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# Towards a better understanding of *Botrytis cinerea* population genetics: how agricultural environments shape population structure and consequences for disease management.

Anne Sophie A. S. Walker

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Anne Sophie A. S. Walker. Towards a better understanding of *Botrytis cinerea* population genetics: how agricultural environments shape population structure and consequences for disease management.. 16th International Botrytis Symposium,, Jun 2013, Bari, Italy. hal-01190680

**HAL Id: hal-01190680**

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Submitted on 5 Jun 2020

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in Agricoltura "Basile Caramia"

On behalf of



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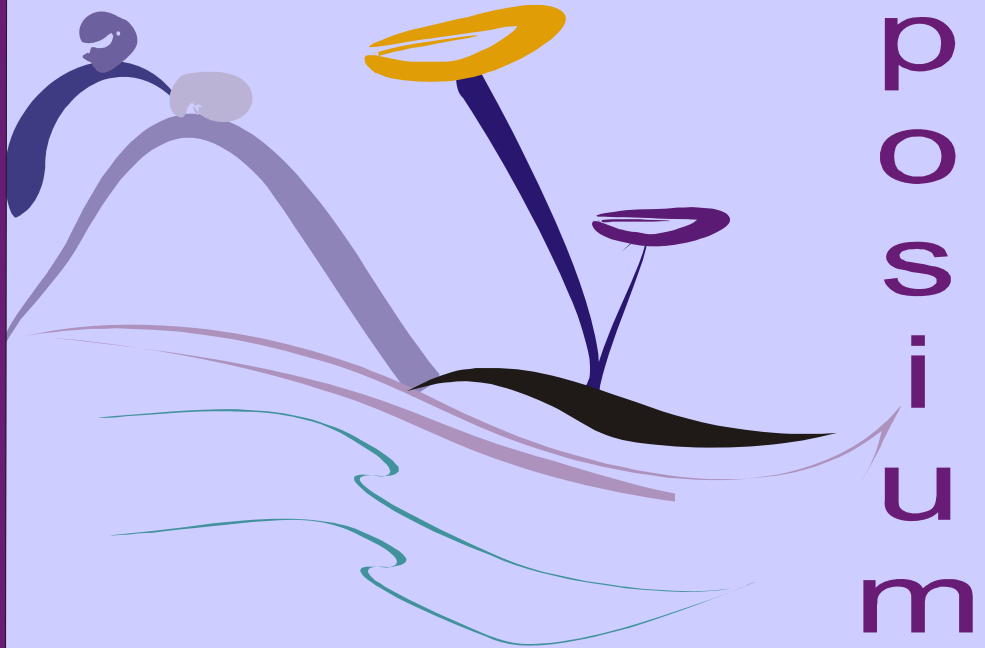
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XVII International

# Botrytis



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Abstract book



June 23-28 2013 Locorotondo (BA), Italy

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## **Welcome to the XVI International Botrytis Symposium**

The XVI International Botrytis Symposium has been organized by the Section of Plant Pathology of the Department of Soil, Plant and Food Sciences of the University "Aldo Moro" of Bari (Italy) (DISSPA) in cooperation with the Research, Experimentation and Education Centre in Agriculture "Basile Caramia" (CRSFA).

The Symposium will be held from 23 to 28 June 2013 in Locorotondo (Bari, Italy), a small and old town looking out over the Itria Valley (Valle d'Itria), one of the most beautiful landscapes and touristic attractive areas all over the Puglia region (Southern Italy).

Like in the past meetings, the Symposium will bring together scientists representing a broad spectrum of countries, topics, research approaches, crops, and agricultural practices all over the world. Skilled and young researchers with different backgrounds and interests, all focused on Botrytis and related fungi, will share knowledge, expertise, and ideas.

The XVI International Botrytis Symposium is based on invited key-note lectures, short oral presentations and posters concerning all the most relevant aspects of Botrytis and related fungi: identification, management, ecology and epidemiology, biology, genetics, chemical and biological control, post-harvest management, -omics, post-genomics and host-pathogen interactions. The Scientific Committee and the Organizing Committee of the Symposium did all their best to combine an exciting scientific programme with the pleasant and friendly hospitality, excellent foods and wines, beautiful landscapes, and historical traditions characterizing the Itria Valley and the Puglia region.

The Organizing Committee

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## Session 1

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# Botrytis, Industry and the Food Chain

*Chairperson:*

**Masato Soma**

*Sumitomo Chemical Agro Europe s.a.s., France*

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## KN.1.1 *Botryotinia fuckeliana* (*Botrytis cinerea*): a review on worldwide economic impact on crops and pest management

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*Botryotinia fuckeliana* (de Bary) Whetz. (an. *Botrytis cinerea* Pers.) is considered the second most significant fungal pathogen in molecular plant pathology based on both scientific and economic impacts (Dean R. *et al.*, Molecular Plant Pathology, 2012, 13 (4), 414-430) and can infect more than 200 plant species. It is one of the major causes of both qualitative (taste, aroma, oxydasic casse in wine) and quantitative (yield losses in fruit and vegetable crops and ornamentals) degradation; its economic impact is mainly on wine- and table-grape, solanaceae (tomato, pepper, eggplant), cucurbits, strawberry, lettuce and other salad vegetables, beans, bulbs and ornamentals.

