes biosynthesis capacities, and how these capacities contribute to its necrotrophic and polyphageous way of life.

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06.3 SECRETED PROTEOME FROM *BOTRYTIS CINEREA*

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Botrytis cinerea is a filamentous fungus known to infect over two hundred different plants. It is capable of infecting berries, tomatoes and other commercial crops resulting in extensive damage. Enzymes secreted by *B. cinerea* play a vital role in the infection process, aiding in the penetration of the cell wall and in providing a source of nutrition to the fungus. The focus of this research is to obtain a profile of the proteins secreted by *B. cinerea*. Secreted proteins are normally present in low concentration and the extract contains interfering compounds, factors which present a challenge to the analysis.

A method was developed to collect the secreted proteins of *B. cinerea*. The proteins were proteolytically digested in solution using trypsin, and the resulting peptide mixture was loaded onto a capillary column packed with C18 beads using positive nitrogen pressure. The peptides were then separated by reverse phase liquid chromatography and analysed by tandem mass spectrometry on a linear ion trap mass spectrometer. The raw data generated by the mass spectrometer was converted to peak lists, which were searched using the Mascot algorithm. Protein identifications were made using the ProVALT algorithm in which the False Discovery Rate was used to identify statistically significant proteins. The presence of the signal peptides on proteins and their putative functions were determined using the signal P algorithm.

In preliminary studies we were able to identify around ninety secreted proteins from *B. cinerea*. Those proteins identified at a 1% false discovery rate and having signal peptides indicative of secretion, were considered as secreted. Among the proteins identified by MS were found cell wall degrading enzymes, proteases and hypothetical proteins with unknown functions. The secretion of unique proteins helps us to understand fungal pathogenicity and its host interactions. Studies to identify proteins from *B. cinerea* grown under various conditions, designed to help us understand the pathogenic behaviour of the fungus, are underway.

06.4 TRANSCIPTOMIC ANALYSIS OF PGIP-SPECIFIC RESISTANCE PHENOTYPES AGAINST *BOTRYTIS CINEREA* REVEALS NOVEL ROLES OF PGIPS IN PLANT DEFENSE RESPONSES

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Plant polygalacturonase-inhibiting proteins (PGIPs), as a result of their inhibition of fungal endopolygalacturonases (ePGs), are believed to prolong the existence of molecules capable of eliciting plant defense responses. *In vitro* evidence has supported this hypothesis, but extensive data to elucidate the specific mechanisms and pathways involved are lacking. Moreover, the need for *in vivo* methodology to evaluate this inhibition interaction has been highlighted when it was reported that a PGIP from grapevine reduces BcPG2 from *B. cinerea* in *Nicotiana benthamiana* leaves without any evidence for *in vitro* interaction.

Overexpression and silencing studies of PGIP encoding genes provide ample proof that PGIPs confer resistance phenotypes to their respective transgenic plant backgrounds. Recently, PGIP activity, ePG inhibition and decreased *Botrytis* susceptibility could be correlated in tobacco plants overexpressing *Vvpgip1* from *Vitis vinifera* L. Transcriptomic analysis and hormone profiling on some of these lines provided novel insights into the roles of PGIPs. These lines were shown to exhibit altered cell wall metabolism under non-infecting conditions, pointing to cell wall strengthening in response to the constitutively expressed PGIP. Following *Botrytis* inoculation, the analysis focused on the early defense-related responses at the point of infection and immediately surrounding areas and therefore provides data on the local response. *Botrytis*-responsive genes were upregulated in all backgrounds, confirming a successful infection/induction response. Differential upregulation between the control and transgenic lines was observed for both branches of the lipoxygenase (LOX) pathway. The 9-LOX and 13-LOX branches of this pathway were induced to a greater extent in the transgenic lines. These data corroborate the role for PGIP in defense signalling and significantly adds to our understanding of the role of PGIP in plant-pathogen interactions.

06.5 TRANSCRIPT PROFILING OF THE TOMATO (*SOLANUM LYCOPERSICUM*) RESPONSE TO THE NECROTROPHIC FUNGUS *BOTRYTIS CINEREA*

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Botrytis cinerea causes severe crop losses in tomato glass cultivation every year, and no resistant cultivars are commercially available. The tomato (*Solanum lycopersicum*) mutant *sitiens*, reduced in the plant hormone abscisic acid (ABA), is much more resistant to *Botrytis* than the WT tomato. Audenaert *et al*. (2002) showed that ABA interacts with the SA-dependent resistance mechanism of tomato, and physiological data suggest that H_2O_2 originating from the oxidative burst plays an important role in resistance of *sitiens* plants during early stages of plant infection (Asselbergh *et al.*, 2007). However, the oxidative burst is known to be involved in resistance against biotrophic fungi, but was shown to favour the infection of necrotrophic fungi (Govrin and Levine, 2000). In order to further investigate these opposing results and to gain a better insight in the resistance mechanism of the *sitiens* mutant, we assessed the interaction between tomato and *Botrytis* at the transcriptome level. Using microarrays containing 13440 tomato ESTs (TOM1 array, BTI), we monitored gene expression 8 hours upon *Botrytis* infection in wild-type and *sitiens* tomato plants. Analysis revealed a few hundred genes that are significantly differentially expressed in *sitiens* plants at this early stage of infection. Many of them are well-known defence response genes such as PR proteins and cell wall precursors, others are involved in amino acid metabolism and give exciting new insights in possible defence strategies of tomato against necrotrophs. Currently, we are using virus-induced gene silencing (VIGS) to knock down expression of genes selected from the microarray study to characterise their function in disease resistance.

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06.6 UNRAVELLING GENE REGULATORY NETWORKS GOVERNING THE *ARABIDOPSIS* RESPONSE TO *BOTRYTIS CINEREA* INFECTION

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The interaction between the host plant, *Arabidopsis thaliana*, and the necrotrophic fungal pathogen, *Botrytis cinerea* is complex. We have shown that the outcome of this interaction is governed by genetic variation in both *Arabidopsis* and *B. cinerea*. *Arabidopsis* accessions vary in their susceptibility to this pathogen, and *B. cinerea* isolates vary in their ability to cause disease. Given the genetic basis underlying disease, we are interested in how *Arabidopsis* cells recognise *B. cinerea* infection and mount a coordinated defence response. Drop inoculation of leaves generates a gradient of secondary metabolism and gene expression changes and these spatial patterns differ after infection with different *B. cinerea* isolates, indicating different isolates can be recognised to different degrees or produce varying amounts and/or type of a signal.

We used global gene expression profiling to identify several hundred *Arabidopsis* genes with significant changes in expression in response to pathogen infection. Several of these expression profiles have been confirmed using quantitative PCR or GUS reporter gene fusions. We have focused on two groups of genes – those encoding potential regulatory components and those encoding potential secondary metabolism enzymes. RNAi and/or T-DNA insertion lines have been used to assess the effect of knocking out these genes on susceptibility to *B. cinerea* and hence identify genes with a key role in defence against this pathogen. Local networks are being built around these key players.

We have also carried out a high-resolution time series experiment using CATMA arrays to analyse gene expression profiles of infected and uninfected leaves every 2 hr over a period of 48 hrs. Bayesian State space modelling of this extensive data set will enable us to infer gene regulatory networks activated in response to *B. cinerea* infection. An iterative cycle of experiment and modelling will be used to validate these models. The system will be perturbed using RNAi/T-DNA knockouts and data from the knockouts used in retraining the models. The goal is to generate validated predictive models of the gene regulatory networks operating in the host during *B. cinerea* infection.

6. *BOTRYTIS*"-OMICS"

POSTERS

P6.1. Philip Young, John Becker and **Melané Vivier**

The mevalonate- and terpenoid pathway is induced in response to *Botrytis cinerea* infection in both tobacco and grapevine

P6.2. **José J. Espino**, Judith Noda, Nélida Brito and Celedonio González

Analysis of *Botrytis cinerea* secretome during first stages of infection

P6.3. **Evelyn Silva-Moreno**, Carolina Aguayo and Pablo D.T. Valenzuela

Identification and expression analysis of adhesion and germination genes involved in the infection process of *Botrytis cinerea*

P6.4. Francisco Javier Fernández-Acero, María Carbú, Carlos Garrido, Inmaculada Vallejo and **Jesús Manuel Cantoral**

Dissecting the *Botrytis cinerea* proteome

P6.1 THE MEVALONATE - AND TERPENOID PATHWAY IS INDUCED IN RESPONSE TO *BOTRYTIS CINEREA* INFECTION IN BOTH TOBACCO AND GRAPEVINE

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The necrotrophic fungus *Botrytis cinerea* is the causal agent of grey mould in over 200 plant species. Several genetic factors may contribute to the outcome of the plant-fungal interaction. *Solanum tuberosum* microarray slides were utilised to evaluate the localised transcriptomic response of *Nicotiana tabacum* to infection by this pathogen. Upregulation of several *Botrytis*-responsive genes confirmed that a successful infection/induction on tobacco leaf tissue occurred. At 24 hours post-inoculation (hpi), several genes of the terpenoid and lignin biosynthetic pathway were upregulated. Although the induction of the lignin biosynthetic genes were less pronounced at 48 hpi, the genes encoding enzymes involved in the mevalonate pathway and terpenoid formation were, nonetheless, upregulated to substantial levels at this time point. Most notably, the annotation vetispiradiene synthase was induced to levels more than 50- and 8-fold that of their uninfected counterparts at 24 and 48 hpi, respectively. Sequence comparison indicated that this annotation shared the highest homology with tobacco 5 epi-aristolochene synthase, a sesquiterpene synthase involved in the formation of tobacco sesquiterpene phytoalexins. This gene acts at a branch point in the isoprenoid pathway, committing the terpenoid pathway to the formation of the aforementioned compounds. The gene encoding squalene monooxygenase, acting in the branch responsible for sterol production, was however not induced. This may indicate that, in tobacco, following *Botrytis* infection, the formation of sesquiterpene phytoalexins is favoured over sterol production in the terpenoid pathway. The data derived from the transcriptomic tobacco analyses was further investigated using quantitative Real-Time PCR. Real-time PCR was further used to determine the expression levels of the grapevine homologues of the terpenoid pathway genes in *Botrytis*-infected detached grapevine leaves.

P6.2 ANALYSIS OF *BOTRYTIS CINEREA* SECRETOME DURING FIRST STAGES OF INFECTION

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The extracellular proteome, or secretome, of phytopathogenic fungi is a key component of the virulence capacity of these organisms. It contains a diverse range of proteins involved in host cell wall degradation, surface penetration, host cells death, plant compounds detoxification or tissue maceration and colonisation. Especially interesting constituents of this set are those proteins secreted at the beginning of the infection, during the adherence and germination of conidia on the plant surface, since they may play essential roles in the establishment of a successful infection. Analysis of these proteins at the early stages of infection could be a good approach to understand the interaction between these fungi and its hosts.

