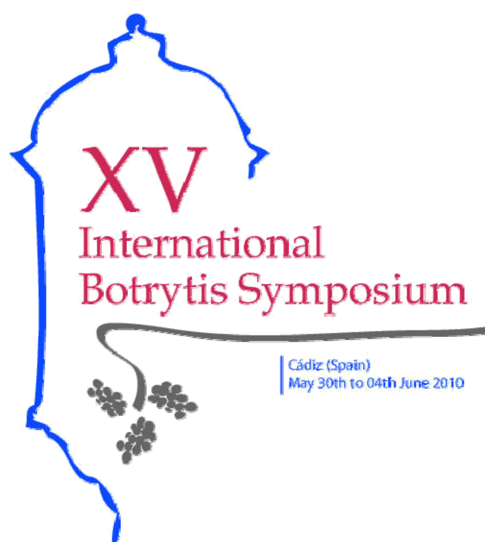
XV International *Botrytis* Symposium

30th May – 4th June 2010
Faculty of Philosophy and Art
University of Cádiz
Cádiz, Spain

UCA Universidad de Cádiz

JUNTA DE ANDALUCIA

GOBIERNO DE ESPAÑA — MINISTERIO DE CIENCIA E INNOVACIÓN



XVth INTERNATIONAL *BOTRYTIS* SYMPOSIUM

ABSTRACT BOOK

30th May- 4th June 2010
Cádiz, Spain

In memory of our colleague Maria Davis, who tragically passed away on February 12, 2010. Maria was a respected member of the Botrytis community. Her death has shocked the community and especially those who collaborated and communicated with her.

WELCOME XVth INTERNATIONAL BOTRYTIS SYMPOSIUM

Message from the *Botrytis* 2010 Organising Committee

On behalf of the organising committee, we wish to extend a warm welcome to the "XV International Botrytis Symposium", to be held at the University of Cadiz (Arts School, old town campus "Facultad de Filosofía y Letras") and thank the attendants for the effort made travelling from different countries around globe to our town.

The Phoenicians visited these lands more than three thousand years ago, bringing with them grapes, wine technology and probably many new species of *Botrytis*. Centuries later, the works of William Shakespeare gave us a good idea of the popularity of sherry wines. Now, from this ancient sherry land, we hope that we will be able to create an environment for meeting old friends, create new ones, share scientific experiences and enjoy the food and wines from the well-known Sherry area.

The University of Cadiz is a young and dynamic institution, which is happy to accept challenges as being host of long established and successful meetings, like this one. We wish you an enjoyable and successfully meeting at Cadiz "Tacita de Plata", a town fully rich in history, which is preparing for the bicentennial of the 1812 Spanish Constitution.

Please, enjoy this beautiful spot of Andalucía, in the South-West coast of Spain, full of light and sea resonances. We wish you a fruitful outcome from the "XV International Botrytis Symposium".

Prof. Dr. Isidro González Collado
Prof. Dr. Jesús M. Cantoral Fernández

ORGANIZATION

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Secretary: Dr. Jesus M. Cantoral Fernández

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Dr. Paul Tudzynski (Germany)
Dr. Jan A. L. van Kan (Holland)
Dr. Muriel Viaud (France)
Dr. Melane Vivier (South Africa)
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Ministerio Ciencia e Innovación

CONGRESS PROGRAMME

Time	***Sunday 30th May***	***Monday 31st May***	***Tuesday 1st June***	***Wednesday 2nd June***	***Thursday 3rd June***	***Friday 4th June***
9:30		9:00-9:30 **Opening Ceremony** **Session 1:** **Invited lecture** **Chairperson** Dr. France Dewey (Molly)	**Session 3:** **Invited lecture** **Chairperson** Dr. Amnon Lichter	**Session 6:** **Invited lecture** **Chairperson** Dr. Paul Tudzinsky	**Session 6:** **Invited lecture** **Chairperson** Dr. B. Tudzinsky	**Session 7:** **Invited lecture** **Chairperson** Dr. Ann Powell
10:00 - 10:45		Short oral presentations: O1.1 – O1.2 – O1.3	Short oral Presentation O3.4 – O3.5 – O3.6 – O3.7	Short oral presentations: O6.1 – O6.2 – O6.3	Short oral presentations: O6.5–O6.6–O6.7	Short oral presentations: O7.2 – O7.3
11:00		*Coffee break + poster viewing*				
11:30		**Invited lecture** **Chairperson** Dr. Jaime Auger	**Session 4:** **Invited lecture** **Chairperson** Dr. E. Monte	**Invited lecture** **Chairperson** Dr. Muriel Viaud	**Session 7:** **Invited lecture** **Chairperson** Dr. Melané Vivier	**Invited Lecture** Dr. Carmen Glez. Bosch
12:00 - 12:45		Short oral Presentations: O1.4 – O1.5 – O1.6	Short oral Presentation: O4.1 – O4.2 – O4.3	Short oral presentations: O6.4 + **Invited lecture** Dr. F. Pieckenstain	Short oral presentation: O7.1 + **Invited lecture** Dr. T. Mengiste	Concluding remark Next meeting announcement
13:00		*Lunch + poster viewing*				Closing Lunch/Meal
14:30		**Session 2:** **Invited lecture** **Chairperson** Dr. Jan Van Kan	**Session 5:** **Invited lecture** **Chairperson** Dr. Fernandez-Acero (Kiko)	End day Session	End day sessions	
15:00 - 16:00	Registration (15:00 - 18:00)	Short oral Presentations: O2.1 – O2.2 – O2.3	Short oral Presentation: O5.1 – O5.2 – O5.3		**Official conference excursion** **Guided Sherry Wine Tasting in El Consejo Regulador** (Regulating Council) Visit to Wine cellar, **Bodegas Domecq**	
16:00		*Coffee break + poster viewing*				
16:45		**Session 3:** **keynotes** **Chairperson** Dr. Amnon Lichter	Short oral Presentation: O5.4 – O5.5	17.00 Bus Departure to Jerez Visit to Garvey wine-cellar		
17:15 - 17.30		Short Oral Presentations: O3.1 - O3.2 - O3.3 – O3.4 End of day sessions	Short oral Presentation: O5.6-O5.7-O5.8 End of day sessions	El Corregidor vineyard		
18:00				Dr. Alberto García de Lujan The current situation of Botrytis in Sherry area		
Evening		Governor Welcome Reception cocktail	Guided Town tour, cocktail	Dinner Cocktail	Gala Dinner in B. Domecq	

TABLE OF CONTENT

Session 1: *BOTRYTIS* IDENTIFICATION, MANAGEMENT, ECOLOGY AND EPIDEMIOLOGY

Invited lecture:

I1.1 First report of *Botrytis* associated with Spring Sickness in *Hemerocallis* (daylilies) in the UK
Robert Grant-Downton, Josefina Rodriguez-Enriquez, Sarah J. Gurr and ***Frances M. (Molly) Dewey***

Short Oral Communications:

O1.1 Multiple fungicide resistance in *Botrytis cinerea* strains from commercial vineyards and strawberry fields
Michaela Leroch *and Matthias Hahn*

O1.2 Predicting botrytis losses through disease monitoring and extrapolating progress curves
Gareth N. Hill *and Robert M. Beresford*

O1.3 Characterisation of Hungarian *Botrytis cinerea* Group I isolates
Éva Fekete, Erzsébet Fekete, Levente Karaffa, László Irinyi and ***Erzsébet Sándor***

Invited lecture:

I1.5 Correlation between the *Botrytis cinerea* population composition and the latent infection in 4 table grape cvs. in Chile
Jaime Auger*, Claudio Rodríguez and Marcela Esterio*

Short Oral Communications:

O1.4 Botryticides affect grapevine leaf photosynthesis.
A. N Petit, N. Vaillant-Gaveau, M. L Panon, C. Clément and F. Fontaine

O1.5 Quantification of a stable *Botrytis* antigen in wines using *Botrytis* Lateral Flow Devices
Frances M. (Molly) Dewey *and Sarah Jane Gurr*

O1.6 Proteomics reveals features of the adaptive response of the model fungus *Botrytis cinerea* to heavy metals
Semcheddine Cherrad, Vincent Girard, Dominique Job, Nathalie Poussereau and Sébastien Vacher

Posters:

P1.1 Evolution of abundance and genetic structure of *Botrytis cinerea* airborne inoculum in the South East of France over 2.5 years
Christel Leyronas, Magali Duffaud, Josselin Montarry and Philippe Nicot

P1.2 Differences between *Botrytis cinerea* populations causing grey mould and noble rot in two Hungarian vineyards
Zsuzsanna Váczy, Kálmán Zoltán Váczy, Levente Karaffa and Erzsébet Sándor

Session 2: BIOLOGY AND GENETICS OF *BOTRYTIS* and *SCLEROTINIA*

Invited lecture:

I2.1 The *Botrytis cinerea* Mating Type loci
Jan A.L. van Kan, *José Duarte, Ester Dekkers, Paul S. Dyer and Linda M. Kohn*

Short Oral Communications:

O2.1 Apoptotic-like cell death in *Botrytis cinerea*: a case study on the putative inhibitor of apoptosis gene BcBIR1
Amir Sharon, ***Neta Shlezinger****, Alin Finkelshtein and Ido Hatam*

O2.2 Mutations leading to overexpression of drug efflux transporters are responsible for multidrug resistance phenotypes of *Botrytis cinerea* field strains
Andreas Mosbach*, Michaela Leroch, Matthias Kretschmer, Dennis Mernke, Anne-Sophie Walker, Sabine Fillinger and Matthias Hahn*

O2.3 Natural field populations of *Botrytis cinerea* from vineyards from Castilla y León in Spain: physiological and population genetics characterization
Thais M. M. Costa, Raquel Guijarro, Marta Hernández, Iñigo Zabalgogueazcoa and ***Ernesto P. Benito***

Posters:

P2.1 Molecular characterisation of the mating type (MAT1) locus in *Botryotinia fuckeliana* (*Botrytis cinerea*)
Rita Milvia De Miccolis Angelini, Caterina Rotolo, Stefania Pollastro and Francesco Faretra

P.2.2 Phenotypic and molecular characterization of fungicide-resistant field isolates of *Botryotinia fuckeliana* (*Botrytis cinerea*)
Rita Milvia De Miccolis Angelini, Caterina Rotolo, Stefania Pollastro and Francesco Faretra

P.2.3 Mutasynthesis, an approach to generate new unnatural natural products from *Botrytis cinera*
José Manuel Botubol, Isidro G. Collado, Antonio J. Macias-Sánchez, and Rosario Hernández-Galán

P2.4 Caracterisation of the new species *Botrytis pseudocinerea* living in sympatry with *Botrytis cinerea* in French populations
Anne-Sophie Walker1, Sabine Fillinger and Elisabeth Fournier

P.2.5 Lack of evidence for an important role of *Botrytis cinerea* hydrophobins in the asexual state
Andreas Mosbach, Michaela Leroch, Andreas Böhm, Kurt Mendgen and Matthias Hahn

P.2.6 Characterisation of *Botrytis cinerea* in raspberry in Serbia
Brankica Tanovic and Mirko Ivanovic

P.2.7 In planta assays highlighted significant differences between *Botrytis cinerea* and *Sclerotinia sclerotiorum*
Geneviève Billon-Grand , Nathalie Poussereau and Jeffrey A. Rollins

P2.8 The osmosensing signal transduction pathway from *Botrytis cinerea* regulates cell wall integrity and, together with the Bmp3 MAP kinase, melanin biosynthesis
Weiwei Liu, Marie-Christine Soulié, Claude Perrino and Sabine Fillinger

P2.9 New advances in mannitol metabolism in the fungal plant pathogen *Botrytis cinerea*
Thierry Dulermo, Christine Rascle, Geneviève Billon-Grand, Elisabeth Gout, Richard Bligny and Pascale Cotton

P2.10 Phenotypic and genotypic characterization of chilean *Botrytis cinerea* isolates with different levels of fenhexamid sensitivity
Marcela Esterio, Cecilia Ramos, Anne-Sophie Walker, Sabine Fillinger, Jaime Auger and Pierre Leroux

P2.11 Use of GFP for live-cell imaging in *Botrytis cinerea*
Anna Minz, Ido Hatam, Neta Shlezinger and Amir Sharon

P2.12 Post-transcriptional gene silencing in *Botrytis cinerea*
José J. Espino, *Nélida Brito and Celedonio González*

Session 3:
DISEASE MANAGEMENT 1

Invited lecture:

I3.1 Air-phase control of *Botrytis cinerea* after harvest
Amnon Lichter *and Avinoam Daus*

Short Oral Comunications:

O3.1 Fenhexamid resistance in *Botrytis cinerea*: Target modifications and fungicide detoxification
Alexis Billard*, Pierre Leroux, Sabine Fillinger, Jocelyne Bach, Pauline Solignac, Hélène Lachaise, Roland Beffa and Danièle Debieu*

O3.2 Effects of climate and mixed bunch rot infections on *Botrytis cinerea* (grey mould) of grapes: Implications for global climate change and disease management.
Christopher C Steel*, Lindsay A Greer, Suren Samuelian and Sandra Savocchia*

O3.3 Development of resistance to pyrrolnitrin and associated fitness costs in the fungal plant pathogen *Botrytis cinerea*
M. Bardin*, S. Ajouz, M. El Maâtaoui and P. C. Nicot*

O3.4 Chitosan treatment for the control of postharvest gray mold of table grapes
Gianfranco Romanazzi

Invited lecture:

I3.2 Resistance to fungicides: new weapons in a never-ending battle
Sabine Fillinger*, Anne-Sophie Walker, Pierre Leroux and Danièle Debieu*

Short Oral Comunications:

O3.4 Implementation of a low-input fungicide strategy in grapes in relation to the pattern of grey mold development
M. Fermaud*, J. Roudet, P. Sauris, L. Druelle, G. Blanc et P. Lagouarde*

O3.5 DNA-based network of airborne inoculum tracking: an additional tool for managing Botrytis leaf blight of onion
Odile Carisse*, David-Mathieu Tremblay and Luc Brodeur*

O3.6 Effectiveness of fungicides and an essential-oil-based product in the control of grey mould in raspberry in Serbia
Brankica Tanovic*, Jovana Hrustc, Mirko Ivanovic, and Goran Delibasic*

O3.7 Monitoring of fungicide resistance in *Botryotinia fuckeliana* (*Botrytis cinerea*) on grapevine and strawberry in South Italy
Caterina Rotolo, Rita Milvia De Miccolis Angelini, ***Stefania Pollastro*** *and Francesco Faretra*

Posters:

P3.1 Exploring the resistance mechanisms towards respiratory inhibitors in field strains of *Botrytis cinerea*, the causal agent of gray mold
Anne-Sophie Walker, Anaïs Lalève, Michel Gredt, Michaela Leroch and Pierre Leroux

P3.2 Phenotypic characterization of *Botrytis cinerea* isolates with different levels of sensitivity to fenhexamid
Marcela Esterio, Laura Pozo, Cecilia Ramos and Jaime Auger

P3.3 Fitness of *B. cinerea* fenhexamid-resistant isolates in Thompson Seedless cv.: Field study
Marcela Esterio, Sebastián Cáceres, Cecilia Ramos and Jaime Auger

P3.4 Effect of pyrimethanil fungicide applied through thermofogging in the control of *Botrytis cinerea* in apple cv. Fuji
Sylvana Soto, Mauricio Lolas, Claudia Moggia and Luis Neubauer

P3.5 Stem-end rot of kiwifruit, caused by *Botrytis cinerea* during cold storage and chemical control strategies
Blancaluz Pinilla, Claudia Corvalán, Victor Navia and Benjamín Valiente

P3.6 Fitness measurements of HydR3$^+$ fenhexamid resistant strains in *Botrytis cinerea*
Alexis Billard, Pierre Leroux, Sabine Fillinger, Hélène Lachaise, Roland Beffa and Danièle Debieu

P3.7 Functional characterisation of the *Botrytis cinerea erg28* gene involved in the C4 demethylation process of the ergosterol biosynthesis pathway: role of an anchorage protein
Alexis Billard, Jocelyne Bach, Sabine Fillinger, Pierre Leroux, Hélène Lachaise, Roland Beffa, and Danièle Debieu

P3.8 Development of isocaryolane derivatives as antifungal agent leads. Biotransformation of some isocaryolane and related compounds by the phytopathogenic fungus *Botrytis cinerea.*
J. Ascari, M. A. D. Boaventura, J. A. Takahashi, R. Hernández-Galán, A. J. Macías-Sánchez, I. G. Collado

P3.9 Designing hybrid molecules as potential new fungicides against *Botrytis cinerea* based on the reported genome data
Antonio Ruano, Beatriz O´Reilly, Gabriela Mancilla, Rosario Hernández-Galán, Antonio J. Macias-Sánchez, and Isidro G. Collado

P3.10 Importance of treatments during different phenological stages to control Botrytis bunch rot in Lleida vineyards
Carlos Calvo, Phil Elmer, Inmaculada Viñas, Josep Usall, Xavier Ochoa de Eribe and Neus Teixidó

P3.11 Current sensitivity to botrycides in Chile: Multidrug Resistance (MdR1)
Marcela Esterio, Cecilia Ramos, Anne-Sophie Walker, Sabine Fillinger, Jaime Auger and Pierre Leroux

Session 4:
DISEASE MANAGEMENT 2 (Biological Control)

Invited lecture:

I4.1 Everybody against *Botrytis*: biocontrol cooperation between *Trichoderma* and plants
Rosa Hermosa, Belén Rubio, Eugenia Morán-Diez, Anamariela Tijerino, Michelina Ruocco, Matteo Lorito and ***Enrique Monte***

Short Oral Communications:

O4.1 The effect of curing temperature on *Botrytis allii* infection of onion bulbs
Chope, G. A.*, Adikaram, N.K.B, Downes, K. and Terry, L. A.*

O4.2 Biological control of *Botrytis cinerea* using *Trichoderma* spp.
Ana María Rincón, *Felipe Guevara, María Jesús Rodríguez-Palero, Antonio C. Codón and Tahía Benítez*

O4.3 Biocontrol of *Botrytis* bunch rot in grapevine using *Candida sake* CPA-1 treatments
Neus Teixidó, *Teresa Paula Cañamás, Josep Usall, Cristina Solsona, Elena Cases, and Inmaculada Viñas*

Posters:

P4.1 Biological control of tomato stem canker disease caused by *Botrytis cinerea*, using *Trichoderma* isolates
Azadeh Eivazi, Mohammad J. Soleiman and Doostmorad Zafari

P4.2 Role of biopolymers from *Rhodotorula glutinis* in biocontrol of the plant pathogen *Botrytis cinerea*
M. Haggag Wafaa, M.Mostafa Enas and A. M. El Azzazy

Session 5:
BOTRYTIS-"OMICS"

Invited lecture:

I5. 1 Proteomic characterization of *Botrytis cinerea* secretome during pathogenicity induction by different plant based elicitors
Francisco Javier Fernández-Acero, *Thomas Colby, Carlos Garrido, Anne Harzen, Victoria E. González-Rodríguez, María Carbú, Ursula Wieneke, Jürgen Schmidt and Jesús Manuel Cantoral*

Short Oral Comunications:

O5.1 Microarray analysis of the early stages of *Botrytis cinerea* development
Michaela Leroch, *Astrid Schamber, Bettina Coenen, Christine Peter and Matthias Hahn*

O5.2 Analysis of changes in expression during growth of *Botrytis cinerea* at low temperature
S. Ish - Shalom *and A. Lichter*

O5.3 T-DNA-mediated Insertional Mutagenesis in *Botrytis cinerea* – a tool to identify new virulence-associated genes –
Julia Schumacher, *Sabine Giesbert, Daniela Odinius, and Paul Tudzynski*

O5.4 Bioinformatic analysis of O-glycosylation in proteins secreted by *Botrytis cinerea*.
Mario González, *Nélida Brito, Celedonio González*

O5.5 Creation of a collection of *Botrytis cinerea* T-DNA transformants for pathogenic development and plant defence studies
Géraldine Mey, *Pascal Bally, Roland Beffa, Raquel González Fernández, Jesús V. Jorrín Novo, Sophie Kaiser, Marie-Pascale Latorse, Julia Schumacher, Paul Tudzynski and Muriel Viaud*

05.6 Proteomics in *Botrytis cinerea*. The simplest 1-DE reveals differences in protein abundance among strains.
Raquel González-Fernández, *Inmaculada Redondo and Jesús V. Jorrín-Novo*

O5.7 Transcriptome of the early steps of *Botrytis cinerea* infection
Patrick Frettinger, *Zsuzsanna Antal, Christine Rascle, Marie-Josèphe Gagey, Catherine Sirven, Jani Kelloniemi, Benoît Poinssot, Adeline Simon, Agnès Cimerman, Muriel Viaud, Fabienne Baillieul, Marc Fermaud, Jeffrey Rollins, Peter Eckes, Christophe Bruel, Géraldine Mey, Nathalie Poussereau and Mathias Choquer*

O5.8 Genome-wide expression profiles uncover infection-specific genes and global gene regulation.
Nora Temme, *Birgitt Oeser, Isidro G Collado, Paul Tudzynski*

Posters:

P5.1 The secretome of *Botrytis cinerea*: adaptation to environmental pH
Semcheddine Cherrad, Cindy Dieryckx, Vincent Girard, Christine Rascle, Genevieve Billon-Grand and Nathalie Poussereau

P5.2 Proteome analysis of the interaction between *Botrytis cinerea* and tomato fruit
Punit Shah, Ann L.T. Powell, Ron Orlando, Carl Bergmann and Gerardo Gutierrez-Sanchez

P5.3 Studying of *Botrytis cinerea* phosphoproteome
Francisco Javier Fernández-Acero, Thomas Colby, Carlos Garrido, Anne Harzen, María Carbú, Victoria E. González-Rodríguez, Ursula Wieneke, Jürgen Schmidt and Jesús Manuel Cantoral

P5.4 Modifications on *Botrytis cinerea* conidial proteome during germination
Victoria E. González-Rodríguez, Thomas Colby, Carlos Garrido, Anne Harzen, Maria Carbú, Jürgen Schmidt, Jesús Manuel Cantoral and Francisco Javier Fernández-Acero

P5.5 RNA interference in *Botrytis cinerea*: Genome identification of RNA-dependent RNA polymerase, Dicer-like and Argonautes, and their expression during early stages of development
Catalina Urrejola, Amir Shmaryahu, Carolina Aguayo, Pablo Valenzuela, Matthias Hahn, Evelyn Silva.

Session 6:
HOST-PATHOGEN INTERACTIONS 1: Fungal virulence factors

Invited lectures:

I6.1 The role of SAPK signalling in differentiation and pathogenicity of *Botrytis cinerea*
Jens Heller, Leonie Kokkelink, Caroline Michielse, Nora Temme, ***Paul Tudzynski***

Short oral communications:

O6.1 The NO detoxifying flavohemoglobin BCFHG1 is not a virulence factor in the fungal necrotroph *Botrytis cinerea*
Juan Luis Turrión-Gómez and ***Ernesto P. Benito***

O6.2 The biosynthesis of the polyketide toxins excreted by the phytopathogen fungus *Botrytis cinerea*
Rosa Durán-Patrón*, Javier Moraga, Michelli Massarolli, Rosario Hernández-Galán, and Isidro G. Collado*

O6.3 The monomeric GTPase Rheb: a development regulator required for successful plant infection
Heber Gamboa-Meléndez, Bénédicte Gayrin, Tim Rollenske, Marie-Josèphe Gagey, Geneviève Billon-Grand, Michel Droux and ***Géraldine Mey***

Invited lectures:

I6.2 Secondary metabolism in *Botrytis cinerea*: the grey and the pink sides of a pathogen
Muriel Viaud*, Julia Schumacher, Bérengère Dalmais, Javier Moraga,Guillaume Morgant, Pascal Le Pêcheur, Angélique Gautier, Jean-Marc Pradier, Sabine Fillinger, Pierre Leroux, Bettina Tudzynski and Isidro Gonzalez Collado*

Short oral communications:

O6.4 "VELVET"- A virulence factor in *Botrytis cinerea*
Julia Schumacher*, Jana Pfeiffer, Javier Moraga, Muriel Viaud, Isidro G. Collado and Bettina Tudzynski*

Invited lectures:

I6.3 Botrydial causes host cell death by inducing the hypersensitive response and by interacting with host signaling pathways that mediate defense responses against pathogens
Franco R. Rossi, Andrés Gárriz, María Marina, Matías F. Romero, María E. Gonzalez, Isidro G. Collado and ***Fernando L. Pieckenstain***

I6.4 Gα-controlled signalling pathways and their role in developmental processes and virulence of *Botrytis cinerea*
Julia Schumacher, Karin Harren, and ***Bettina Tudzynski***

Short oral communications:

O6.5 Functional analysis of the *Botrytis cinerea Bcbip1* bZip transcription factor
Emmanuelle Galland, Pascale Cotton, Christine Rascle, Geneviève Billon-Grand, Nathalie Poussereau, Roland Beffa, Marc-Henri Lebrun and ***Mathias Choquer***

O6.6 Triggering cryptic sesquiterpenes biosynthesis in *Botrytis cinerea*
Javier Barúa, Cristina Pinedo, Josefina Aleu Casatejada, Rosa Durán-Patrón, ***Antonio J. Macías-Sánchez****, Rosario Hernández-Galán, and Isidro G. Collado*

O6.7 The cerato-platanin-like protein BcSPL1 is required for full virulence in *Botrytis cinerea* and is able to induce necrosis in plant tissues,
Marcos Frías*, Nélida Brito, and Celedonio González*

Posters:

P6.1 Galacturonic acid catabolism in *Botrytis cinerea*
Lisha Zhang *and Jan A. L. van Kan*

P6.2 Investigation the role of BcPIE1 gene in the virulence of *Botrytis cinerea*
Zsuzsanna Antal*, Muriel Viaud, Christophe Bruel*

P6.3 Preliminary study of a *Botrytis cinerea* extracellular β –xylosidase
Nélida Brito, ***Judith Noda****, José J. Espino, Marcos Frías, Mario González and Celedonio González*

P6.4 The methylammonium permease (MEP) gene family of *Botrytis cinerea*: expression and functional analysis
Raúl Martín-Domínguez*, Jinny A. Paul, Michael H. Perlin and Ernesto P. Benito*

Session 7:
HOST-PATHOGEN INTERACTIONS 2: Plant defence

Invited lectures:

I7.1 Analyses of *Botrytis cinerea* defense phenotypes highlight the importance of studying the plant cell wall in plant-pathogen interactions
Eric Nguema-Ona, John P. Moore, Erik Alexandersson, Daniel Jacobson and ***Melané A. Vivier***

Short oral communications:

O7.1 Fine mapping of the RBPG1 locus which controls the response to *Botrytis cinerea* endopolygalacturonases in *Arabidopsis thaliana*
*Lisha Zhang****, Panagiota Tagkalaki, Devlin Tjoitang, Ilona Kars, and Jan A. L. van Kan***

Invited lectures:

I7.2 Mechanisms of plant innate immunity to necrotrophic fungi
Tesfaye Mengiste and Kristin Laluk

I7.3 Ripening and rotting: Tomato fruit ripening and susceptibility to *Botrytis cinerea*
Dario Cantu, Barbara Blanco-Ulate, KaLai Lam, Gerardo Gutierrez, Punit Shah, Alan Bennett, John Labavitch, Carl Bergmann, and ***Ann Powell***

Short oral communications:

O7.2 Contrasted responses of *Botrytis cinerea* strains developing on tomato plants grown under different nitrogen nutrition regimes
F. Lecompte, M. A. Abro, ***Philippe Nicot***

O7.3 Increased susceptibility of non-ripening Cnr tomato fruit to *Botrytis cinerea* and the plant cell wall implications
Barbara Blanco-Ulate*, KaLai Lam, Dario Cantu, Alan Bennett, John Labavitch and Ann Powell*

Invited lectures:

I7.4 Priming of plant defenses against *Botrytis cinerea* by hexanoic acid treatment,
Begonya Vicedo, Mª de la O Leyva, Ivan Finiti, Víctor Flors, Mª Dolores Real, Pilar García-Agustín and ***Carmen González-Bosch***

Posters:

P7.1 Genomics of the grapevine - pathogen interactions: *Botrytis cinerea* virulence factors & molecular mechanisms of induced resistance
Benoît Poinssot*, Jani Kelloniemi, Patrick Frettinger, Mathias Choquer, Adeline Simon, Zsuzsanna Antal, Marie Claire Héloir, Xavier Daire, Christine Rascle, Marie-Josèphe Gagey, B. Dalmais, JM Pradier, Fabienne Baillieul, Muriel Viaud*

P7.2 The role of cuticle permeability in the defence response of *sitiens*, an abscisic acid deficient tomato mutant, against the necrotrophic pathogen *Botrytis cinerea*
Hamed Seifi*, Katrien Curvers, Juan Carlos Cabrera, Pierre Van Cutsem, Frank Van Breusegem and Monica Höfte*

ABSTRACTS

XV
International
Botrytis Symposium

Session 1:
BOTRYTIS **IDENTIFICATION, MANAGEMENT, ECOLOGY AND EPIDEMIOLOGY**

I1.1 First report of *Botrytis* associated with Spring Sickness in *Hemerocallis* (daylilies) in the UK

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Spring Sickness in daylilies is responsible for considerable damage to these popular ornamental plants worldwide, particularly in new varieties. As the name indicates, the disease is prevalent in Spring during cold, wet conditions. Symptoms include stunting, curling and severe distortion of the leaves which frequently develop necrotic patches, often causing holes and serrations of the leaf tissue. Basal portions of the leaves and the stem-base can become affected, in severe cases resulting in widespread necrosis, chlorosis and death. The causal agent of the disease in the US and UK is not known but adverse weather conditions and bulb mites have been implicated.