The actual economic impact is not easy to estimate, because of the broad range of host crops. However, the global cost of crop protection against the pathogen has been estimated around \$ 310 million (Phillips McDougall, 2011), mainly in France and Italy for grapevine and China and Japan for vegetables; other important countries are Spain, Chile, U.S.A., Germany, Australia and The Netherlands. According to an estimation reported by the Genoscope website (Genoscope - Centre National de Séquençage, France; [http://www.genoscope.cns.fr/spip/Botrytis-cinerea-estimated-losses.html?var\\_recherche=botrytis](http://www.genoscope.cns.fr/spip/Botrytis-cinerea-estimated-losses.html?var_recherche=botrytis)), *B. fuckeliana* is responsible for about 20% of the host crop losses and their cost is estimated at € 10-100 billion per year.

During the last 30 years, agrochemical companies have provided the market with fungicides having different modes of action. Sumitomo Chemical contributed with the dicarboximide procymidon (1976), the N-phenyl-carbamate diethofencarb (1987) and, recently, with amino-pyrazolinone fenpyrazamine (world premiere in Italy in 2012). Beside these, the main Botryticide families are: benzimidazoles, thiophanates, phenylpyrroles, anilino-pyrimidines, 2,6-dinitroanilines, hydroxyanilides and SDHIs.

In the last decades, an increasing interest in biological control led to an in depth study of several biological control agents (BCAs). *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Aureobasidium pullulans*, *Trichoderma harzianum*, *Ulocladium atrum*, *Pichia guillermondi*, *Bacillus mycooides*, *Clonostachys rosea*, *Fusarium semitectum*, *Saccharomyces cerevisiae* and *Candida sake* are only few examples. Some of them have already been introduced to the market by crop protection companies.

*B. fuckeliana* is well known for its relatively easy acquisition of resistance to fungicides. Thus, in addition to the continuous effort to discover and develop new fungicides with new modes of action, integrated pest management using suitable anti-resistance strategies is needed to satisfy market demands in terms of high quality, food safety, low residues and sustainability of production processes. An overview of grey mold control throughout the world will be described, focusing also on the new challenge of fungicide resistance management.



## **P.1.1 Reduction of *Botrytis* incidence in long journey chains for Rose**

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*Botrytis* infection is considered the number one disease affecting floral crops (e.g., rose, gerbera and eustoma) resulting in huge uncalculated economic losses. *Botrytis* infection begins with deposition of conidia during flower development and the disease symptoms are visualized later on the petals as small quiescent lesions. If the conditions are favorable (very humid, >93% RH), then the lesions become necrotic and infect the petals. Production areas of cut flowers and consumer markets are distant. Thus, roses from South America are transported to USA or Europe, roses from Kenya and Ethiopia are transported via Western Europe to Eastern Europe – and Asian countries. Due to sustainability issues and lower costs, a lot of focus is currently being placed on flower transport by sea containers. Even though, long transport is executed at low transport temperatures *Botrytis* development still threatens the successful transport execution to distant markets.

The aim of this study was to assess the impact of dry and wet transport to distant markets at low temperatures of three rose cultivars. The effect of dry and wet transport on *Botrytis* development during the distribution and distribution plus retailer chains were evaluated. The impact of dry and wet transport on vase life, the effect of transport time, cultivar and temperature on *Botrytis* development and further vase life were also evaluated.

Three rose cultivars ‘Red Naomi!’, ‘Aqua!’ and ‘Avalanche+’ were tested. Long journeys at low temperatures for reefer transport were simulated. Thus, roses were dry or wet stored for 4, 7, 10, 14 and 21 days at 0.5°C and for 7 and 14 days at 2°C and RH of 90-95%. Flower opening stage (1-5 scale), visual symptoms of *Botrytis* (0-4 scale), number of bent necks, wilted flowers and number of stems with flower damage and leaf damage were determined. Quality assessment was carried out at the start of vase life (day 0) and after 7 days of vase life.

Results showed that the type of transport (dry or wet), transport time and cultivar influence the *Botrytis* damage level, affecting the number of flowers in poor condition at the start of vase life. Dry transport of roses was significantly better to reduce *Botrytis* development during transport and subsequent vase life. Longer transport times resulted in increased *Botrytis* development. The ‘Red Naomi!’ variety was the most sensitive cultivar to *Botrytis* development.