We have devised a method to prepare and isolate the proteins secreted by *B. cinerea* during the germination of conidia, in conditions that resemble the plant environment. Basically the protocol consists of germinating the conidia for 16 hours in a synthetic medium enriched with low-molecular weight plant compounds, those able to cross a dialysis membrane, and then precipitating twice the proteins from this medium. PAGE of the precipitate showed a relatively high amount of fungal secreted proteins which are not contaminated with plant proteins. 2D electrophoresis of the precipitate showed more than 40 proteins, mainly in the acidic region, with molecular weights in the range of 10-60 kDa. Germination of the conidia in different vegetable extracts produced secretomes with significant differences, as was germination in various synthetics media. The most abundant protein in all conditions has been identified as an aspartic protease, and the corresponding gene has been mutated by gene replacement. Phenotypic characterisation of the mutant is being carried out and will be presented. The rest of the intense spots in the 2D PAGE gel are being identified.

P6.3 IDENTIFICATION AND EXPRESSION ANALYSIS OF ADHESION AND GERMINATION GENES INVOLVED IN THE INFECTION PROCESS OF *BOTRYTIS CINEREA*

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Botrytis cinerea, also known as the "grey mould fungus," causes serious pre- and post-harvest diseases in at least two hundred plant species, including agriculturally important crops and harvested commodities, such as grapes, tomatoes, strawberries, cucumbers, bulb flowers, cut-flowers and ornamental plants. The disease cycle starts with a conidium landing on the host surface. Following attachment, the conidium germinates on the host surface under moist conditions and produces a germ tube that develops into an appressorium that penetrates the host surface and invade the tissue.

Genome-wide analysis provides a new and powerful way of investigating fungal pathogens. Previously we have described the construction and analysis of a cDNA library from *B. cinerea* (Silva *et al.*, 2006) that allowed us to identify several genes putatively involved in pathogenicity.

Herein, we identify *B. cinerea* genes which are preferentially expressed during adhesion and germination and which have orthologs in other ascomycetes. Similarity searches against this cDNA library detected putative orthologs of several genes involved in adhesion and morphogenesis conidiation such us *np-1, arg-2, tec-1, swi-1, mbs-1, sap-4, hyd-1, emp-1* and *mhp-1.* Work is in progress to study the expression of these genes during infection as well as to analyse specific mutants.

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P6.4 DISSECTING THE *BOTRYTIS CINEREA* PROTEOME

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Botrytis cinerea has been described as one of the most devastating phytopathogenic fungi. This is due to the wide versatility of this fungus that is capable to infect a wide range of crops at any plant stage and at any plant structure (leaves, fruits, etc.). Several virulence and pathogenicity factors are produced by the fungus during the infection process. The role of those factors (genes, proteins or toxins) has shown to vary between strains. Experiments with knock out mutants showed that a gene that is essential for the virulence of one specific strain, is expendable in another one. We have selected the analysis of the proteins produced by the fungus under specific growth conditions, i.e. the proteome, to search for proteins involved in different steps of the infection cycle. Differences between proteomes could be ascribed to differences in protein functionality and expression, which are responsible for the phenotype of the strains. Recently, a research programme to study the protein 2-DE profile of *B. cinerea* has been initiated. As a first step to study the fungus proteome, the process to obtain protein extracts, protein separation by 2-DE, and protein identification by MALDI M/S and ESI-IT, was optimised. Twenty-one protein spots were positively identified, which represent the 34% of analysed spots, and the 5.5–5.8% of detected spots. An important number of the identified spots corresponded to MDH, GADPH and cyclophilin, which had been related to virulence in other organisms. Then, an expression proteomic study was initiated to find differences between *B. cinerea* strains differing in virulence and toxin production. The protein profile from cellular extracts of two *B. cinerea* phytopathogenic isolates, *B. cinerea* 2100 (that presents high virulence and toxin production) and *B. cinerea* 1.11 (that does not produce toxins and shows low virulence level), were compared by using two-dimensional gel electrophoresis. From the comparison between proteome profiles, twenty eight spots were selected for identification. Among others, an important number of spots were identified as MDH and GADPH, indicating the possible role of these housekeeping enzymes as pathogenicity/virulence factors.

Currently, we have initiated an ambitious molecular and proteomic approach. We are characterising the expression of extracellular proteins (secretome) with different plant elicitors as carbon source. So far, the 2-DE gels show a low number of spots (3-15) distributed on a wide pH range (3-10). At the same time, using technologies based on ADN/ARN, the role in pathogenicity of several proteins is being elucidated.

7. HOST-PATHOGEN INTERACTIONS

Botrytis cinerea causes serious losses in more than 200 crop species worldwide. It is most destructive on mature or senescent tissues of dicotyledonous hosts and on harvested commodities. The pathogen often gains entry at an earlier stage in crop development and remains quiescent for a considerable period before rapidly rotting tissues when the environment is conducive and the host physiology changes. Molecular-genetic and physiological research has in recent years unraveled a lot of information on the attack mechanisms exploited by the pathogen and the defense mechanisms deployed by the host plant. The interaction of *B. cinerea* with its hosts appears to be a subtle balance in which multiple factors collectively determine whether the pathogen thrives or the plant survives.

This session will discuss the recent views on the biochemical and genetic factors that determine whether or not *B. cinerea* will successfully invade and colonise a plant, with attention for both partners in the interaction. Paul Tudzynski will provide an overview of fungal pathogenicity factors, whereas Jan van Kan will provide an overview of plant defense responses and resistance mechanisms.

ORAL SESSION

Keynote + Chairperson:

O7.1. Jan A.L. van Kan

Host-pathogen interactions: the plant side of the coin

O7.2. **Ann L.T. Powell**, Dario Cantu, Ariel R. Vicente, Molly Dewey, Alan B. Bennett and John M. Labavitch

The susceptibility of ripe tomato fruit to *Botrytis cinerea* is influenced by the structure and composition of the extracellular plant cell wall

O7.3. **Abré de Beer** and Melané Vivier

A small peptide from *Vitis vinifera*, shows antifungal activity towards the necrotrophic plant pathogen *Botrytis cinerea*

O7.4. **Bob Asselbergh**, Soraya França, Katrien Curvers, Frank van Breusegem and Monica Höfte

Resistance to *Botrytis cinerea* in the abscisic acid-deficient *sitiens* tomato mutant is mediated by rapid and extensive hydrogen peroxide accumulation leading to cell wall fortification in the epidermis

O7.5. **Steve Van Sluyter**, Jan van Kan, Filomena Pettolino, Antony Bacic and Elizabeth Waters

Botrytis protease interactions with grape berry proteins

O7.6. Maripaz Celorio-Mancera, L. Carl Greve, Caroline Roper, Cecilia Aguero, Abhaya Dandekar, Bruce Kirkpatrick, Ann L.T. Powell and **John M. Labavitch**

Plant inhibitors that are effective against polygalacturonases (PGs) produced by *Botrytis cinerea* inhibit PGs produced by bacteria and insects

O7.7. Paul Tudzynski

Host-pathogen interaction: pathogenicity determinants in *Botrytis cinerea*

O7.8. Nora Temme and Paul Tudzynski

ROS signalling in *Botrytis cinerea*

O7.9. **Julia Schumacher** and Bettina Tudzynski

The calcineurin-responsive zinc finger transcription factor CRZ1/CRaZy of *Botrytis cinerea* is required for growth, cell wall / membrane integrity, stress response, and full virulence on bean plants

O7.10. Cristina Pinedo, Jean-Marc Pradier, Mathias Choquer, Muriel Viaud and **Isidro G. Collado**

Identification of the *Botrytis cinerea* sesquiterpene cyclase involved in botrydial production

O7.11. **Leonie B. Kokkelink**, Bettina Tudzynski and Paul Tudzynski

Small GTPases in *Botrytis cinerea* – unravelling their role in signalling networks and early steps of pathogenicity

O7.12. Judith Noda, Nélida Brito, José J. Espino and **Celedonio González**

The *Botrytis cinerea* endo-ß-1,4-xylanase Xyn11A displays necrotising activity which is independent of its enzymatic activity on xylan

O7.13. **Yaite Cuesta Arenas**, Eric Kalkman, Alexander Schouten, Peter Vredenbregt, Mirjam Dieho, Beatrice Uwumukiza and Jan van Kan

Functional analysis of *Botrytis cinerea* NEP-like proteins

07.1 HOST-PATHOGEN INTERACTIONS: THE PLANT SIDE OF THE COIN

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Until a few years ago, it was generally considered that host plants play a rather passive role in the interaction with necrotrophs. The pathogen lands on the host surface, kills the underlying tissue, invades the host cells and expands from its primary infection site. The pathogen is not inhibited because it can effectively counteract any host defence mechanisms that may occur. Recent research has revealed that host plants in fact play a much more active role in disease than previously anticipated, and the interactions between plants and necrotrophic fungi are more complex and subtle than commonly appreciated. The ability to induce (programmed) cell death appears to play a pivotal role in the success of *Botrytis* species. I will discuss the role of the host plant in the interaction, with emphasis on the following aspects:

- Formation of Reactive Oxygen Species including Nitric Oxide.
- Senescence, autophagy and programmed cell death.
- Cell death induction determining host specificity of the pathogen.
- Improving plant resistance by transformation with genes interfering with cell death.

I will subsequently discuss the defense responses that plants activate during infection by *Botrytis* species and their contribution to successful resistance mechanisms, with focus on:

- Antifungal metabolites and pathogenesis-related proteins
- Phytohormone-mediated defence pathways
- Studies on gene expression in *Arabidopsis thaliana*
- PGIPs, plant proteins that inhibit fungal endopolygalacturonases

Finally, I will illustrate with some examples that *Botrytis* species must be able to counteract the antifungal activity of plant defence compounds to be a successful pathogen.

07.2 THE SUSCEPTIBILITY OF RIPE TOMATO FRUIT TO *BOTRYTIS CINEREA* IS INFLUENCED BY THE STRUCTURE AND COMPOSITION OF THE EXTRACELLULAR PLANT CELL WALL

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Fruit ripening includes processes that result in a soft, ripe plant organ that is palatable to numerous organisms, but this developmental program also coincides with increased susceptibility to pathogens. While the release of mature seeds is facilitated by the consumption of ripe fruit by seed dispersing animals and pathogens, healthy human diets include generous portions of fully ripened intact fruit. Therefore, understanding what aspects of ripening can be modified so that fruit ripen optimally to contribute to the human diet while not retaining an increased susceptibility to pathogens, is an important goal.

Fruit softening is a significant process during ripening and is associated largely with the disassembly of the major structural components of the plant extracellular compartment, the cell wall polysaccharides (CWs). The roles of plant extracellular enzymes and proteins in the breakdown and relaxation of the structural network of wall polysaccharides, have been described for many fruit, including tomato. In addition to their structural roles for the textural properties of fruit, plant CWs are an obstacle that necrotrophic pathogens encounter during host tissue penetration and colonisation. CW degrading enzymes (CWDEs) are produced by many of these pathogens, and the plant CWs are the targets of these virulence functions.