From surface sterilized infected leaf tissues we repeatedly isolated a filamentous fungus that could not be made to sporulate in culture despite growth on many different types of media under different regimes of temperature and light. Tests with a near genus-specific *Botrytis*-Lateral Flow device (*Botrytis*-QuickStix, EnviroLogix, Maine, US and Forsite Diagnostics, UK) of washings of cultures of this fungus and extracts from infected leaves indicated strongly that it is a species of *Botrytis*. Young sterile plantlets of *Hemerocalli*s infected with plugs of mycelium of the fungal isolate died quickly. Sequencing of an ITS region confirmed that this was a *Botrytis* species. Further sequencing of *NEP1, HSP60* and *G3PDH* genes as well as its morphology indicated that this isolate was most closely related to *B.ellipitica* but not one that had been previously described. This is apparently the first report of a *Botrytis* species being the cause of Spring Sickness in *Hemerocallis* in Europe or the United States but there are reports from Korea of *B. elliptica* infecting *Hemerocallis.*

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O1.1 Multiple fungicide resistance in *Botrytis cinerea* strains from commercial vineyards and strawberry fields

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The grey mould fungus *Botrytis cinerea* causes losses of important fruit and vegetable crops worldwide. Fungicide treatments are effective for plant protection, but bear the risk of resistance development. Monitoring of *Botrytis cinerea* strains in vineyards from the Champagne and German wine road region revealed both an increasing occurrence of MDR (multidrug resistance) strains (Kretschmer et al., 2009), and specific (target site) resistance against individual fungicides. In the vineyards three different MDR phenotypes were distinguished according to their fungicide resistance spetra. MDR1 strains show increased resistance levels against fludioxonil and cyprodinil, and MDR2 strains against fenhexamid and iprodion. MDR3 strains result from recombination of MDR1 and MDR2 strains and show higher broad-spectrum resistance. Field experiments confirmed that in vineyards fungicide treatment leads to strong selection of MDR strains. In addition, preliminary data indicate that MDR1 strains can reduce the protection by fungicides against grey mould. In 2008-2009, an increasing population of strains with both MDR1 and specific cyprodinil resistance was observed in Germany, which indicates that MDR strains can undergo further mutations leading to additional resistance levels.

In contrast to 2-3 fungicide treatments against *Botrytis* in vineyards, treatments in commercial strawberry fields often occur weekly (up to 8 times per season), after beginning of flowering, resulting in repeated use of the same fungicides. A monitoring of *Botrytis cinerea* strains isolated from German strawberry fields showed the high occurrence of multiple fungicide resistant strains, due to a combination of specific and MDR resistance mechanisms. Up to 86% of these strains showed a novel, stronger MDR1-like phenotype (called MDR1^{+}), probably due to even stronger constitutive overexpression of the AtrB efflux pump.. Altogether, the populations in some strawberry fields are more than 90% resistant to fenhexamid, azoxystrobin and cyprodinil, and to more than 60% to iprodione. These findings indicate that chemical control of *B. cinerea* in some strawberry fields is severely compromised by fungicide resistance.

First population genetic analyses were performed to analyse the genetic diversity of the strains. The results of IGS-RFLP marker analyses clearly show a less genetic diversitiy in contrast to isolates from vineyards. Compared to the vineyard isolates from France and Germany, the German strawberry isolates were genetically distinct, similar to the genetic distance between the previously described group II (*B. cinerea sensu stricto*) and group I ('*B. pseudocinerea*') subspecies. Taken together, our data indicate that on grapes and strawberries, *B. cinerea* populations with restricted genetic exchange are selected for different patterns of fungicide resistance.

O1.2 Predicting *Botrytis* losses through disease monitoring and extrapolating progress curves

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Botrytis bunch rot (botrytis) epidemics, caused by *Botrytis cinerea,* can be effectively modelled using the logit equation: ln((% *severity* + 0.1) / (100.1 - % *severity*)). By fitting a progress curve to disease observations early in the epidemic, a certain level of predictive ability can be achieved in order to estimate harvest severity. However, due to the effect of other variables, such as weather factors, these predictions are not always very accurate.

Certain parameters can be adjusted to better model the epidemic, and thus enhance the predictive ability. One such parameter is the asymptote. This determines the theoretical maximum of the epidemic. While in the above equation this is set at 100%, in practice epidemics do not reach this level. By lowering the asymptote to a more appropriate level, one can reduce overestimations of harvest severity when predicting from early observations. The optimal asymptote is that which gives the highest R^2 for the regression of logit severity over time.

The problem with this approach is knowing what asymptote to set without knowing what the harvest severity will be. Using empirical relationships between the optimal asymptote for an epidemic and weather variables, such as wetness duration, it is possible to estimate the optimal asymptote and subsequently harvest severity.

Relationships have been found between weather and optimal asymptote which allow asymptote to be estimated early in the epidemic, increasing the accuracy of harvest severity predictions, even from a single disease observation. However, if a single observation is used to predict harvest severity it is also essential to know the slope of the epidemic, something that can not be calculated from a single point. In order to estimate a second disease observation, an 'average epidemic' was calculated. This was done by finding the optimal asymptote retrospectively for epidemics from 30 site-years, logit-transforming each disease observation using each site-years optimal asymptote and performing a single linear regression on all the transformed data. Using this it is possible to determine where a single logit-transformed disease observation is along the average epidemic, estimate an additional disease observation and back-transform using the recently calculated optimal asymptote to gain an additional data point in order to calculate the slope of the epidemic.

O1.3 Characterisation of Hungarian *Botrytis cinerea* group I isolates

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Botrytis cinerea (anamorph of *Botryotinia fuckeliana*) causes gray mold on a high number of crop plants. However, *B. cinerea* was proposed to be a species complex. Initially, two sympatric sibling species or transposon types were described: 1) *transposa* that contained two transposons *Boty* and *Flipper* and 2) *vacuma* which contained no transposons. Later molecular studies of different nuclear genes have suggested that *B. cinerea* populations grouped in two different clades in the different gene phylogenies, Group I and Group II, with restricted gene flow between different cryptic genetic groups. To date, *vacuma* transposon type has been detected within Group I and all transposon types (*vacuma*, *transposa*, *flipper*-only, and *boty*-only) have been detected in Group II. DNA polymorphism and vegetative incompatibility studies revealed that the genetic diversity is lower in Group I. The GroupI and GroupII have also been shown to coincide with resistance to the fungicide fenhexamid, and synonymously known as FenR (resistant) = Group I and FenS (sensitive) Group II. The two groups also show differences in morphology, phenology and host range characters too.

More than two hundred *B. cinerea* isolates were collected from infected rape, strawberry and raspberry in Hungary in 2008. Single-spore isolates were prepared for DNA isolation. Dot-blot analysis was used for the detection of transposable elements. Purified PCR products were subjected to automatic sequencing. Mycelial incompatibility was tested by observing the interaction zone between paired colonies of wild-type *B. cinerea* strains on MEA amended with NaCl. To evaluate the resistance for fenhexamid, the mycelial growth of *B. cinerea* strains was measured on a solid minimal medium containing 0, 2, and 6 mg l^{-1} fenhexamide, respectively.

In this work we found that both Group I and II types of *B. cinerea* occurred in the Hungarian isolates. The identification of Group I strains based on PCR-RFLP of *Bc-hch* gene and sequence analysis of β-tubulin gene. That group earlier was characterized by the absence of the transposable elements *Boty* and *Flipper*. However, all the Hungarian Group I isolates contained the transposable element *Boty* and one isolate also contained *Flipper*, indicating a more diverse genetic structure of this cryptic species than earlier described and the possibility of information exchange between the two group. Vegetative compatibility results indicated, that majority of Group I isolates formed a unique Vegetative Compatibility Group (VCG), and several VCGs were detected within group II. Moreover VCGs overlapped between the two groups supporting the possibility of information exchange between Group I and Group II strains. The fenhexamid resistance studies supported previous results as fenhexamid resistant strains could only be detected in Group I isolates. However, 3 Group I strains were sensitive for this fungicide. We concluded that fenhexamid resistance can not be used for determination of Group I *B. cinerea* isolates.

I1.2 Correlation between the *Botrytis cinerea* population composition and the latent infection in 4 table grape cvs. in Chile

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In order to learn about the population composition of *B. cinerea* of the major table grape cultivars in Chile, Thompson Seedless (TS), Crimson Seedless (CS), Sugraone (Sg) y Red Globe (RG), and their relationship with the predominant infection levels of the fungus during bloom (F) v/s preharvest (PC), samples were collected from plants from these cultivars located in the Central Valley of Chile. They were subjected and not subjected to a fungicide-botrycide (pt+/pt-) (pt+: cyprodinil & fludioxonil, kresoxim-methyl & boscalid, boscalid & pyraclostrobin and fenhexamid during flowering, pre-veraison, veraison and preharvest, respectively). The parameters evaluated were the following: i) infection level in flowers and flower remains in F and berries in PC (n=300 samples /phenological stage), and ii) genotypic correspondence based on the presence or absence of transposable *Boty* and *Flipper* elements (PCR-duplex and specific primers), in 100 isolates of *B. cinerea* randomly selected by cultivar (n=20 / phenological stage or type of tissue: (n=10/program)).

The infection level was variable and higher in pt- than in pt+, with statistically significant differences among cultivars only in some periods ($p \leq 0,05$). In F, no differences were detected among cultivars, however, there was a higher response to fungicides in some of them (TS and RG > Sg and CS). In PC, the infection levels were significantly different among cultivars ($p \leq 0.05$), being higher in TS and CS. There was a differential response among cultivars, where only in TS and CS the final PC levels are not significantly different to the ones of F ($p > 0.05$). In Sg and RG this behaviour was not observed. These results reaffirm the importance of making preventative foliar sprays in F within the TS and CS control program, but it is not so in Sg and RG.

Of the total amount of isolates (n=400), 83.3% corresponded to the *transposa* genotype (Boty$^+$/Flipper$^+$), 10% to *flipper* (Boty$^-$/Flipper$^+$), 4,2% to *vacuma* (Boty$^-$/ Flipper$^-$) and 2,5% to *boty* (Boty$^+$/Flipper$^-$). In general, in the 4 cultivars, differences were noticed in the genotype proportions between pt+ and pt- ($p > 0.05$). The cultivars that showed the greatest proportion of *transposa* isolates were Sg and RG (92 and 87%) and *flipper* isolates in TS and CS (23 and 9%), cultivars with association between the F and PC infection levels. Although the flipper isolates are not the most predominant in Chile, this association might suggest that its presence plays a relevant role in the *B. cinerea* infections in two of the major cultivars in the country's export table grape market, issue that must be analyzed in future studies.

O1.4 Botryticides affect grapevine leaf photosynthesis

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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 number of applications per growing season has to be limited. To reduce them, a better knowledge of fungicide effects is necessary.

Therefore, the toxicity of two botryticides, fludioxonil (fdx) and fenhexamid (fhd), on grapevine leaves (*Vitis vinifera* L. cv. Pinot noir) was investigated by measuring variations of leaf photosynthetic parameters and correlated expression of photosynthesis-related genes.
Treatments were carried out in vineyard at the end of flowering, a key stage in infection by *B. cinerea*. Our results demonstrated that net photosynthesis was similarly affected after both fdx and fhd applications. Moreover, the mechanism leading to photosynthesis alteration seems to be the same for both fungicides. Stomatal limitation to gas exchange remained unchanged following treatments indicating that inhibition of photosynthesis was mostly attributed to nonstomatal factors. In addition, neither Rubisco carboxylation efficiency and the ability to regenerate ribulose 1,5-bisphosphate nor loss in PSII activity were involved in the fungicidesinduced depression of photosynthesis. Nevertheless, the analysis of gene expression encoding a PsbP subunit of photosystem II (*psbP1*), a chlorophyll *a/b* binding protein of photosystem I (*cab*) and a Rubisco small subunit (*rbcS*), showed a repression of these genes following fdx and fhd treatments. The lowest levels of gene expression may participate in decreased photosynthetic activity. To our knowledge, this is the first study of photosynthesis-related gene expression following fungicide stress.

O.1.5 Quantification of a stable *Botrytis* antigen in wines using *Botrytis* lateral flow devices

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It is well known that *Botrytis cinerea* is repsonsible for both the 'good ' and the 'bad' of wine making . Berries with late season infections (Noble rot) are highly sought after; they are used for making high value dessert wines. By contrast, infections that occur earlier in the season are destructive; they are the primary cause of Bunch rot. Besides reducing crop yeilds juice from bunch rotted berries generally lowers the quality of the resulting wines and has an adverse effect on the colour of both red and white wines. However, it is possible that the poor vinification properties of juice from berries with bunch rot are not the result of *Botrytis* alone but are due to the presence of other fungi such as species of *Aspergillus* and *Penicillium*. It has long been suspected that juice from berries with low levels of infection of *Botrytis* alone can result in table wines of good quality but proof is difficult.

Previously we raised a near-gensu-*Botrytis*-specific monoclonal antibody (BC-12.CA4, Meyer & Dewey, 2000) that detects a highly stable antigen that is not metablized during fermentation and is present in the resulting finished wines. This antibody has been used by two independent commercial companies to develop rapid, user-friendly, semi-quantitative immunochromatographic assays commonly known as Lateral Flow assays/devices. One lot, developed by EnviroLogix, Maine, USA, are designed for use in the lab or winery whereas the others, developed by Forsite, UK, are designed for use in the field. The devices were tested in parallel, to determine their ability to quantify levels of *Botrytis* antigens in a range of red and white Table Wines. The results from tests with both devices were comparable and support data previously obtained by ELISA on a range of similar wines (Dewey, 2004). The signal intensities (SI) of the positive test line from the devices developed by EnviroLogix were spread over a greater range with SI values ranging from 0 to 47 , whereas those from the Forsite devices ranged from 0.7 to 36 and in general were lower. However, both were consistent in detecting high levels of *Botrytis* antigens in some wines and low levels in others. The highest levels of *Botrytis* antigens were found in European Red wines wines and the lowest among Australian wines with the exception of a Pinot Noir. Results from tests on expensive, prize winning-English white sparkling wines were interesting, 6 out of 10 contained significant levels of *Botrytis* antigens, SI values ranging from 24 to 40.

These tests have shown that both types of devices can be used to determine the levels of *Botrytis* in finished wines and this, in turn, reflects the level of *Botrytis* rot present in the grape berries used to make the wines. Such devices should prove informative and useful to the wine industry.

O1.6 Proteomics reveals features of the adaptive response of the model fungus *Botrytis cinerea* to heavy metals

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The rapid development of human activity and many various industries has led to a significant accumulation of heavy metals in the environment. The presence of these elements in the atmosphere, water, soil and sediment causes a serious pollution problem. Many metals in their ionic form are directly or indirectly involved in many biological processes. While some (called micronutrients) are essential for cell metabolism (Iron (Fe), Copper (Cu), Nickel (Ni), Zinc (Zn) and Cobalt (Co)), others are considered toxic even at low concentrations (Mercury (Hg), Lead (Pb), Cadmium(Cd)). However, whatever the metal considered (essential or not essential), they are all toxic at high concentrations.

The mechanisms of interaction between metals and microorganisms have been extensively studied in bacteria and some yeasts. In the case of filamentous fungi, the work remains focused on the ability to accumulate metals and their use in bioremediation. In recent years, the use of biological organisms has become an alternative to conventional physicochemical methods used for heavy metal extraction in polluted environments. Different groups of microorganisms (algae, fungi [yeasts and molds] and bacteria) were used to set various heavy metals (Soares, 2002).

In our study we are interested in understanding the features of metal interactions with fungi and mechanisms used by the fungal cell in response to metal stress. The model chosen for this study is *Botrytis cinerea*. It is an ascomycete fungus found in soil and can infect a large number of plants including grapevines. Moreover, this fungus has the capacity of effective biosorption against heavy metals such as zinc (Zn^{+2}), lead (Pb^{+2}) and cadmium (Cd^{+2}) (Akar et *al.* 2005; Akar and Tunali, 2006).

By a proteomic approach, we present the first results of the adaptive response of *B.cinerea* toward different metals. The fungus is exposed to various concentrations of metal "X", and secreted proteins are separated by two-dimensional electrophoresis, then identified by mass spectrometry.

P1.1 Evolution of abundance and genetic structure of *Botrytis cinerea* airborne inoculum in the South East of France over 2.5 years

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Botrytis cinerea can be highly damageable for crops cultivated under greenhouse. Understanding the parameters that influence the presence of inoculum and its genetic structure could help to predict the outbreak and prevent the development of epidemics in the greenhouses. Studies in a variety of geographic situations have shown that inoculum can vary greatly both quantitatively and in terms of its genetic structure. However, little is known about the long term evolution of inoculum over a single site. The objective of our study is to characterise such variations and to determine if the abundance and the genetic characteristics of *B. cinerea* airborne inoculum can be linked to local and meso-climatic parameters.

Airborne inoculum of *B. cinerea* was sampled twice a month from September 2007 to March 2010 using a high throughput jet sampler located outdoors, in the vicinity of experimental greenhouses of INRA-Avignon. For each sampling date the airborne concentration was quantified (CFU m^{-3}) over a 24-hour period with the help of a selective medium and the climatic data (air temperature, wind speed, relative humidity) were recorded. Isolates were collected and single-spored and their genotype was determined using nine micro-satellites markers designed by Fournier *et al.* (2002).

In total, 65 samplings were carried out over the 2.5-year period. The highest concentrations were encountered in autumn and winter and the lowest during summer, with a 100-fold difference between the highest and the lowest values. Only three sampling dates provided no viable inoculum. These three days (in June and July) were characterized by high temperatures and low relative humidity. Preliminary analyses showed no significant correlation between inoculum concentration and local air temperature, relative humidity and wind speed. Further statistical analyses will be made taking into account parameters of air masses that brought the inoculum on the sampling site.

To date, 222 of the 1355 isolates collected have been characterized. Twenty-two of them were identified as pseudo-cinerea (based on a specific allele at the Bc6 microsatellite marker) and were excluded from further analysis. The microsatellite loci were polymorphic with a number of alleles varying from two (Bc4) to twenty-two (Bc6). We discriminated 156 different multilocus genotypes. Multicopy genotypes were represented by two to six isolates. Airborne population of *B. cinerea* showed a high level of diversity : the Simpson's diversity index was 0.99 and the Simpson eveness index was 0.95. The estimated multilocus linkage disequilibrium was significant but low (r_d= 0.14, P<0.001), suggesting a sexual reproductive mode mainly with a low of clonality. The analyses will be pursued with the rest of the isolates collected at others dates and the evolution of the genetic structure of airborne populations of *B. cinerea* will be further characterized.

Reference :

Fournier *et al.* 2002. *Mol. Ecol. Notes* 2 :253-255

P1.2 Differences between *Botrytis cinerea* populations causing grey mould and noble rot in two Hungarian vineyards

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Botrytis cinerea Pers.:Fr., the anamorph of *Botryotinia fuckeliana* (de Bary ex de Bary) Whetzel causes grey mould on a high number of crop plants in the temperate zones worldwide including grapes. In grape vineyards, the frequent occurrence of *B. cinerea* prior to harvest results in serious losses of fruits and deterioration of wine quality. *Botrytis* infection requires moist conditions and if the weather stays wet, the malevolent form, “grey rot” can destroy crops of grapes. Grapes typically become infected with *Botrytis* when they are ripe, but when then exposed to drier conditions become partially raisined and the form of infection brought about by the partial drying process is known as “noble rot”. Grapes when picked at a certain point during infection can produce particularly fine and concentrated sweet wine. The realisation of grape protection for these different wine making technologies is difficult. *B. cinerea* is already resistant against most fungicides, thus making alternative strategies for its control essential. A clue to these strategies lies in the understanding of the genetic structure and dynamics of its populations.

In the course of our work we have investigated the genetic properties of grape pathogenic populations of *B. cinerea* through three sequential years in the area of Eger and Tokaj, Hungary. Eger, the region of Bull’s Blood is well known from its dry wines, Tokaj is famous for its botrytised wine called aszú of Tokaj. 300 isolates from these two wine regions and from three years were sampled. Based on the sequence of the β-tubulin, presence or absence of transposon elements *boty* and *flipper* and a minisatellite from the intron of an ATPase, MSB were the data analyzed.

Very high variability was found among the investigated sequences. The presence of *flipper* transposon element alone in the isolates was a frequent occurrence in both territories as well. A corresponding analysis of the fixation index revealed that the noble roted population from Tokaj exhibited some differentiation from both the noble rotted and gray moulded populations from Eger. We could also detect some differentiation between the different vintages and geographically isolated populations.

In summary, our data underline a significant genetic variability between populations from these territories and these differences could arise by geographical barriers and by dissimilar grape protection technologies as well.

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Session 2:
BIOLOGY AND GENETICS OF *BOTRYTIS* + *SCLEROTINIA*

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I2.1 The *Botrytis cinerea* mating type loci

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Botrytis cinerea is a heterothallic ascomycete with two mating types, MAT1-1 and MAT1-2. Sexual development in ascomycetes is usually controlled by a *MAT* locus that contains genes encoding transcription factors, either of the class of alpha-domain proteins or high mobility group (HMG)-domain proteins. By convention, the alpha-domain gene is named *MAT1-1-1* and the HMG-domain gene is named *MAT1-2-1*. Ascomycete species that are homothallic contain both genes, physically linked in the same region of the genome. The two *B. cinerea* strains of which the genome was sequenced, B05.10 and T4, are of opposite mating type and their genome sequences have revealed novel features in the structure of the MAT loci.

Incomplete fragments of the *MAT1-2-1* and *MAT1-1-1* genes were detected that border the *MAT* loci of the MAT1-1 and MAT1-2 isolates, respectively. Both of these fragments encode truncated, non-functional proteins. This structure strongly suggests that *B. cinerea* has evolved from a homothallic ancestor containing complete *MAT1-1-1* and *MAT1-2-1* genes at the same locus. The MAT1-1 and MAT1-2 alleles have arisen by the loss of either the HMG-domain or alpha-domain sequences, leaving the disabled gene fragments seen in the current *MAT* loci. Two additional genes were detected, designated *MAT1-1-5* and *MAT1-2-3*, that have not previously been reported from other fungi. Homologs of *MAT1-1-5* are present in other leotiomycetes (including *Sclerotinia sclerotiorum*), whereas the *MAT1-2-3* gene is exclusively present in species of the genus *Botrytis*. Knockout mutants in *MAT1-1-5*, generated by gene replacement, appeared to be sterile. In crosses of such a mutant with the wild type MAT1-2 strain SAS405, there was normal development of primordia and stipes, but the dikaryon failed in differentiating the cap structure in which the asci and ascospores develop.

B. cinerea is unusual in that some isolates are capable of 'dual mating'. This refers to the observation that most isolates act in a standard heterothallic, cross-fertilizing fashion (MAT1-1 or MAT-1-2). Some isolates, however, can mate with both MAT1-1 ánd MAT1-2 isolates. Certain dual mater isolates can even self-fertilize and are truly homothallic. The *MAT* locus of five homothallic *B. cinerea* isolates was analysed. Four of those contain a MAT1-2 locus, without any sequence of the MAT1-1 locus being detected. Remarkably, one homothallic isolate contains a MAT1-1 locus, without any sequence of the MAT1-2 locus being detected. We conclude that dual mating and homothallism is controlled by sequences outside the *MAT* locus.

O2.1 Apoptotic-like cell death in *Botrytis cinerea*: a case study on the putative inhibitor of apoptosis gene *BcBIR1*

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Recent studies support the possibility that plants might provoke fungal PCD as a mechanism to deter pathogens. To investigate this possibility, we have determined the conditions and methods for detection of apoptotic-like cell death in *B. cinerea,* a cosmopolitan plant pathogen capable of infecting over 200 plant species. Next we studied the role of putative apoptotic genes in regulation of PCD and pathogenicity in this fungus.

Apoptotic-like cell death was determined by several criteria including production of ROS, nuclei morphology and TUNEL. Using these assays, we characterized apoptotic-like cell death during growth and in response to several treatments, including exposure to the plant defense compound hexanoic acid. Homologs of human and yeast apoptotic genes were identified and characterized. Knockout or over expression of these proteins had various effects on fungal apoptotic response as well as on apoptosis-associated features such as viability, aging, vegetative reproduction and resistance to stresses.