In conclusion, this study shows that transport of roses in water or wet transport at low temperatures is not necessary and even can have a negative impact on the quality of the product compared to dry transport. These results showed that *Botrytis* development can be substantially reduced in three varieties of roses by using dry transport at low temperatures. Transport and handling costs in addition can be substantially reduced

## P.1.2 *Botrytis* bunch rot impact on phenolic and sensory quality of Merlot grapes, musts and wines

Bénédicte Lorrain<sup>#1</sup>, Isabelle Ky<sup>#1</sup>, Marc Fermaud<sup>2</sup>, Michael Jourdes<sup>1</sup>, Grégory Pasquier<sup>1</sup>, Jean Roudet<sup>2</sup>, Laurence Gény<sup>1</sup>, Patrice Rey<sup>2</sup>, Bernard Donèche<sup>1</sup> and Pierre-Louis Teissedre<sup>1</sup>

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This study was aimed at defining an acceptable grey mould infection threshold level below which phenolic and sensory quality of Bordeaux red wines remain unaffected. The pathogen effect on phenolic composition of Merlot grapes was quantitatively assessed, notably for proanthocyanidin and anthocyanin composition. After wine making, the impact of different percentages of *Botrytis*-affected grapes (0, 5, 10, 15 and/or 20%) was assessed on the chemical, phenolic and sensorial quality of derived musts and wines.

In two seasons, 2009 & 2010, grey mould development was assessed in the experimental vineyard. In 2009, under dry summer conditions, a low disease incidence at harvest (23 Sept.), *i.e.* 7%, led us to artificially inoculate with *B. cinerea* healthy mature berries (“mat” incubated 8 days). On 21 Oct., over-matured berries, naturally infected by the pathogen (“overmat”) showed a mean disease age of 9 days. In 2010, conducive climatic conditions led, at maturity (3 Oct.), to a mean disease incidence of 23% associated with a mean disease age of 4 days for infected berries.

At maturity or overmaturity, in skins of rotted berries (naturally or artificially infected), the concentrations of all the phenolic compounds decreased drastically. These compounds included anthocyanins and proanthocyanidin monomers, dimmers and trimmer. The mDP, *i.e.* mean degree of polymerization, of the proanthocyanidin polymeric fraction was also affected in these skins. Chemical analyses of musts and wines made with different percentages of rotted berries showed a moderate pathogen impact on their phenolic composition. In the must, the anthocyanin depletion rate was higher in 2009 (“mat”) than in 2010. This would confirm that recently contaminated grapes may show a lower laccase activity than older rotted berries.

Importantly, a significant loss of quality in wines containing rotted grapes was evidenced by sensory analyses from the threshold as low as 5% of botrytized berries upward. This depreciation was mainly due to off-flavours, *i.e.* organoleptic deviations, such as “damp earth”, “vegetal/herbal like”, “mushroom”. Moreover, in botrytized wines (2010), a significant decrease in astringency and in the mDP was perceived, confirming that molecular size of proanthocyanidins could affect their bitterness and astringency.

In conclusion, *B. cinerea* drastically affects the phenolic and organoleptic properties of grape skins and derived wines. The phenolic variations and the resulting negative impact in grapes, associated musts and wines may be related to the *B. cinerea* laccase oxidative activity which may vary according to both severity and age of grey mould symptoms. Thus, prophylactic measures at early stages in the vineyard, evaluation of the sanitary status of the harvested grapes and berry sorting are crucial, even under low disease pressure.

Ky *et al.*, 2012. Assessment of grey mould (*Botrytis cinerea*) impact on phenolic and sensory quality of Bordeaux grapes, musts and wines for two consecutive vintages, *Australian J. Grape Wine Res.* 18, 215-226. # Both authors contributed equally.



## Session 2

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# Identification, Ecology and Epidemiology

*Chairpersons:*

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**Jaime Auger**

*Laboratorio de Fitopatología Frutal, Facultad de Ciencias Agronómicas, Universidad de Chile*

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## KN.2.1 An overview of *Botrytis* spp. populations causing gray mold in table grapes, kiwifruits and blueberries in Chile

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Gray mold (*Botrytis* spp.) causes considerable economic losses on table grapes (*Vitis vinifera*), kiwifruits (*Actinidia deliciosa*), and blueberries (*Vaccinium* spp.) in Chile, due to favorable environmental conditions and widespread planting of susceptible cultivars. Despite the economic impact of gray mold in these crops, until recently, there was little information about the predominant *Botrytis* spp. (*Bc*) populations in Chile.

The first study was conducted to evaluate the genetic diversity of *Bc* and to investigate the frequency of *Bc* genotypes in 67 isolates randomly collected from the most important fruit crops (grapes, tomato, kiwifruits, and blueberries), based on the detection of transposable elements. Lately, during the course of the different studies, significant differences in *Bc* genotype frequencies were observed among the host samples.

From table grapes (cv. Thompson Seedless), 457 isolates were recovered from three different Chilean growing zones and characterized genetically. In all locations and during all phenological stages, *transposa* isolates were the most common genotype (93.6%), followed by *boty* (4.9%) and *vacuma* (1.5%). In other studies *flipper* were detected only in flower remainders.

From kiwifruit, 279 single spore *Bc* isolates recovered from 'Hayward' flowers and fruits. *Vacuma* was the predominant genotype during blossom (36%) and post-harvest (69.2%); *transposa* had low frequency during blossom (20%), it increased at harvest (84%) but was only 7.7% at post-harvest. *Flipper* isolates had a low frequency in all the analyzed period, except for the pea size stage (24%), and *boty* isolates were only recovered in a high proportion during blossom (32%), but not detected at post-harvest.