To determine if CW disassembly by endogenous fruit enzymes and proteins influences susceptibility to the necrotrophic fungus, *Botrytis cinerea*, transgenic tomato fruit with suppressed expression of the ripening-associated polygalacturonase (*PG*) and/or expansin (*LeExp1*), were evaluated. The simultaneous suppression of *PG* and *LeExp1* (-*PG-Exp*) resulted in uninfected fruit that were firmer, with reduced pectin solubilisation and depolymerisation, compared to the control or the -*PG* or the -*LeExp1* fruit. Cell wall swelling, typically noted in ripening fruit, was reduced in -*PG-Exp* fruit as observed *in vitro* and by electron microscopy. The fully ripe -*PG-Exp* fruit had an ca. 70% reduction in susceptibility to *B. cinerea*, whereas the suppression of either *PG* or *LeExp1* alone did not alter susceptibility as measured by tissue maceration and fungal biomass accumulation. When CWs were added as carbon sources for the *in vitro* liquid culture of *B. cinerea,* growth was 3-fold greater on CWs from ripe control fruit than on CWs from ripe -*PG-Exp* fruit, suggesting a direct effect of the fruit CW composition and integrity on the vigour of fungal growth.

Phenolic compounds and lignin accumulate near inoculation sites in ripe -*PG-Exp* tomato fruit, and thus, indicate that induced plant responses are involved in the restriction of *B. cinerea* infection development. Fruit gene expression changes in green fruit inoculated with *B. cinerea* and thus suggests that the pathogen is capable of inducing responses in green fruit before the ripening-associated softening functions are expressed. Comparisons of induced responses in green and red fruit in the presence and absence of PG and *LeExp1*, will demonstrate how the ripening-associated fruit softening processes influence the responses of fruit to *B. cinerea* infections.

07.3 A SMALL PEPTIDE FROM *VITIS VINIFERA*, SHOWS ANTIFUNGAL ACTIVITY TOWARDS THE NECROTROPHIC PLANT PATHOGEN *BOTRYTIS CINEREA*

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Plants are constantly challenged by plant pathogens and have developed a wide array of defense systems to prevent these pathogens from establishing themselves and causing disease. Plants have especially developed strong defense systems to protect their reproductive systems, thus ensuring survival for the next generation. These defense systems consist mainly of preformed defense substances like cell wall degrading enzymes, chemical compounds and small antifungal peptides. Small antifungal peptides play an important role in the protection of seeds during seed development and seed germination. These antifungal peptides are typically secreted into the soil environment during seed germination to form a protective barrier around the developing seedling, protecting it from soil-borne pathogens.

We have identified a nucleotide sequence encoding for a putative plant defensin peptide from *Vitis vinifera*. The complete coding sequence of *VvAMP1* was isolated from cDNA derived from Pinotage berry tissue. Sequence analysis revealed that this 234 bp gene encoded for a 77 amino acid peptide with homology to the superfamily of plant defensins, a group of peptides well documented for their broad spectrum of antifungal activity. Southern blot analysis revealed the presence of two copies of *VvAMP1* within the *V. vinifera* genome. The expression profile of *VvAMP1* was determined by Northern blot analysis conducted on total RNA isolated from various grapevine tissues including the different developmental stages of berry ripening. *VvAMP1* showed a strict tissue specific, developmentally regulated expression pattern, with *VvAMP1* only being expressed in grape berries and only at the onset of véraison, continuing till the end of berry ripening. Localisation of GFP directed by the VvAMP1 signal peptide showed that this peptide is targeted to the apoplastic regions. High levels of GFP were also observed in the xylem and in the guard cells of the stomata, a favourite entry point for fungi. Recombinant production and biochemical characterisation of this peptide has showed that it is active against the plant pathogen *B. cinerea*, causing 50% growth inhibition at concentrations of 12-13 μg/ml. VvAMP1 also severely altered the spore germination rate, growth rate and hyphal morphology of *B. cinerea*. Overexpression of *VvAMP1* is currently being evaluated *in planta* for its potential to confer disease resistance to commercially important crop species.

07.4 RESISTANCE TO *BOTRYTIS CINEREA* IN THE ABSCISIC ACID-DEFICIENT SITIENS TOMATO MUTANT IS MEDIATED BY RAPID AND EXTENSIVE HYDROGEN PEROXIDE ACCUMULATION LEADING TO CELL WALL FORTIFICATION IN THE EPIDERMIS

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Plant defence mechanisms against necrotrophic pathogens, such as *Botrytis cinerea*, are complex and appear to differ from those that are effective against biotrophs. The production of plant secondary metabolites and the ability to control cell death are considered as major determinants of resistance to *B. cinerea*. On the other hand, defence reactions that have a central role in resistance against biotrophic pathogens, such as the production of reactive oxygen species (ROS) linked to a hypersensitive reaction (HR), are generally believed to have a negative effect on defence against *B. cinerea*.

ABA-deficiency in tomato (*Solanum lycopersicum)* leads to high levels of resistance to different pathogens, including *B. cinerea*. Histo-chemical detection of hydrogen peroxide (H_2O_2) with diaminobenzidine revealed that in ABA-deficient *sitiens* tomato, which is highly resistant to *B. cinerea*, accumulation of H_2O_2 at the site of infection was earlier and stronger than in the susceptible wild type (WT). The importance of early H_2O_2 accumulation in the resistance of *sitiens* was shown by treatment with the antioxidants catalase or ascorbate, which respectively removed the early H_2O_2 accumulation partially or completely, and increased disease susceptibility in *sitiens* to an intermediate or excessive level, respectively. Similarly, blocking the production of H_2O_2 with diphenilene iodonium, a known inhibitor of ROS-generating NADPH oxidases, restored susceptibility in *sitiens* to WT levels.

Microscopic assessment showed that in WT, H_2O_2 started to accumulate 24 h post inoculation (hpi), mainly in the mesophyll cell layer, and was associated with the formation of primary necrosis and subsequent spreading cell death. In *sitiens*, H_2O_2 accumulation was already observed at 4 hpi at fungal penetration sites, and quickly thereafter, neighbouring leaf epidermal cells extensively accumulated H_2O_2 in their anticlinal walls. By using different stains, it was shown that the H_2O_2 accumulation resulted in cell wall modification by protein cross-linking and incorporation of phenolic compounds. From 12 hpi, intracellular H_2O_2 was also observed in *sitiens* in HR-like cells, but the reaction remained limited to the epidermal cell layer. The temporal and spatial distribution of H_2O_2 accumulation in both genotypes was confirmed on cross-sections, and in addition, the ultra-structural location of cell wall degradation by *B. cinerea* was evaluated by using the pectin-binding monoclonal antibodies JIM5 and JIM7. *B. cinerea* was able to degrade WT cell walls in the epidermal as well as in the mesophyll cell layer, whereas the degradation in *sitiens* was mostly restricted to the outer periclinal walls of epidermal cells, which indicates the effectiveness of rapid epidermal anticlinal wall reinforcements in hindering colonisation of the underlying tissue. Although plant defence-related ROS formation facilitates necrotrophic colonisation, our data show that ABA-deficiency results in a timely hyperinduction of H_2O_2-dependent defences in the epidermal cell wall, that can effectively block early development of *B. cinerea*.

07.5 *BOTRYTIS* PROTEASE INTERACTIONS WITH GRAPE BERRY PROTEINS

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Thaumatin-like (TL) proteins and chitinases inhibit *Botrytis in vitro* and are the predominant proteins in ripe grapes. Our group and others (Marchal *et al.*, 1998) have found that *Botrytis cinerea* infected grapes have significantly less protein than uninfected grapes. The lower protein concentrations in infected grapes may be caused by reduced expression of TL proteins and chitinases during grape development, by proteolytic enzymes from *Botrytis*, or by grape proteases. We have used mass spectrometry to investigate *Botrytis* proteases and have screened 10 *Botrytis* protease knock-out mutants for differential inhibition by grape TL proteins and chitinases.

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07.6 PLANT INHIBITORS THAT ARE EFFECTIVE AGAINST POLYGALACTURONASES (PGS) PRODUCED BY *BOTRYTIS CINEREA* INHIBIT PGS PRODUCED BY BACTERIA AND INSECTS

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Necrotrophic fungal pathogens, such as *Botrytis cinerea*, secrete cell wall digesting enzymes (CWDEs), including polygalacturonases (PGs), that hydrolyse the homogalacturonan pectins within the protective plant cell wall. Plants, particularly in reproductive tissues (*i.e.*, flowers, fruits), contain extracellular proteins that inhibit these PGs but do not inhibit most endogenous plant PGs. These PG inhibiting proteins (PGIPs) selectively inhibit some but not all of the PG isoforms produced by *B. cinerea*. Several studies have now established that manipulation of PGIPs in plant tissue (tomato, grape, *Arabidopsis*) alters plant susceptibility to infection by *B. cinerea.* Studies in our group have shown that the same PGIPs that inhibit *B. cinerea* PGs also inhibit the PGs of plant bacterial pathogens and insect pests and, thus, may be useful for enhancing crop plant tolerance of attacks from organisms of quite diverse taxonomic groups.

The bacterium, *Xylella fastidiosa,* can be introduced into xylem vessels of grape vines by insect vectors. The *X. fastidiosa* population then spreads throughout the host via the xylem and causes Pierce's disease, an economically significant disease for the grape industry in California. The pathogen produces a PG that has been shown to be a Pierce's disease virulence factor. Transgenic grapevines expressing the pear fruit PGIP that was shown to enhance tomato vegetative and fruit tissue tolerance to *B. cinerea* infection have increased tolerance of infection by both *B. cinerea* and *X. Fastidiosa*. Therefore, it is not surprising that the pear PGIP also inhibits the bacterial pathogen's PG.

The tarnished plant bugs, *Lygus hesperus* and *L. lineolaris*, cause extensive tissue damage and seed and commodity loss in many crop plants, including alfalfa and cotton. The insect uses a lacerate and flush type of feeding, involving modest mechanical damage to plant tissues and the secretion of substantial amounts of a CWDE-rich saliva onto damaged tissues. The insects' salivary secretions contain several PG isoforms. We have shown that introduction of *L. hesperus* PG into the floral tissues of these two crop plants causes flower developmental arrest and abscission, the same responses made by flowers on which *L. hesperus* has fed. We have ascertained that PGIPs in protein extracts from alfalfa and cotton germplasm collections inhibit *B. cinerea* and *L. hesperus* PGs and we have shown that tomato and pear fruit PGIPs inhibit lygus PGs.

Our results suggest that mechanisms used by fungal pathogens, such as *B. cinerea*, to break down plant tissues also are employed by bacterial pathogens and insect pests. One component of interaction between plants and these pathogens and pests has been the plant PGIPs that serve to blunt the effects of the PGs that each of these diverse organisms uses to complete its life cycle. Our hope is that awareness of the factors influencing PGIP interaction with and inhibition of these PGs can inform work aimed at enhancing crop plant tolerance of multiple pests and pathogens.