One of our main candidates is *BcBIR1*, a homolog of *Saccharomyces cerevisiae* IAP-like gene *BIR1*. IAPs (inhibitor of apoptosis proteins) prevent apoptosis in human through inhibition of caspases. *BcBIR1* was cloned and transgenic strains with reduced or over expression were generated and characterized. BcBIR1 over-expressing strains accumulated more biomass in liquid cultures, had reduced sensitivity to nutrient stress, and were more virulent than wild type while the deletion strains exhibited the opposite trend with increased sensitivity to apoptosis-inducing conditions and reduced virulence. Overall, these results suggest that BcBIR1 has anti-apoptotic activity and is essential for virulence, supporting the hypothesis that induced fungal cell death might be associated with reduced disease spreading.

O2.2 Mutations leading to overexpression of drug efflux transporters are responsible for multidrug resistance phenotypes of *Botrytis cinerea* field strains

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The "multiple drug resistance" (MDR), is a great medical problem for chemical treatment of cancer or microbial infections. MDR 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 so-called MDR transporters can result in the increased export and thereby reduced sensitivity to many different natural or synthetic drugs.

In French and German winegrowing regions, increasing *B. cinerea* populations with MDR phenotypes have been observed. Three types of MDR strains with different fungicide resistance spectra were distinguished. MDR phenotypes were correlated with increased fungicide efflux activities and constitutive overexpression of genes encoding drug efflux transporters. MDR1 strains showed high mRNA levels of *atrB* encoding an ABC transporter, whereas MDR2 strains showed high transcript levels of *mfsM2* encoding an MFS transporter. In MDR1 strains, *atrB* overexpression was found to be due to single activating mutations in the transcription factor Mrr1 that controls *atrB* and other ABC transporters. Promoter-reporter gene constructs of *atrB* are currently analysed by in vitro studies to identify the binding site of Mrr1. In MDR2 strains, *mfsM2* overexpression was correlated with two unique rearrangements, due to insertion of retrotransposon-like gene fragments into the *mfsM2* promoter. An *mfsM2* promoter-reporter gene fusion confirmed that these rearrangements are responsible for *mfsM2* overexpression, presumably by insertion of a novel transcription factor binding site. MDR3 strains had both mutations of MDR1 and MDR2 strains and showed overexpression of both *atrB* and *mfsM2*.

Disruption of either *atrB* or *mrr1* resulted in complete loss of the MDR1 phenotype, while expression of a constitutively active *mrr1* allele from an MDR1 strain conferred to a sensitive strain an MDR1 phenotype. Similarly, disruption of *mfsM2* in an MDR2 strain led to loss of MDR2 phenotype, and transformation of a sensitive strain with a construct leading to overexpression of *mfsM2* created strains with MDR2 phenotypes, confirming that these genes are responsible for the MDR phenotypes.

A monitoring for fungicide resistance of *B. cinerea* in German strawberry fields revealed the existence of MDR1-like strains which showed further increased overexpression of *atrB* and higher levels of multidrug resistance than MDR1 strains from vineyards. The molecular basis of this "super-MDR1" phenotype is currently under investigation.

O2.3 Natural field populations of *Botrytis cinerea* from vineyards from Castilla y León in Spain: physiological and population genetics characterization

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Botrytis cinerea (teleomorph *Botryotinia fuckleiana*) is a necrotrophic plant pathogenic fungus that infects more than 200 plant species. It causes the gray mold disease in a number of economically important hosts, among them *Vitis vinifera*. In the Castilla y León region of Spain, grapevine culturing and wine industry have acquired an enormous relevance for the development of areas in which agriculture is the main economy driving force. In this region there are numerous wine producing areas where different grape varieties, very often autochthonous and well adapted to their environments, are grown and high quality wines with specific and peculiar characteristics are produced. Some of these areas, called "Denominaciones de Origen" (DO) maintain high standards during the whole process of wine production, including the utilization of grapes only from very specific varieties cultured in registered vineyards. These cultural and processing practices impose certain limitations which could influence the way in which the genetic diversity of both the host and the pathogen is structured in the area of interest. We are mainly interested on the characterization of the natural populations of *B. cinerea* in the wine producing areas in Castilla y León, on the quantification of their genetic variability and on the determination of the way in which this genetic variability is structured in those populations.

In order to gain basic information needed to investigate these questions we first made a survey of *B. cinerea* in vineyards from Castilla y León in 2007 and collected about 500 field isolates from symptomatic and non-symptomatic grape bunches. About 250 isolates are being characterized physiologically and genetically. Infection assays on bean allowed us to detect large differences in pathogenicity between isolates. Large differences were also observed in the aggressiveness of different isolates on several *Vitis* varieties. When considering the sample of the isolates analyzed as a whole, the average aggressiveness on variety "Verdejo" was lower than on variety "Tempranillo", indicating that the first one has a certain degree of natural resistance towards *B. cinerea*.

Genetic analysis of the fungal isolates collection is being carried out by two means. First, the isolates are being checked for the presence of the transposons *Boty* and *Flipper*. Second, genetic variability is being quantified and analyzed by scoring a total of 250 AFLP markers in each isolate. The population structure units considered are DOs (geography) and, when possible, vineyard within them, and the *Vitis* variety from which the isolate was obtained. A description of the field isolates population under study will be presented and results will be discussed in the context of population structure, mode of reproduction and population differentiation.

P2.1 Molecular characterisation of the mating type (*MAT1*) locus in *Botryotinia fuckeliana* (*Botrytis cinerea*)

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In *Botryotinia fuckeliana* (de Bary) Whetz. (an. *Botrytis cinerea* Pers.), like in other filamentous ascomycetes exhibiting an heterothallic breeding system, sexual compatibility is controlled by a single mating type (*MAT1*) locus with two alternative idiomorphs (*MAT1-1* or *MAT1-2*) determining sexual identity. Homothallic behaviour (*MAT1-1/2*) was occasionally observed in both field and monoascosporic isolates of the fungus which were fertile with reference strains of opposite mating types.

The structural organization of the *MAT1* locus was investigated in strains of the fungus having different sexual behaviour. Genome regions including the *MAT1* genes and flanking sequences were sequenced and analyzed by using a PCR-based approach in the reference strains of opposite mating type, SAS56 (*MAT1-1*) and SAS405 (*MAT1-2*), and their monoascosporic progeny.

The mating type-specific sequences in *MAT1-1* and *MAT1-2* idiomorphs consisted of 2,515 bp and 2,777 bp, respectively. As expected, flanking sequences of the *MAT1* locus were almost identical in isolates of different mating type. Two open reading frames (ORFs) were identified in each idiomorph. The *MAT1-1* idiomorph included the mating type gene designated *MAT1-1-1* (1,161 bp) encoding a protein of 354 amino acids with an alpha-box DNA binding motif, and the *MAT1-2* idiomorph included the *MAT1-2-1* (1,236 bp) gene encoding a high mobility group (HMG)-domain protein (376 a.a.). Each idiomorph also included an ORF encoding predicted proteins of 377 a.a. (*MAT1-1-2*) or 197 a.a. (*MAT1-2-2*) showing high homology (77-80%) with two ORFs identified in the *MAT1* locus of the closely-related homothallic species *Sclerotinia sclerotiorum* (Lib.) de Bary. Portions of the *MAT1-1-1* (670 bp) and the *MAT1-2-1* (92 bp) genes were found in the opposite mating-type and extended in the flanking regions.

Idiomorph-specific PCR primer pairs were designed and used to explore the structure of the *MAT1* locus in ascospore progeny and field isolates showing homothallic behaviour. All the analysed isolates carried exclusively the *MAT1-1* or the *MAT1-2* specific genes and the organization of the *MAT1* locus did not differ from those of heterothallic strains.

Transcription of the mating type genes (*MAT1-1-1* or *MAT1-2-1*) was confirmed by RT-PCR in sclerotia of reference strains (SAS56 and SAS405) grown under carpogenesis-promoting conditions.

Comparative analysis of the *MAT1* locus in *B. fuckeliana* and *S. sclerotiorum* suggests that homothallism evolved from heterothallism in the family *Sclerotiniaceae*.

P2.2 Phenotypic and molecular characterization of fungicide-resistant field isolates of *Botryotinia fuckeliana* (*Botrytis cinerea*)

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Acquired resistance to fungicides is frequently experienced in *Botryotinia fuckeliana* (de Bary) Whetz. (an. *Botrytis cinerea* Pers.). Phenotypic and molecular characterization of field isolates resistant to pyrimethanil, fludioxonil, fenhexamid, boscalid or trifloxystrobin were carried out through colony growth tests and analysis of DNA sequence variations in the genes coding for the target proteins.

Three levels of resistance to anilinopyrimidines (APs) were distinguished: low (EC_{50} = 0.3-1 μg ml^{-1}; MIC = 3-10 μg ml^{-1}), moderate (EC_{50} = 1-10 μg ml^{-1}; MIC ≥ 100 μg ml^{-1}) and high resistance (EC_{50} = 30-100 μg ml^{-1}; MIC > 100 μg ml^{-1}). Molecular characterization of the genes encoding key-enzymes of methionine biosynthesis (cystathionine γ-synthase, cystathionine β-lyase, cystathionine β-synthase and cystathionine γ-lyase), potentially involved in AP-resistance, revealed single nucleotide polymorphisms (SNPs) which were, however, not associated to resistance.

All fludioxonil-resistant isolates showed EC_{50} (0.1-1 μg ml^{-1}) and MIC (1-3 μg ml^{-1}) values significantly higher than wild-type sensitive isolates (EC_{50} = 0.02-0.03 μg ml^{-1}; MIC = 0.2 μg ml^{-1}). No association between SNPs found in the *Daf1* gene sequence and phenotypical response to fludioxonil was observed.

At least three groups of *B. fuckeliana* field isolates were discriminated according to their response to fenhexamid: sensitivity (EC_{50} = 0.03 μg ml^{-1}; MIC = 0.3 μg ml^{-1}), low (EC_{50} = 2.7 to 9.5 μg ml^{-1}; MIC > 100 μg ml^{-1}) and high (EC_{50} and MIC > 100 μg ml^{-1}) resistance. Molecular characterization of the 3-keto-reductase gene (*Erg27*) revealed two SNPs leading to amino acid changes (F26S and N369D) in low resistant mutants. The replacements at position 412 of a phenylalanine with isoleucine (F412I), valine (F412V) or serine (F412S) were associated with a high level of resistance.

Boscalid-resistant isolates displayed EC_{50} and MIC values higher than 100 μg ml^{-1}, (wild-type sensitive isolates EC_{50} = 0.3 μg ml^{-1} and MIC = 10 μg ml^{-1}). Gene sequence analysis of the iron-sulphur (Ip) sub-unit of the succinate dehydrogenase complex revealed that SNPs at position 272, resulting in a histidine to tyrosine (H272Y) or arginine (H272R) replacement, were associated to the resistance. Only one mutant showing a lower level of resistance (EC_{50} = 15 μg ml^{-1} and MIC > 100 μg ml^{-1}) carried a different amino acid substitution (N230I).

All QoI-resistant mutants showed a high level of resistance (EC_{50} and MIC > 100 μg ml^{-1}) as compared to the wild-type sensitive strains (EC_{50} = 0.03-0.1; MIC = 1 μg ml^{-1}) and all carried the G143A mutation in the mitochondrial *cytb* gene. QoI-resistance was always maternally inherited in ascospore progeny of sexual crosses.

P2.3 Mutasynthesis, an approach to generate new unnatural natural products from *Botrytis cinerea*

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Semi-synthesis, total synthesis and combinatorial biosynthesis have succeeded in introducing greater diversity into natural products. However, betters results can be arise by combining chemical and biological approaches utilising the strengths of each approach. A very interesting strategy combines chemical synthesis with the biosynthesis using genetically engineered microorganism. This approach has been termed mutational biosynthesis (MBS) or in short mutasynthesis (Kennedy et al. 2008, Kirschning et al. 2007). Recently, mutasynthesis has experienced a renaissance since the number of fully sequenced biosynthetic gene clusters coding for natural products has substantially increased.

The sequencing of the genomes of the B05-10 and T4 strains of *B. cinerea* at the Broad Institute and the Genoscope, respectively (Fillinger et al. 2007), revealed an abundance of novel biosynthetic gene clusters.

In addition to other relevant results, two secondary metabolism genes, *bcbot1* and *bcbot2*, involved in botrydial biosynthesis, have been characterized and a sesquiterpene ciclase and P450 monooxigenase identified (Siewers, et al. 2005; Pinedo et al. 2008). The lack of botrydial synthesis in *bcbot1* and *bcbot2* mutants seems to be compensated by the overproduction of the polyketide type toxin botcinic acid.

Here, we present preliminary results in the synthesis of analogues of 2,4,6,8-tetramethyl-3,4-dihydroxydeca-8(9)-enolide, a hypothetical intermediate in the biosynthesis of polyketides toxins by *B. cinerea*.

Feeding of disrupted in key genes strains with those synthetic analogues, can help to a more fully understood of the complete biosynthetic pathway and represents a new approach for the diversification of this kind of natural products.

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P2.4 Characterisation of the new species *Botrytis pseudocinerea* living in sympatry with *Botrytis cinerea* in French populations

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Botrytis cinerea Pers. ex Fr, the anamorph of *Botryotinia fuckeliana* (de Barry) Whetzel, is a ubiquitous fungus causing gray mold on many crops, including vegetables, ornamental plants and fruits and especially grapevine. This disease was recently found to be caused by a complex of two related fungal species living in sympatry: *Botrytis* group II (=*B. cinerea sensu stricto,* the most abundant species in the complex) and *Botrytis* group I (=*B. pseudocinerea* as proposed name), which is present in weak abundance in French vineyards.

Morphological and biological criteria, as well as pathogeny on tomato and green bean, were investigated between the two species without revealing significant differences. Nevertheless, isolates from group I were found to be naturally resistant to the fungicide fenhexamid and highly sensitive to fenpropidin in comparison to group II wild-type strains. Moreover, molecular analysis revealed high polymorphism in many genes and enabled to place this new species within the *Botrytis* genus previously established by Staats *et al* (2005). No correlation was found with the grouping previously proposed to structure *B. cinerea* populations and using the presence of the transposable elements (TE) *Boty* and *Flipper*. Indeed, group I strains are all free from these TE (*vacuma* type), whereas group II strains can harbour none (*vacuma*), one, two or ripped copies (*transposa*) of these TE. Population analyses using microsatellite markers from Fournier *et al* (2002) enabled to assess respective genetic diversity in these two species and pinpointed private alleles from *Botrytis* group I. At last, as no viable progeny was produced from crosses between group I and group II strains, we conclude that *Botrytis* group I is a distinct species that occupies similar ecological niches as *B. cinerea sensu stricto*.

P2.5 Lack of evidence for an important role of *Botrytis cinerea* hydrophobins in the asexual state

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Conidial germination in *B. cinerea* can be induced by several chemical and physical stimuli. While carbon sources are required for germination on hydrophilic surfaces, conidia are able to germinate in pure water on artificial hydrophobic surfaces. 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 hydrophobic contact-induced germination, we sought to construct mutants devoid of hydrophobins in the spore wall. Hydrophobins are small, cysteine-rich, secreted proteins that were found to render the surfaces of spores and aerial mycelia of many filamentous fungi, e.g. *Aspergillus nidulans* and *Magnaporthe grisea*, hydrophobic. In the *B. cinerea* strain B05.10 genome sequence, three hydrophobin genes were initially identified, one encoding a class I hydrophobin (Mpg1), and two encoding class II hydrophobins (Mhp1, Mhp2). Further hydrophobin-like sequences were later found in the improved *B. cinerea* strain T4 genome sequence. However, all except of one of these showed either low overall hydrophobicity, or an unusual spacing of the eight cysteine residues, and are thus probably no functional hydrophobins.

Gene expression studies by RT-PCR revealed that the three hydrophobin genes are expressed in spores, germlings and both saprophytic and parasitic mycelium. 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 none of them exhibited an "easily wettable" phenotype which is often observed with fungal hydrophobin mutants. Analysis of the conidial surface of *B. cinerea* by scanning electron microscopy revealed neither rodlet layers, as in the case of *A. nidulans* conidia, nor any differences between wild type and the hydrophobin double mutants. Finally, hydrophobin triple mutants were generated, using a phleomycin resistance marker, which are currently under investigation. At present, there is no evidence for a role of hydrophobins for the water repellent properties of *B. cinerea* conidia and aerial mycelium, in contrast to other ascomycetous fungi.

P2.6 Characterisation of *Botrytis cinerea* in raspberry in Serbia

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Botrytis cinerea Pers. Fr., the anamorph of *Botryotinia fuckeliana*, is a pathogen that causes grey mould on a wide variety of plants worldwide. It is one of the main factors that influence berry production in Serbia. Yield losses in commercial fields exceed 50%, especially during periods of rainy, wet weather during flowering and harvest. In addition, the fungus causes significant losses during shipping and marketing. Many studies reported that this fungus can exhibit great phenotypic diversity. Recent studies on populations of *B. cinerea* provided a new finding that this species is composed of two sympatric species, *transposa* and *vacuma*. Since the management of gray mould control involves chemical and biological methods, use of organic systems, and protection programs based on disease monitoring and prediction, information on genetic structure of the pathogen population could help us to develop effective strategies for the control. Therefore, the aims of this study were to determine whether two sympatric species *transposa* and *vacuma* are present in pathogen populations in raspberry crops in Serbia and to characterize some biological features of the isolates from both subpopulations: morphology of the colony, growth rate, sporulation, sclerotia production, and sensitivity to fungicide fenhexamid.

A hundred and thirty isolates of *Botrytis cinerea*, derived from raspberry fruits originating from six commercial fields in raspberry growing area in Serbia, were studied using conventional mycological and molecular methods. It was found that at the beginning of the growth on PDA medium, all the isolates form white, uniform, aerial micelium with an entire margin. Morphological differences among the isolates appeared after period of incubation of six days. After 30-days incubation period, the isolates were distributed into eight morphological types – four mycelial and four sclerotial. Most of the isolates had colonies of sclerotial type (81.5%), and the most frequently found was S3 type which formed large irregularly placed sclerotia. This type of isolate was dominant in five of six populations investigated and represented 45 - 65% of the isolates. The least frequently found was mycelial type M3 (0.7% of the isolates) characterized by mycelial masses. The isolates expressed significant differences in growth rate and virulence.

Presence of transposons *Boty* and/or *Flipper* was detected in isolates originating from all the investigated localities. It was discovered that *B. cinerea* population in raspberry in Serbia, beside genetically isolated sympatric species *transposa* (43.1%) and *vacuma* (10.8%), contains additional two – *boty* (44,6%) and *flipper* (1.5%), with only one transposon (*Boty* or *Flipper*, respectively) in the genome. The difference between *vacuma* isolates and isolates containing transposons in terms of growth rate, morphology or virulence was not recorded. The investigation reveiled that all the isolates from raspberry in Serbia, either *transposa*, *vacuma*, *boty* or *flipper*, belonged to genetical Group II *B. cinerea.*

P2.7 *In planta* assays highlighted significant differences between *Botrytis cinerea* and *Sclerotinia sclerotiorum*

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Botrytis cinerea and *Sclerotinia sclerotiorum* are known to produce oxalic acid and a range of cell-wall degrading enzymes such as cellulase, polygalacturonases and proteases during pathogenesis (**1**). We observed that *B. cinerea* (BO5.10 strain) produces less oxalic acid than *S. sclerotiorum* (strain S5) when infecting sunflower cotyledons. On the other hand, we found that both fungi produce ammonia during the colonization of sunflower cotyledons but the ammonia level is higher with *B. cinerea*. Thus the resulting ambient pH of *S. sclerotiorum*-infected tissues remained acidic during the colonization process while the resulting ambient pH of *B. cinerea*-infected tissues started acidic but became neutral by the end of the colonization process. Strong differences were found in proteases expression levels between the two fungi during colonization, probably due to differences in ambient pH (**2**, **3**, **4**).

Effects of the deletion of oxaloacetate acetyl hydrolase in *S. sclerotiorum* were studied during the infection process on sunflower cotyledon. The Δoah1 mutant does not produce oxalic acid and is strongly affected in its pathogenicity on sunflower cotyledons since scarcely 20% of sunflower cotyledons were colonized. However a strong increase in production of cell-wall degrading enzymes, cellulase and proteases, was noticed. The resulting ambient pH of Δoah1 mutant-infected tissues surprisingly followed the same behavior as the one observed during the *B. cinerea* infection process: it started acidic then became neutral by the end of colonization.

We showed in this study that *Botrytis cinerea* and *Sclerotinia sclerotiorum* display a strong difference in ambient-pH regulation modulation during pathogenesis. We suggest that this difference could be due to differences in metabolism such as a lack of oxalic acid production associated with an increase in specific cell-wall degrading enzymes.

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P2.8 The osmosensing signal transduction pathway from *Botrytis cinerea* regulates cell wall integrity and, together with the Bmp3 MAP kinase, melanin biosynthesis

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Mitogen activated protein kinase (MAPK) signal transduction pathways are ubiquitous among eukaryotic organisms with evolutionary conserved modules. They coordinate the adaptation of cellular functions to changing environmental conditions. Concerning saprophytic or (animal or plant-) pathogenic life styles, fungal MAPK pathways have been extensively studied. Although generally classified as osmotic and cell wall integrity pathways, functional divergences have been observed for HOG1- and SLT2-related MAPK pathways. In this study we show that the osmotic signal transduction cascade is the principal pathway involved in cell wall integrity in the phytopathogenic ascomycete *Botrytis cinerea*. The deletion mutants of the upstream histidine kinase Bos1 and of the MAPK Sak1 showed modified tolerance to cell wall degrading enzymes and cell wall interfering agents, as well as increased staining of ß1-3 glucan and chitin compared to the wild-type. In addition the Sak1 MAPK was phosphorylated upon cell wall challenging. Sak1 interfered with the phosphorylation status of the SLT2-type MAPK Bmp3 hinting to cross talk between both MAPK pathways. The coordination of melanin biosynthesis by both signal transduction pathways involving the Bos1 histidine kinase and the Sak1 and Bmp3 MAPKs through transcriptional control of biosynthesis genes corroborates cross talk between Sak1 and Bmp3 pathways.

P2.9 New advances in mannitol metabolism in the fungal plant pathogen *Botrytis cinerea*

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Mannitol is one of the most abundant polyols occurring in nature. It is usually the most abundant soluble carbohydrate within the mycelium. In fungi, several physiological functions have been ascribed to D-mannitol, such as reservoir of reducing power, carbon storage compound accumulated. Mannitol contributes to stress tolerance in fungi.

The metabolic pathway for mannitol biosynthesis and catabolism takes place in Ascomycotous fungi through the mannitol cycle, involving two pathways. The direct reduction of fructose-6-phosphate into mannitol-1-phosphate (mannitol-1-phosphate) involves a mannitol-1-phosphate dehydrogenase (MPD, EC 1.1.1.17). Mannitol-1-phosphate is then dephosphorylated into mannitol via a mannitol-1-phosphate phosphatase. This last reaction was described as irreversible, consequently mannitol degradation is supposed to occur through oxidation of mannitol to fructose via a reversible mannitol dehydrogenase (MTDH, EC 1.1.1.138). Recent reports have challenged the existence of a mannitol cycle. In *Stagonospora nodorum*, MPD is necessary for mannitol catabolism, whereas MTDH is not. A mannitol phosphorylation pathway allowing conversion of mannitol into mannitol-1-phosphate might exist.

In order to redefine the mannitol pathway in *Botrytis cinerea*, we used a targeted deletion strategy of genes encoding two proteins of mannitol metabolism, a mannitol dehydrogenase (BcMTDH) and a mannitol-1-phosphate dehydrogenase (BcMPD). Mobilization of mannitol and quantification of *Bcmpd* and *Bcmtdh* gene transcripts during development and osmotic stress confirmed a role for mannitol as temporary and disposable carbon storage compound. In order to study metabolic fluxes, we followed conversion of labelled hexoses by wild type and *Bcmpd* and *Bcmtdh* mutant strains by *in vivo* NMR spectroscopy. Altogether our data revealed that glucose was essentially converted into mannitol through BcMPD pathway, whereas fructose was mainly converted into mannitol by BcMTDH. Moreover, the existence of a mannitol phosphorylation pathway was suggested by NMR investigations for the first time. This last finding definitively challenged the existence of the originally postulated mannitol cycle in favor of two simultaneously expressed pathways. Finally, physiological and biochemical studies conducted on double deletion mutants (*Bcmpd/Bcmtdh*) showed that mannitol was still produced despite a complete alteration of both mannitol biosynthesis pathways. This strongly suggests that one or several additional undescribed pathways could participate to mannitol metabolism in *B. cinerea*.

P2.10 Phenotypic and genotypic characterization of chilean *Botrytis cinerea* isolates with different levels of fenhexamid sensitivity

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Chilean *Botrytis cinerea* isolates with different levels of sensitivity to fenhexamid were phenotypically analyzed and sequenced for the *erg27* gene that encodes the 3 ketoreductase enzyme (n=46). The fenhexamid highly resistant isolates ($HydR3^{+}$) (n=15) showed EC_{50} values > 5 µg mL^{-1} for conidial germination and > 2 µg mL^{-1} for mycelial growth. The isolates with slight to moderate resistance ($HydR3^{-}$) (n=5) showed EC_{50} values for conidial germination and mycelial growth that fluctuated between 1 and 2 µg mL^{-1} and 0.4 and 3 µg mL^{-1}, respectively. The fenhexamid sensitive isolates (HydS) (n=26) showed EC_{50} values for myceliar growth of < 0.1 µg mL^{-1}. In addition, of the totals amount of isolates analyzed, 9 of them presented phenotype MdR1 multi-drug resistance.

In competition tests between isolates from the phenotypes $HydR3^{+}$ *vs*. HydS, when the $HydR3^{+}$ phenotype also showed the ImiR1 + BenR1 phenotype, it was more competitive than the HydS phenotype, reaching a 60% higher level of development compared to the HydS. However, in the $HydR3^{+}$ phenotype that is characterized for also having the ImiR1 + BenR1 + AniR1 phenotype, the competitiveness above the HydS was 100%. On the contrary, the HydS + MdR1 phenotype became more competitive than the $HydR3^{+}$ phenotype.

The *erg27* alleles of $HydR3^{-}$ and $HydR3^{+}$ isolates were sequenced and compared to the fenhexamid resistant French isolates. All $HydR3^{+}$ isolates showed a modification in the C-terminal end at position 412 of the protein (transmembrane domain), mutation that causes the fenhexamid resistance. The $HydR3^{-}$ isolates showed six point mutations in the sequenced region of gene *erg27*, corresponding to amino acid changes between positions 199 and 408 of the protein, and three of them had not been described before.