By the other side, in a recent study *Bc* isolates were collected from flowers and fruits of two blueberries varieties (Legacy and Brigitta) (n=189), located in the three major growing zones of this crop in Chile. *Vacuma* genotype was predominant (57.4%) and 83.5% of the isolates corresponded to group II (*Botrytis cinerea sensu stricto*), independent of the variety, phenological stage and the growing zone. On group I (*Botrytis pseudocinerea*, 16.5%), the highest proportion corresponded also to *vacuma* genotype (71.4%) and the remaining percentage was only *boty*.

The results of the current report strongly support previous finding that the pathogen's host shapes its genetic structure, despite the absence of strict host specificity. The knowledge of population genetic structure on the attacked hosts is fundamental for developing more effective and rational control strategies.

## KN.2.2 Not a necrotroph but an endophyte? Implications for the epidemiology of *Botrytis cinerea*

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Many *Botrytis* species pass extended periods in quiescent or endophytic growth, without causing external symptoms. The majority of these have restricted host ranges, such as *B. allii* or the recently discovered *B. deweyae* in *Hemerocallis*. In contrast, *B. cinerea* has an exceptionally wide host range and is damaging to crops during aggressive necrotic growth. However, there are several routes by which this phase of growth may arise.

The aggressive spread of established mycelium between neighbouring plants or tissues is best seen as mycelia growth. Aside from this it is possible to provide evidence for a number of successively more endophytic life-cycles. (1) Simple spore to necrotic lesion to sporulation. In most hosts this requires both a wound or senesced tissue and a nutrient source, and possibly multiple spores. This would be typical of stem lesions in tomato originating at pruning wounds, for example. (2) Spore infection leading to a quiescent phase in a small number of dead host cells, followed by necrotic spread and sporulation when plant defences are reduced or hormonal signals change. This has been shown in grape, for example. (3) Spore infection of lightly defended tissues such as tepals followed by endophytic growth to fruits in which latent infection is established, followed by necrotic spread and sporulation as the fruit ripens. Blackcurrant and strawberry are examples. (4) Spore or seed infection followed by systemic endophytic growth through root, stem and new leaves, converting to widespread necrotic growth on plant maturity - presumably as plant defences weaken. This has been demonstrated in *Primula*, lettuce and other species. (5) There is some evidence that flower tissues in a systemically infected plant may give rise to infected seed, implying a potentially fully endophytic life-cycle.

Many, but not all, apparently healthy host species sampled in the natural environment are found to carry extensive infection, inaccessible to surface sterilization, by *B. cinerea* in leaf, stem and root tissues. Co-existing species may have zero and high levels of infection, further suggesting that this does not simply reflect the ubiquity of *B. cinerea* spores. Internal root, stem, or leaf infection is common in wild growing *Taraxacum officinale*, *Senecio vulgaris*, *Centaurea nigra* and *Achillea millefolium* (Asteraceae) but was completely absent from an 18-month old *Gerbera* crop nearby. In *Lactuca sativa* (Asteraceae), *Cyclamen persicum* and *Primula × polyantha* (Primulaceae) there is leaf, root, stem and seed infection, possibly from generation to generation. However, leaf or stem infection of healthy *Rubus fruticosus* agg., *Sorbus aucuparia*, *Fragaria × ananassae* and *Potentilla fruticosa* (all Rosaceae) appears rare or absent as does leaf, stem or root infection of *Bellis perennis* and *Tussilago farfara* (Asteraceae).

The bulk of *B. cinerea* endophytic infection at any one time is invisible; it only causes disease when the tissue it occupies dies. We see the fungus as a problem because we too want to eat plant parts without defences. Good *Botrytis* IPM means understanding the causes of damage and the epidemiology applicable to each crop. It may not be possible to eliminate infection – and non systemic fungicides will be ineffective on endophytic infection but it may be possible to avoid conditions which cause existing infection to become aggressive, to eliminate seed infection, and to encourage benign endophytes which prevent damage by *Botrytis* in otherwise favourable conditions.

## O2.1 Similar but not the same: fungicide resistance, genetic diversity, taxonomy and population dynamics of *Botrytis* spp.

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Repeated fungicide treatments are common to protect fruit and vegetable crops against grey mould. Consequently, there is a strong selection pressure on the *Botrytis* populations in the fields. In addition, the host plant, cultural practice and climatic conditions are believed to be important factors that determine population dynamics. In German strawberry fields, which are heavily sprayed against *Botrytis*, high resistance frequencies against all specific botryticides were observed. A novel, stronger efflux-mediated multidrug resistance phenotype, called MDR1h was discovered, caused by a small deletion in a drug efflux regulator. MDR1h strains often contained multiple target resistance mutations and seriously compromised fungicide control efficiency. Preliminary population studies indicated that all MDR1h isolates are derived from a single founder cell, and that they rapidly invade fungicide treated fields.