07.7 HOST-PATHOGEN INTERACTION: PATHOGENICITY DETERMINANTS IN *BOTRYTIS CINEREA*

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Concerted efforts of the rapidly growing *Botrytis* community have made *B. cinerea* to one of the best-investigated fungal pathogens; it is becoming the model system for the study of necrotrophy. Availability of molecular methods and the genome project speeded up functional analyses in the last years. A large set of knock out mutants and the increasing use of "omics" approaches helped to improve our understanding of the complex and highly variable life-style of this pathogen; still our knowledge is limited. I will briefly discuss the results of molecular analyses of "classical" pathogenicity determinants, i.e. the candidate gene approach (cell-wall degrading enzymes, toxic metabolites, etc.). Only very few true pathogenicity factors have been identified/confirmed so far by this approach. Also the status of forward genetic approaches will be briefly reviewed. A major topic will be the role of the reactive oxygen species (ROS) status for the interaction (as seen from the fungal side), and the impact of collaboration/cross-talk of the major signalling cascades on the early infection process. A promising approach for the identification of new pathogenicity determinants is the use of mutants lacking signalling components/transcription factors, with central impact on specific steps in early pathogenesis, in combination with transcriptome and proteome analyses.

07.8 ROS SIGNALLING IN *BOTRYTIS CINEREA*

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Reactive oxygen species (ROS) play a major role in plant-pathogen interactions: phytopathogenic fungi are exposed to the host plant's oxidative burst. So they possess effective ROS-detoxification systems like catalases and peroxidases. They can even produce ROS themselves: NADPH-oxidases reduce oxygen to superoxide radicals; superoxide dismutases convert these radicals to hydrogen peroxide. *Botrytis cinerea* causes the oxidative burst on its host plants. Its virulence correlates with the intensity of this plant defence reaction induced. The grey mould might even depend on the induction of the oxidative burst to achieve full pathogenicity. To analyse ROS signalling in *B. cinerea*, hypothetical components of the stress-activated MAPK (SAPK) cascade were identified and characterised.

The bZIP transcription factor Bap1 homologous to yeast's Yap1 is a regulator in the oxidative stress response (OSR) in *B. cinerea*. Its deletion resulted in decreased resistance to H_2O_2 and menadione. Expression of genes (*cat2*, *trr1*, *glr*) encoding anti-oxidising proteins was induced in the wild type under oxidative stress but not in the Δ*bap1* mutant. Macroarray analyses revealed additional Bap1-dependent genes. The SAPK BcSak1 is activated under oxidative and osmotic stress and under specific fungicides. BcSak1 is, in contrast to Bap1, essential for pathogenicity and conidiogenesis (Segmüller *et* al., 2007), however, Δ*bcsak1* and Δ*bap1* mutants share at least one target gene *cat2*, indicating a BcSak1-Bap1 cross talk. Downstream components of the SAPK might cooperate with Bap1 in gene regulation. Characterisation of the transcription factor BcAtf1 possibly activated by BcSak1, will reveal its role in cooperated gene regulation with Bap1 via dual activation. Further putative sensing components of the SAPK cascade are the class X histidine kinase BcHk1 that might act as a negative regulator of BcSak1 and the response regulator BcSkn7. Its role in OSR gene regulation as well as its cross-talk function between different signalling pathways is under examination.

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07.9 THE CALCINEURIN-RESPONSIVE ZINC FINGER TRANSCRIPTION FACTOR CRZ1/CRAZY OF *BOTRYTIS CINEREA* IS REQUIRED FOR GROWTH, CELL WALL/ MEMBRANE INTEGRITY, STRESS RESPONSE, AND FULL VIRULENCE ON BEAN PLANTS

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Recently, we have shown that the α-subunit BCG1 of a heterotrimeric G-protein which plays an important role during the infection of host plants by the grey mould fungus *B. cinerea,* is an upstream activator of the Ca2+/calmodulin-dependent calcineurin-phosphatase, in particular in regulation of secondary metabolism (production of the phytotoxin botrydial).

In order to identify the transcription factors downstream of BCG1 and calcineurin in this signalling cascade, we cloned the *B. cinerea* homologue of CRZ1/ CRaZy (Calcineurin-Responsive Zinc finger transcription factor), the mediator of calcineurin function in yeast. Complementation- and GFP-localisation studies in yeast showed that BcCRZ1 is able to complement the corresponding yeast mutation, and that the sub-cellular localisation of the GFP-BcCRZ1-fusion product in yeast cells is dependent on calcium levels and the activity of calcineurin.

Bccrz1-deletion mutants are not able to grow on minimal media. On other plant compound-containing media, growth rate, conidiation, and sclerotia formation are impaired. Addition of Mg2+ to the medium restores specifically growth rate and conidiation, but not the ability to form sclerotia. Moreover, the mutants are affected in cell wall and membrane integrity since they are more sensitive to the treatment with cell wall degrading enzymes and membrane disturbing compounds such as SDS.

Interestingly, BcCRZ1 seems to be dispensable for the conidia-derived infection of bean plants, whereas mycelium of the deletion mutants is strongly impaired in its ability to penetrate the intact surfaces of bean leaves and tomato fruits.

Northern analyses have shown that the expression of BCG1- and calcineurin-dependent genes is also down-regulated in Δ*bccrz1*-mutants, confirming our suggestion that this transcription factor acts downstream of calcineurin in *B. cinerea*. Since Δ*bccrz1*-mutants are still responding to calcineurin-inhibitors, we conclude that BcCRZ1 is not the sole signalling effector of calcineurin.

07.10 IDENTIFICATION OF THE *BOTRYTIS CINEREA* SESQUITERPENE CYCLASE INVOLVED IN BOTRYDIAL PRODUCTION

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Botrytis cinerea (teleomorph: *Botryotinia fuckeliana*) is the causal agent of grey mould diseases in a broad range of dicotyledonous plants. As a typical necrotroph, its infection strategy includes killing of host cells and feeding on dead tissue; it secretes cell wall degrading enzymes and toxic metabolites, inducing cell death in advance of the invading hyphae. Amongst isolated metabolites, botrydial (**1**), a tricyclic sesquiterpene, and botcinins (before named botcinolides) displayed a high phytotoxic activity. Botrydial (**1**) reproduces the characteristic symptoms of *B. cinerea* infection, and its accumulation *in planta* has been demonstrated during infection process. All these compounds are unspecific phytotoxins which fits well to the broad host range of this pathogen. Although evidence that botrydial is a strain-specific virulence factor, has been reported, no unequivocal proof for an essential role of these toxins in the pathogenicity of *B. cinerea* has been found.

During the screening of calcineurin-dependent (CND) genes of *B. cinerea*, a secondary metabolism gene cluster was identified. Inactivation of one of the clustered genes, *Bcbot1/cnd5* encoding a P450 monooxygenase, demonstrated that it was essential for botrydial synthesis. Here, we present the functional characterisation of the *S1/Cnd15* gene, that is part of the cluster and encodes a putative sesquiterpene cyclase. Study of secondary metabolites produced by the *S1/Cnd15* null mutant, evidenced that the gene *S1/Cnd15* is involved in the first step of botrydial pathway, and correspond to the botryane skeleton cyclase.

Gene inactivation was realised in the B05-10 *ku70* modified strain, that allows a high rate of homologous recombination. The resulting B05-10 *ku70 cnd15*Δ mutant did not show any significant defect in saprophitic growth or virulence. The extracts obtained from fermentation broth of isolates B05-10 *ku70* (reference strain) and mutant strain B05-10 *ku70 cnd15*Δ, were separated by column chromatography and studied by extensive spectroscopic methods, specifically ^{1}H-NMR and ^{13}C-NMR. Botrydial (**1**) and its derivatives **2** and **3** were isolated and its structures characterised from the reference strain, while, from the null mutant, neither botrydial (**1**) nor some botryane derivatives were isolated or detected. Curiously, a high amount of 3-*O*-acetyl botcinic acid (**4**) and botcinin A (**5**) were isolated from the *S1/Cnd15* null mutant.

07.11 SMALL GTPASES IN *BOTRYTIS CINEREA* – UNRAVELLING THEIR ROLE IN SIGNALLING NETWORKS AND EARLY STEPS OF PATHOGENICITY

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In *Botrytis cinerea*, several components of signalling pathways have been identified which are involved in pathogenicity, e.g. members of the cAMP signalling (Schulze Gronover *et al*., 2004). Yet, so far there is little knowledge about the impact of small GTPases, belonging to the Ras superfamily, on pathogenicity in *B. cinerea*, and about connections to other signalling pathways.

We generated knock-out mutants of several small GTPase encoding genes from *B. cinerea*: two genes encoding Rho type GTPases, *bccdc42* and *bcrac*, and two Ras homologues, *bcras1* and *bcras2*, and characterised them in pathogenicity assays, epidermis penetration experiments, germination assays and microscopy, to determine the role of these small GTPases in pathogenicity and early infection stages.

bcras1 and *bcrac* deletion mutants are viable, but show a similar severe phenotype, characterised by an undirected hyphal growth with abnormal branching pattern, a lack of sporulation and full apathogenicity, indicating that BcRas1 and BcRac act in one signalling pathway.

In contrast, the Δbccdc42 mutants grow properly on rich media and are able to form conidia. These conidia are able to germinate, although the mode of germination on different nutrients is affected. Δbccdc42 mutants cause a retarded infection and seem to have a penetration defect.

Strains with a deletion in the *bcras2* gene show a retarded growth on different media but are also able to conidiate. The infection development and germination process is delayed, interestingly the latter could be partially restored by the addition of cAMP, indicating a link between BcRas2 and cAMP signalling.

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07.12 THE *BOTRYTIS CINEREA* ENDO-ß-1,4-XYLANASE XYN11A DISPLAYS NECROTISING ACTIVITY WHICH IS INDEPENDENT OF ITS ENZYMATIC ACTIVITY ON XYLAN

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The endo-ß-1,4-xylanase Xyn11A has been previously shown to be required for virulence in *Botrytis cinerea*. Targeted inactivation of the *xyn11A* gene resulted in a reduced virulence phenotype that was restored by transformation with the wild-type gene (Brito *et al.*, 2006). However, it is not clear whether the contribution of Xyn11A to virulence could be due to its endo-ß-1,4-xylanase activity or to a possible necrotising activity previously reported for similar xylanases from other fungi (Furman-Matarasso *et al.*, 1999). Now we have expressed Xyn11A in *Pichia pastoris* and have purified it form the culture filtrate. The isolated enzyme showed high activity towards xylan, with an optimum pH of 5 and an optimum temperature of 35ºC, and was also able to elicit necrosis and production of reactive oxygen species in tomato or tobacco when infiltrated in the leaves. We have also designed, expressed and purified four variants of the protein with point mutations in either of the two glutamate residues of the active site, which are essential for activity. Neither of the four enzymes retained any detectable endo-ß-1,4-xylanase activity, but all of them were still able to elicit necrosis on tomato and tobacco leaves. The ability of these four protein variants to complement the *xyn11A* mutant is being studied and will be reported.

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Furman-Matarasso, N., Cohen, E., Du, Q. S., Chejanovsky, N., Hanania, U. & Avni, A. (1999). A point mutation in the ethylene-inducing xylanase elicitor inhibits the beta-1-4-endoxylanase activity but not the elicitation activity. *Plant Physiology* 121:345-351.