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P2.11 Use of GFP for live-cell imaging in *Botrytis cinerea*

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Fluorescent probes are instrumental in cell biology research; live-cell imaging of fluorescently labeled cells enable end point protein localization and whole cell visualization, as well as study of dynamic cellular processes. Imaging studies in *Botrytis cinerea* have been limited, partly due to lack of appropriate fluorescent probes. Here we report on the development of GFP-based probes to study cell biology in *B. cinerea.*

GFP-labeled histone (H1-GFP) was used to follow cell cycle progression. Time lapse microscopy of germinating spores revealed simultaneous division of nuclei in the germinating spores and emerging germ tubes. Early events of pathogenic development were visualized *in planta* using transgenic strains expressing the H1-GFP or GFP-HPH fusion proteins. An EndoG-GFP fusion protein was used for visualization of mitochondria. This and other constructs were also useful in detecting cellular responses to stress or during development. Altogether, our data show that GFP, and possibly other fluorescent probes, can be used to promote live cell imaging studies in *B. cinerea.*

P2.12 Post-transcriptional gene silencing in *Botrytis cinerea*

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Post-transcriptional gene silencing (PTGS) consists of a set of pathways which results in the inhibition of expresion of an specific gene (1). This mechanism is present in a wide number of eukaryotic organisms and has been applied as a functional genomic tool to generate null mutants without inactivating the corresponding gene (2). There are many studies of gene function analysis in fungi using gene silencing with success. Such are the cases of *Venturia inaequalis* (3) or *Magnaphorthe oryzae* (4). In *B. cinerea,* gene disruption or replacement have been the main strategies used to inactivate specific genes, but this method requieres protoplast transformation, checking interruption at the specific locus without ectopic integration of the transforming DNA, and isolation of homokariotic transformants. These concerns could be overcome using PTGS as a method to inactivate genes in *B. cinerea.*

As a first approach, we addressed the inactivation the endogenous gene *nia*D, coding for nitrate reductase, by transforming the wild type strain B05.10 with different *nia*D fragments flanked by two promoters in opposite orientation. The transcribed complementary RNAs would form a dsRNA which would initiate PTGS and would silence *nia*D expression. About 50% of transformants showed reduced nitrate reductase activity, with respect to the wild type, and 40% of these silenced strains showed a reduction in nitrate reductase activity of more than 50%.

An alternative approach was then developed to silence the GFP gene in two different strains expressing this reporter protein previosuly constructed in our lab. In this case, the strains were transformed with constructions displaying two inverted fragments of the GFP gene separated by a loop region, so that the transcribed RNA could form a loop-stem structure to initiate PTGS. The level of GFP expressed by all the transformants was quantified by Western blot. In this approach, we observed that more than 90% of the assayed strains showed levels of GFP lower than the wild-type. Moreover, a reduction of more than 50% in GFP expression was observed in 80% of the silenced strains.

Choosing the right construct, this method could be presented as a very usefull tool to identify pathogenicity factors through gene silencing in *Botrytis cinerea.*

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Session 3:
DISEASE MANAGEMENT 1

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I3.1 Air-phase control of *Botrytis cinerea* after harvest

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A novel air-phase methodology was developed to control *B. cinerea* after harvest in the storage facilities. The methodology is based on an apparatus that is able to clean the air from particles and microrganisms and to release into the air disinfecting agents generated by electrolysis of a salt solution from a natural source. The technology was able to prevent development of *B. cinerea* on agar plates as well as table grapes and other fruit types. The results so far support the potential of the technology at various scales without inflicting damage to the fruit.

O3.1 Fenhexamid resistance in *Botrytis cinerea*: Target modifications and fungicide detoxification

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Since 2000 the Sterol Biosynthesis Inhibitor (SBI) fungicide of class III, fenhexamid, a hydroxyanilide, is widely used in French vineyards against *Botrytis cinerea*, the causal agent of grey mould. This compound inhibits the 3-keto reductase activity of the Erg27 protein involved in the C4 demethylation process during ergosterol biosynthesis (Debieu *et al.,* 2001). *B. cinerea* strains resistant to this hydroxyanilide, named HydR3$^+$ & HydR3$^-$, occurred in 2005 in French vineyards. HydR3$^+$ has a higher IC_{50} value (>10ppm) than HydR3$^-$ (<0.5ppm) (wild type IC_{50}<0.03). The majority of resistance phenomena in fungal plant pathogens are due to target modifications. Sequence analysis of the *erg27* gene showed several mutations. The HydR3$^+$ alleles differ by three amino acid substitutions at position 412, where the amino acid phenylalanine is substituted either by serine, valine or isoleucine. The HydR3$^-$ isolates have half a dozen mutations scattered overall the gene (Fillinger *et al.,* 2008*)*. We have generated artificial mutants by site directed mutagenesis *via* homologous recombination in order to assess the resistance factors and enzyme characteristics conferred by the mutations. The generated strains exhibit resistance factors and reduced affinities of 3-keto reductase towards fenhexamid comparable to the natural HydR3 strains, confirming target gene mutations as resistance mechanism. We also took advantage of these isogenic strains to evaluate if HydR3 mutations could impact on fitness parameters.

An original case of resistance was found in a species relative to *B. cinerea*, named *Botrytis pseudocinerea*. Strains belonging to *B. pseudocinerea* were originally named HydR1 because of their natural resistance to fenhexamid. They were shown to metabolise fenhexamid (Suty *et* al., 1999). In the aim to identify and characterise the gene(s) involved in fenhexamid metabolisation in *B. pseudocinerea*, synergism experiments using esterase, oxidase, glutathion S-tranferase and cytochrome P450 inhibitors were carried out to refine among all potential enzymes currently involved in drug metabolism. A strong synergism was only observed with the sterol C14 DeMethylation Inhibitors (DMIs) prochloraz and tebuconazole, inhibiting the DMI target Cyp51 protein. The synergy between fenhexamid and DMIs on *B. cinerea* suggests that a protein similar to Cyp51 could be responsible for fenhexamid metabolisation. Gene expression measurements were conducted on 60 *B. cinerea* genes similar to *cyp51*. The gene with the highest similarity, called *cyp67*, revealed to be induced by fenhexamid treatment in a *B. pseudocinerea* strain. We constructed *cyp67* knockout mutants in *B. pseudocinerea.* They exhibit an increase of fenhexamid sensitivity compared to the parental strain showing that *cyp67* encoding a cytochrome P450 is responsible, at least partially for the *B. pseudocinerea*'s natural resistance to fenhexamid. Differences between Cyp67 protein composition or its regulation in *B. cinerea* and *B. pseudocinerea* may account for their respective fenhexamid susceptibilities.

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O3.2 Effects of climate and mixed bunch rot infections on *Botrytis cinerea* (grey mould) of grapes: Implications for global climate change and disease management

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Bunch rot of grapes is commonly associated with *Botrytis cinerea*, however circumstantial evidence gathered over several years from grape growers indicated that bunch rot problems were due to other fungal pathogens. Bunch rot incidence and severity were quantified from a number of vineyards in the Hunter Valley (200 km north of Sydney, characterised as sub-tropical with warm and humid conditions during berry maturity) over three growing seasons from 2006 to 2009. Data were examined with respect to climate records for the November to February period, corresponding to the phenological stages of berry set to maturity for the southern hemisphere. The long term average weather conditions in the Hunter Valley for these months includes 41.2 days where the mean temperature exceeds 30°C and rainfall of 316 mm. From the vineyards surveyed, *B. cinerea* was absent in the 2006/7 season. This season was hotter (62 days >30°C) and drier (176 mm rain) than the long term average and the predominant bunch rotting organism recorded was *Greeneria uvicola,* responsible for bitter rot of grapes. This contrasted with the 2007/8 season which was cooler (19 days >30°C) and wetter (579.2 mm rain) than the long term average. In this season *B. cinerea* was recorded at the six sites examined and was the predominant bunch rot organism. The climate of the 2008/9 growing season was closer to the long term average with 374 mm of rain and 53 days where the temperature exceeded 30°C. In this season both *B. cinerea* and *G. uvicola* were recorded from the vineyards surveyed. Ripe rot (*Colletotrichum acutatum*), a bunch rot frequently associated with bitter rot, was only rarely isolated from the vineyards investigated.

In order to further investigate the susceptibility of grape berries to *B. cinerea* and other bunch rot pathogens, a series of inoculation experiments were conducted with detached berries (cv Cabernet Sauvignon) using *B. cinerea*, *G. uvicola* and *C. acutatum* under controlled environmental conditions at either 20 or 27°C and assessing berry infection 5 days after inoculation. More berries were infected by *B. cinerea* at 20ºC (92%) than at 27ºC (65%) while *G. uvicola* infection was favoured at 27ºC. There was little difference in the infection of grape berries by *C. acutatum* at either temperature. Further experiments involved co-inoculating berries with mixtures of bunch rot pathogens. The colonisation of grape berries by *B. cinerea* was not affected by co-inoculation with either of the two other bunch rot pathogens at 20ºC but at 27ºC was reduced by 56% in the presence of *C. acutatum* and by 30% in the presence of *G. uvicola*. Conversely, infection by *C. acutatum* and *G. uvicola* was reduced by co-inoculation with *B. cinerea* at 20ºC but not at 27ºC. *G. uvicola* failed to colonise any berries at 20ºC when co-inoculated with *B. cinerea*.

While our study has been limited to three bunch rot pathogens, it can be concluded that disease incidence and type is influenced by climatic conditions. During years with warmer temperatures and less than average rainfall, bunch rots such as bitter rot are likely to predominate in the Hunter Valley while cooler and wetter years are likely to lead to a higher occurrence of grey mould. Our observations have implications for disease management and climate change scenarios, particularly those that predict altered rainfall patterns and solar radiation in grape growing regions of the world.

O3.3 Development of resistance to pyrrolnitrin and associated fitness costs in the fungal plant pathogen *Botrytis cinerea*

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Gray mould, caused by *Botrytis cinerea,* is a severe disease on a wide range of crops. Disease control generally relies on chemicals, although biological control strategies have been intensively studied over the last decades. This pathogen can withstand a wide variety of fungitoxic compounds including fungicides and natural molecules. This capacity to adapt to different stress might, potentially, compromise the durability of biological control methods. Knowledge on the potential development of resistance to biological control agents can help to devise and improve resistance management strategies. In this work, efforts have been focused on the antibiotic pyrrolnitrin produced by various bacteria described as potential biological control agents against *B. cinerea.* The objective of the study was to estimate the risk of loss of efficacy of pyrrolnitrin-producing biological control agent due to selection pressure exerted by pyrrolnitrin.

To evaluate a possible decrease in sensitivity to pyrrolnitrin, ten successive generations of 5 isolates of *B. cinerea* were produced *in vitro* in the presence of a sub-lethal dose of the antibiotic (10µg L^{-1}). For one isolate, a significant reduction in the sensitivity to pyrrolnitrin at the 5th generation was observed with a resistance factor (RF) of ca. 11. The production of 10 additional generations for 4 of these isolates, with increasing doses of pyrrolnitrin (from 100 to 4000 µg L^{-1}), resulted in the development of variants of *B. cinerea* with high levels of resistance to the antibiotic (RF > 1000) and a reduced sensitivity *in vitro* to the pyrrolnitrin-producing bacterium *Pseudomonas chlororaphis* ChPhzS24. Comparison of the pyrrolnitrin-resistant mutants and their sensitive parent isolates for mycelial growth, sporulation and aggressiveness on plant tissues revealed that the high level of resistance to pyrrolnitrin has resulted in a high fitness cost. Additional cytohistological investigations revealed that while the sensitive isolate spread throughout the petiole and rapidly invaded the stem *via* the abscission zone, the pyrrolnitrin-resistant mutant failed to extend beyond petiole to invade the stem. Moreover, the pyrrolnitrin-resistant mutant formed abnormal mycelium and chlamydospore-like cells. This study provides evidence that a fungal plant pathogen is able to gradually build-up resistance to an antibiotic produced by a biocontrol agent and that this resistance is accompanied by a dramatic loss of fitness.

O3.4 Chitosan treatment for the control of postharvest gray mold of table grapes

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Gray mold, caused by *Botrytis cinerea* Pers., is the main postharvest decay of table grapes. The disease affects the crop in the field and even more so during its long-distance transport and storage at low temperatures. Postharvest conditions allow the pathogen to develop as it is able to grow at low temperatures, and it benefits from the high moisture that is needed to avoid desiccation of the grape berries and their stalks. Usually, cold storage at 0 °C is followed by a 2-4 day period at room temperatures (around 20 °C) when the grape bunches are exposed while on sale, which is known as the shelf life. Under commercial conditions, table grapes are sprayed in the field with fungicides to control gray mold, and after harvest they are stored with sulfur dioxide releasing pads. However, these applications are not allowed in organic agriculture, so alternatives are needed for the control of postharvest gray mold. Among the available alternatives, there is the possibility of applying resistance inducers, one of which is chitosan, a natural animal-derived biopolymer, obtained by partial deacetylation of chitin from crab shells. Treatment with chitosan considerably reduced postharvest gray mold of table grapes, both with preharvest spraying and postharvest dipping. The application of chitosan can induce resistance responses in the host tissues. However, this biopolymer also showed antifungal activity, as it can reduce the growth of several postharvest pathogens, included *B. cinerea*. Chitosan formed a film on the surface of the treated berries, with a thickness that varied according to the acid solution used to dissolve the powder. Chito Plant, a commercial chitosan formulation that is easy to dissolve in water, was equally effective in the control of gray mold of table grapes as chitosan acetate, which proved to be the best combination when the biopolymer was dissolved in different acids.

I3.2 Resistance to fungicides: new weapons in a never-ending battle

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The principal strategy to prevent grey mould in vineyards and on other economically important crops is fungicide treatment. Over the past 50 years, chemical companies have developed multiple compounds active against *Botrytis cinerea*, related species and diverse plant pathogenic ascomycetes. These compounds representing *ca.* 10 chemical classes, target specific cellular functions (e.g. respiration, sterol biosynthesis etc.) and most of them have to cope with problems of resistance development. *B. cinerea* is considered as a high-risk pathogen due to its ability to rapidly acquire resistance to nearly all fungicides. Rotations and/or combinations of treatments with different modes of action are supposed to minimize the risk of resistance. Such strategies allow keeping treatment efficacies for most molecules over long time periods, delaying the development of specific resistances. With the observation of increasing frequencies of multiple-drug-resistant (MDR) strains – favoured by applications of different modes of action in the same season – other problems have to be faced. What is the cost of specific and multiple resistance respectively? What are the chances for resistant strains to survive and to propagate in the vineyard? How can they be controlled in the era where society and regulatory agengies claim the reduction of pesticides?

In order to precisely address these questions, it is essential to identify the molecular bases of fungicide resistance phenomena. Once the mutation responsible for resistance is identified, the evaluation of fitness cost can be conducted on isogenic strains constructed through reverse genetics. However the bottleneck of all these studies is the identification of the resistance allele. Not always sequencing of the fungicide-target encoding gene is sufficient to identify the responsible mutation, but long-lasting physiological and genetic studies are necessary. The availability of the *B. cinerea* genome sequence combined to high-throughput technologies considerably “widened” this bottleneck over the past few years. Based on this molecular knowledge field populations can be easily analyzed with respect to resistance allele frequencies and dispersion, allowing to refine treatment recommendations. In the near future, it should be possible to identify emerging resistances “in the act” by whole genome sequencing therefore accelerating the whole research process.

O3.4 Implementation of a low-input fungicide strategy in grapes in relation to the pattern of grey mold development

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A low-input fungicide strategy was developed in order to control the grey mould pathogen, *Botrytis cinerea*, on grapevine. Field trials were carried out from 2004 to 2009 on the cv. Merlot noir at two vineyards near Bordeaux (Médoc and Entre-deux-Mers). Using a statistical modelling approach, the strategy was based on the evaluation and characterization of the risk of an epidemic from the phenological stage "veraison" onwards. Under the assumption of standard climatic variables, our model was able to predict both the severity and timing associated with disease development, but more importantly the periods when there was a low risk of symptom expression on grape clusters. The results, in terms of disease control, using one fungicide (active ingredient: fenhexamid) allowed us to test whether a fungicide spray was required or not between the veraison stage and harvest. During this period, we were able to abstain from spraying against the pathogen in *ca*. half of the cases studied out of a total of 10 "site x year" situations. The absence of treatment corresponded to low disease expression mainly due to dry climatic conditions during summer, particularly in the years 2005 and 2009. Furthermore, when the spraying was necessary, postponing the application was demonstrated generally as relevant and a time-lag between 15 and 28 days (post-veraison) was effective depending on the site and the year. The optimized strategy demonstrates the outcome of both limiting fungicide use and also of relaxing the current rules which are based on precise phenological stages.

O3.5 DNA-based network of airborne inoculum tracking: an additional tool for managing *Botrytis* leaf blight of onion

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In eastern Canada and north-east United States *Botrytis squamosa* Walker is a damaging pathogen of onion and Botrytis leaf blight is the key disease for scheduling fungicide sprays. The biology of *B. squamosa* and epidemiology of Botrytis leaf blight have been well documented (1,3). Critical disease levels and forecasting systems were develop to time initiation and interval between fungicide sprays. Analysis of these disease risk indicators demonstrated that monitoring-based predictors were more reliable than weather-based predictors and among them airborne inoculum monitoring was the most reliable risk indicator (2). This is largely explained by the polycyclic nature of the disease with conidia as the main source of both initial and secondary inoculum.

It is generally admitted that it is difficult to use airborne inoculum monitoring as risk indicator because of problems related to quantification of fungal spores. Traditionally, spores identification and quantification relied on light microscopy counts. Microscopic spore counts are time-consuming and need substantial expertise to accurately identify the fungal spores. To circumvent this problem, a TaqMan real-time quantitative PCR (qPCR) assay was developed (4). The test is highly specific and the detection limit is 2 conidia per air sample (sampler rod). Based on Receiver Operating Characteristic (ROC) curve analysis, the use of the qPCR essay to quantify the spores significantly improved management the accuracy of management decisions based on airborne inoculum.

A network of spore samplers was implemented in the onion production area of Southwest of Montreal (Canada). Onion growers are informed of need for fungicide spray based on disease and airborne spore monitored at the field and the farm level, respectively and on sporulation index calculated from regional weather data. Data on disease level, airborne inoculum and sporulation index are sent to growers weekly, three times per week, and every day, respectively. Considering that a network of spore samplers is now established, the next step will be to enhance the value of air-sampled DNA by providing information on other onion pathogens and on the presence of fungicide-resistance genes.

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O3.6 Effectiveness of fungicides and an essential-oil-based product in the control of grey mould in raspberry in Serbia

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Control of *Botrytis cinerea* in raspberry in Serbia is mainly based on the use of chemicals. The most commonly used fungicides in raspberry farms in Serbia are: iprodione, vinclozolin, and pyrimethanil. However, occurence of resistance to pesticides with specific mode of action, as well as residues of pesticides in food imply the necessity to find a suitable alternative. Application of substances of natural origin as crop protectants could be a convenient solution, safe for both human health and the environment. Antimicrobial properties of certain essential oils have already been known for a long time, but their efficacy as crop protectants has not been well documented.

In order to compare effectiveness of an essential oil based product and some fungicides in *Botrytis cinerea* control, field experiments were conducted in two commercial raspberry fields. The experiments consisted of four fungicide and two oil product treatments (0.5% and 1% emulsion) and untreated control, in randomized block design with four replicates per treatment. Fungicides including: fenhxamid, vinclozolin, benomyl, and pyrimethanil were applied three times, until the beginning of ripening, while the tea tree oil product (Timorex 66 EC) was applied four times - until the beginning of harvest. The effect of the tested products was assessed 20 days after the last fungicide treatment according to the intensity of fruit infection. Afterwards, the pathogen was isolated from infected fruits and identified based on colonial and conidial morphology and by PCR amplification of an expected 0.7 kbp DNA fragment using *B. cinerea*-specific primer pair $C_{729+/729-}$. Sensitivity of 10 randomly chosen isolates to all the fungicides and tea tree oil was determined in radial growth experiment on PDA medium supplemented with a range of concentrations of the relevant product.

At both localities, the highest efficacy was achieved by pyrimethanil (97.4% and 98.2%) and fenhexamid (93.6% and 97.6%), while the efficacy of tea tree oil, applied at both concentrations, was less than satisfactory (13.3% – 55.9%). Sensitivity of *B. cinerea,* based on EC-50 values, was as follows: for vinclozolin – 0.14 mg/l to 0.20 mg/l, for benomyl – 0.16 mg/l to 0.46 mg/l, for pyrimethanil – 0.22 mg/l to 3.81 mg/l, for fenhexamid – 0.06 mg/l to 0.19 mg/l, and 383.3 mg/l to 1500.6 mg/l for tea tree oil product.

O3.7 Monitoring of fungicide resistance in *Botryotinia fuckeliana* (*Botrytis cinerea*) on grapevine and strawberry in South Italy

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Botryotinia fuckeliana (de Bary) Whetzel (an. *Botrytis cinerea* Pers.), inducing grey mould on numerous economically-important crops, is generally recognized as a pathogen at high risk of acquired resistance to fungicides. In South Italy, an intensive use of fungicides is required for instance on strawberry grown under greenhouse-like conditions and in table-grape vineyards covered with plastic films to delay the harvesting time.

The response of *B. fuckeliana* field-populations to the most commonly used fungicides to control grey mould (anilinopyrimidines, phenylpyrroles, hydroxyanilides and carboxamides) and to the broad-spectrum QoIs (used against downy and powdery mildews) was monitored during 2008-2009 in 18 vineyards and 8 strawberry commercial fields in Southern Italy. Conidia were sampled at the harvest time from naturally infected fruits and plated on appropriate media amended with single fungicides at concentration (1 mg l^{-1} pyrimethanil, 0.3 mg l^{-1} fludioxonil, 0.4 and 4 mg l^{-1} fenhexamid, 1 mg l^{-1} boscalid, 1 mg l^{-1} trifloxystrobin) discriminating between wild-type sensitive and resistant phenotypes.

Pyrimethanil-resistant isolates were detected at high frequency (12-93%) especially in fields intensively or exclusively (3-7 sprays/season) treated with anilinopyrimidines (APs). Double resistance to APs and phenylpyrroles were found on strawberry at a frequency (0-7%) significantly lower than on grapevine (up to 50%) where the cyprodinil+fludioxonil mixture is more commonly used.

Isolates showing reduced sensitivity to fenhexamid was recovered in 3 vineyards and 5 strawberry fields at a very low frequency, generally ranging from $2 \cdot 10^{-4}$ to $8 \cdot 10^{-3}$, with the only exception of a strawberry field in which it was as high as 2.7%.

Boscalid-resistant conidia were detected in all the monitored strawberry fields, sometimes at a high frequency (up to 73%); resistant mutants were detected in less than 30% of the vineyards at frequencies ranging from $5 \cdot 10^{-5}$ to $6 \cdot 10^{-2}$. The highest frequency of boscalid-resistant conidia were recorded when the fungicide was applied 1-3 times per season, alone or in mixture with the QoIs kresoxim-methyl or pyraclostrobin.

QoI-resistant mutants were recovered in 11 vineyards and in all the strawberry fields at a frequency ranging from $1.5 \cdot 10^{-4}$ to $4.7 \cdot 10^{-2}$.

Multiple resistant phenotypes to different fungicides (2 to 6, including benzimidazoles and dicarboximides) in different combinations were detected. The implementation of effective anti-resistance measures through restriction of the number of seasonal sprays is strongly recommended in IPM strategies against gray mould.

P3.1 Exploring the resistance mechanisms towards respiratory inhibitors in field strains of *Botrytis cinerea*, the causal agent of grey mold

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Respiratory inhibitors are among the fungicides most widely used for fungal disease control on crops. Most are QoIs (*e.g.* strobilurins) and SDHIs (*e.g.* carboxamides), inhibiting respectively the cytochrome *b* of mitochondrial complex III and the succinate dehydrogenase of mitochondrial complex II. A few years after the approval of respiration inhibitors for use on grapevine, field mutants of *Botrytis cinerea,* resistant to one or both of these modes of action were isolated in France and Germany. However, little was known about the mechanisms underlying this resistance in field populations of this fungus. Such knowledge would facilitate resistance risk assessment. The aim of this study was to investigate the mechanisms of resistance occurring in *B. cinerea* populations.

Multidrug resistance (MDR), linked with the overexpression of membrane transporters, was identified in strains with low to moderate resistance to several respiratory inhibitors. Highly specific resistance to QoIs was correlated with a single mutation of the *cytb* target gene leading to the G143A change. Length polymorphism of this gene may also have occurred due to an evolutionary process controlling selection for resistance. Resistance to SDHIs was characterized by six phenotypes, with various patterns of resistance and cross-resistance to carboxamides. Several amino-acids changes, some of them specific to *B. cinerea,* were identified within SdhB (Y272Y/R/L, P225T/L and N230I) and SdhD (H132R), respectively constituting the iron-sulfur protein and an anchor protein of the succinate dehydrogenase complex. Another, yet uncharacterized mechanism of resistance was also recorded. This diversity of resistance mechanisms makes resistance management difficult and must be taken into account when developing strategies of *Botrytis* control.

P3.2 Phenotypic characterization of *Botrytis cinerea* isolates with different levels of sensitivity to fenhexamid

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B. cinerea isolates with moderate and high resistance to fenhexamid have been recently reported in table grape in the Chilean Central Valley. In order to phenotypically characterize these isolates, the behaviour of isolates with different sensitivity levels to the fungicide (n=12) was evaluated: 1) sensitive to fenhexamid ($EC_{50} \leq 0{,}084$ μg ·mL^{-1}), 2) intermediate resistance (0,084 μg ·mL^{-1} > EC_{50} < 0,2 μg ·mL^{-1}); 3) resistant (0,3 μg ·mL^{-1} > EC_{50} < 3,9 μg ·mL^{-1}); and 4) highly resistant ($EC_{50} \geq$ 3,9 μg ·mL^{-1}). The parameters evaluated were the following: a) myceliar or sclerotial aspect (Malta Agar, 20º C); b) sporulation level; c) sclerotia forming capacity; d) myceliar growth rate (MGR) at 15, 20 and 25º C; e) *in vitro* levels of sensitivity to iprodione, fludioxonil, tebuconazole and cyprodinil, based on myceliar growth or conidial germination depending on the fungicide, and f) evaluation of the virulence capacity on inoculated berries with and without injury at 0 and at 20º C.

All the isolates showed a lower MGR at 15º C; at 20 and 25º C the behaviour of the isolates was similar, obtaining in both temperatures the highest MGR. The sensitive isolates and the intermediate resistance isolates were classified as myceliar while the resistant and highly resistant isolates were classified as sclerotial.

At 20º C the sensitive isolates were less virulent. Although the virulence was less at 0º C, the isolates with a certain level of resistance were more virulent than the sensitive ones. The isolates with different levels of sensitivity to Fenhexamid were resistant to iprodione; the highly resistant to fenhexamid were also resistant to cyprodinil. All the isolates behaved as sensitive to tebuconazole and fludioxonil.