Multiple gene sequencing and whole genome sequencing revealed a surprising genetic diversity of *Botrytis* strains on strawberries. All MDR1h strains belong to a distinct group, called *Botrytis* group Sa, closely related to *B. cinerea* and *B. fabae*. PCR markers were developed for rapid identification of the main dicot infecting species (*B. cinerea*, *B. fabae*, *B. pseudocinerea*, *B. calthae*), and three new groups (Sa, Sb, Sc). Different patterns of occurrence of each *Botrytis* species or group were found, depending on host plant, fungicide treatments, time of season, and geographic region. While *B. cinerea* is common on many hosts, *Botrytis* group Sa strains are largely absent from vineyards, but found worldwide in strawberry fields. In German soft fruit fields, Sa strains showed significantly higher fungicide resistance frequencies than *B. cinerea* strains, and were found to be selected by fungicide treatments. In contrast, overall low resistance frequencies were observed for *B. pseudocinerea*. These results have practical implications and will be used for a better prediction of fungicide resistance frequencies and the development of anti-resistance management strategies against *Botrytis*.

A survey of several cultivated and wild host plants revealed different patterns of distribution of *Botrytis* species and groups. The host plants of the host specific *B. fabae* (*Vicia faba*) and *B. calthae* (*Caltha palustris*), were infected sometimes predominantly by broad host range species (*B. cinerea*, *B. pseudocinerea*, group Sa), and sometimes mainly by the specialists. Artificial infection tests confirmed different host preferences of the dicot-infecting *Botrytis* species and genotypes. In conclusion, grey mould populations are assemblages of diverse species and genotypes, with different host preferences and physiological properties. Comparative genomics approaches will be used to uncover the molecular basis of the differences in host specificity in *Botrytis* spp..

Leroch *et al.*, 2013. Appl. Environ. Microbiol., 77, 2887-2897.



## O.2.2 Use of Lateral Flow Devices for the estimation of *Botrytis* antigens in dessert wines

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The presence of *Botrytis cinerea* (Noble rot) is desirable for the production of late harvest desert wine. The amount of *Botrytis* present influences the sensory and chemical properties of the resulting wine. However, quantifying the amount of *Botrytis* present and differentiating *Botrytis* from other fungi that are present on the surfaces of mature fruit is problematic. This study investigated two commercially available lateral flow devices (LFD, immunochromatographic devices) for the detection of *Botrytis* antigens in wines, one produced by EnviroLogix, Portland, ME, USA and the other by Forsite Pocket Diagnostics, York, UK. Both devices employ the same *Botrytis* monoclonal antibody, BC-12.CA4, that does not recognize other possible co-infecting or superficial fungi.

Twenty-seven dessert wines from Australia (from 1984 to 2009 vintages) and France (from 2001 to 2005 vintages) were examined in this study. A Californian Semillon dessert wine (Dolce, 2010 vintage) made from late-harvest grapes infected with *B. cinerea* was used as a standard. On completion the intensity of the test lines were determined using the respective custom made readers.

Both types of devices were easy to use. Tests were rapid (10 min). Results were comparable and repeatability was good. Historical data for the precise incidence of *Botrytis* in vineyards is generally lacking but based on circumstantial evidence and discussion with winemakers the levels of antigens detected in one set of Australian wines from one winery, correlated with the perceived amount of *Botrytis* in the vineyard (9 vintages over the period from 1984 to 2008). Future work will examine this technology in comparison to other measures of *Botrytis* estimation in vineyards such as visual assessments.

### O.2.3 A novel *Botrytis* species, *B. deweyae*, is associated with the ‘spring sickness’ foliar disease of cultivated daylilies (*Hemerocallis*)

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A number of distinct *Botrytis* species infect various petaloid monocotyledons. Typically these *Botrytis* pathogens are specialized to individual host genera and several of these species cause important damage to horticultural and agricultural crops. For example, *B. elliptica* (fire blight, lily blight) causes major damage to lily bulb (*Lilium*) crops and several *Botrytis* species infect *Allium* crops (such as onion), for instance *B. squamosa*.

We investigated a mysterious foliar disease of cultivated *Hemerocallis* (daylily) called ‘spring sickness’, which has never been confidently associated with a specific pathogen. Foliage developing in winter and spring affected by this disease typically shows symptoms of necrosis, chlorosis and distortion. This usually weakens the plant but sometimes results in death of the entire growing point. It is an emergent disease, having been first reported in the 1960s but has recently become more prevalent in cultivated hybrids.

After rigorous surface-sterilisation of infected leaf material, from samples taken over a period of 4 years, we isolated a non-sporulating filamentous fungus repeatedly. These isolates did produce melanised sclerotia. Given their morphology and that the symptoms of ‘spring sickness’ resembled some *Botrytis* infections of developing spring foliage of other monocotyledons, such as smoulder (*B. narcissicola*) on daffodils, we investigated this possibility. Strong positive results from tests with the *Botrytis*-specific monoclonal antibody BC-12.CA4 suggested these isolates were likely to be *Botrytis*.

Confirmation of their identity as *Botrytis* was provided by analysis of *ITS* sequence as well as 5 other sequences known to be phylogenetically informative at the generic level. Phylogenetic analysis demonstrated that these isolates constitute a distinct species most closely related to *B. elliptica* and *B. squamosa*.