07.13 FUNCTIONAL ANALYSIS OF *BOTRYTIS CINEREA* NEP-LIKE PROTEINS

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Necrosis and ethylene-inducing proteins (NEP) have been described in bacteria, oomycetes and fungi, and have been proposed to act as phytotoxin in dicotyledons. However, they have not been shown to exhibit any phytotoxic activity in monocotyledons. The mechanism of action has thus far not been elucidated. This ever-expanding family of related proteins is characterised by a highly conserved sequence motif (GHRHDWE) of unknown function. The *Botrytis cinerea* genome contains two genes, encoding NEP-like proteins, namely BcNEP1 and BcNEP2. Knockout mutants in the *Bcnep1* or *Bcnep2* gene do not show a reduced virulence on tomato or *Nicotiana benthamiana*. To study the *in planta* expression pattern of *Bcnep* genes, constructs of Green Fluorescent Protein (GFP), under control of the native promoters of either *Bcnep1* or *Bcnep2*, were transformed to *B. cinerea*, and GFP expression was followed in time, after infection on flower petals, using confocal laser scanning and wide-field fluorescence microscopy.

Both BcNEP proteins have been produced by heterologous production in *Pichia pastoris*. Infiltration of the purified proteins into *Nicotiana benthamiana* leads to a rapid induction of ethylene in a dose-dependent manner. BcNEP1 is capable of inducing ethylene at much lower concentrations than BcNEP2. Both proteins contain a number of cysteine residues, which are predicted to form disulfide bridges. They also possess several potential post-translational modification motifs for phosphorylation, N-glycosylation, O-glycosylation. In order to study which amino acid residues are important for the phytotoxic activity of the *B. cinerea* NEP proteins, site directed mutagenesis was performed. Preliminary results suggest that mutation of certain cysteine residues or post-translational modification motifs does not lead to total loss of function, whereas mutation of the conserved heptapeptide motif completely abolishes the phytotoxic activity. A comprehensive update of the results will be presented.

7. HOST-PATHOGEN INTERACTIONS

Posters

P7.1. **Sandiswa Mbewana**, Albert Joubert and Melané Vivier

Transgenic tobacco with altered lignin levels: analysis of possible fungal resistance phenotypes

P7.2. **Marthèlize Tredoux**, Abrè de Beer, Lizel Mostert and Melanè Vivier

The evaluation of the antifungal activity of a *Vitis vinifera* antifungal peptide

P7.3. **Alida Venter**, Albert Joubert and Melané Vivier

The evaluation of a range of *Vitis* polygalacturonase-inhibiting proteins (PGIPs) for their antifungal activity against *Botrytis cinerea*

P7.4. **Punit Shah**, Shreyal Patel, James Atwood III, Gerardo Gutierrez-Sanchez, Ron Orlando and Carl Bergmann

Mass spectrometric characterisation of the post translation modification of *Botrytis cinerea* endopolygalacturonase 3 (BcPG3)

P7.5. Jacinto Rámirez-Fernández, Cristina Pinedo, Rosario Hernández-Galán, Muriel Viaud and **Isidro G. Collado**

The versatile toxin production mechanism of *B. cinerea*: an overview and revision of polyhydroxilated metabolites

P7.6. A. Gioti, **Jean-Marc Pradier**, E. Fournier, P. Le Pêcheur, C. Giraud, D. Debieu, J. Bach, P. Leroux and C. Levis

A *Botrytis cinerea* emopamil binding domain protein affecting virulence belongs to a eukaryotic superfamily which has expanded in euascomycetes

P7.1 TRANSGENIC TOBACCO WITH ALTERED LIGNIN LEVELS: ANALYSIS OF POSSIBLE FUNGAL RESISTANCE PHENOTYPES

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Lignification is a common response to infection and wounding. It provides an effective physical barrier against pathogen colonisation and growth. Recently, our group has observed that transgenic tobacco lines overexpressing an antifungal protein, showed an upregulation of lignin biosynthesis, even in the absence of infection. The lines overexpressed the grapevine polygalacturonase-inhibiting protein (VvPGIP1) and have been shown to display a PGIP-specific resistance phenotype against *Botrytis cinerea*. A genome-wide transcriptional analysis of these tobacco lines indicated that a *cinnamyl alcohol dehydrogenase* (*CAD*), as well as other lignin biosynthetic genes, was upregulated in healthy uninfected tissue. Biochemical evidence corroborated the indication of increased lignin deposition in their cell walls. These reinforced cell walls might thus be "primed" before pathogen ingress, contributing to the observed decrease in disease susceptibility observed in lines accumulating high levels of PGIP.

The aim of this study was to confirm the observed phenotype by studying and overexpressing the *CAD* gene in tobacco. The objective is to understand one of the mechanisms by which PGIP reduced disease susceptibility and to establish a phenotype linked to lignin deposition in stable *CAD* transgenic lines.

The CAD encoding gene was isolated from tobacco (*Nicotiana tabacum* c Petite Havana SR1), cloned into a plant expression vector, under the control of a constitutive promoter and transformed back into tobacco. Gene integration and copy number was determined by PCR analysis and Southern blots. Gene expression was determined by northern blots. CAD activity assays as well as lignin content determination proceeded. The transgenic lines will be comprehensively analysed to determine the resistance phenotypes when infected with *B. cinerea*.

P7.2 THE EVALUATION OF THE ANTIFUNGAL ACTIVITY OF A *VITIS VINIFERA* ANTIFUNGAL PEPTIDE

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A putative antifungal peptide encoding gene (*VvAMP1)* was previously isolated from *Vitis vinifera* and shown to belong to the plant defensin family of peptides. Purified VvAMP1 showed varying degrees of activity against a number of pathogens. A spectrophotometric antifungal assay was used to determine the antifungal activities against *Botrytis cinerea, Phomopsis viticola, Phaeomoniella chlamydospora, Phaeoacremonium aleophilum* and *Cylindrocarpon liriodendri.* The VvAMP1 peptide inhibited the growth of *B. cinerea* with IC_{50} values of 13 μg/ml. Preliminary results suggest similar growth inhibition of *P. viticola* at 5 μg/ml and *C. liriodendri* at 7 μg/ml. The *VvAMP1* gene was also overexpressed in *Vitis vinifera* cultivars Sultana and Red Globe to evaluate its antifungal effectiveness *in planta*. Genetic characterisation of transgenic grapevine lines confirmed integration and expression of the transgene. Western blot analysis will confirm the production of the VvAMP1 peptide in the grapevine tissues. Selected populations of the transgenic grapevines, as well as untransformed controls will be hardened off and infected with *Botrytis* to follow the infection in the various genotypes. A time-course whole plant infection will be conducted to determine the antifungal peptide's ability to protect the plants against fungal infection. The plant material will also be used to study the mode of action of the peptides *in planta* in interaction with fungal pathogens.

P7.3 THE EVALUATION OF A RANGE OF *VITIS* POLYGALACTURONASE-INHIBITING PROTEINS (PGIPS) FOR THEIR ANTIFUNGAL ACTIVITY AGAINST *BOTRYTIS CINEREA*

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Several aspects regarding polygalacturonase-inhibiting proteins (PGIPs) infer or confirm their role in defense against phytopathogenic fungi. These proteins are capable of inhibiting an extensive range of endopolygalacturonases (ePGs) from not only fungal, but also of insect origin. The inhibition potential of these proteins may extend to a much wider range of organisms, to date most of those tested have been shown to be inhibited to some degree. Small *pgip* gene families seem to have been maintained through evolution, providing plants with PGIPs with distinct inhibitory capabilities, which may also be regulated by separate signal transduction pathways. Furthermore, the structure of PGIP allows it to be targeted to the cell wall, where it interacts with ePGs.

Numerous genetic backgrounds in which *pgip* genes were overexpressed have been afforded a degree of protection from fungal virulence, more specifically the necrotrophic fungus *Botrytis cinerea*. These include not only the model plants *Nicotiana tabacum* and *Arabidopsis thaliana*, but the economically important fruit crops tomato and grapevine. In the case of the latter, an increased tolerance to a bacterial pathogen of grape was also observed.

A grapevine PGIP, designated VvPGIP1, has been isolated from *Vitis vinifera* cv. Pinotage. It has been shown that over-expression of the *Vvpgip1* gene in tobacco reduced the disease susceptibility of transgenic tobacco when infected with *Botrytis cinerea*. Subsequently, 37 additional grapevine *pgip*-encoding genes, from *Vitis* and non-*Vitis* grapevine species, known to be highly resistant and even immune to fungal infection, were isolated. These 37 PGIPs were grouped into 14 different sets based on the structure of their active domains. A selection of these genes were analysed further for their inhibition profiles. Overexpression in tobacco allowed for the *in planta* evaluation of the different PGIPs against *B. cinerea* infection.

P7.4 MASS SPECTROMETRIC CHARACTERISATION OF THE POST TRANSLATION MODIFICATION OF *BOTRYTIS CINEREA* ENDOPOLYGALACTURONASE 3 (BCPG3)

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Botrytis cinerea is a widespread plant pathogen that causes grey mould or soft rot on host tissues and is responsible for significant economic loss. *B. cinerea* must breach the plant cell wall during the infection process, which is partially accomplished by secreted enzymes that degrade cell walls. Polygalacturonases (PG) are one of the initial protein families secreted by *B. cinerea* in a successful infection of the host. Six *B. cinerea* genes encoding PGs have been studied and the results indicate that each gene is differentially expressed depending on factors such as the stage of infection, plant species and temperature.

The characterisation of the location and structure of the carbohydrate moieties of *B. cinerea* endopolygalacturonase 3 is described here. The glycoprotein is first analysed by Matrix Assisted Laser Desorption/Ionisation Mass spectrometery (MALDI-MS) to obtain the molecular masses of BcPG3, prior to, and following treatment with the enzyme Endoglycosidase H. From the theoretical mass of the protein obtained from the genome and our MALDI-MS, we were able to calculate the approximate mass of the *N*-linked and *O*-linked glycans. The location of the *N*-linked glycans in the protein was determined following trypsin digestion and analysis of the resulting peptides by Quadruple Time of Flight mass spectrometry using a stepped orifice voltage technique, while the structures of the N-linked carbohydrates were obtained from ESI MS/MS data. Two *N*-linked glycosylation sites were identified which are occupied by high mannose *N*-linked glycans. The location of *O*-linked glycans on the protein BcPG3 was obtained by trypsin degradation, Beta-Elimination and Michael Addition with Dithiothreitol (BEMAD) followed by LC-MS/MS analysis on an ion trap mass spectrometer. The *O*-linked sugars obtained after beta elimination were purified and permethylated and studied by Fourier Transform Ion Cyclotron Resonance mass spectrometer (FTICR).

The use of a variety of mass spectrometry based techniques was necessary for the comprensive determination of the locations and structures of the carbohydrates that modify the original peptide backbone following translation. Because such modifications have effects on the structure, half-life, and activity of the proteins they modify, a complete characterisation of all PTMs on BcPG3 is necessary to fully understand the interaction of this enzyme with other biologically significant macromolecules.

P7.5 THE VERSATILE TOXIN PRODUCTION MECHANISM OF *B. CINEREA*: AN OVERVIEW AND REVISION OF POLYHYDROXILATED METABOLITES

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The initiation of disease by *Botrytis* species depends on a complex sequence of biological events, involving host and environmental sensing, chemical and physical interactions between the fungal propagules and the host surface, and microbial interactions on the surface of the host. *Botrytis cinerea* has a broad habitat range, is not restricted with regard to host or tissues, and uses various infection strategies to cope with different conditions. The infection of host plants by *Botrytis* spp. is mediated by numerous extracellular enzymes and metabolites, each of which may play a role in different stages of the infection process.