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P3.3 Fitness of *Botrytis cinerea* fenhexamid-resistant isolates in Thompson Seedless cv.: Field study

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The field adaptability of fenhexamid sensitive (*Bc*fS) and resistant (*Bc*fR) *Botrytis cinerea* isolates was evaluated in a Thompson Seedless cv. Table grape vineyard located in the Chilean Central Valley. With this purpose, 36 clusters with (*Bc*fR) and 36 with (*Bc*fS) strains during flowering (F) and preharvest (PC) – two periods that are considered critical for table grape infections in Chile – were inoculated. One half of them was inoculated and later (24 h) treated with fenhexamid (T1) and the other half was first treated and later inoculated (T2). After or before the treatments, the clusters were subjected to cyprodinil & fludioxonil applications in veraison and preharvest (T1) and in flowering and veraison (T2). The control treatments were an equal amount of clusters inoculated with both strains but not treated with fenhexamid. Later, the clusters were harvested and maintained at 0º C for 45 days; 180 *B. cinerea* isolates were recovered from these clusters. Parameters evaluated: i) fenhexamid, cyprodinil and fludioxonil *in Vitro* sensitivity levels (n=180) (EC_{50} fungicide values); and in *Bc*fR isolates, determination of resistant fenhexamid phenotype through the *erg27* gene sequencing; ii) genotypic correspondence through duplex PCR and specific primers (Boty and Flipper transposons) (n=180); and iii) genetic affiliation (RAPDs) on a set of 45 *B. cinerea* isolates selected from the total recovered.

From the total isolates recovered from the clusters inoculated in F and P, only 3% (n=6) showed resistance to fenhexamid (EC_{50}>10µg mL^{-1}), which corresponds to phenotype $HydR3^{+}$. 30 and 48.9% of the total isolates analyzed showed a slight resistance to cyprodinil and high resistance to fludioxonil (EC_{50}=0,0478µg ·mL^{-1}, EC_{50}=2,21 µg ·mL^{-1}, respectively). All the isolates from clusters inoculated with *Bc*fR, were sensitive to fludioxonil and cyprodinil, while the fludioxonil resistant isolates were sensitive to fenhexamid (BcfS). These results become the first report on negative crossed resistance between hydroxyanilides and phenylpyrroles in *field conditions*, which suggests that the use of the latter ones could become an efficient preventative measure to control field fenhexamid-resistant strains.

In postharvest 96% of the isolates recovered corresponded to *transposa* genotype (*Boty*$^{+}$/*Flipper*$^{+}$) (n=172), and 4% to *vacuma* (*Boty*$^{-}$/*Flipper*$^{-}$) (n=8). The 8 *vacuma* isolates belonged to Botrytis Group II (*Botrytis cinerea sensu stricto*) (PCR-RFLP). In the genetic affiliation dendrograms, a larger grouping between the *Bc*fR isolates and the inoculated fenhexamid-resistant isolate (Jaccard similarity coefficient=0.75) was detected. This grouping was smaller in the case of the recovered sensitive isolates compared to the inoculated ones (cs=0.655). In addition, in the recovered *B. cinerea* isolates, a significant grouping among the *Bc*fR and the *Bc*fS (*F*st= 0.3487) was detected and a moderately significant grouping was detected among the *vacuma* and *transposa* isolates (*F*st= 0.1483). These results show that the *Bc*fR isolates are more homogeneous compared to the sensitive ones and although the adaptability of the resistant isolates is low due to the small number of recovered isolates, it would be higher during flowering than in preharvest. This finding confirm the importance of the current use of fenhexamid during preharvest.

P3.4 Effect of pyrimethanil fungicide applied through thermofogging in the control of *Botrytis cinerea* in apple cv. Fuji

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Botrytis cinerea (teleomorph: *Botryotinia fuckeliana*) causes significant fruit losses in cold stored apples and pears. Postharvest fungicide treatments are needed and they are applied as a drenching, immersion or spray. The possibilities of chemical control have been narrowed due to factors such as the restrictions of some fungicides in certain markets and the presence of resistant races to specific fungicides. Pyrimethanil is a fungicide that impedes the secretion of enzymes by the fungus and it is used for *B. cinerea* control in apples. Thermofogging allows disseminating the fungicides in the atmosphere inside of a cold chamber room in form of stable fog, so active residues are kept on the apple surface restricting the fungal infection. Therefore, the objectives of this study were to evaluate the effectiveness of thermofogging system for fungicide treatments in post-harvest and to compare the effectiveness of pyrimethanil applied through thermofogging or drenching in the control of *B. cinerea* in Fuji apples. Consequently, 3 groups of 100 apples were inoculated or not with *B. cinerea* and then subjected to pyrimethanil or thiabendazol as a drenching and pyrimethanil as a thermofogging. The treated fruit, inoculated and noninoculated, was stored at 0°C for 3 months and after grey rot incidence registered. The level of grey rot in inoculated and noninoculated control treatments was 85.2 and 12.4%, respectively. The inoculated treatments with pyrimethanil, by drenching or thermofogging, showed a 13.7 and 56.4%, respectively, being significantly lower than the inoculated control. This fungicide applied by drenching showed a significantly better performance than thermofogging. For noninoculated fruit, there was not any significant difference between them (1 and 4,6%, respectively) but both were significantly lower than the control. Thiabendazol (35,5% for inoculated apples) was significantly less effective than pyrimethanil in *B. cinerea* control, but showed less rot than the control. Therefore, pyrimethanil applied by drenching or thermofogging to commercial harvested Fuji apples allows a significant protection from *B. cinerea* infections, so the level of grey rot is significantly reduced after 3 months of cold storage.

P3.5 Stem-end rot of kiwifruit, caused by *Botrytis cinerea* during cold storage and chemical control strategies

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Chile is the third world-wide producer of kiwifruit, with around 15,000 hectares, concentrated between the Libertador Bernardo O'Higgins and Maule Regions at the Central Valley of the country. Even though Chile has managed to situate itself strongly in the international markets, it faces a tough competition of countries like New Zealand, also located in the South hemisphere. One of the factors that mainly affect the quality and condition of the fruits during cold storage, is the stem-end rot disease, caused by *Botrytis cinerea* (teleomorph: *Botryotinia fuckeliana*). Generally their symptoms appear after three months of cold storage at 0ºC in Chile and during sea shipment to the different importing markets. This rot inflicts direct loss by fruit discharge, without any commercial value, and indirect loss by the necessity to adopt control measures. *B. cinerea* colonizes blossom debris, such as sepals, remaining latent adhered to these in the peduncle cavity until harvest. The fungus does not die at temperature of 0º C and does not produce conidia in the dark conditions of the cold room. Nevertheless, the fungus forms white mycelium that contacts nearby sound fruits, forming a "nest" of rotting kiwifruits. Depending on the level of contamination and the duration of the kiwifruit storage, *B. cinerea* can rot a significant number of fruits by stored bin. Therefore, a different control strategy was developed, using kiwifruits just harvested, which were treated with iprodione or fenhexamide fungicides, leaving a control without chemical treatment. Later, half of the kiwifruits treated or not with fungicide were inoculated with a conidia suspension (50,000 conidia of *B. cinerea* per ml) at the stem-ends. Inoculated or non inoculated kiwifruit were stored at 0ºC for 5 months. Analyzed data showed that both fungicide treatments significantly decreased the number of kiwifruits affected by stem-end rot compared with the control, after 5 month of cold storage.

P3.6 Fitness measurements of HydR3+ fenhexamid resistant strains in *Botrytis cinerea*

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Botrytis cinerea is a devastating pathogen. In France, to control this grey mould on vinegrapes, one of the widely used fungicide is the Sterol Biosynthesis Inhibitors (SBI) fenhexamid, a hydroxyanilide. Biochemical analyses have identified the target enzyme, the 3-keto reductase encoded by the *erg27* gene, involved in the C4 demethylation processes of sterol biosynthesis. Since its introduction, annual *Botrytis cinerea* monitoring deployed on french vineyards allowed to detect several types of strains resistant to this hydroxyanilide, called respectively HydR3$^+$ and HydR3$^-$. These strains exhibit various resistance levels according to their development stages (i.e germ tube elongation or mycelial growth). HydR3$^+$ strains have a higher IC_{50} value (>10ppm) than HydR3$^-$ (<0.5ppm) (wild type IC_{50}<0.03) for both development stages. Sequence analysis of the *erg27* gene showed several mutations, the HydR3$^+$ alleles correspond to a « complex » SNP, where the amino acid phenylalanine (TTC) at the position 412 was substituted by serine (TCC), valine (GTC) or isoleucine (ATC). Since HydR3 strain occurrence in 2005, HydR3$^+$ resistance grew up slowly to reach a rate of 11 % in French vineyards in 2009, but did not attempt to fenhexamid efficiency. In a same area, huge fluctuations of HydR3$^+$ frequencies can be observed. This observation in addition to moderate growth parameters of HydR3$^+$ strains relative to the sensitive ones suggests a putative impact of HydR3$^+$ mutations on *Botrytis'* fitness. We have generated artificial HydR3$^+$ mutants by site-directed mutagenesis, *via* homologous recombination. These isogenic strains were used to quantify precisely under controlled conditions the impact of the allelic mutations on fitness. Classical parameters (Sporulation capacity, radial growth, sclerotic production, frozen resistance, competitivity, agressivity…) were quantified at three different temperatures. Large differences were observed on some characters between mutant and parental strains, in particular on sclerotia production and mycelial growth, underlining a possible effect of F412 mutations on *Botrytis* survival.

P3.7 Functional characterisation of the *Botrytis cinerea erg28* gene involved in the C4 demethylation process of the ergosterol biosynthesis pathway: role of an anchorage protein

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The physicochemical properties of sterols make them indispensable for cells in all kingdoms. Cholesterol in mammals, campesterol, sitosterol, and stigmasterol in plants; in fungi the major sterol is ergosterol. The biosynthesis pathway of ergosterol in *Botrytis cinerea* regroups 15 steps from the squalen. Some of them have been extensively studied among fungi like the C14 demethylation or the Δ8-7 isomerisation step, target of DMI (C14 DeMethylation Inhibitor) and amine fungicides respectively. Other crucial steps are the C4 demethylations, these reactions sequentially involve 3 enzymes, an oxidase Erg25p, a decarboxylase Erg26p, a 3-keto reductase Erg27p and an anchorage protein Erg28 as described in *Saccharomyces cerevisiae*. *S. cerevisae ΔErg28* mutants exhibit drastic phenotypes. Unsaponifiable lipid content analysis showed a severe reduction of ergosterol levels, accumulation of both carboxylic and 3-keto sterol intermediates, as observed in the case of *S. cerevisiae Δerg26* & *Δerg27* mutants respectively. The presence of numerous transmembrane domains and protein interaction analysis carried out in yeast have shown that this protein interacts strongly with Erg26p and Erg27p, but also with more than the half of the other Erg enzymes. Erg28p is involved in the anchoring of specific enzyme to facilitate interactions and enzymatic reactions, in order to maximize efficiency and minimize the accumulation of toxic intermediates.

Biochemical and molecular studies made in our lab allowed us to identify the *B. cinerea* 3-keto reductase Erg27p as the target of the fenhexamid fungicide (Debieu et al., 2001). *Erg28* gene was identified in the *B. cinerea* genome. Is Erg28p function conserved in *B. cinerea*? Do the same sterol intermediates accumulated in *B. cinerea ΔErg28* mutants*?* Is Erg28p a reasonable target for a new putative botryticide or a wide fungicide? In order to give answers to theses questions improving knowledge about the C4 demethylation in the *B. cinerea* phytopathogenic fungus, and especially about the role of Erg28p towards Erg27p, *B. cinerea ΔErg28* mutants were constructed. They showed interesting phenotypes: growth, agressivity and conidia reduction, sterol content is largely disturbed, but differs from those observed in *S. cerevisae ΔErg28* mutants. Massive consequences on *erg* gene expression were analysed and surprisingly susceptibility to different SBIs (Sterol Biosynthesis Inhibitors) does not strictly correlate. The role of this protein in the C4 demethylation and in whole the ergosterol biosynthesis pathway of *B. cinerea* will be discussed.

Debieu, D., Bach, J., Hugon, M., Malosse, C. and Leroux, P. 2001. The hydroxyanilide fenhexamid, a new sterol biosynthesis inhibitor fungicide efficient against the plant pathogenic fungus *Botryotinia fuckeliana* (*Botrytis cinerea*). *Pest Management Science* 57(11): 1060-1067.

P3.8 Development of isocaryolane derivatives as antifungal agent leads. Biotransformation of some isocaryolane and related compounds by the phytopathogenic fungus *Botrytis cinerea*

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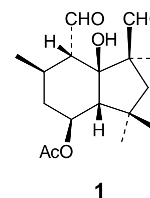
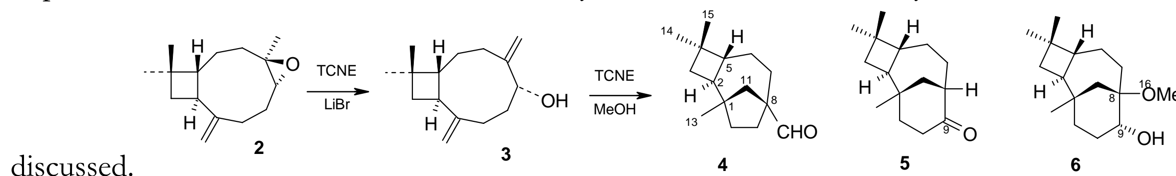
Plant pathogens such as *B. cinerea* cause serious economic losses.[1] There is a considerable need to develop fungicides with novel modes of action to combat resistant strains of organisms[2] and with specific rather than general antifungal activity.[3] These novel agents should ensure compatibility with the use of biological crop protection agents and not cause damages to the crop, farmer, consumer and the environment.[4]

Part of the interaction of *B. cinerea* with a host plant involves the action of the low molecular weight phytotoxin, botrydial (**1**), on the plant. [5] Inhibition of the production of this phytotoxin can lead to a decrease in the symptoms of the disease. Furthermore, there is evidence that the production botrydial (1) during the idiophase of fungal growth has a limiting effect on fungal growth.[6] Therefore, a disturbance on the delicate balance of secondary metabolites produced by the fungus can lead to the control of the phytopathogen. A method of fungal control which has been explored by our research group is to use a non-phytotoxic analogue of botrydial (**1**) or analogues to biosynthetic intermediates *en route* to **1**. Preparation of sesquiterpene derivatives with several carbon skeletons have been aimed at this end.[5]



Compounds with the isocaryolane skeleton (**5,6**) have been obtained by rearrangement of (-)-*trans*-caryophyllene and caryophyllene oxide (**2**) with several electrophilic reagents, through the intervention of a caryophylladiene intermediate (**3**).[7] Some derivatives have been shown to exert inhibition on the growth of *B. cinerea*. In this communication we evaluate the biotransformation by the fungus *B. cinerea* of suitable isocaryolane derivatives like compound **6**. The oxidation patterns on the carbon skeletons, activity of the resulting compounds against the fungus and impact on the balance of the secondary metabolites with botryane skeleton will be discussed.



[†] J. A. acknowledges a fellowship to CAPES.

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P3.9 Designing hybrid molecules as potential new fungicides against *Botrytis cinerea* based on the reported genome data

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Advances in genomic studies, in combination with increased access to DNA sequencing, are providing a wealth of information about how natural products are assembled, mechanisms by which natural product gene clusters can be manipulated to yield new product diversity, and the genetic potential of individual organisms.

Botrytis cinerea secondary metabolism genes by looking for conserved domains of the relevant « key enzymes » *i.e.* Terpene Synthases, Polyketide Synthases (PKS), Non-Ribosomal Peptide Synthases (NRPS) and Dimethylallyl Tryptophan Synthetases (DMATS) have been investigated. Genomic data revealed that *B. cinerea* has 43 key enzymes some of them are specific of this phytopathogen. The sequencing of the genomes of the B05-10 and T4 strains of *B. cinerea* at the Broad Institute and the Genoscope, respectively (Fillinger et al. 2007), revealed an abundance of novel biosynthetic gene clusters, the majority of which were unexpected on the basis of previous fermentation analyses of this and closely related species and strains.

In addition to other relevant results, two secondary metabolism genes, *bcbot1* and *bcbot2*, involved in botrydial biosynthesis, have been characterized and a sesquiterpene ciclase and P450 monooxigenase identified (Siewers, et al. 2005; Pinedo et al. 2008).

Pathogenicity tests confirmed that the toxin botrydial acts as a strain-dependant virulence factor. Indeed the sesquiterpene cyclase (*STC1)* inactivation in T4 led to a defect in colonization while the same mutation in the B05-10 strain had no effect on virulence. The lack of botrydial synthesis in *bcbot1* and *bcbot2* mutants seems to be compensated by the overproduction of the polyketide type toxin botcinic acid (a toxin not produced in T4 strain), indicating that botrydial and botcinic acid may have a redundant function in virulence.

The known of the secondary metabolism genes and enzymes involved in the toxins biosynthesis produced by *Botrytis* have revealed to be an interesting strategy to design new selective and efficient fungicides to control these pathogens.

Two new biological targets have been described, which can permit us undertake the design of new fungicides by synthesis of hybrid molecules that carry two entities: one as an inhibitor of mevalonate route (botrydial toxin) and the other one inhibitor of polyketide route (botcinin toxins). Here, we present preliminary results in the design of hybrid molecules as a new fungicides generation based in the inhibition of both molecular targets.

P3.10 Importance of treatments during different phenological stages to control *Botrytis* bunch rot in Lleida vineyards

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Bunch rot, caused by *Botrytis cinerea* is an important disease of grapevine, responsible for significant damages in vineyards worldwide. Disease control depends on pathogen's biology since this latter is capable to adapt its growth to different environmental conditions and, in several cases, it colonises vine tissues as an opportunistic saprophyte during early season leading to latent infections, in addition to the pathogenic infection developed during berry maturation. Pathogen's development in both cycles is closely linked to the vineyard phenology and weather conditions during the season. Taking into account occurrence of fungicide resistance in *Botrytis cinerea* and the negative effects of fungicides and their residues on human health, there is an increasing interest in reducing the use of chemical fungicides, trying to obtain alternatives to chemical treatments and minimise the amount and number of applications.

This study tries to determinate the best moments during vine season to control *B. cinerea* in field conditions, identifying which stages in the phenology of the vines are more susceptible to *Botrytis* infection and lead to a higher decay levels at harvest.

Seven different treatments using the same fungicide were applied to plots of seven vines each (cv. Macabeu) replicated six times, in two different vineyards during 2009 season. Six moments during the season were selected, from early bloom to pre-harvest. Treatments had different calendars of fungicide applications so each treatment controlled *Botrytis* in a different stage of the vineyard phenology. Iprodione was the employed chemical, a generalist fungicide commonly used in Lleida area. Applications were carried out simulating the spraying methodology of local vineyard managers. At harvest time, incidence of rots and percentage of decayed berries per bunch (severity) were measured.

Incidence and severity of disease expression were significantly reduced by all the treatments in both studied vineyards. Most effective treatments were those that applied fungicide, during early and full bloom. Treatments from veraison to harvest, which are the most common in Lleida area, were the less effective to control *B. cinerea.*

P3.11 Current sensitivity to botrycides in Chile: Multidrug Resistance (MdR1)

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Chilean *Botrytis cinerea* isolates were analyzed *in vitro*, regarding their sensitivity levels to different fungicides (benzimidazoles, dicarboximides, hydroxyanilides, fenylpirrols, strobilurins, carboxamides, phenyl-piridinamines and IBEs, among others). It was considered that resistant isolates were present when the germ tube length was not affected at the following concentrations: 2.5 $\mu g \cdot mL^{-1}$ in iprodione; fenhexamid, phenotype HydR3^{-} / HydR3^{+} above 0.4 and 4 $\mu g \cdot mL^{-1}$, respectively; 1 and 0.5 $\mu g \cdot mL^{-1}$ for pyrimethanil and cyprodinil, respectively; in fludioxonil 0.2 $\mu g \cdot mL^{-1}$; above 10 $\mu g \cdot mL^{-1}$ in azoxystrobin, 0.5 $\mu g \cdot mL^{-1}$ in boscalid, 1 $\mu g \cdot mL^{-1}$ for tebuconazole and 0.08 for fenpropidine. Tolnaftate was used as a positive control for the identification of MdR isolates which are the sole to develop on this compound.

Of the total amount of isolates analyzed (n=46), more than 70% showed resistance to iprodione, 32% was resistant to fenhexamid (HydR3^{+}); more than 50% to anilinopyrimidines (cyprodinil and pyrimethanil); 15, 20 and 22% to tebuconazole, fludioxonil and azoxystrobin, respectively and none of the isolates showed resistance to boscalid. The nine isolates that were resistant to fludioxonil also were resistant to iprodione, tolnaftate and fenpropidine and were sensitive to pyrimethanil, tebuconazole and fenhexamid. This behaviour corresponds to phenotype MdR1 (multidrug resistance). This is the first report for *B. cinerea* isolates with multidrug resistance in Chile, therefore it is important to consider periodical monitoring to study its evolution and control.

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Session 4:
DISEASE MANAGEMENT 2 (Biological Control)

XV
International
Botrytis Symposium

I4.1 Everybody against *Botrytis*: Biocontrol cooperation between *Trichoderma* and plants

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Trichoderma is a fungal genus that includes species in current use as biological control agents due to their ability to antagonize other fungi. *Trichoderma* spp. produce antibiotics and cell wall degrading enzymes, but they also have the capacity to compete for space and nutrients in the rhizosphere, to stimulate defenses to biotic and abiotic stresses in plants and to promote plant growth. Genes encoding some of the antifungal enzymes have been cloned and inserted into plants, where they frequently provide a high level of resistance to plant pathogenic fungi and tolerance to adverse environments. In combination with direct effect to the pathogen structure and activity, *Trichoderma* spp. have been found to immunize the plants through induced or acquired systemic resistance and the control of the levels of plant response to pathogens. As a result, *Trichoderma* commercial formulations maintain high crop yields by buffering the effect of biotic and abiotic stresses eventually affecting the crop and/or the natural suppressiveness of the soil.

In this scenario, direct biocontrol against *Botrytis cinerea* was effective in crops such as tomato, strawberry, tobacco or beans, treated with single or combined *Trichoderma* strains. In addition, *B. cinerea* has resulted a good target for *Trichoderma* extracellular proteins and culture filtrates with biocontrol activity, as well as a perfect foliar/fruit pathogen model to explore the mechanisms of stimulation of plant immunity when *Trichoderma* colonize the root system.

Different examples of cooperation between *Trichoderma* and plants in the biocontrol of *B. cinerea* disease demonstrate that *Trichoderma* helps the plant to fight against one of its most important microbial enemies.

O4.1 The effect of curing temperature on *Botrytis allii* infection of onion bulbs

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Onion bulbs are stored in order to meet consumer demand for year-round supply. Losses of marketable onions in store can occur due to pathogen attack. In temperate climates, such as the UK, onions are not cured in the field, but are subjected to forced air ventilation in purpose-built facilities at temperatures of around 28ºC at 65-75% RH for a period of up to six weeks. The purpose of curing is to dry the thin outer layers of the bulb to form one or more complete outer skins. These outer skins act as a barrier against water loss and infection from fungal pathogens such as *Botrytis allii* (neck rot), *Aspergillus niger* (black mould) and *Fusarium oxysporum* (basal rot), and bacterial pathogens such as *Pseudomonas*.

Incidence of neck rot disease caused by *Botrytis allii* has been reduced in the UK since the introduction of forced air curing. It has been proposed that reducing curing temperature from 28 to 20ºC will result in energy savings; however, the effect of the lower temperature on subsequent disease incidence in store must be investigated.

Onions were inoculated with a spore suspension of *B. allii* or water (controls) and cured at 20 or 28ºC for six weeks, and subsequently cold-stored at 1ºC for six months. Disease incidence and severity was recorded after curing and after storage. The effect of temperature on growth of *B. allii* was investigated in detail using a custom temperature block capable of maintaining a gradient of 20ºC between the top and bottom of the block in 1ºC increments.

Minimal disease was present in non-inoculated onions cured at either temperature. In inoculated onions, curing at 28ºC delayed onset of disease until after cold storage. For inoculated onions cured at 20ºC, disease incidence remained stable during time, but disease coverage increased during cold storage. In culture, *B. allii* grew over a broad temperature range (1– 32ºC, optimum 20ºC) but sporulated within a more narrow range (12 – 31ºC, optimum 21ºC).

Taken together, these results suggest that curing onions at 28ºC can delay the onset of disease, compared with curing at 20ºC, but this treatment does not kill the pathogen. Where high disease pressure is not present, there is no difference between the two curing temperatures. Possible mechanisms for the marked difference between infection rates of onions immediately after curing at 20 and 28ºC are discussed.

O4.2 Biological control of *Botrytis cinerea* using *Trichoderma* spp.

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Several filamentous fungal species that belong to *Trichoderma* genus have been widely used as biocontrol agents against phytopathogenic fungi. The antagonism exerted by *Trichoderma* has been atributed to several complex mechanisms, including competition, antibiosis, interference with pathogenicity factors, mycoparasitism and, more recently, induction of plant defence responses. Many strains of *Trichoderma* have been used in trials aimed at biological control of *B. cinerea*, most of them in vineyards.

In vitro assays against different isolates of *Botrytis cinerea* have shown that *Trichoderma harzianum* CECT2413 and *Trichoderma atroviride* IMI206940 are able to directly antagonize this plant pathogen and to protect the grapes against Botrytis infection. Genetically modified *Trichoderma* strains with enhanced mycoparasitic abilities (overproducing antibiotic secondary metabolites and hydrolytic enzymes) derived from the former strains are even more effective. Application of wild type strains in field trials in vineyards of Jerez resulted in a reduction of *Botrytis* infection of grapes, with no alteration of the chemical composition of musts, rate of must fermentation, wine quality or biological aging of Sherry wines. This effectiveness was improved when these strains were applied together with low doses of a new synthetic botrycide. For this reason, selection of *Trichoderma* mutants resistant to this compound as well as to some normally used chemical fungicides (including benomyl-based and copper-based fungicides) has been carried out. The mutants were considerably more tolerant to these toxics than different *Botrytis cinerea* strains tested and some of them showed cross-resistance to several commercial fungicidal compounds. This strategy could lead to the design of more suitable integrated control protocols.

O4.3 Biocontrol of *Botrytis* bunch rot in grapevine using *Candida sake* CPA-1 treatments

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Bunch rot, caused by *Botrytis cinerea* is an important disease of grapevine. There is increasing interest in the use of biocontrol agents (BCAs) and other environmental safety methods alternative to chemical fungicides. Biosuppression of *B. cinerea* in vineyards using BCAs has been inconsistent compared with results in controlled conditions due to variable field environment. Research efforts have been directed towards the development of formulation strategies capable of enhancing the activity of BCAs. Stress adaptation methods of *C. sake* have been developed to improve the BCA role under field conditions and formulation processes. Mild heat adapted cells showed greater thermotolerance than non adapted at laboratory conditions.

The aim of this study was to evaluate the effectiveness of combining different formulation strategies on the establishment, survival, persistence and efficacy of *C. sake* CPA-1 applied in the field in order to control *B. cinerea* in grapes.

In this three-season study, different preparation strategies of the BCA *C. sake* cells were applied at flowering, pea sized berries, veraison and before vintage in order to evaluated efficacy to control *Botrytis* at harvest. Strategies used included liquid formulation, mild heat adaptation and additives. The compound FUNGICOVER® (FC) an edible coating was evaluated as a potential additive for *C. sake* treatments.