The distinction of this new species was further demonstrated by studies of its morphology. Formation of diagnostic macroconidia was highly suppressed except under conditions of near-UV light with the exclusion of other spectra, and infected *Hemerocallis* material was not associated with macroconidia development. This explains why this species has remained cryptic until now and suggests that the diversity of *Botrytis* has been underestimated.

Pathogenicity assays on excised *Hemerocallis* leaf material and whole sterile *Hemerocallis* plantlets demonstrated that this species could rapidly induce leaf tissue necrosis and death of plantlets, supporting our assertion that *B. deweyae* is important in triggering development of ‘spring sickness’ symptoms. We speculate that continual in-breeding in cultivated *Hemerocallis* hybrid lines may have been important in triggering the emergence of this new disease, which may have rapidly evolved from an endophytic ancestor promoted by conditions created in cultivation.

## O.2.4 Aerobiology of *Botrytis cinerea* in raspberry, strawberry and grape: analysis of airborne inoculum progress curves.

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Analysis of disease progress curves is a common activity in quantitative epidemiological research and has been used to design and evaluate various disease management strategies. Similarly, for airborne diseases, the concentration of airborne inoculum when assessed at several times could be used to characterize epidemics and to design efficient control programs. To facilitate *Botrytis cinerea* monitoring, a new qPCR assay was developed. The assay was designed to accurately quantify *B. cinerea* conidia from air samples.

Airborne *B. cinerea* conidium concentration (ACC) was monitored two to three times weekly in strawberries, raspberries and grapes plantings in 2010 and 2011. A total of 22 airborne inoculum progress curves (IPC) were constructed and analyzed using curve descriptors derived directly from the data: the maximum ACC ( $Y_{max}$ ), ACC at the beginning of the flowering period ( $Y_f$ ), and area under the inoculum progress curves standardized for the epidemic duration (AUIPC<sub>std</sub>); and descriptors derived from fitting growth models:  $r_G$ ,  $r_E$ ,  $r_L$ ,  $r_S$ ,  $r_R$ ,  $r_{abs}$ , and  $m_R$ , which represent the rate estimated from the Gompertz, exponential, logistic, sigmoid, and Richards models, weighted mean absolute rate, and shape parameter from the Richards model, respectively and the time at which ACC was one half of the maximum ( $x_0$ ).

The structure of *B. cinerea* inoculum progress curves was examined by conducting multivariate principal components analyses. The dimensionality of the data was reduced into two principal components (PC) accounting for 86.50% of the variation (54.83% and 31.67% for PC1 and PC2, respectively). Regardless of the models, all descriptors derived from growth models were associated with the PC1 with factor loadings of 0.94, 0.68, 0.94, -0.83, 0.94, 0.94, -0.79, and -0.61, for  $r_G$ ,  $r_E$ ,  $r_L$ ,  $r_S$ ,  $r_R$ ,  $r_{abs}$ ,  $m_R$ , and  $x_0$ , respectively. All descriptors derived directly from the data were associated with the PC2 with factor loading of 0.91, 0.90, and for 0.79, for AUIPC<sub>std</sub>,  $Y_{MAX}$ , and  $Y_f$ , respectively. Based on PCA analysis, airborne inoculum progress curves monitored in strawberry and raspberry plots were distinct from curves monitored in grape plots and characterized by AUIPC<sub>std</sub>,  $Y_{MAX}$ , and  $Y_f$  varying from 42.8-40.5, 942-753, and 20-28, respectively, which were lower than for grapes with values of 166, 2966, and 132, respectively. Similarly, time to 50% of the maximum ACC was lower in strawberry and raspberry with values of 38.5 and 35.9 as compare to 49 days after the beginning of flowering, respectively. In conclusion, the airborne inoculum curves from strawberry and raspberry were different than curves from grapes.

Overall, IPC from strawberry and raspberry crops were characterized by mean absolute rate ranging between 0.014 and 0.080 and AUIPC<sub>std</sub> ranging from 12.30 to 98.39 while IPC monitored in grape crops were characterized by lower mean absolute rate with values ranging between 0.006 to 0.012 and higher AUIPC<sub>std</sub> with values ranging from 50.95 to 335.14. The low but constant progress of *B. cinerea* airborne inoculum observed in the grape crops is suggestive of successive infection-sporulation cycles.

## O.2.5 Comparison of methods for quantification of botrytis bunch rot in white wine grape varieties

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Botrytis bunch rot (BBR), caused by the fungus *Botrytis cinerea*, is one of the most economically damaging diseases in wine grapes in Australia and New Zealand. Although quantification of BBR is important for both grape growers and wine makers, the widely used method for visual estimation of BBR severity is time consuming and can be highly subjective.

The reliability of the visual estimation method was tested by having participants from both research and industry backgrounds measuring BBR severity on 20 digital images of diseased bunches. Participants were asked to assess the same 20 images four months later in a random order and their results for the two assessments were compared. Various alternative quantification methods were compared with the visual estimation method using naturally infected white wine grape bunches. These methods included near-infrared spectroscopy (NIR), mid-infrared spectroscopy (MIR), quantitative PCR (qPCR) and specialised image analysis software (RotBot).