Several secondary metabolites showing phytotoxic and apoptotic activities have been identified in culture filtrates of *B. cinerea.* There is no evidence for the production of host-specific toxins by *B. cinerea*, which is in accordance with the broad host range of this pathogen. The fungus produces two series of phytotoxic metabolites: a family of characteristic sesquiterpene metabolites which contain the basic botryane skeleton, principally botrydial and dihydrobotrydial; and polyketide lactones known as botrylactone and botcinolides.

During the screening of calcineurin-dependent (CND) genes of *B. cinerea*, a secondary metabolism gene cluster was identified. Inactivation of some of the clustered genes, *Bcbot1/cnd5* and *S1/Cnd15*, demonstrated that they were essential for botrydial synthesis. Inhibition of botrydial biosynthesis was accompanied by a higher production of polyketide toxins. The toxin production of these mutants revealed a versatile production mechanism, producing higher amounts of polyketide toxins, while the wild type strain produced only botryane toxins (botrydial and derivatives). This seems to indicate that *B. cinerea* have different infection strategies and a possible host adaptation.

The higher production of polyketide toxins from the mutant strain B05-10 *ku70 cnd15*Δ, led us to investigate the molecular structures of those polyketide toxins. Here, we present an overview of this toxin type and a revision of the chemical structures of some botcinolides reported.

P7.6 A *BOTRYTIS CINEREA* EMOPAMIL BINDING DOMAIN PROTEIN AFFECTING VIRULENCE BELONGS TO A EUKARYOTIC SUPERFAMILY WHICH HAS EXPANDED IN EUASCOMYCETES

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A previous transcriptomic analysis of 3.032 fungal genes identified the *BcPIE3* gene, up-regulated early *in planta* (Gioti *et al.*, 2006). Here, *BcPIE3* was disrupted to determine its implication in pathogenicity. BcPIE3 is a virulence factor, since the ΔBcPIE3 mutant was blocked during colonisation of tomato and bean leaves, giving lesions reduced by at least 74 %. BcPIE3 shares significant structural similarities to mammalian Emopamil Binding Proteins (EBPs) within the emopamil binding domain (EBD). Mammalian EBPs function as sterol isomerases, but analysis of the sterol content and growth inhibition experiments with the ΔBcPIE3 strain indicated that BcPIE3 is dispensable for ergosterol biosynthesis. The systematic identification of proteins containing the EBD domain in public databases showed that they constitute a protein superfamily, present only in eukaryotes. Phylogenetic analysis showed that the ancestral EBD-coding gene was duplicated in the common ancestor of animals and fungi after its split from plants. We present evidence that the EBP phylogenetic clade of this superfamily has further expanded exclusively in Euascomycetes, especially in the *B. cinerea* genome which contains four copies. Its first characterised fungal member, BcPIE3, has a function related to pathogenic interactions.

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8. OPEN SESSION: BOTRYTIS MANAGEMENT, INDUSTRY AND THE FOOD CHAIN

Oral Session

Chairperson for Open Session: Paul Fourie

Keynote:

O8.1. Stéphane La Guerche, Brunhilde Dauphin, Pierre Sauris, Dominique Blancard and **Philippe Darriet**

Botrytis cinerea and other bunch rot complexes : impact on musts and wines off-flavours

O8.2. **Stéphane La Guerche**, Laure de Senneville, Dominique Blancard and Philippe Darriet

Botrytis cinerea strains and the level of (-) geosmin produced by *Penicillium expansum*

O8.3. Stella M. Zitter and **Wayne F. Wilcox**

Physical modes of action of fungicides used for control of Botrytis bunch rot of grapes

O8.4. **Anne-Sophie Walker**, Sabine Fillinger, Danièle Debieu, Matthias Hahn and Pierre Leroux

Resistance of *Botrytis cinerea* to fungicides: still a problem in vineyards?!

O8.5. **Matthias Kretschmer**, Anne-Sophie Walker, Michaela Leroch, Melanie Wiwiorra, Sabine Fillinger, Pierre Leroux, Henk-jan Schoonbeek and Matthias Hahn

High frequency occurence of multiple fungicide resistance in *B. cinerea* field strains from French and German vineyards

O8.6. Hélène Lachaise

Life cycle management of a botryticide

O8.7. **Jan-Cor Brink** and Paul Fourie

Fungicide spray cover in grapevine canopies and control of *Botrytis cinerea*

O8.8. Sybrand A. van Zyl, Jan-Cor Brink and **Paul H. Fourie**

The use of surfactants to improve control of *Botrytis cinerea* on grape leaves

O8.9. **Anne-Noëlle Petit**, M.L. Panon, N. Vaillant-Gaveau, F. Mazeyrat-Gourbeyre, F. Baillieul, C. Clément and F. Fontaine

Treatment efficacy against grey mould of the grapevine and defence mechanisms activation

O8.10. Kobus Hartman

Botrytis control: can it meet the latest demands of the producer, retailer, consumer and agrochemical supplier?

O8.11. Alfons Sagenmüller

Sharing success through Food Chain Partnership based on integrated control of *Botrytis*

08.1 *BOTRYTIS CINEREA* AND OTHER BUNCH ROT COMPLEXES : IMPACT ON MUSTS AND WINES OFF-FLAVOURS

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The development of *Botrytis cinerea* on grapes is known to have a great importance on the flavour composition of musts and wines. When colonising overmature grapes berries, and under specific climatic conditions, the fungus greatly modifies grape composition. This process, identified as noble rot, permits the elaboration of quality wines with complex and distinctive aroma. However, *B. cinerea* is usually one of the major causes of damage of a number of grape components and thus of deterioration in wine quality. During the recent years, several aromatic defects with a mushroom, moldy or earthy character, combined with the more or less visible development of rots on grapes, have been underlined in grapes and wines from various vine regions of France and Europe. The importance of these damages on the quality of wines from numerous cultivars (Cabernet Sauvignon, Semillon, Gamay, Chenin, Pinot noir) has led to a detailed study aiming firstly at the characterisation of the compounds responsible for these off-flavours, and secondly, at the specification of their biological origin and the conditions of their expression in the vineyard.

Using various gas chromatography techniques, volatile compounds with mushroom, mossy, or earthy odours could be identified and were assayed in grapes and wines presenting these defaults. Some compounds, such as 2-heptanol, 2-octen-1-ol, 2-methylisoborneol, fenchol and fenchone, were detected in the grapes and musts, but were not found in wines, whereas others were present in grapes and musts and remained after alcoholic fermentation, and could be prejudicial to the quality of wine [1-octen-3-ol, (-)geosmin]. Sometimes, also fungal compounds were found in wines at higher levels than in grape juice. (-) geosmin, with a powerful damp earthy, beetroot odour, was the main defect found in numerous grapes and wines.

When analysing the microflora present on rotten grape bunches, the presence of *Botrytis cinerea* was always evident. This fungus is known for its ability to produce the potent 2-methylisoborneol with earthy and camphoreous odour. But the main fungal off-flavours in wines, were related to grapes presenting bunch rot complexes between *Botrytis cinerea* and secondary invaders belonging to various species, especially from the *Penicillium* genus. The implication of these fungi on the formation of several aromatic defects was analysed. Particularly, through the study of (-)geosmin biosynthesis by *Penicillium expansum*, the biological and metabolic role of *Botrytis cinerea* in the bunch rot complexes was emphasised.

08.2 *BOTRYTIS CINEREA* STRAINS AND THE LEVEL OF (-) GEOSMIN PRODUCED BY *PENICILLIUM EXPANSUM*

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Geosmin, which causes an off-flavour of some rotten grapes, has been implicated in wine defects. It's biosynthesis by *P. expansum* was demonstrated in grape juice and on crushed grapes, which had been pre-cultured with some *B. cinerea* strains. Therefore, the *B. cinerea* / *P. expansum* model was studied in depth regarding the role of *B. cinerea* strain on *P. expansum* geosmin synthesis.

One hundred and fifty six *B. cinerea* strains were isolated from the centre of 120 matured, rotten Gamay grape bunches, collected at various sites in the Beaujolais region. All *B. cinerea* strains were characterised by morphological criteria. The strains were maintained on different media (GJ, Czapek, CYA, Malt agar) and pre-cultivated on Grape Juice medium (GJ) in order to assess their capacity to induce geosmin synthesis by *P. expansum*. Irrespective of the medium, no geosmin was detected above the detection threshold (i.e. 5 ng/Petri dish) in *B. cinerea* cultures. Thirty four (22%) *B. cinerea* strains were able to induce high geosmin production, up to 494 ng/l, by *P. expansum* in grape juice ([bot +] phenotype). For the remainder of the strains tested, labelled with the [bot -] phenotype, no geosmin was detected above 5 ng/l. The significant proportion of *B. cinerea* [bot +] strains postulates that they are necessary for geosmin synthesis by *P. expansum* on grapes.

The metabolism of amino acids from grape juice, by *B. cinerea*, is a parameter conducive to geosmin production by *P. expansum*. However, the amino-acid and ammonium concentrations in grape juices pre-cultured with *B. cinerea* [bot -] and [bot +] strains were very similar, implying that other factors are involved as well. Indeed, an ethanol-precipitable fraction, probably a polysaccharide, synthesised by *B. cinerea* [bot -], but not [bot +] strains, was shown to inhibit geosmin production by *P. expansum*.

08.3 PHYSICAL MODES OF ACTION OF FUNGICIDES USED FOR CONTROL OF BOTRYTIS BUNCH ROT OF GRAPES

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With respect to fungicides, the term "physical mode of action" is typically used to denote the activity of a material in the sense of its temporal or physical placement relative to a pathogen infection event. Knowledge of specific fungicide properties allows users to deploy them most intelligently and efficiently. Our objective was to better characterise these properties for various fungicides currently used to control *Botrytis cinerea* on grapes.

Fungicides (applied as commercially formulated product) and rates (USA label equivalents) were: boscalid (420 mg/L), cyprodinil (563 mg/L), fenhexamid (600 mg/L), iprodione (1,200 mg/L), pyraclostrobin+boscalid (182+359 mg/L), pyrimethanil (826 mg/L), and trifloxystrobin (113 mg/L). To investigate their ability to control quiescent infections long after initiation, potted Pinot noir vines were inoculated at flowering with conidia of an anilinopyrimidine (AP)- and dicarboximide-sensitive isolate of *B. cinerea,* and clusters were sprayed individually at veraison (60 and 54 days after inoculation in 2005 and '06, respectively); half also were sprayed 15 days later. Clusters were harvested 7-10 days after the final spray date, and quiescent infection frequency determined using the freezing technique. In both years, a single application of fenhexamid, cyprodinil, or pyrimethanil at veraison provided 87-98% control of quiescent infections initiated at flowering; a second application 2 wk after the first often provided modest additional benefit. Iprodione provided 62 and 72% control following a single application at veraison, but was statistically equivalent to the above materials following the second application. Trifloxystrobin, pyraclostrobin+boscalid, and boscalid solo provided generally fair to poor control under these conditions. To determine the ability of fungicides to provide residual protection of internal infection courts (e.g., as exposed by injury), clusters of potted Chardonnay (2005) or Pinot noir (2006) vines were sprayed at fruit set, bunch closure and veraison. Then, 3 berries per cluster were injected with *B. cinerea* spores either 2 or 3 wk after the final spray, and disease was evaluated at harvest. In both years, fenhexamid, pyrimethanil, cyprodinil and iprodione provided virtually complete control of internal infections following both inoculations, with no spread of disease to uninoculated berries. Pyraclostrobin+boscalid allowed a substantially greater frequency of necrotic, sporulating berries in 1 of the 2 years, although it largely prevented spread to uninoculated fruit. Trifloxystrobin was only weakly effective in protecting internal tissues, but largely prevented disease spread to uninoculated berries. All materials provided complete control when berries were surface-inoculated with a spore suspension either 2 or 3 weeks after the final (veraison) spray. In a final experiment, fenhexamid, trifloxystrobin, cyprodinil and iprodione reduced resporulation equivalently (about 50% relative to the water check) after previously-untreated, sporulating berries were washed free of conidia and sprayed, whereas pyraclostrobin+boscalid and boscalid solo had no significant effect on resporulation.