Results indicated that applications of different preparations of *C. sake* resulted in colonisation of bunches under field conditions. Population sizes of *C. sake* supplemented with the food film FC showed survival rates significantly higher than the rest of treatments. No improvements were observed in survival rates with formulation and heat adaptation strategies. In general, yeast treatments significantly reduced the incidence of *B. cinerea* with respect to control treatment. *C. sake*+Fungicover treatment was the most effective yeast treatment with rot reduction levels of 90%.

The potential of *C. sake* for biocontrol of *Botrytis* bunch rot of grapevine has been demonstrated. FC has a beneficial effect on the BCA, improving the persistence of *C. sake* cells on the host and its efficacy to levels comparable to fungicide treatment. It is possible to broaden the spectrum of use of BCAs using different formulation strategies and to thereby develop practical uses under field conditions.

*O4.4 The current situation of *Botrytis cinerea* in the vineyard from Sherry area

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Botrytis cinerea is currently found in the vineyard of Jerez, and its attacks depend on environmental conditions of each year, especially during the ripening period of the grapes. The lesions over the surface of the fruits, due to the action of other parasites, have also an important effect in the infection caused by *Botrytis cinerea*.

The cultivar "Palomino fino" is the main and majority for making of Sherry wine. This cultivar is characterized by compact bunch and thin skin. Nevertheless, the attacks of the fungus are not usual because the weather is dry during the ripening period and bunches present a good state of healthiness because the growers are very careful controlling other pathogens that could damage the skin of the fruit. In this region, closed to the Atlantic Ocean, it is necessary to control the level of humidity, what can affect severely to the vineyard.

In the province of Cádiz, the growers crop other cultivars different to cv "Palomino fino". These vineyards are focussed in the production of other kind of wines, i.e. white, rose and red wines. They do not belong to the Denominations of Origin "Jerez-Xérès-Sherry" and "Manzanilla - Sanlúcar de Barrameda" and these cultivars show different level of resistance against *Botrytis cinerea*.

* *This communication will be presented into the visit to El Corregidor vineyard*

P4.1 Role of biopolymers from *Rhodotorula glutinis* in biocontrol of the plant pathogen *Botrytis cinerea*

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The ability of biocontrol agents to control disease is dependent on colonization of plant surfaces. A huge variety of biopolymers, such as polysaccharides, polyesters, and polyamides, are naturally produced by microorganisms. *Rhodotorula glutinis* previously isolated from strawberry phyllosphere was assessed for *in vitro* antagonism against *Botrytis cinerea.* Here, the structure of extracellular polymers production by *Rhodotorula glutinis* was studied by measuring their polysaccharide and protein production. The results revealed that the presence of exopolymers in the growth medium was essential for biofilm formation. The biopolymers were purified with several precipitation steps using ethanol and cetyl-trimethyl-ammonium bromide. Carbohydrate analysis using various color reactions, infrared spectroscopy, and high performance liquid chromatography (HPLC) revealed that the biopolymer is a heteropolysaccharide, which is consist of various sugars such as glucose, galactose, mannose and xylose. In addition, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of total proteins in filtrate showed also different bands. Purified polymers collected from the exponential and stationary phases inhibited the growth of the fungus tested, indicating that growth suppression was due to extracellular antifungal metabolites present in culture filtrates. In order to standardize the biopolymers, some cultural conditions like different incubation temperatures (20.0, 25.0, 30.0 and 37.0°C), carbon sources (glucose, glycerol, starch and sucrose), pH (6.0, 7.0, 7.5, 8.0 and 9.0) , nitrogen source (KNO_3, $NaNO_3$, $(NH_4)_2.SO_4$, NH_4NO_3, NH_4Cl, urea, casein, yeast extract and incubation time in hours (24, 48, 72, 96 and 120) were determined. During fermentation, growth, pH and antibiotic production were monitored at 12 h intervals. Under natural field conditions, *Rhodotorula glutinis* and polymers exhibited a significant high activity against development of *Botrytis* disease on strawberry. Polymers showed a strong potential in reducing disease incidence

P4.2 Biological control of tomato stem canker disease caused by *Botrytis cinerea,* using *Trichoderma* isolates

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Tomato stem canker disease caused by *B. cinerea* is one of the most devastating disease on tomato production in greenhouses. Since the biocontrol effect of *Trichoderma* species against some of other fungal pathogens of plants have been provided, in this study effect of ten *Trichoderma* species including *T. asperellum, T. harzianum, T. orientalis, T. koningiopsis, T. atroviridae. T.ceramicum, T.brevicombactum, T.koningii, T. viride ,T. viridescens* on this pathogen been investigated. In order to determine the antagonistic effect of the *Trichoderma* species against *B. cinerea* in greenhouse conditions tomato seeds, super strain B variety grown in greenhouse , were sprayed by conidial suspensions of *Trichoderma* species (1×10^6 CFU/ml) and then inoculated with *B. cinerea* conidia suspension (1×10^5 CFU/ml) 72 hrs afterwards. The experiment was conducted in completely randomized design with three replicates, and disease severity index were evaluated after 15 days of inoculation. Among the *Trichoderma* species which been tested the most effective biocontrol species were: *T. brevicompactum, T. viridescens, T. koningii, T. atroviridae, T. harzianu* . In dual culture method which been performed in vitro, the extra cellular and volatile fungus metabolites had the most inhibited effect on pathogen mycelial growth rate. The results indicated that *T. brevicompactum, T. viridescens, T. harzianum* species have significantly reduced disease severity in treated plants, comparing control.

Session 5:
BOTRYTIS-"OMICS"

I5.1 Proteomic characterization of *Botrytis cinerea* secretome during pathogenicity induction by different plant based elicitors

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Botrytis cinerea has been adopted as a model biological system in molecular phytopathology. This is due, among other reasons, to the amazing variety of infection strategies used by *B. cinerea.* This biological machinery is controlled by a set of genes/proteins called pathogenicity/virulence factors. Most of these factors have been described as secreted proteins, and thus the study of this subproteome, the secretome, under changing circumstances can help us to understand its roles, possibly revealing new loci for the fight against the pathogen.

Our research has used 2-DE, MALDI TOF/TOF-based approach to establish the proteins secreted to culture media supplemented with different carbon sources and plant-based elicitors (in this study: glucose, cellulose (CMC), starch, pectin, and tomato cell walls (TCW). An improved method to precipitate the proteins based on DOC-TCA/phenol extraction was optimized. After 2-DE separation and protein staining, the secretome profiles induced by the five different carbon sources were visualized showing clear differences in the *B. cinerea* response to glucose, starch, pectin, CMC and TCW, indicating that each assayed carbon source elicites a specific secretion response. 2-DE gels of secretomes of cultures with glucose show 33 ± 17.45 spots. Using starch as the sole carbon source we observed 23.67 ± 3.09, and in case of pectin 26.67 ± 6.94, CMC 53.67 ± 14.08 and TCW 94 ± 21.35 spots, respectively. These data show that the secretion of proteins is similar in glucose, starch and pectin, but that many more spots are detectable after CMC treatment and still more when TCW is the sole carbon source.

Seventy six spots were identified, yielding 95 positive hits that correspond to 56 unique proteins, including several known virulence factors (i.e. pectin methyl esterases, xylanases and proteases). The observed increases in secretion of proteins with established virulence-related functions indicate that this in vitro-induction/proteome mining approach is a promising strategy for discovering new pathogenicity factors and dissecting infection mechanisms in a discrete fashion.

O5.1 Microarray analysis of the early stages of *Botrytis cinerea* development

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Germination of *Botrytis cinerea* conidia is a crucial step for fungal development. We previously showed that the germination process can be induced by several stimuli. Carbon source-induced germination is dependent on a cAMP-dependent signalling pathway, whereas for germination of conidia on hydrophobic surfaces without nutrients, an intact Ste11-Ste7-Bmp1 MAP kinase cascade is required. Using video microscopy we found, that conidia show a nutrient-dependent pre-germination swelling within the first hours of incubation. In addition, nuclear division was observed already before germination. These findings suggest that initiation of germination-related metabolism occurs long before germ tube appearance.

To analyse gene expression of germination on a global level, *B. cinerea* full-genome microarrays were used for analyses of different germination stages of the wild type (B05.10) and the Δ*bmp1* MAP kinase deletion mutant. The *bmp1* mutant was chosen because it is unable 1. to germinate on hydrophic surfaces, 2. to form appressoria, and 3. to penetrate into host tissues and to cause necrotic infections. Using apple wax coated petri dishes and minimal medium with 10 mM fructose, an optimised and rather synchronised conidial germination was achieved, resulting in an almost complete germination after 3h. For microarray hybridisation following stages were used: 0h: Dormant stage; 1h: Pre-germination conidial swelling; 2.5h: 75% germination; 4h: appressoria; 15h: saprophytic mycelium.

The results showed that greatest changes of gene expression occur between 0 and 1 hour, and between 4 and 15 hours. Approximately 30% of the genes that are specifically upregulated during the first few hours encode for secreted proteins. In the *bmp1* deletion mutant, up to 55% of these secreted proteins show only a very weak expression. These data indicate a peak of secretory activity during the early germination stages, and a significant reduction of the *bmp1* deletion mutant in secretory activity in comparison to the wild type.

In addition to interesting *bmp1*-regulated genes that will be targets for functional analyses, our current aim is to examine the global metabolic changes in the early steps of germination. Genes that are activated during particular developmental stages will be functionally categorised. A correlation of functionally related genes with their patterns of stage-specific expression will be a first step to understand the molecular mechanisms that are responsible for spore germination and development.

O5.2 Analysis of changes in expression during growth of *Botrytis cinerea* at low temperature

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B. cinerea is a major postharvest pathogen, mainly due its exceptional ability to develop at low temperature, which is the major postharvest tool used to maintain the quality of fresh produce. The aim of this research is to understand the physiological and genetic factors which allow *B. cinerea* to develop at low temperature. Growth was characterized for BO5.10, T4 and field strains which showed variability in development at low temperature. The major hypothesis is that genes which are up regulated at low temperature may be involved in the mechanisms of cold tolerance. Two sets of genes originated from cDNA subtraction or selected from the literature, were verified to be cold-induced by qPCR. Microarray analysis was performed on 4-plex Nimblegen microarray chips which compared expression between 4°C and 22°C at 1, 4, 10 and 24 h. Many of the genes which changed significantly ($P \leq 0.01$), do not have assigned function. Of the annotated genes, many are not known to be involved in cold response in other systems. Diverse cellular functions are activated with notable increase in amino acid and metal ion transporters and the 26S proteosome system. These results reinforce the hypothesis that cold response demands drastic changes in various cell activities but it appears that unlike other organisms, low temperature does not impose significant stress responses on *B. cinerea*.

O5.3 T-DNA-mediated insertional mutagenesis in *Botrytis cinerea* – a tool to identify new virulence-associated genes –

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Agrobacterium tumefaciens-mediated transformation (ATMT) has been proven to be a valuable method to obtain insertional mutant libraries and to identify virulence-associated genes in several fungal pathogens such as in *Magnaporthe grisea* and *Fusarium oxysporum*.

By using an ATMT approach in *B. cinerea* B05.10 we generated a library with 2,350 transformants which carry random integrations of a hygromycin resistance cassette flanked by LB and RB sequences. By performing Southern blot analyses of randomly chosen transformants it was shown that most of the ATMT strains contain single copies of the T-DNA sequence. A first virulence screen of all transformants on detached tomato leaves resulted in the identification of 560 less virulent strains. 160 of these have been undergone a second screening on primary leaves of *Phaseolus vulgaris*, and the less virulent phenotype has been confirmed for 133 strains. Up to now, the T-DNA insertion sites in 50 transformants have been identified by TAIL (Thermal Asymmetric Interlaced)-PCR analyses, illustrating that T-DNA integrations preferably occur in non-coding regions.

Eleven genes, tagged by a single T-DNA insertion either in the coding or non-coding region have been chosen to validate the procedure. So far, knock-out approaches for seven candidate genes have been completed successfully: five knock-out strains are impaired in virulence on *P. vulgaris* as observed for the corresponding ATMT mutants; for two genes, the confirmation of the ATMT phenotype has failed. However, our data demonstrate that the screening of an ATMT library is suitable to identify new genes involved in the *B. cinerea* – host interaction.

O5.4 Bioinformatic analysis of *O*-glycosylation in proteins secreted by *Botrytis cinerea*

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Among the proteins secreted by *Botrytis cinerea* it is common to find regions for which multiple *O*-glycosylation sites are predicted *in silico*. Now that the genome of this organism has been sequenced, it possible to asses the presence of such regions in the whole set of putatively secreted proteins. To address this question, a list of 2779 protein sequences, annotated as containing signal sequence, was obtained from the *B. cinerea* T4 genome database and studied. From these, the 2125 sequences longer that 100 amino acids were submitted to the NetOGlyc 3.1 server (http://www.cbs.dtu.dk/services/NetOGlyc/) for the prediction of *O*-glycosylation in serine or threonine residues, and 1113 sequences were found displaying varying degrees of glycosylation. 285 of these sequences, however, either were not predicted to have signal peptide by the NetOGlyc server, or were predicted to be glycosylated within the signal peptide itself, and were therefore discarded. The final set of putative *O*-glycosylated, secretory proteins is composed of 828 members. The analysis of this set revealed two main types of proteins: 1) a group in which the putatively glycosylated residues are distributed randomly along the sequence, and 2) a group displaying regions with a high density of *O*-glicosylation. These latter regions were found mainly in the N-terminal end of the proteins, in some cases in an intermediate position, and only in a few cases in the C-terminal region. Intriguingly, some proteins of unknown function were found for which a high density of *O*-glycosylated residues was predicted in most of the sequence.

O5.5 Creation of a collection of *Botrytis cinerea* T-DNA transformants for pathogenic development and plant defence studies

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Botrytis cinerea infection success rely on a battery of weapons –degrading enzymes, reactive oxygen species (ROS) producing and scavenging enzymes, toxins– to decompose the plant tissues and trigger the programmed cell death, which is exploited by the parasite to further colonize its hosts. The contribution of the different plant defence mechanisms to *B. cinerea* susceptibility, their rerouting for the benefit of the parasite or their inhibition by the effectors produced by the pathogen started to be explored with the non-natural host *Arabidopsis thaliana* and needed to be extended to major host crops.

The goal of the project is to develop, within the Plant-KBBE proposal, a collection of *B. cinerea* tagged mutants and to validate the creation of this library with the characterization of the mutant lines whose infectious cycle is affected. As a first step, the *Agrobacterium tumefaciens*-mediated transformation (ATMT) will be employed to enlarge the existing mutant library (Tudzynski *et al.*, unpublished). The growth and the conidiation capacity of the transformants will be assayed both *in vitro* and *in planta*. In parallel, the insertion sites of the T-DNA will be identified using TAIL-PCR and the genome sequences (Broad Institute, Genoscope). These data will be organized into a genome-orientated database of *B. cinerea* tagged mutants, as it was previously developed for the plants *A. thaliana* and *Oryza sativa* and the fungal pathogen *Magnaporthe oryzae*. These resources will be available for the scientific community as a support for fundamental research.

As a second step, the mutant library will be exploited with a specific focus on the mutants whose parasitic development is hampered. For this purpose, biochemical mutant signatures will be defined on the basis of *in vitro* assays for the lytic enzyme secretion, the ROS production and the resistance to ROS. Representatives of all mutant signatures will be further analyzed at the molecular level. For this purpose, comparative studies of the wild-type virulent strain *vs* the non pathogenic mutant lines will be performed, based on transcriptomic and proteomic approaches both *in vitro* and *in planta*, using grapevine berries as a model host.

The expected results should lead to the identification of genes and gene products mediating essential functions for *B. cinerea* pathogenicity and/or plant defence traits. As a perspective, the conservation of the newly identified functions among pathogenic models, both biotrophic and hemibiotrophic, will be tested with the purpose of developing new broad spectrum solutions for disease management. Moreover the defence properties, that prevent the development and the spread of the mutant lines, could be used as a basis to develop new strategies to control plant disease development based on the elicitation or the priming of plant defence reactions. Altogether, these studies fall in the research for a more sustainable agriculture system, increasing and maintaining crop yield together with producing healthier crops, free of fungal residues and toxins.

O5.6 Proteomics in *Botrytis cinerea.* The simplest 1-DE reveals differences in protein abundance among strains

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In the post-genomics era, Proteomics has became in a powerful tool which can contribute to understand biology, infection strategies, and live cycle of plant pathogenic fungi, to identify virulence factors, and, on the basis of them, to develop crop protection strategies (reviewed in Gonzalez-Fernandez *et al*., 2010; *Journal of Biomedicine and Biotechnology*, *in Press*). Within BOTBANK project, we are using such approach to characterize and validate a collection of mutants of *B. cinerea* whose infectious cycle is affected (Mey *et al*., this meeting). In this work, we present a preliminary gel-based proteomic analysis using one-dimensional electrophoresis (1-DE) and MALDI-TOF/TOF MS/MS analysis for protein identification from mycelium and media (secretome) of seven wild-type strains of *B. cinerea* with different virulence.

Wild-type strains of *B. cinerea* B05.10, T4, CECT 2100, CECT 2850, CECT 2996 and CECT 20518, and BOLC (isolated from lentil infected plants) were used. Fungal mycelia (three biological replicates) were inoculated in modified Czapek-Dox minimal medium for 6 days at 21ºC with agitation in darkness. The protein precipitation from mycelium and media was carried out by TCA/acetone-phenol/methanol method with some modifications optimized in our laboratory. The SDS-PAGE was performed using the Criterion System with precast Criterion Stain Free Gels (Bio-Rad). The protein band pattern was analyzed using the Image Lab software (Bio-Rad). The bands were cut out and digested with trypsin. Tryptic peptides were analyzed by MALDI-TOF/TOF MS/MS analysis. A PMF search and a combined search (+MS/MS) were performed in nrNCBI database of proteins using MASCOT.

The protein profile showed significative and quantitative differences both in mycelium and in secretome among strains. Several proteins were identified in mycelium protein extracts, some of them have been reported to be involved in pathogenicity in *B. cinerea* or in other phytopathogenic fungi, such as malate dehydrogenase, woronin body major protein, esterase, peptidyl-prolyl cis-trans isomerase and PCI5 protein or implicated in fungal growth and differentiation, such as nucleoside diphosphate kinase. Similarly, several proteins involved in plant cell wall degradation were identified in secretome, such as β-glucosidase, tripeptidyl-peptidase 1, pectin methylesterase, pectin esterase, endo-polygalacturonase, glucan 1,3-β-glucosidase, cutinase or esterase. The abundance of all these proteins was different among isolated. Thus, using this technique, it is possible to distinguish between genotypes of different wild-type strains and identify proteins involved in the pathogenicity mechanisms, providing relevant information, especially in the case of comparative proteomics with a large numbers of samples.

Work is now in progress in different directions: i) analysis of spores in wild-type strains; ii) analysis of mutants, obtained by *Agrobacterium tumefaciens*-mediated transformations (ATMT) and affected in infectious cycle; and iii) use of gel-free proteomics approaches.

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O5.7 Transcriptome of the early steps of *Botrytis cinerea* infection

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A recent project from GENOPLANTE (federative programme for plant genomics research in France), called "SafeGrape" and involving four associated research teams[1,2,3,4], is partly aimed at getting a better knowledge on the interaction between grapevine (*Vitis vinifera*), a major crop for French agriculture, and one of its major pathogens, *Botrytis cinerea*. In this frame, transcriptomic analyses were conducted to identify genes that may putatively function as key determinants of the *B. cinerea* infection process. Early steps of infection, including conidial germination and unicellular appressorium formation were followed *in vitro* on a hydrophobic membrane mimicking the plant surface. Samples were collected at four times along a developmental kinetic with tissues inriched for spores with germination tubes, unicellular appressorium and epiphytic mycelium. Surviving conidia were also used as control. Penetration and colonization were followed on grape berries with the collaboration of M. Fermaud[5] and on grape leaves. Hybridizations of cDNAs were done by PartnerChip (http://www.partnerchip.fr/) on the Nimblegen chips, designed using the set of approximately 15000 predicted genes in the *B. cinerea* genome (URGI, INRA, Evry, France).

Apart from the SafeGrape project, a new collaboration was begun with M. Choquer[1], N. Poussereau[1] and J. Rollins[6], for comparing the transcriptomes of the *B. cinerea* and *Sclerotinia sclerotiorum* infection cushions. These complex pluricellular appressoria are developed by both fungi, and are supposed to be involved in fungal penetration of hard surfaces. Tissues enriched for infection cushions were obtained from spores germinated on the surface of a hydrophilic cellophane membrane in the presence of exogenous nutrients (assay), and they were not formed in agitated liquid cultures (control). For the *B. cinerea* side, hybridizations were done on the same Nimblegen chips using the transcriptomic platform of BAYER S.A.S. in Lyon[1].

Based on the *Botrytis* genome, micro-array analyses allowed us to describe functional category changes of the regulated genes, comparing different growth conditions *in vitro* (teflon and cellophane) and *in planta* (grape berries and leaves). Variations in the number of regulated genes and their patterns of regulation were studied. As some genes are similarly regulated during infection and during *in vitro* infection-associated development, they were considered to be core genes associated with infection. Others however, appear specific to an early step of infection. Candidate genes identified as specifically expressed during conidia germination, unicellular or pluricellular appressorium formation, or penetration will be studied by reverse genetics in order to validate their function. Early steps of infection are key targets in the aim of pathogen control. Search for specific *B. cinerea* markers will provide the information needed for developing new control strategies or fungicide treatments.

O5.8 Genome-wide expression profiles uncover infection-specific genes and global gene regulation

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Microarray analyses (*Botrytis cinerea* 4-plex arrays, Roche NimbleGen Systems) were used to discover gene expression at different stages of infection as well as to analyse transcriptional regulation of Δbcatf1 transcription factor strain.
Early infection stages of a pathogen on its host are a complex challenging research field as the process of pathogenesis is not only dependent on the driving force of the invading microbe but also on the power of resistance of the host organism. Thus these first steps of host-pathogen interaction are very often the crux that decides on successful infection. Knowledge of the regulation of *Botrytis cinerea's* early infection steps (conidiospore germination > penetration > host invasion) *in planta* is still limited and of great interest for the understanding of fungal pathogenesis. Gene expression profiles of these different stages of infection obtained from a microarray approach can now be used to answer questions concerning sensing and signalling systems important for the host surface recognition, for the germination of the attached fungal spore, for regulation of host tissue penetration and finally for host plant killing. These data provide the opportunity to identify novel infections-specific genes that were not known to be involved in the infection process so far.
In *Botrytis cinerea* BcAtf1 regulates genes expression upon oxidative stress dependent on the SAPK BcSak1 but also controls diverse differentiation processes independent of the MAP kinase. Microarray analysis presented the transcriptom alteration due to the loss of *bcatf1*. Deregulation of developmental and metabolic target genes explains to some extend the phenotype of the *bcatf1* deletion strain and might cause its uncontrolled enhanced growth not only in axenic culture but also on different host plants and tissues. Those data also uncovered its repressing function on secondary metabolite production revealing a novel function of the transcription factor.

P5.1 The secretome of *Botrytis cinerea* : adaptation to environmental pH

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Micro-organisms must adapt to environmental change to survive, and this is particularly true for fungal pathogens such as *Botrytis cinerea*. *B. cinerea* is found both in the environment and in diverse plant hosts. Their ambient pHs varie considerably, and therefore we have examined the response of *B. cinerea* to changes in ambient pH using a proteomic approach.

The expression of secreted proteins induced by controlled growth conditions, i.e. complete synthetic medium at pH 5.0, was identified by two-dimensional gel electrophoresis and image analysis software. All reproducible and statistically significant spots were identified by peptide mass fingerprinting, thereby extending our 2-DE map of the *Botrytis cinerea* to a total of 310 identified proteins. Proteins expressed in *B. cinerea* cells growing at pH 5.0 or 7.0 were compared by 2-DE. Qualitative and quantitative differences were observed related to the protein patterns whereas at both pH (5.0 and 7.0), *Botrytis* was equally virulent on apple or chicory.

Discussion will be done on the possible factors regulating secreted protein expression by the pH in *Botrytis*. In addition, our data suggested that different fungal proteins could be involved in the infection process depending on the external pH.

P5.2 Proteome analysis of the interaction between *Botrytis cinerea* and tomato fruit

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Botrytis cinerea secretes virulence factors to accomplish its colonization of targeted plant tissues. Among these factors, an abundant and large family of *B. cinerea* enzymes targets the polysaccharides within the plant cell wall, a complex and carbon-rich structure of polysaccharides, proteins and phenolic compounds that is the plant's primary barrier of defense against pathogens. The enzymes produced by the fungus play an important role in the early stages of infection by hydrolyzing and decomposing the polysaccharides within the host cell wall. In this study, shotgun proteomics was used to characterize fruit and fungal proteins in infected tissues in order to identify proteins that are especially relevant to this host-pathogen interaction. Specifically, we employed proteomic analysis of proteins released into the microenvironment of *B. cinerea* infection sites of mature green, red ripe wildtype and non-ripening *rin* mutant tomato fruit to identify both the proteins produced by mature green, red ripe and *rin* mutant fruit in response to infection as well as the proteins released by *B. cinerea*. We have identified proteins of both the host and the pathogen when only limited *Botrytis* growth on MG fruit or when aggressive colonization of RR or *rin* fruit has occured.

Red ripe and mature green tomatoes were inoculated with *Botrytis cinerea*. After 5 days the tomatoes infected with *B. cinerea* were gently agitated in buffer with 1.5M NaCl to collect solubilized proteins. The buffer solution was then filtered and desalted. The collected proteins were separated on one dimensional SDS-PAGE gel, followed by in-gel digestion. Peptides were then analyzed by LC-MS/MS on a linear ion trap mass spectrometer. Data was searched using Mascot algorithm. Proteins were identified by using a target database created by combining the *B. cinerea* BO5.10 (Broad Institute, MA), and T4 databases (Genoscope, France) with a tomato protein database (SOL Genomics Network, Cornell University, NY). A decoy database was constructed by reversing the sequences in the target database. Statistically significant proteins were determined for all of the samples at a 1% protein FDR using ProteoIQ Software.

Results show that many of the same fruit proteins can be collected from both the red ripe and non-ripening *rin* mutant tomato fruit during successful infections by *B. cinerea*. However, the unsuccessful infection of mature green fruit by *Botrytis* results in the accumulation of defense and pathogen response-related fruit proteins that are not as abundant in infections of red ripe or *rin* fruit. In contrast, *B. cinerea* secretes similar proteins independent of the ripening stage of the fruit host. The pattern of protein expression and the putative function of the identified proteins argue for their role in plant-pathogen interactions. This study provides a characterization of the *B. cinerea*-tomato interaction proteome using shotgun proteomics.