Differences between assessors for the visual estimation method were significant ( $P < 0.05$ ) but differences within assessors over time were not significant ( $P = 0.134$ ). Linear regressions showed significant relationships ( $P < 0.001$ ) between the visual estimation method and all alternative quantification methods.

Each method had advantages and disadvantages. The scanning of samples using NIR and MIR was quick and highly sensitive. NIR penetrates farther into the sample than MIR, potentially giving a more accurate assessment for samples that are not completely homogeneous; however, MIR is more sensitive. Sample preparation for both these methods was less labour intensive than the DNA extractions required for qPCR. Running the qPCR reaction also took longer than NIR and MIR scanning. One advantage of qPCR is that it quantifies *B. cinerea* DNA, which is directly related to fungal mass. It is unclear what NIR or MIR is quantifying and the relationship with BBR is purely empirical. The RotBot method required no sample preparation or expensive equipment and the software was able to batch-process images rapidly. However, this method only quantifies visible BBR symptoms.

In summary, the results obtained by the visual estimation method were found to vary significantly between assessors but not for the same assessor over time. All methods tested were found to be reliable, accurate and objective alternatives to the visual estimation method. The choice of method would depend on the intended purpose and whether the additional time needed to achieve higher accuracy is justified.

## O.2.6 Seasonal and regional variation of *Botrytis* in New Zealand vineyards

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Genetic diversity of *Botrytis* in New Zealand vineyards was surveyed over the period 2008 to 2012 from five wine growing regions. Isolates were gathered from symptomless flower buds immediately prior to flowering and, from the same vines, from diseased fruit at harvest. The isolates collected in spring represent the total genetic diversity of *Botrytis* associated with fruit in the vineyard, whereas the isolates collected in autumn comprise only that part of the *Botrytis* population that is associated with disease at harvest.

The species and transposon diversity found in New Zealand closely matched that reported from European vineyards. Both *B. cinerea* and *B. pseudocinerea* were present, with *B. cinerea* being the most common species. Within *B. cinerea*, isolates containing both the Flipper and Boty transposons were more common than those with only one transposon, or with neither.

Despite the genetic similarity between the New Zealand and European populations, and despite the fact that *Botrytis* produces huge numbers of air-borne spores, strong geographic structure to the New Zealand vineyard population was detected. *B. pseudocinerea* was common in the two Auckland vineyards sampled (about 14% of the isolates sampled at flowering) but infrequent elsewhere (between 0% and 2% of the isolates sampled at flowering). Within *B. cinerea*, isolates containing only the Flipper transposon were more common in the Auckland region (up to 22% of the isolates sampled at flowering) than elsewhere (between 0% and 4% of the isolates sampled at flowering); isolates containing only the Boty transposon were more common in the Waipara region (up to 54% of the isolates sampled at flowering) than elsewhere (between 0% and 9% of the isolates sampled at flowering).

There was also strong seasonal variation in the vineyard *Botrytis* diversity. Even in those vineyards where *B. pseudocinerea*, Boty-only and Flipper-only isolates were common at flowering, they were rarely detected in association with diseased fruit at harvest. These results suggest that *B. pseudocinerea*, and isolates with one or both of the Flipper and Boty transposons missing, are less capable of causing disease than *B. cinerea* or isolates with both transposons present.

To better understand the field observation of an apparent correlation between transposon type and pathogenicity, progeny of crosses between isolates containing different transposons are being examined for segregation of transposon type. Isolates containing a single transposon, both Flipper and Boty transposons, or neither transposon, are being tested for pathogenicity.

## O.2.7 Electrochemical biosensor for early detection of *Botrytis* infections in the field using immunological and PCR based identification techniques

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Internal defects of onions, particularly neck rot, caused by *Botrytis* species result in considerable losses in the field and during storage. A rapid, sensitive and reliable method to assess pathogen load of infected onions could help to prescribe an effective curing regime. Onion growers would then have the ability to predict and manage the storage potential of their bulbs. Hence, this research aimed to develop a biosensor for early detection and identification of *Botrytis* infections in onions.

An enhanced sensitivity electrochemical immunosensor based on a screen-printed gold working electrode (SPGE), with onboard carbon counter and Ag/AgCl pseudo-reference electrode was developed for the detection and quantification of *Botrytis* species. The sensor utilised a direct sandwich enzyme-linked immunosorbent assay (ELISA) format with *Botrytis* specific monoclonal antibody immobilised on the gold working electrode using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) / N-hydroxysuccinimide (NHS) activated thiolated self-assembled monolayer. A polyclonal antibody conjugated to the electroactive enzyme horseradish peroxidase (HRP) was then applied for signal generation. Electrochemical measurements were conducted using 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB)/H<sub>2</sub>O<sub>2</sub> as the enzyme mediator/substrate system.

The developed biosensor was capable of detecting latent *Botrytis* infections 24 h post-inoculation with a linear range from 1500 to 0.5 µg *Botrytis* per gram plant tissue and a limit of detection as low as 160 ng *Botrytis* g<sup>-1</sup>. The developed sensor format provides a new approach for simple and sensitive in-field and in-store detection of *Botrytis* infections caused by *B. allii*, *B. aclada*, *B. byssoidea* and *B. cinerea*.