All materials provided excellent protection of surface infections, whereas the two AP fungicides, iprodione, and fenhexamid also provided good to excellent protection of internal tissues and control of quiescent infections 2 months after their initiation. For fenhexamid, these activities were unexpected but occurred consistently.

08.4 RESISTANCE OF *BOTRYTIS CINEREA* TO FUNGICIDES: STILL A PROBLEM IN VINEYARDS?!

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On grapevine, the control of *Botrytis cinerea* is mainly achieved through chemical fungicides. In French vineyards, especially in Champagne, spray programmes selected resistance to almost all the botryticides, despite the large variety of available modes of action, their restricted use and the regular registrations of novelties.

Concerning old fungicides, such as anti-microtubule botryticides like benzimidazoles (carbendazim) and phenylcarbamates (diethofencarb), or dicarboximides (iprodione, procymidone and vinclozolin), resistance was selected in the early 80's and was correlated to mutations in their respective target genes. According to genotype and local selection pressure, these strains may still frequently be found in populations.

Fungicides affecting respiration are old multi-site toxicants (thiram) or uncouplers (fluazinam) and are not important in resistance. Boscalid is a recent carboxamide which inhibits the mitochondrial complex II. Resistance to this fungicide was described at low frequency for the first time in 2006 in Germany and was associated with at least two distinct target alterations in the *sdh B* gene, conferring medium to high level of resistance according to the genotype.

Anilinopyrimidines (cyprodinil, mepanipyrim, pyrimethanil) inhibit methionine biosynthesis but their primary target site remains unknown. In few situations, resistance of commercial significance has been recorded.

Amongst the sterol biosynthesis inhibitors, fenhexamid is the only fungicide effective against grey mould on grapevine. It inhibits the 3 keto-reductase encoded by the *erg27* gene which is involved in sterol C4-demethylations. In the past few years, various phenotypes, specifically resistant to fenhexamid, were selected in the French vineyards. The most frequent ones with the target change F412I/S/V exhibited high resistance levels, whereas several phenotypes with low to medium resistance levels showed alterations at several distinct positions. Despite the medium to high resistance levels associated with these phenotypes, practical impact is still limited, because their frequencies are still low to moderate in the populations.

At last, the main problem in Champagne vineyard and also in German ones is the emergence of MultiDRug (MDR) resistant phenotypes, less sensitive to various unrelated fungicides. Up to three phenotypes (MdR1 MdR2, MdR3) were described according to their spectra of cross resistance; MdR3 is probably a natural hybrid between MdR1 and MdR2. This unspecific resistance is determined by over-production of membrane transporters, recently identified for each phenotype. Since resistance levels are generally low to moderate, field efficacy is still correct, but the following years will be determinant since these strains represent up to 50% of the population in the Champagne vineyard, for example.

08.5 HIGH FREQUENCY OCCURENCE OF MULTIPLE FUNGICIDE RESISTANCE IN *B. CINEREA* FIELD STRAINS FROM FRENCH AND GERMAN VINEYARDS

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Botrytis cinerea is a world-wide occuring plant pathogen which usually needs to be controlled by fungicides. Continuous chemical treatments lead to the selection of resistant strains. Fungicide resistance occurs most often by mutations of the fungicide target proteins, but increased fungicide degradation or reduced uptake due to membrane modifications have also been observed (Leroux *et al.*, 2002). Another type of resistance is termed multi drug resistance (MDR). MDR, a great medical problem in cancer or microbial cells, is often caused by mutations leading to the overexpression of ABC- or MFS-type membrane efflux transporters. Due to their low substrate specificity, overexpression of these MDR transporters can result in the increased export and reduced sensitivity to many different drugs.

In French vineyards, *B. cinerea* strains with a low to moderate level of resistance to chemically different fungicides (MDR phenotype) have first been observed in 1994. Since 1999, MDR strains constitute a rapidly increasing population which in 2005 accounted for 50% of the total population of the Champagne region. In Germany, more than 20% MDR strains were found in 2006 in the Palatine region. An analysis of the French and German MDR strains revealed that all strains could be classified into three MDR groups according to their fungicide resistance spectrum. MDR3 strains probably represent the progeny of natural crosses between MDR1 and MDR2 strains: (i) MDR3 strains occur with a much lower frequency than MDR1 or MDR2 strains in the field; (ii) the spectrum of fungicide resistance of MDR3 strains is the composite of the fungicide resistance of MDR1 and MDR2, respectively; and (iii) gene expression studies revealed that in MDR3 strains, both sets of efflux transporter genes show increased expression that are indivually overexpressed in MDR1 and MDR2 strains, respectively.

Genetic studies indicated that three types of MDR strains are selected by the current spray programmes used in Champagne or Germany. Genetic analysis show that the genotypes are diverse and migrate at wide scale, due to the large amount of asexual spores produced at vintage, making them likely to appear continuously in fungicide treated fields.

Uptake experiments with ^{14}C-labeled fludioxonil were performed with germlings of strains with a MDR1 phenotype. Compared to a susceptible laboratory strain, the MDR1 strains showed a reduced initial uptake of the drug. Addition of the uncoupler CCCP leads to an increased ^{14}C- fludioxonil uptake in the MDR strains. This is a clear evidence for a functional correlation between MDR and increased, energy-dependent efflux transport activity.

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08.6 LIFE CYCLE MANAGEMENT OF A BOTRYTICIDE

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Botrytis cinerea is a fungal pathogen, affecting a broad range of crops, especially high value crops such as grapes, soft-fruit, pome and stone fruit, vegetable and ornamental plants. Without effective control, before and after harvest, *Botrytis* infections regularly cause economic losses on crops not only in terms of quantity but equally, if not more important, in terms of quality of harvested produces. In that context, the objective of life cycle management is to offer customers a long term strategy for *Botrytis* control including sustainable technical solutions through adapted use recommendations. To do so, various studies are continuously conducted in the laboratory as well as in the field to define and adapt anti-resistance strategies, optimise product positioning as well as to design new formulations.

Botrytis cinerea is know to be a high risk pathogen in term of resistance development, therefore definition of an adapted anti-resistance strategy is a key factor for long term effectiveness of a botryticide product. Early in the development process, the risk of resistance development is assessed and the sensitivity base line is established. In parallel, long term trials are initiated in order to demonstrate, over several years at the same location, the effectiveness of the anti resistance strategy in order to anticipate any risk of resistance development. After market introduction, sensitivity of the pathogen populations is monitored, on annual basis, in practical conditions to detect any potential shift in sensitivity. Once resistant strains have been identified, laboratory trials are carried out at the locations where these strains were detected to check the impact of such isolates on the field efficacy of the product and adapt the resistance strategy. Cross resistance with other active ingredients and fitness studies provide additional information to manage the risk of resistance development.

Based on the biological mode of action and the biological properties, trials are implemented to evaluate the behaviour of the product under practical conditions and thus define the best product positioning within treatment programmes. During the life cycle of the product, complementary experiments are regularly carried out to optimise this positioning according to environmental evolution: introduction of new botryticides into the market, more stringent regulations, new consumers demand and new findings in *Botrytis* population structure.

Finally, continuous investigations are performed to evaluate the interest of new additives or new techniques in formulation with the aim of improving technical properties of the product: rain-fastness, long lasting effect, active ingredient bioavailability. The objective of these studies is to propose more effective and easy to use solutions for the market.

In conclusion, life cycle management of a botryticide is a continuous process designed to provide better solutions to answer the problems of the customers.

08.7 FUNGICIDE SPRAY COVER IN GRAPEVINE CANOPIES AND CONTROL OF *BOTRYTIS CINEREA*

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Optimisation of fungicide spray deposition on target sites (i.e. susceptible tissue) is an essential requirement for effective disease management. In South Africa, *Botrytis cinerea*, the causal pathogen of Botrytis bunch rot, is mostly associated with pedicels, rachises, laterals and berry bases, and not with berry skins as previously understood. Laboratory studies showed that, provided sufficient coverage of inner bunch parts was achieved, fungicides effectively controlled *B. cinerea* at all growth stages. The same efficacy was, however, not achieved with the same fungicides using conventional spraying methods in vineyards. In order to study the optimisation of spray cover in vineyards, a spray cover assessment protocol was developed. Bunches and leaves of table (Waltham Cross) and wine (Chenin blanc) grapes were sampled and precision-sprayed at various stages with different volumes of a mixture of fenhexamid (Teldor® 500 SC, Bayer) at the recommended dose and Yellow Fluorescent Pigment® (400 g/L, EC; South Australian Research and Development Institute) at 0.2 L/100 L. Sprayed plant material is illuminated under black light and visualised using a stereo microscope at 10-30× magnification. Digital photos are taken and image analyses are performed with Image-Pro Plus software. Quantitative analysis involved removal of green channels from the image, followed by quantification of foreground elements (deposited pigment) of the binarised image. For qualitative analysis, a combined Euclidian distance map and skeleton is created on the binarised image, with absolute white indicating the furthest distance from a particular foreground element. Subsequent analysis of grey-scale values indicates spray deposition quality. This protocol was used to determine the minimum spray coverage levels needed for effective *B. cinerea* control in vineyards. One day after spray application, leaves or bunches were dusted with dry air-borne conidia of *B. cinerea* in a settling tower and incubated for 24 h at high relative humidity (98%). The amount of *B. cinerea* infections was determined by means of isolations onto paraquat medium. The fluorescent pigment coverage needed for 75% control of *B. cinerea* infection (benchmark values) was subsequently calculated. Subsequent spray trials indicated that current best-practice spray application in vineyards resulted in sub-optimal deposition, which would most likely lead to control failure under high disease pressure conditions. In order to study means of improving spray cover with existing spray application technology, a series of spray trials were conducted at best-practice recommendations, but with a range in spray volumes of which the spray mixture concentrates were amended accordingly. The fungicide + pigment mixture was applied with commercial air blast and air shear spray applicators in table (Waltham Cross) and wine (Chenin blanc) grape vineyards at various stages. In terms of quantitative and qualitative deposition, the air shear sprayer performed markedly better at low volume (250-500 L/ha), whereas the air blast sprayer performed more consistently over a range of volumes; most likely due to correct nozzle selection.