P5.3 Studying the *Botrytis cinerea* phosphoproteome

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Botrytis cinerea is one of the most important plant pathogenic fungi, being the responsible of major crops losses in Europe. In Andalucía, it has special interest the fungal effects when tomato, grape or strawberry crops are infected. The fungus possesses different set weapons available to invade plant tissues transforming plant biomass into fungal biomass. "Pathogen-host interactions database" (www.phi-base.org/query.php) is considered as a catalogue of pathogenicity/virulence factors that have been experimentally verified in different organisms. From 34 genes of *B. cinerea* listed, the deletion of 20 of them produces phenotypes catalogued as "loss of patogenicidad" or "reduced virulence". More than the half of them codify for components of different signalling cascades, revealing its relevance as a bridge between environmental conditions and fungal metabolism.

Most of the signalling cascades are based on the post-translational modifications of its components. In brief, the proteins belonging to signal transduction pathway used to be present in the cell prior to the perception of the elicitor. They are activated by conformational changes or mainly by post-translational modifications. Hundreds of post-translational modifications have been described, such as acetylation, glycosylation and ubiquitylation, amplifying the complexity of each proteome. Among all post-translational modifications, phosphorylation has been studied most intensively due to some studies suggest that one-third of all proteins are modified by phosphorylation. We present the study of *Botrytis cinerea* phosphoproteome. Different pathogenicity “stages” were induced from glucose as a control of the constitutive view, through cellulose, and finally by using de-proteinized tomato cell walls as the closest approximation of an “*in planta”* view. Phosphoproteins were isolates using MOAC protocol, and the proteins were studied by 2-DE and MALDI/TOF-TOF. Our data support the hypothesis that each assayed carbon source produce different phosphoproteins 2-DE profiles. At present, we had identified 768 different protein spots. An important number of spots were identified as “*Botrytis cinerea* hypothetical protein” that will be annotated by blast searches. On the other hand, we had found knew virulence/pathogenicity factors, i.e. peptidyl-prolyl cis-trans isomerase, NADPH cytochrome P450 oxidoreductase; while the role of the rest of them remains undilucidated. The present research is a part of a wide approach to *B. cinerea* virulence factors from proteomics, genomics and metabolomics tecqniques.

P5.4 Modifications of the *Botrytis cinerea* conidial proteome during germination

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Botrytis cinerea is a phytopathogenic fungus that causes significant yield losses in a substantial number of crops around the world. It is considered as a very complex species in which molecular differences among populations have been described by several authors. In the last few years, many advances regarding the infection process developed by this fungus have been made, although neither the molecular basis of the synthesis and production of different virulence factors, nor the mechanisms of phytopathogenicity used by this fungus are yet well understood. Several approaches have been applied to unravel its mechanisms of infection. These studies have revealed the complexity and wide variety of infection strategies used by *B. cinerea*. Thus, several virulence factors, such as enzymes involved in penetrating plant tissues, have been shown to be produced by this fungus and research efforts have been made to elucidate which of them are essential for full virulence of the fungus. This study presents an initial approach to characterize the proteins content of the main structure of resistance: conidia. They constitute the principal inoculum of the fungus in the fields. We have identified the proteins from conidia during the first stages of germination and production of germination tubes. After 2-DE, the majority of the spots were found from 5 to 8 of pI values, presenting a Mr from 14 to 105 kDa. After protein identification will be done, our main aim is to find those proteins involved in conidial germination, adhesion and primary plant lesion.

P5.5 RNA interference in Botrytis cinerea: Genome identification of RNA-dependent RNA polymerase, *Dicer*-like and *Argonautes*, and their expression during early stages of development

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Gene silencing by means of RNA interference (RNAi) is a conserved process throughout the kingdoms of eukarya, and has been one of the major discoveries in biology in the past twenty years. Its mechanisms have been described in a variety of organisms, and it have been shown be related with different cellular processes, such as control of expression of gene involved in development genes and the maintenance of genome integrity against transposons and viruses.

In the present work we have identified *Neurospora crassa* orthologues of the principal components of RNAi in the genome of *Botrytis cinerea*: Three RNA-dependent RNA polymerases, two Dicer-likes and two Argonautes. By bioinformatics tools, sequence analyses of functional domains have been performed, suggesting that *B.cinerea* contains the main components of the two RNA silencing-related pathways, previously described in *N. crassa*: quelling and meiotic silencing by unpaired DNA (MSUD), related to vegetative and sexual development, respectively. Using the microarray technology, we found that the main components of the RNAi machinery have different transcriptional activity during the early stages of development of the fungus, suggesting that gene silencing by RNAi in *B.cinerea* should be active and could be involved in the regulation of gene expression.

Session 6:
HOST-PATHOGEN INTERACTIONS 1: Fungal virulence factor

I6.1 The role of SAPK signalling in differentiation and pathogenicity of *Botrytis cinerea*

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In *Botrytis cinerea* -in contrast to other phytopathogenic fungi like e.g. *Magnaporthe grisea* and *Cochliobolus heterostrophus*- the stress-activated protein kinase (SAPK:Hog homolog, Bcsak1) is of central importance for differentiation and virulence: Δbcsak mutants do not form conidia, but produce more sclerotia, and they are impaired in penetration and colonization of host tissue (Segmüller et al. 2007). The cascade is involved in response to osmotic stress, but it is not the major player in oxidative stress response (OSR). Detailed analyses of the expression profile of OSR genes, including a functional analysis of the AP1-homologous transcription factor Bap1, indicate that oxidative stress is not a major factor in the host pathogen interaction, inspite of the oxidative burst caused by *B.cinerea* (Temme and Tudzynski 2009). Available data on downstream factors of this MAPK cascade (transcription factors , target genes) will be presented. In addition, cross-talk to other cascades, the impact of small GTPases on differentiation and MAPK signalling,and the role of the ROS generating NADPH-oxidase (NOX) complex in this regulatory network will be discussed.

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O6.1 The NO detoxifying flavohemoglobin BCFHG1 is not a virulence factor in the fungal necrotroph *Botrytis cinerea*

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Botrytis cinerea is a necrotrophic plant pathogen with a wide host range that colonizes senescent or wounded tissues but is also able to infect healthy plants. Experimental evidence supports the ability of the fungus to exploit the hypersensitive cell response of healthy plant tissues to promote its colonization. As a necrotroph *B. cinerea* survives in this hostile environment and is certainly exposed to toxic reactive oxygen species (ROS) and to NO. The pathogen would be expected to benefit from mechanisms limiting damage caused by ROS and by NO and NO-derived reactive nitrogen species (RNS). We are interested in the understanding of the *B. cinerea* mechanisms facilitating survival of the fungus under nitrosative stress conditions and during its interaction with the host plant. We therefore decided to investigate NO metabolism in *B. cinerea* focusing, as a first step, on the analysis of the NO detoxification capacity of the fungus.

Flavohemoglobins constitute a group of proteins involved in the metabolism of nitric oxide (NO). Genome analysis reveals that *B. cinerea* has a single flavohemoglobin coding gene, *Bcfhg*1. Its expression is developmentally regulated, with maximum expression levels during germination of conidia, and is enhanced very quickly upon exposure to NO of germinating conidia, but not of mycelium growing and branching actively. Expression *in planta* parallels the expression pattern during saprophytic growth with maximal expression occurring during the very early stages of the infection process. *Bcfhg*1 complements the *Saccharomyces cerevisiae yhb*1 mutation, indicating that the encoded enzyme has NO dioxygenase activity. Biochemical and functional characterization of *ΔBcfhg*1 mutants in comparison with the wild type strain demonstrate that BCFHG1 shows a high affinity for its substrate, appears to represent the main inducible NO detoxification system and confers protection against nitrosative stress in *B. cinerea*. However, and contrary to what could be expected, the ability of the *ΔBcfhg*1 mutant strains to infect different hosts is not affected, indicating that BCFHG1 it is not a virulence factor in this fungus. It can therefore be concluded that either the fungus possesses a high level of natural resistance to exogenous NO or that it does not experience a strong nitrosative stress during its interaction with the host plant tissues.

Which are the physiological processes in which NO detoxification by a flavohemoglobin in this fungus is relevant is at the moment an open question. Our results suggest that the physiological functions of the flavohemoglobin in *B. cinerea* could be more related to its involvement in the modulation of endogenous NO levels produced by the fungus during specific developmental stages. Our research is aimed now at the characterization of the endogenous NO production systems in *B. cinerea*.

O6.2 The biosynthesis of the polyketide toxins excreted by the phytopathogen fungus *B. cinerea*

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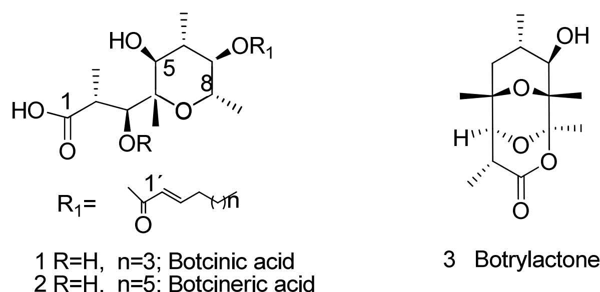
Botrytis cinerea is a well known pathogen of a number of commercial crops and produces many structurally diverse metabolites. There is no evidence for the production of host-specific toxins by this fungus, which is in accordance with the broad host range of this pathogen. *Botrytis* produces two series of phytotoxic metabolites: a family of characteristic sesquiterpene metabolites which contain the basic botryane skeleton, principally botrydial and dihydrobotrydial and two polyketide lactones types: botcinins and botrylactone.[1-3] The higher production of polyketide toxins from the botrydial sesquiterpene cyclase nock out mutant strain *bcbot1*, led us to reinvestigate the structure and biosynthesis of these polyketide compounds. On the other hand, the similarity of botrylactone spectroscopic data with those of botcinins and the finding of common sterochemical features for both compounds[4] indicated a possible common biosynthetic origin.



Feeding experiments with ^{13}C and ^{2}H-labelled acetate, malonate, and L-[methyl]methionine indicated that 3-acetylbotcineric acid and botrylactone are a malonate-derived polyketide whose methyl groups originate from L-[methyl]methionine.

The labelling pattern of isolated botcinins and botrylactone was analysed and these data were consistent with a common origin of both polyketides compounds. Given that methyl transferase activities present within the polyketide synthase catalyze the formation of carbon-carbon bonds at activated methylene groups, the biosynthesis of botcinin and botrylactone seems to involve the formation of a C9 polyketide precursor, which is methylated at activated methylene. The decarboxylation of C1 at the starter unit gives botcinic acid and derivatives while the displacement of the carboxyl moiety by acetyl coenzyme A followed by cyclization gives botrylactone.

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O6.3 The monomeric GTPase Rheb: a development regulator required for successful plant infection

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Phytopathogenic fungi may fulfill their nutritional needs by first mobilizing their storage compounds, to initiate the differentiation of the penetration structures, then by using the simple elements drawn from the host during the later stages of the infection process. Our studies focused on the amino acid triggered regulation mechanisms and their involvement in the fungal development control, using the necrotrophe *Botrytis cinerea* and the hemibiotrophe *Magnaporthe grisea* as models.

The monomeric GTPase Rheb (Ras Homologue Enriched in Brain) controls the growth of eukaryotic cells *via* the activation of the conserved TOR kinase complex, in response to the extracellular amino acid availability, and the regulation of the arginine and lysine import rates. In order to identify Rheb-controlled functions in both models, we used different strategies to impair the GTPase activity (RNA interference, promoter replacement, creation of hyper/hypo active mutants).

Reducing *Rheb* expression level affected *B. cinerea* conidiation capacity and its virulence on different host systems. Whether the pathogenicity defect was associated with the inability to penetrate the plant surface and/or to produce the effector molecules that enable *B. cinerea* to kill the hosts and decompose the plant tissues (oxalic acid, ROS, lytic enzymes) was analyzed.

Interestingly, the virulence of the *B. cinerea* RNAi mutant strains varied according to the nutrient conditions used to produce the inoculums. In parallel, their amino acid content, measured using reversed chromatography (HPLC), was different from the wild-type strain's one. The complementation experiments of the *S. cerevisiae* Rheb deficient strain suggested that the differential accumulation of certain amino acids could partly result from the increase in their import rate.

The link between the deregulation of the amino acid uptake and metabolism and the pathogenicity defect of the RNAi mutants was further investigated. In parallel, a comparative study of *Magnaporthe grisea Rheb* mutant strains was initiated to determine whether the GTPase functions are conserved among fungal phytopathogens. The expected results should give insights on how the amino acid triggered regulation mechanisms support the parasitic development.

I6.2 Secondary metabolism in *Botrytis cinerea*: the grey and the pink sides of a pathogen

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Botrytis cinerea genome revealed putative secondary metabolism genes that could encode the biosynthesis of approximately 40 different metabolites including many polyketides (21), non ribosomal peptides (8) and terpenes (9). The role of most of these metabolites remains elusive but they may contribute to the fungal fitness, pathogeny and protection against biotic and abiotic stresses. The most well-known are the unspecific phytotoxins *i.e.* botrydial (sesquiterpene), botcinic acid (polyketide) and its botcinins derivatives. The botrydial biosynthetic gene cluster (*BcBOT1* to *BcBOT5*) was previously identified and BcBOT2 was characterized as the sesquiterpene cyclase (presilphiperfolan-8b-ol synthase) necessary for the first step of the synthesis. More recently, genes involved in botcinic acid biosynthesis were identified. Gene inactivation and chemical analysis indicated that two PolyKetide Synthases (PKS) are necessary to produce the two ketide moieties of the toxin. Virulence of both botrydial deficient mutants *(bcbot2Δ)* and botcinic acid deficient mutants (*pksΔ)* was similar to the B05-10 wild-type strain. In opposite, *pksΔ/bcbot2Δ* double mutants were severely affected in virulence suggesting that the two toxins have redundant functions during plant tissues colonisation.

Among the secondary metabolism gene clusters that were predicted from the genome sequence, one was very similar to the bikaverin biosynthesis cluster that was only identified in *Fusarium* species so far. In the majority of *B. cinerea* strains, the bikaverin-like cluster is not functional because of several mutations including the complete deletion of the *PKS* gene (*BcBIK1*). Nevertheless, in rare pinkish strains isolated from various host plants, the cluster is functional and bikaverin pigment could be chemically detected. These data raise questions about the evolutionary origin of the cluster in *B. cinerea* and about its role in the pinkish strains.

O6.4 "VELVET" – A virulence factor in *B. cinerea*

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In fungi, the *velvet* gene, or *ve*, is involved in the regulation of diverse cellular processes, including control of asexual and sexual development as well as secondary metabolism. The characterization of *velvet* began more than 50 years ago, when E. Käfer obtained the first *veA* mutant in *A. nidulans*. Later, it was shown that this global regulator is conserved in numerous fungal species and its impact on differentiation and biosynthesis of secondary metabolites has been studied in different fungi such as in *Fusarium fujikuroi*. However, not much is known about the function of *velvet* during fungal pathogenesis.

To investigate the role of *velvet* in the *B. cinerea* – host interaction, we identified the *velvet*-like genes *bcvel1* and *bcvel2,* both expected to be components of the *velvet* protein complex. The knock-out of *bcvel1* resulted in mutants which are not significantly affected in conidial germination and in growth rates on standard media and on media containing different stressors (osmotic, oxidative, cell wall and membrane stress). The deletion mutants produce more conidia than the wild type in the light. In continuous darkness sclerotial development is prevented and instead conidia are formed in contrast to the wild type. Hence, we could demonstrate the role of *bcvel1* in regulation of light-dependent differentiation. Like in *Aspergillus* species, the *velvet* homologue in *B. cinerea* activates sexual and inhibits asexual development.

Notable is the effect of the *bcvel1* mutation on virulence: the mutants do infect primary leaves of *Phaseolus vulgaris* resulting in small necrotic spots but they are incapable of growing invasively. Hence, no further lesion development occur and consequently the mutants fail to reproduce. Strikingly, the defect in virulence is not associated with the loss of phytotoxin production as botrydial and botcinic acid are still produced by Δ*bcvel1* mutants in submers cultures. Furthermore, we observed expression of botrydial biosynthetic genes in primary lesions of *bcvel1* mutants.

I6.3 Botrydial causes host cell death by inducing the hypersensitive response and by interacting with host signaling pathways that mediate defense responses against pathogens

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Necrotrophic plant pathogens such as *B. cinerea* kill host tissues and feed on the remains. *B. cinerea* secretes a variety of nonspecific phytotoxic metabolites that provoke host cell death. Among them, the tricyclic sesquiterpene botrydial is known to be toxic to several plant hosts and seems to act as a strain-specific virulence factor.

The hypersensitive response (HR), almost universally accepted as a form of programmed cell death, is typically triggered upon recognition of a pathogen-encoded avirulence protein by a resistance protein of the host plant. This response provides resistance to biotrophic pathogens by restricting their access to living tissues. However, the HR enhances virulence of necrotrophic pathogens, by increasing tissue damage. In fact, *B. cinerea* is able to promote the host HR and thus takes advantage of this cell death process. Moreover, it has been proposed that HR induction by *B. cinerea* is mediated by elicitors that act as virulence factors. However, the identity of such HR elicitors has not been clearly established. The aim of this work was to determine if the well-known sesquiterpene botrydial produced by *B. cinerea* merely acts as a 'plain toxin' or if, on the contrary, is able to induce the host HR.

For this purpose, biochemical and molecular indicators of the HR were analysed on leaves of the model plant *Arabidopsis thaliana* treated with botrydial. Aniline-blue staining and epifluorescense microscopy revealed that botrydial induced callose deposition as early as 3 h after treatment (a.t.), this effect being more noticeable 24 h a.t. Autofluorescence analysis of leaves treated with botrydial revealed that this toxin also induced the accumulation of phenolic compounds in host tissues, as evaluated both 3 and 24 h a.t.. Moreover, rapid accumulation of reactive oxygen species in botrydial–treated leaves was evidenced by the redox-sensitive dye dihydrofluorescein diacetate. In addition to the above-mentioned biochemical markers of the HR, botrydial was found to cause a rapid and transient increase in mRNA levels of *Athsr3* This gene is known to be induced early during the HR of *A. thaliana* elicited by pathogen attack and is considered as a molecular marker of the HR . In this way, it can be concluded that botrydial is able to induce the HR in plant tissues, this tricyclic sesquiterpene probably being one of the effectors involved in HR elicitation by *B. cinerea* on its hosts.

Additional evidence that botrydial is not a 'plain toxin' and that depends on host cell machinery to exert its toxicity was obtained by analyzing the effects of botrydial on genetically modified plants affected in signaling pathways mediated by salicylic acid and jasmonic acid. Botrydial caused an increase in mRNA levels of PR1 and PDF1.2, two pathogenesis related proteins whose expression is respectively mediated by salicylic acid and jasmonic acid signaling. In addition, *A. thaliana* and tobacco (*Nicotiana tabacum*) plants defective in salicylic acid signaling were more resistant to botrydial than wild type plants. On the contrary, and interestingly, *A. thaliana* plants defective in jasmonic acid signaling were more sensitive than wild type plants to botrydial toxicity. Thus, botrydial toxicity was found to depend on host signaling pathways mediated by salicylic acid and jasmonic acid, both hormones seeming to play opposed roles in the development of host cell death induced by this toxin.

I6.4 Gα-controlled signalling pathways and their role in developmental processes and virulence of *Botrytis cinerea*

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Comparative analysis of mutants affected in different signalling components gives us the possibility to get a better insight into the regulation network and interconnections between different signalling pathways. Thus, the Gα subunit BCG1 regulates manifold processes of growth, virulence and differentiation by controlling at least two different signalling pathways - the cAMP and the Ca^{2+}/calcineurin pathway (Schumacher et al., 2008a, b). While the deletion of the Gα subunit BCG1 resulted in loss of secondary metabolite production, the adenylate cyclase and PKA mutants still produce the mycotoxins botrydial and botcinic acids. On the other hand, the mycotoxin biosynthetic genes were shown to be down-regulated under calcineurin-inhibiting (cyclosporine A treatment) conditions and in Δ*bccrz1* mutants with a deletion of the calcineurin responsive transcription factor CRZ1, confirming a direct link between BCG1 and the calcineurin signalling pathway. Detailed studies on the role of single components of both cascades, such as the adenylate cyclase and PKA subunits from the cAMP pathway, and calcineurin, calcipressin and the transcription factor CRZ1 from the calcineurin signalling pathway, will give a preliminary model of interconnected signal-
ling processes in this fungus.

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O6.5 Functional analysis of the *Botrytis cinerea* *Bcbip1* bZip transcription factor

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The *bip1* bZip transcription factor was first studied in the rice blast fungus. It is essential for *Magnaporthe grisea* penetration and pathogenesis on barley and rice, as it regulates a distinct set of appressorium specific genes in this fungus (1). Its CDS in *M. grisea* is limited to the DNA-binding bZIP domain whereas its CDS in other fungal species encodes a supplementary C-terminal domain of unknown function. In order to elucidate its role in other fungi, functional analysis of *Bcbip1* was investigated in *Botrytis cinerea* (2, 3). *Bcbip1* cDNAs were produced from mRNAs harvested at different fungal stage development, and their sequencing revealed that this transcription factor is subjected to alternative splicing in *B. cinerea.* Three categories of isoforms were predicted for *Bc*BIP1 : a complete isoform (bZip + C-terminal domain), and two truncated isoforms (bZip alone or even no bZip). *In vitro* and *in planta* expression profiles analysis of *Bcbip1* transcripts and the construction of a knock-out mutant for this gene in *B. cinerea* will be presented.

Keywords : Appressorium, bZIP transcription factor, alternative splicing, knock-out mutant.

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O6.6 Triggering cryptic sesquiterpenes biosynthesis in *Botrytis cinerea*

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The gray mold *Botrytis cinerea* Pers. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) is an important plant pathogen causing serious pre- and postharvest losses in more than 230 crop species worldwide. Despite the recent introduction of new antifungal compounds, *B. cinerea* is difficult to control because of its broad host range, high genetic variation, and ability to survive as a saprophyte or for extended periods as sclerotia in crop debris.

The sequencing of the genomes of the B05-10 and T4 strains revealed an abundance of novel biosynthetic gene clusters, the majority of which were unexpected on the basis of previous fermentation analyses of these and closely related species. Genomic data revealed that *B. cinerea* has 43 key enzymes some of them are specific of this phytopathogen. So, the following key enzymes have been reported: Non-Ribosomal Peptide Synthase (NRPS) (8); Polyketide synthases (PKs) (22); Dimethylallyl tryptophan synthetase (DMATS) (1); Sesqui-, Di-terpene cyclases (6 + 2). With this information now available, we have undertaken a chemical epigenetic fermentation methodology in order to find cryptic sesquiterpenic molecules and to deep in the study of the secondary metabolism of this phytopathogen.

As a result, we have identified a new sesquiterpene family, which has not been previously described in *B. cinerea*. Additionally, some interesting data about the biosynthesis of sesquiterpenes in *B. cinerea* have been obtained and will be summarized in this communication.

O6.7 The cerato-platanin-like protein BcSPL1 is required for full virulence in *Botrytis cinerea* and is able to induce necrosis in plant tissues

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The *B. cinerea* protein BcSPL1 belongs to cerato-platanin family, whose members are small proteins showing phytotoxic activity. BcSPL1 is the eighth most abundant protein in the secretome obtained from *B. cinerea* shortly after germination, and has been found, to our knowledge, in every condition of all the studies about the *B. cinerea* secretome. Expression analysis of *bcspl1* were carried out by Q-RT-PCR under different conditions both in planta and in axenic culture, and we found expression in all media tested, with the highest levels found in infected tomato leaves. Expression was also studied for a second, quite similar, cerato-platanin gene found in the *B. cinerea* genome, which we have called *bcspl2*. In this case no significant expression was found in any of the media tested. We have generated two *bcspl1* mutants by gene replacement, and both have been characterized by PCR, Southern-blot, and 2D-electrophoresis. The two mutant strains show a decreased virulence on tomato and tobacco leaves, as well as in several other fruits and vegetables. However, the phenotype of the mutants was identical to that of the wild type in every other aspect studied: growth rates in different media, colony morphology, conidiation, etc. To study the effect of isolated protein BcSPL1 on plants, the gene was expressed in *Pichia pastoris* and the recombinant protein was purified to homogeneity. The isolated protein was able to induce clear necrosis symptoms when infiltrated in tomato, tobacco and Arabidopsis leaves, in just a few hours. Infiltration in leaves also caused the induction of the tobacco defence genes *PR1, PR5, HIN1* and *HSR203J*, assayed by Q-RT-PCR, of which the two latter are considered to be a markers of the hypersensitive response. Additional symptoms of hypersensitive cell death were also observed in tissues treated with BcSPL1: induction of reactive oxygen species, plasma membrane permeabilization and induction of autofluorescence.

P6.1 Galacturonic acid catabolism in *Botrytis cinerea*

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D-galacturonic acid (GalA) is the major component of pectin, which can be degraded by plant pathogens; GalA potentially is an important carbon source for microorganisms living on decaying plant material. For bacteria, a catabolic pathway of GalA has been described, which consists of five enzymes converting GalA to pyruvate and glyceraldehyde-3-phosphate. A different catabolic pathway is proposed in filamentous fungi. In *Hypocrea jecorina*, GalA is converted to pyruvate and glycerol via D-galacturonate reductase, L-galactonate dehydratase, 2-keto-3-deoxy-L-galactonate aldolase, and glycerol dehydrogenase.

The *Botrytis cinerea* genome contains a D-galacturonate reductase gene (*Bcgaa*A), a L-galactonate dehydratase gene (*Bcgaa*B), and a 2-keto-3-deoxy-L-galactonate aldolase gene (*Bcgaa*C). The three genes were cloned into a protein expression vector and the enzymatic activity was determined for each gene separately. The heterologous simultaneous expression of *Bcgaa*A, *Bcgaa*B, and *Bcgaa*C in an *E. coli ΔuxaC* mutant which cannot grow on GalA is performed to determine whether the catabolic pathway from *B. cinerea* can restore the growth deficiency in *E.coli*. Targeted gene replacement of *BcgaaA, BcgaaB, BcgaaC* or both *BcgaaA* and *BcgaaC* resulted in *ΔgaaA, ΔgaaB, ΔgaaC* mutants and *ΔgaaAC* double knock-out mutants that displayed significantly reduced growth when D-galacturonic acid was used as the sole carbon source. The mutants showed similar virulence as the wild-type stain B05.10 on tomato leaves, indicating that GalA is not the main carbon source for *B. cinerea* growth during infection on tomato leaves. The virulence will be tested on other pectin-rich plants and tissues.

P6.2 Investigation the role of *BcPIE1* gene in the virulence of *Botrytis cinerea*

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A macro-array transcriptomics study of the fungus *B. cinerea* during infection of *Arabidopsis thaliana* leaves lead to the identification of few genes showing strong expression in the early phase of the interaction (Gioti *et al.*, 2006). This expression pattern suggests that the products of these genes may play a role in the establishment of the fungus pathogenicity. One of these genes, *BcPIE1*, encodes a protein with unknown function, but whose sequence analysis revealed homology to the homeobox protein PAH1 of *Podospora anserina*. As transcriptional regulators, known fungal homeoproteins are involved in the control of various patterns of development like hyphal morphology, microconidiogenesis, pathogenicity and sexual cycle. The goal of our investigation is to gather information about the *BcPIE1* gene and its protein product, and to explore their role in the fungus virulence.