08.8 THE USE OF SURFACTANTS TO IMPROVE CONTROL OF *BOTRYTIS CINEREA* ON GRAPE LEAVES

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Poor fruit and foliar disease control is often attributed to insufficient quantitative coverage on susceptible grapevine tissue. Moreover, control failure during high disease pressure situations might also be attributed to poor qualitative coverage. Spray adjuvants, specifically surfactants, have the potential to improve the quality of fungicide applications by effecting uniform distribution of fungicide on plant surfaces. In order to study whether such a qualitative improvement of spray deposition would lead to improved disease control, laboratory experiments were conducted on artificially inoculated grape (cv. Chardonnay) leaves. Prior to inoculation with 5 mg dry conidia of *Botrytis cinerea* in a spore settling tower, leaves were sprayed to the point of run-off with 1 ml of a mixture of fenhexamid, a fluorescent pigment, as well as selected commercial surfactants to manipulate the deposition quality of a given quantity of deposited spray. Following an incubation period of 24 h at high relative humidity, 20 leaf discs per leaf (6 leaves per treatment) were isolated onto Petri dishes with paraquat-amended water agar. Plates were rated 1 week later for development of *B. cinerea* from isolated leaf discs. Spray cover on leaves was assessed with a spray assessment protocol using fluorometry, photomicrography and digital image analyses. Quantitative and qualitative spray deposition values as effected by the different spray treatments were correlated with the respective infection levels. The experiment was repeated once. *Botrytis* incidences on the adaxial and abaxial sides of water sprayed leaves averaged 90.4% and 95.8%, respectively. Despite full spray cover of leaves, applications with fenhexamid alone could not completely prevent infection and resulted in 34.6% and 40.8% *Botrytis* incidences on the adaxial and abaxial sides of leaves, respectively. Through the addition of certain surfactants, *Botrytis* incidences were significantly lower (2.9-17.1% and 10.0-30.8%, resepectively), while some surfactants did not differ from the fungicide-only treatment. Infection values correlated favourably with quantitative (-0.54 and -0.56, respectively) and qualitative (0.59 and 0.70, respectively) spray cover values on adaxial and abaxial sides. These results clearly show that an improvement in the quality of fungicide spray deposition would lead to increased control of *B. cinerea* on grape leaves.

08.9 TREATMENT EFFICACY AGAINST GREY MOULD OF THE GRAPEVINE AND DEFENCE MECHANISMS ACTIVATION

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Grey mould of grapevine, caused by the fungus *Botrytis cinerea*, is a serious disease which affects both the quantity and the quality of wine production. The most common method to control this disease in vineyards is the use of chemical fungicides. Treatments against grey mould have considerably decreased the disease. However, these fungicides have been known to generate residues in grapes and wine, and to increase the percentage of *Botrytis* strains resistant to fungicides. Additionally, they can alter carbon and/or nitrogen metabolisms of the plants. For all these reasons, the three applications per growing season have to be limited. To reduce them, a better knowledge of fungicide effects is necessary.

Firstly, our work was to evaluate the contribution of each treatment stage on the control of *B. cinerea* in vineyard. Three preventative applications are generally recommended: at the end of flowering (stage A), at bunch closure (stage B) and at the beginning of berry ripening (stage C). Fungicide spraying was performed at stages A, B or C during several years. Disease intensity, attack frequency of *B. cinerea* and treatment efficiency, were measured at harvest. Our results revealed that the treatment during stage A is the most efficient to control grey mould.

The second objective was to determine if the efficiency of the fungicides at stage A was partly due to a stronger stimulation of defence responses. Therefore, expression of defence-related genes and chitinase activity were quantified in clusters at the three treatment stages. Nevertheless, activation of defence mechanisms was only noticed in clusters treated at stage C. Our results therefore do not seem to validate the hypothesis that induction of defence mechanisms was higher at stage A. Several hypotheses may explain the best efficiency of the fungicides at stage A: (i) direct anti-fungal activity against *B. cinerea*, (ii) potentiation of plant defence mechanisms, (iii) or both.

08.10 *BOTRYTIS* CONTROL: CAN IT MEET THE LATEST DEMANDS OF THE PRODUCER, RETAILER, CONSUMER, AND AGROCHEMICAL SUPPLIER?

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Botrytis is a progressive defect that can steal a substantial margin off potentially saleable fresh produce.

Along with other post-harvest disorders, such as browning, scald, shrivelling, bitter pit, and cold damage – to name but a few – it contributes substantially to the loss in salability of fruit after dispatch. *Botrytis* is one of the major reasons for the majority of fresh fruit out of South Africa still being sold on consignment basis instead of at a firm price.

The unit of sale of South African fruit is a pallet – not a carton. Retailers are so sensitive to progressive defects such as *Botrytis* that they will reject entire pallets when more than one box in the pallet show signs of decay; this in spite of the fact that the tolerance for *Botrytis* could be 1 - 2%, depending on the fruit type.

Control measures are limited and often not very practical. This problem is further exacerbated by the fact that *Botrytis* almost entirely manifests just before or after harvest. Seemingly sound fruit is dispatched only to arrive spoilt at their destination.

The presentation addresses the risks associated with existing *Botrytis* control strategies as perceived by the producer, retailer, consumer and agrochemical suppliers. Although *Botrytis* affects a wide range of crops, the focus is limited to pome fruit, stone fruit, table grapes and mention is made of citrus.

The goal of the presentation is to demonstrate the practicalities facing stakeholders in the supply chain when trying to keep fruit decay-free, while simultaneously managing all the perceptions of the various role players in an attempt to put them at ease. Furthermore, the aim is to discuss other factors, in addition to the purely scientific facts, that could influence the marketability of fresh produce in a modern society.

08.11 SHARING SUCCESS THROUGH FOOD CHAIN PARTNERSHIP BASED ON INTEGRATED CONTROL OF *BOTRYTIS*

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In recent years, consumers have become very conscious about what they are eating and how the food has been produced. Fuelled by numerous food scares such as BSE, dioxin and microbiological contamination and by regular campaigns against crop protection products, consumers have become increasingly aware of healthy nutrition and environmentally-friendly production. At one end of the food chain, the companies who directly supply food to consumers are confronted with stricter food laws, international trade requirements and increasing consumer demands. At the other end of the chain, the food producers are faced with the challenge of coping with additional requirements imposed by the retailers, such as compliance with certification standards, sustainable agricultural practices or even prescriptive lists of crop protection products.

Bayer CropScience has created Food Chain Partnership, a business philosophy with the objective of generating added value for the entire food chain by facilitating partnerships, with a focus on key crops. A 'tool box' has been developed, offering a comprehensive package of products and services. These range from seeds and innovative crop protection products, through expertise on crop production and storage to advice on country-specific agricultural and regulatory requirements and global crop trade.

Food Chain Partnership leads to the implementation of integrated crop solutions. For example, the partnership formed with fruit exporters from Turkey to provide premium quality grapes with no trade hurdles was achieved through integrated crop cultivation tailored to meet the needs of individual growers. Specific 'tools' for integrated control of *Botrytis cinerea* include products from different chemical groups, such as fenhexamid, an hydroxyanilide, and pyrimethanil, an anilinopyrimidine, and life cycle management of its botryticide products to offer growers a long-term strategy for disease control. Also, 'Cinerea®', a computer modelling tool to evaluate factors (farmers'practices and environmental characteristics) that influence the development of *B. cinerea.*

Bayer CropScience brings success to the growers by making availabe an integrated programme for season-long control of *B. cinerea* ensuring high quality fruit. The maintenance of high quality is ensured by post-harvest treatment with the Typhoon® system for control of *B. cinerea* and other diseases during storage and transport. Success for the processors (grape juice and wine) is also achieved by providing fruit that meets their quality requirements by being disease-free and without contaminants and off-flavours such as geosmin and laccase. The retailer is also successful by knowing that what is being sold meets the required MRL and other quality standards.

Only by linking all the partners, can consumer demands for safe, fresh, food at affordable prices all the year round be met. By facilitating partnerships along the food chain, Bayer CropScience is making a significant contribution to healthy nutrition.

8. OPEN SESSION: *BOTRYTIS* MANAGEMENT, INDUSTRY AND THE FOOD CHAIN

Poster

P8.1. Elise Sarrazin, Takatoshi Tominaga, Denis Dubourdieu and **Philippe Darriet**

Influence of grape botrytisation in the formation of the typical aromas of Sauternes wines

P8.1 INFLUENCE OF GRAPE BOTRYTISATION IN THE FORMATION OF THE TYPICAL AROMAS OF SAUTERNES WINES

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Botrytised wines are well-known for their exceptional range of aromas. They are produced all over Europe (Germany, Hungary, France, etc.), as well as in Australia and South Africa from grapes that are affected by the fungus *Botrytis cinerea* under specific climatic conditions. However, only a few studies have reported on the volatile compounds involved in their typical aromas. In this study, we present the characterisation of some key odorants of botrytised wines and the influence of *B. cinerea* infection in their formation.

By gas chromatography-olfactometry (AEDA technique) with three experienced judges, 35 main odoriferous zones were screened in botrytised wine extracts. As these wines are well-known for their citrus, fruity, caramel, and honey aromas, odoriferous zones relative to these nuances were studied more specifically. Three odoriferous zones were characterised by caramel nuances and were identified as norfuronol®, furaneol®, and homofuraneol®. Three odorants were reminiscent of honey and one was identified as phenylacetaldehyde, a *B. cinerea* metabolite. In addition, six citrus odoriferous zones were detected. 3-sulfanylhexan-1-ol was shown to be a great contributor to citrus aroma, and the specific volatile thiol extraction made possible the identification of three new volatile thiols in botrytised wines: 3-sulfanylpentan-1-ol, 3-sulfanylheptan-1-ol, and 2-methyl-3-sulfanylbutan-1-ol.

To precise the influence of *B. cinerea* infection in the formation of these odorants, Semillon and Sauvignon blanc grapes were picked on the same plot at different stages in botrytisation. Homofuraneol®, furaneol®, and norfuraneol® were absent in musts and were produced during alcoholic fermentation. Their concentrations were far higher in wines made from botrytised grapes and were shown to be closely related to the desiccation level of the grapes. Before alcoholic fermentation, phenylacetaldehyde was only present in rotten must, confirming its fungal origin. Then, phenylacetaldehyde contents increased significantly in wines made from botrytised grapes, whereas no significant increase was observed in wines made from healthy grapes. All the five volatile thiols were absent in musts and were released during alcoholic fermentation. 3-sulfanylhexan-1-ol levels were drastically higher in wines made from botrytised grapes than from healthy grapes. The same was true for the three new volatile thiols. They were shown to be consistently found in wines made from botrytised grapes, whereas they were present at trace levels in the dry white wines.

Therefore, these results demonstrated the predominant role of *B. cinerea* infection in the genesis of the typical aromas of Sauternes wines. Although the characteristic odorants are already present in wines made from healthy grapes, their concentrations are far higher in wines made from botrytised grapes.

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