A *BcPIE1* disruption cassette has been constructed. Transformants deleted of the target gene are being sought whose phenotype, including their infection ability, will be analysed. *BcPIE1* expression will be studied in mycelium grown both *in vitro* and *in planta*; the selected *in vitro* conditions allow the collection of samples corresponding to early developmental stages of the fungus while the *in vivo* conditions allow that of samples corresponding to both early and late infection stages. In parallel, the localization of the BcPIE1 protein in the cell will be investigated via the study of a mutant strain producing a fluorescent fusion protein. Altogether, the collected data will contribute to a better understanding of the role of *BcPIE1* in the fungus physiology.

Gioti *et al.*, J. Mol. Biol. (2006) 358, 372–386

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P6.3 Preliminary study of a *Botrytis cinerea* extracellular β-xylosidase

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Xylan is the principal component of plant cell wall hemicelluloses. It is a heteroglycan composed of a linear chain of xylopyranose residues bound by β (1→4) linkages, with a variety of substituents linked to the main chain by glycosidic or ester linkages. These substituents are mainly acetate, methyl glucuronate and L-arabinofuranose residues, and the amount of each one depends on the source of xylan. The biodegradation of such complex polymers requires thus a set of enzymes, including endoxylanases (E.C. 3.2.1.8), which randomly hydrolyze the main chain of xylan, producing a mixture of xylooligosaccharides; β-xylosidases (E.C. 3.2.1.37), which liberate xylose from short oligosaccharides; and a variety of esterases and glycanases which hidrolize different substituents from the main chain.

B. cinerea, like other phytopathogenic fungi, secretes a high number of enzymes to the extracellular medium during the infection process to degrade the various plant tissues and defensive barriers. The endoxylanase Xyn11A has been previously shown to be required for full virulence in this organism despite its poor contribution to the secreted xylanase activity and the low xylan content of *B. cinerea* hosts. The main contribution of this enzyme to the infection process is to induce necrosis of the infected plant tissue, independently of its enzymatic activity.

Now, we have generated two knock-out mutants in a gene coding for a β-xylosidase, belonging to family 43 of the glycosyl hydrolases. The putative protein consisting of 520 amino acids shows features of a signal peptide for the first 21 residues, according to SignalP3.0. The mature enzyme has a molecular weight of 54.5 kDa and an isoelectric point of 5.1, and shows high similarity with a xylosidase:arabinofuranosidase from *Aspergillus clavatus* NRRL1. Both mutants were able to growth as the parental strain (B05.10) using xylan as carbon source on solid medium, but the mutation produced a slight decrease in growing on xylose. The extracellular β-xylosidase activity was determined for both mutants and for the B05.10 strain, inducing the cultures with xylose, and showed a decrease of almost 50% for the mutant strains.

Virulence assays with the two mutants are being carried out to determine the influence of this enzyme in the pathogenicity of the fungus.

P6.4 The methylammonium permease (MEP) gene family of *Botrytis cinerea:* expression and functional analysis

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Sensing the availability of essential nutrients, e.g., nitrogen, may constitute the initial signal that *Botrytis cinerea* perceives and that informs it about the presence of a susceptible host. Ammonium detection and transport into the fungal cells is carried out by members of a transmembrane ammonium transporter protein family, the methylammonium permeases (MEP/AMT family). In the yeast, *Saccharomyces cerevisiae*, low nitrogen availability leads diploid cells to grow as pseudohyphae. The yeast triple mutant diploid strain deleted for all the three *mep* genes identified in this organism (*Δmep1Δmep2Δmep3*) is unable to grown on low ammonium (<50 micromolar) agar and, similarly, the mutant cells fail to filament on such media. The growth defect is complemented by each of the two members of MEP/AMT gene family from *Ustilago maydis*, *ump1* and *ump2*. However, only the latter gene has also been shown to complement the pseudohyphal growth defect characteristic of the yeast mutant. In this plant pathogenic fungus, *U. maydis,* a change from yeast-like to filamentous growth is required to cause disease. The *U. maydis ump2* mutant has a budding cellular appearance when grown under nitrogen starvation conditions and colonies lack the filamentous appearance of wild type under the same conditions. This defect was shown to be complemented by the yeast *mep2* gene. The yeast Mep2 protein has been considered to act as the sensor for low ammonium availability and evidence suggests that it is associated with the signal transduction cascades leading to filamentous growth.

Three genes coding for MEP in *B. cinerea* (*bcmep1, bcmep2, bcmep3*) were identified and cloned, with the predicted proteins being most similar to MeaA, MepA and MepB, respectively, the three main ammonium permeases from *Aspergillus nidulans.* The expression patterns of the three *B. cinerea mep* genes during saprophytic growth in the presence of 1 mM NH_4^+ differed. *Bcmep1* was expressed at all developmental stages, with maximal expression during germination of conidia. *Bcmep3* expression was only detected after 20 h of culturing, when the amount of ammonium left in the medium is likely exhausted. Expression of *Bcmep2* could not be detected under the experimental conditions tested at any developmental stage. Expression analyses under higher and lower ammonium concentrations and at different pH conditions are currently being performed.

Functional analysis of the *Bcmep* genes is being carried out by two means. First, the three genes were over-expressed in the *U. maydis ump2* mutant. Interestingly the three genes, to varying degrees, restored filamentous growth ability on low ammonium concentrations, indicating that the three genes each complement the sensor function in *U. maydis*. Second, single mutants altered in each *Bcmep* gene and double mutants deficient in all pair combinations are being obtained by gene replacement. Alterations during saprophytic growth when cultured under different ammonium concentrations and pH conditions as well as during *in planta* growth will be investigated in the mutant strains. These expression and functional analyses will further elucidate the specific roles of each *B. cinerea* MEP in the physiology of this necrotrophic plant pathogen.

Session 7:
HOST-PATHOGEN INTERACTIONS 2: Plant defence

I7.1 Analyses of *Botrytis cinerea* defense phenotypes highlight the importance of studying the plant cell wall in plant-pathogen interactions

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The plant cell wall is a composite structure, consisting of polysaccharides, glycoproteins and phenolic compounds. Several microscopical and analytical techniques have been developed to study different aspects of plant cell wall biology, including its role during plant infection by pathogens. Indeed, the cell wall act as a physical barrier, protecting plant cells against a variety of biotic agents, or can counteract an infection event by expressing several factors able to inhibit pathogen degradative enzymes. Microscopy and analytical profiling allow us to study changes in the composition, structure and/or localisation of cell wall components before, during and after infection by pathogens.

Grapevine (*Vitis vinifera*) plants are susceptible to a wide range of plant pathogens, including *Botrytis cinerea*, which attacks the cell walls of leaves and berries by producing pectin degradating endo-polygalacturonases (PGs). To defend themselves, plants produce counteracting proteins, such as polygalacturonase-inhibiting proteins (PGIPs) which are known to inhibit, and possibly prevent plant infection by forming inhibition interactions with fungal PGs. To investigate PGIP mechanism(s) of action *in vivo*, tobacco and grapevine lines have been generated which stably overexpress a range of grapevine PGIPs. These lines show marked and significant increased resistance to fungal infection. Transcriptomic evaluation of the resistant phenotypes in comparison with the controls have been used as a profiling technology to study the defense mechanism underlying the observed phenotypes. One of the major outcomes of the transcriptomic analysis, when combined with detailed bioinformatical analyses and confirmatory molecular and biochemical tests was the result that in the absence of infection, PGIP overexpressing lines showed differential gene expression that suggested cell wall strenghtening in these lines. The analysis suggested that cell wall remodeling could be an important factor in resistant lines. To confirm this, different microscopical techniques, combined with biochemical and analytical chemistry techniques have been used to further characterise PGIP-specific resistant phenotypes in comparison with controls. Our present hypothesis is that PGIPs, apart from their well-known PG-inhibition function during pathogen infection, might form part of a mechanism preparing the plant prior to infection, possibly involving cell wall reinforcement. Progress towards confirming/clarifying this hypothesis will be presented. The importance of including cell wall analysis in the study of plant-pathogen interactions will be illustrated.

O7.1 Fine mapping of the *RBPG1* locus which controls the response to *Botrytis cinerea* endopolygalacturonases in *Arabidopsis thaliana*

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During infection, *Botrytis cinerea* secretes endopolygalacturonases (BcPGs) that are able to degrade pectin in the cell walls of host plants. Previous research has demonstrated that infiltration of purified BcPGs, produced in the yeast *Pichia pastoris*, into the leaf apoplast could cause tissue maceration, collapse and necrosis development in several plant species. However, not all BcPGs caused similar damage and also not all plant species responded similarly to the same BcPG. We investigated the natural variation in the response of *Arabidopsis thaliana* to four different BcPGs by infiltrating leaves of different *A. thaliana* accessions with the pure enzymes. Considerable variation in responses was observed, ranging from no visible symptoms to full necrosis of the entire infiltrated leaf area. Of the 47 *A. thaliana* accessions tested, Br-0 and Est-0 showed a significantly lower response to the infiltration with BcPGs than any of the other accessions tested. Chemical analysis of cell wall composition and architecture revealed several differences in sugar composition and modification between ecotypes Col-0 (sensitive) and Br-0 (resistant).

Quantitative trait locus (QTL) mapping was performed in a segregating F2 progeny from the cross between accessions Col-0 (total tissue collapse and necrosis upon BcPG infiltration) and Br-0 (no symptoms). A single QTL controlling the response to BcPGs was detected. The Br-0 allele at this locus is recessive and significantly diminishes the response to the BcPGs. The locus was designated RBPG1 and was positioned on chromosome 3 of the *A. thaliana* genome in a 12 cM interval, containing 366 predicted genes. Several new markers were generated based on the single nucleartide polymophisms (SNPs) between Col-0 and Br-0, and a new population was generated to further map the RBPG1 locus, which reduced the interval to 0.15 Mbp, containing 44 predicted genes. We studied the expression pattern of the candidate genes in Col-0 and Br-0 leaves. T-DNA insertion lines of the candidate genes in Col-0 background (sensitive to BcPGs) were infiltrated with BcPGs in order to find mutants that are resistant. We will present the latest progress in our attempts to identify the RBPG1 gene.

I7.2 Mechanisms of plant innate immunity to necrotrophic fungi

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The plant innate immune response is activated through a complex network of regulatory mechanisms. Intracellular disease resistance proteins and surface localized pattern recognition receptors perceive pathogen derived elicitors to active plant immune responses. Pathogens use effectors, toxins and other virulence molecules to suppress plant innate immunity. We study mechanisms of plant innate immunity to important group of fungal pathogens and their interactions with other response pathways. Crucial components of plant immune responses to pathogens were isolated and studied. Botrytis induced kinase1 (BIK1) is a receptor like cytoplasmic kinase that confers resistance to necrotrophic fungi but suppresses resistance to the bacterial pathogen Psudomonas syringae. Recent data show that BIK1 is involved in PAMP triggered immunity by acting downstream of multiple surface localized pattern recognition receptors. PAMPs (Pathogen associated molecular patterns) are molecular signatures characteristic of whole classes of microbial species, and their recognition plays a key role in innate immunity. In addition, the Arabidopsis RING E3 ligase HISTONE MONOUBIQUITINATION 1 is specifically required for defense against necrotrophic fungi. HUB1 shows very low basal expression but is induced at the site of fungal infection. The fungal PAMP chitin induces HUB1 expression suggesting a role in PAMP triggered immunity. Interestingly, HUB1 interacts with MED21 subunit of the Mediator, an evolutionarily conserved transcriptional co-activator complex. I will discuss how these and other proteins function in plant innate immunity to pathogens of different life styles.

I7.3 Ripening and rotting: Tomato fruit ripening and susceptibility to *Botrytis cinerea*

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Fruit ripening is an example of a developmental program that coincides with increased susceptibility to *Botrytis cinerea* (teleomorph: *Botryotinia fuckeliana*). Unripe fruit are largely resistant to *Botrytis*, that is, gray mold does not develop and the fungus does not expand in the tissue but ripe fruit are susceptible to decomposition by the growing fungal mass. In order to determine the mechanisms that limit *Botrytis* growth and infection in green fruit and facilitate gray mold decomposition of ripe fruit, we are identifying the plant functions activated when resistance or susceptibility are expressed and the fungal virulence activities deployed in successful and quiescent infections. Molecular and chemical regulators of fruit ripening have been used to determine how the regulation of ripening modifies susceptibility. The results have suggested that mechanisms that promote the susceptibility of fruit can be distinguished from ripening processes that result in palatable and nutritious fruit.

In tomato, fruit ripening is regulated independently and cooperatively by ethylene and transcription factors, including *NOR*, *RIN* and *CNR*. Mutations in *NOR, RIN, CNR* or interference with ethylene perception and production prevent fruit ripening; however, mutations in only some of the ripening regulators reduce susceptibility to *Botrytis*. We have shown that ripe fruit susceptibility to *Botrytis* depends on *NOR* but only partially on *RIN* or ethylene perception (Cantu et al., 2009). Thus, some, but not all, regulated ripening processes are linked to the increased susceptibility of ripening fruit.

Transcriptome analysis of healthy and *Botrytis* infected unripe and ripe tomato fruit using Affymetrix tomato microarrays has shown that inoculation of unripe fruit activates some fruit ripening associated gene expression changes and induces the expression of genes that are associated with plant responses to pathogens (Cantu et al., 2009). By simultaneously analyzing the *B. cinerea* and the infected unripe or ripe tomato fruit proteomes, 45 *Botrytis* proteins were identified; 32% of the *Botrytis* proteins detected in each infected tissue were the same regardless of the ripening stage, supporting the conclusion that *Botrytis* produces many of the same proteins during attempted infections of unripe fruit or successful infections of ripe fruit. However, in the proteome of unripe fruit inoculated with *Botrytis*, 21% of the 119 tomato proteins detected have pathogen response functions but only a small percentage of the infected ripe fruit proteome has biotic stress response functions. Thus, histochemical, transcriptome and proteome analyses demonstrate that green fruit respond to *Botrytis* by expressing processes that limit pathogen growth and expansion but, paradoxically, also respond to *Botrytis* by precociously expressing ripening genes, indicating that *Botrytis* promotes events that will eventually facilitate infections of ripened fruit. We have demonstrated that the endogenous disassembly of the polysaccharide matrix of the cell wall that occurs during uninfected fruit ripening is crucial for infection since without polygalacturonase and expansin expression during ripening, susceptibility is substantially reduced (Cantu et al., 2008). Thus, the plasticity of plant responses to *Botrytis* during development is demonstrated in green fruit where responses provide resistance before ripening is complete and in ripe fruit where susceptibility is promoted by some, but not all, regulators of ripening processes.

O7.2 Contrasted responses of *Botrytis cinerea* strains developing on tomato plants grown under different nitrogen nutrition regimes

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The nutritional status of a plant is known to influence its susceptibility to pathogens. In the case of *Botrytis cinerea* the role of nitrogen fertilization of various host plants on disease development appears to be variable. A study was carried out to characterize possible variability associated with strains and inoculum density of *B. cinerea* in its ability to infect leaf-pruning wounds and to develop stem lesions on tomato plants as affected by the nitrogen input.

To this end, one-month old plants were subjected to five differential fertigation regimes with nitrogen inputs varying from 0.5 to 20 $mmol.L^{-1}$ NO_3^-, all other major nutrient elements being kept constant, at the following levels: 11 $mmol.L^{-1}$ K, 3.5 $mmol.L^{-1}$ Mg, 3.5 $mmol.L^{-1}$ Ca and 1 $mmol.L^{-1}$ P. The pH was adjusted to 6 in each treatment by addition of H_2SO_4. Plants were grown under those regimes for 3-4 weeks prior to inoculation.

Six strains differing in their aggressiveness to tomato were compared. They all had similar reaction patterns *in vitro* in response to differential nitrogen levels. In tests on plants, overall disease severity was lower for all strains on plants with higher nitrogen inputs, regardless of inoculum concentration. However, differences among strains were observed in the effect of plant nitrogen nutrition on infection and on lesion expansion. Disease onset was delayed on all plants with higher nitrogen inputs, but the response was greater for strains with lower aggressiveness on tomato. The highest contrast among strains was observed with the colonization of stems. The daily rate of stem lesion expansion decreased with increasing nitrogen fertilization levels for the more aggressive strains, while it increased for the less aggressive strain.

Hypotheses to explain these results will be discussed in light of the possible physiological effects of nitrogen fertilization on nutrient availability for the pathogen in the host tissue and of possible production of defence metabolites by the plant.

O7.3 Increased susceptibility of non-ripening *Cnr* tomato fruit to *Botrytis cinerea* and the plant cell wall implications

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During ripening fruit become increasingly susceptible to *Botrytis cinerea* (teleomorph: *Botryotinia fuckeliana*). *Botrytis* does not easily colonize mature green (MG) tomato fruit but when the fruit become red ripe (RR), *Botrytis* rapidly penetrates the pericarp tissue causing extensive maceration. We have shown that fruit softening, a result of the disassembly of the fruit cell wall polysaccharide matrix caused by proteins produced by the ripening fruit and by the infecting *Botrytis* is an important contributor to susceptibility (Powell et al., 2000; Cantu et al., 2007, 2008).

The activation and progression of tomato fruit ripening depends on the interactive network of pathways regulated by transcription factors, including *RIN, NOR* and *CNR*, and by ethylene. Mutations in these transcription factors or in the production or perception of ethylene modify, severely delay, or inhibit ripening. However, *Botrytis* is able to grow on fruit from the non-ripening mutants *rin* and *Cnr,* but not *nor* mutant fruit (Cantu et al., 2009), demonstrating that some, but not all, ripening pathways contribute to susceptibility.

The *Cnr* (colorless non-ripening) mutation, a spontaneous epigenetic change (i.e. hypermethylation) in the promoter of the *SBP-box* (*SQUAMOSA* promoter binding protein-like) gene, results in fruit that fail to soften or ripen to a red color. Despite this substantial inhibition of ripening in the *Cnr* mutant, *Cnr* fruit are significantly susceptible to *Botrytis* at the MG stage, unlike wild type fruit and the other non-ripening mutant fruit analyzed.

The composition and structure of the *Cnr* fruit cell wall polysaccharide matrix is altered compared to wild-type fruit, resulting in reduced cell-to-cell adhesion and mealy textured fruit. The targets of *CNR* transcriptional regulation are not known, but changes in fruit cell wall polysaccharide disassembly activities and perturbed expression of other ripening related genes are consequences of reduced *CNR* expression (Orfila et al., 2001; Eriksson et al., 2004; Ordaz-Ortiz et al., 2009). We examined the expression of >12 fruit cell wall modifying, defense and ripening-related genes during *Botrytis* infections of *Cnr* fruit at developmental stages equivalent to MG and RR wild-type fruit of the non-mutant background, Ailsa Craig. We observed *Botrytis*-induced expression of specific hemicellulose polysaccharide modifying tomato genes, suggesting that *Botrytis* activates fruit functions that target the disassembly of the hemicellulose portion of the fruit cell wall. The hemicelluloses polysaccharides provide links between adjacent cellulose microfibrils. We analyzed the composition and abundance of polysaccharide subfractions of *Cnr* and wild-type fruit cell walls to identify the wall matrix components that are altered by the *Cnr* mutation and/or by *Botrytis* infection. The results identify enzymes, including xyloglucan transhydroxylases that are responsible for the cell wall alterations of infected *Cnr* fruit, and suggest that *Botrytis* infection is not only facilitated by the altered cell walls in *Cnr* fruit but also promotes cell wall modifications by inducing the expression of fruit and *Botrytis* genes whose products disassemble particular wall polysaccharides. Thus, we show that MG fruit resistance depends on *Cnr* and that fruit susceptibility to *Botrytis* is greatly influenced by the fruit cell wall architecture that is affected by *Cnr*.

I7.4 Priming of plant defenses against *Botrytis cinerea* by hexanoic acid treatment

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Botrytis cinerea is a ubiquitous necrotrophic pathogen with a broad host range that causes substantial crop losses. Environmental concerns have prompted governments to promote alternatives to the use of synthetic chemicals to reduce risks and impacts of pesticide use on human health and the environment. The development of novel resistance-inducing chemicals represents an alternative to protect crops against pathogens.

During the last years we have been working on strategies based on natural compounds and we have demonstrated that soil-drench treatment with hexanoic acid protects tomato plants against *B. cinerea*. Hexanoic acid acts as inducer of plant defenses by means of a primingmechanism. Upon infection, the oxylipin 12-oxo-phytodienoic acid (OPDA) and the bioactive molecule JA-Ile were significantly induced in treated plants. Hexanoic acid-induced resistance (Hx-IR) was blocked in the jasmonic acid (JA)-insensitive mutant *jai1* (a *coi1* homolog). In addition, callose deposition was primed and ABA acted as a positive regulator of Hx-IR by enhancing callose accumulation. To better understand the molecular mechanisms beneath hexanoic acid priming the effect on production of reactive oxygen species (ROS) was assessed by histochemical analysis of treated plants upon *B. cinerea* inoculation. In addition, hexanoic acid perception was analyzed by selected genes-corresponding T-DNA mutants in *Arabidopsis*. Hexanoic acid also protected tomato plants against the hemibiotrophic bacterium *Pseudomonas syringae*, indicating a broad-spectrum effect for this new inducer.

In conclusion, these results will shed light into the complexity underlying induced disease resistance. Hx-IR represents an attractive tool for the molecular characterization of the priming phenomenon and for the integrated pest management strategies with the advantage of being a natural compound, already found in many plants.

P7. 1 Genomics of the grapevine - pathogen interactions: *Botrytis cinerea* virulence factors and molecular mechanisms of induced resistance

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Grapevine (*Vitis vinifera*) is confronted to severe diseases mainly caused by phytopathogenic fungi and oomycetes. In this context, our aim is to have a better knowledge on the interaction between grapevine and two of its major pathogens, one necrotroph (*B. cinerea*) and one biotroph (*P. viticola*).

We use transcriptomic and genetic approaches to identify and validate the plant and fungal genes that are key determinants of the infection and defense reactions. On the plant side, the objectives are to decipher the molecular mechanisms involved in resistance toward a necrotroph *versus* a biotroph pathogen and to understand how natural defenses could be stimulated by a derivative of the beta 1,3 glucan laminarin (PS3). On the fungus side, the objectives are to identify *B. cinerea* genes that are essential during the infection process.

First analysis realized at 12 h post-treatment reveals that PS3 and laminarin triggered a common expression profile that is mainly independent of the JA and SA pathways. PS3 up-regulates genes that prepare plant cells to stress exposure. During *P. viticola* infection: PS3 primes a faster expression of genes involved in PAMP perception (PRR genes), grapevine defenses (PR genes) and resistance (R genes). It's also interesting to note that many SA-dependent defense genes are primed by PS3 during the *P. viticola* infection.

Candidate genes thus identified in grapevine and *B. cinerea* will be studied by reverse genetics in order to validate their function. To improve functional genomics in grapevine, different biotechnological tools were developed (antisense or over-expressing Gateway vectors) and regeneration of grapevine transgenic plantlets was optimized in collaboration with the grapevine transformation platform (J. Masson, INRA Colmar, France).

A better knowledge of pathogen infection cycle and grapevine defense mechanisms should allow the development of new control strategies based on: (1) Induction of grapevine resistance triggered by the stimulation of its natural defenses, (2) Identification of specific markers for infection and/or resistance as decision tools to reduce chemical treatments, (3) Identification of new infection-specific targets leading to the development of environmentally friendly fungicides with novel modes of action.

P7.2 The role of cuticle permeability in the defence response of *sitiens*, an abscisic acid deficient tomato mutant, against *Botrytis cinerea*

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Botrytis cinerea, a necrotrophic fungus with broad host range, causes significant losses in tomato greenhouse cultivation every year. We have previously shown that a timely production of hydrogen peroxide (H_2O_2) is involved in resistance to *B. cinerea* in the abscisic acid-deficient *sitiens* tomato mutant. Moreover, it was demonstrated that the fast and localized accumulation of H_2O_2, before the fungal spore penetration, leads to cell wall fortification in the epidermis, effectively arresting spreading of the pathogen (Asselbergh et al. 2007). To explain the rapid defence response in *sitiens*, we investigated the cuticle of the mutant, as it makes up the first barrier in the *sitiens-B.cinerea* interaction. Bessire et al. (2007) recently reported that a more permeable cuticle in an Arabidopsis mutant leads to a strong resistance to *B. cinerea.* They suggested that an increase in leaf permeability leads to the presence of antifungal compounds on the leaf surface. We compared permeability of wild-type and *sitiens* cuticle and found that *sitiens* was more permeable. Therefore, we assessed the correlation between cuticle permeability and resistance/susceptibility response in *sitiens* and wild-type to the necrotroph *B. cinerea.* Disease index and permeability index were calculated for leaf discs of the same leaf and the correlation between them was statistically analyzed. The results show that permeability varies in leaves of both genotypes in different developmental stages and that there is a significant linear correlation between permeability and resistance in *sitiens*, whereas in wild-type, no clear correlation between both characteristics was found. Furthermore, HPLC and mass spectrometry were performed to detect the nature of the compounds in leaf exudates of the two genotypes in a time course after inoculation. Using High Performance Anion Exchange Chromatography with Pulse Amperometric Detection (HPAEC-PAD), we observed one peak in the chromatogram of the infection droplets on *sitiens* leaf at 4 hpi, rising up in successive time points (8, 12 and 16 hpi), while in wild-type this peak was much less pronounced, particularly in early time points. In the MALDI-TOF (Matrix-Assisted Laser Desorption/Ionisation-Time of Flight) spectra, we were able to identify the presence of the plant defensive saponin alpha-tomatine (1034 m/z) and its *B. cinerea* detoxified form β1-tomatine (902 m/z). In addition, to determine any effective antifungal activity of the leaf diffusates, we incubated PDB (Potato Dextrose Broth) droplets on the leaf surface of *sitiens* and wild-type for 18 hours and monitored the germination rate of *B. cinerea* spores until all the spores were germinated; however, no difference in spore germination was observed. The fungistatic effect of leaf diffusates on hyphal growth of the pathogen was assessed by measuring the length of elongating germ tube at different time points. Clear difference was observed between *sitiens*-incubated and wild-type-incubated PDBs, showing a suppressed hyphal development in spores growing in *sitiens*-incubated medium. Moreover, we determined an abnormal hyphal structure in these samples, visible upon the spore germination, using microscopic approach. It seems that the more permeable cuticle in *sitiens* plant facilitates the release of an antifungal compound, presumably alpha-tomatine, with an efficient fungistatic effect. This hyphal-development suppression might either weaken the pathogenicity or give the plant an extended time to boost its defence reaction.

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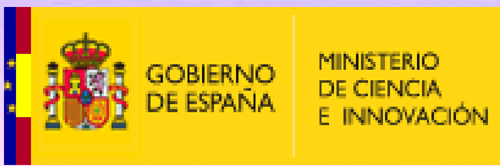
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