The second stage of the development combined the biosensor with a DNA testing procedure in order to detect and discriminate between the *Botrytis* species. Genetic differentiation between the *Botrytis* species associated with neck rot was performed using PCR amplification and restriction fragments length polymorphism (RFLP) differentiation. Real time PCR was used to identify the pathogen-derived DNA with biotin labelled primers specific to *Botrytis* species causing neck rot. NeutrAvidin was immobilised on the gold working electrode as a binding molecule for the amplified pathogen DNA, followed by Streptavidin–Peroxidase polymer for signal generation.

The biosensor was able to detect pathogen DNA from *Botrytis* species associated with neck rot (*B. allii*, *B. aclada* and *B. byssoidea*) after PCR amplification. The DNA based procedure is now being optimised to combine the biosensor with PCR techniques for early detection and identification of *Botrytis* infections in the field.

## P.2.1 Characterization of *Botrytis cinerea* isolates present in forestry nurseries in Chile

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Gray mold is recognized as one of the main phytopathological problem in the world because the ability of the causal agent, the fungus *Botrytis cinerea*, to infect a wide range of host plants of economical importance. Thus, gray mold has been longer studied in fruits and in horticulture, however, it has also been described in woody plant species. In fact, *B. cinerea* is a serious fungal problem in forest nurseries, woody plants being particularly susceptible during the early stages of development. Despite the fact that gray mold is an important phytopathological problem in *Pinus radiata* D. Don and *Eucalyptus globulus* Labill. nurseries, the problem has been scarcely studied. Therefore, this work was conducted as a first approximation to obtain information about the genetic composition of the causal agent of gray mold in forestry nurseries in Chile.

For this study, *B. cinerea* isolates collected from *P. radiata* and *E. globulus* plants cultivated in two nurseries, 5 km apart from each other, located in Los Angeles city, Bio-Bio Region, Chile, were characterized in terms of: (i) determination of vegetative-incompatibility alleles (*Bc-hcb*) (ii) genotyping based on transposon (*Boty* and *Flipper*) detection, and (iii) identification of the most common mutations involved in resistance to fungicides. *B. cinerea* isolates from native plants located near the nurseries such as *Rubus* spp and *Aristotelia chilensis* were also included in the study.

A total of 55 isolates was collected and identified as *B. cinerea* Group II because all isolates carried the *Bc-hcb2* allele. This genetic group corresponds to a phylogenetic species within *Botrytis*, also referred to as *B. cinerea sensu stricto*. Genotyping based on the presence of transposons showed differences between isolates related to the plant host. Thus, *transposa* isolates (containing both transposons) were detected in *P. radiata* and *E. globulus* while *vacuma* isolates (containing neither transposon) were detected in all plants except *E. globulus*. Notably, *boty* isolates (containing just the *Boty* transposon) were detected at high frequency in all plant hosts. Based on transposon content, isolates collected from the four plant hosts were statistically different.

Finally, analyses to detect mutations involved in resistance to fungicides such as benzimidazoles, dicarboximides and QoIs also showed differences in the collected isolates. Isolates collected from native plant hosts did not carry any of the mutations analyzed, while those collected from *P. radiata* presented mutations involved in resistance to benzimidazoles only. Isolates collected from *E. globulus* were shown to carry mutations for all tree fungicides. These results are in line with fungicide control programs, which are more intense in *E. globulus*.

Thus, our results suggest that migration of *B. cinerea* from native plant hosts to nurseries, if it occurs, would not be the origin of gray mold. Migration from other commercial crops could be possible. *B. cinerea* isolates present in forestry nurseries may be adapted to those environments and this is a situation open for evaluation.

This work was supported by DGID-UNAB through a DI-18-11/R grant.

## P.2.2 Molecular identification of *B. cinerea*, *B. paeoniae* and *B. pseudocinerea* in peony (*Paeonia lactiflora*) associated to gray mold in Chile

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Peony (*Paeonia lactiflora*) is an ornamental plant introduced in Chile in the early 90s. Since then, this crop has gained importance, becoming today as one of the main exported flower of Chile. One of the most important phytosanitary problems of peony is gray mold caused mainly by *Botrytis cinerea*, however other species, such as *Botrytis paeoniae*, has been described. Because there are no previous reports in Chile, a phenotypic and molecular characterization was conducted to know the diversity of *Botrytis* spp. associated to peonies decay.

In this study, 17 monosporic isolates of the fungus were obtained from diseased peony leaves. The isolates were collected in seven locations across three regions in Chile. The phenotypic characterization was performed on PDA medium. Four isolates (IBx7, IBx9, IBx12 and IBx16) shared common characteristic which distinguish them from the rest, such as color and aspect of mycelium as well as a longer time required to produce conidia and sclerotia. Isolate IBx15 showed white mycelium and a high number of small sclerotia. Species were first determined through sequencing a region of the glyceraldehyde triphosphate dehydrogenase gene, homology searching and phylogenetic analysis. The later was done using the MEGA2.1 program and sequences related to seven *Botrytis* species were included. We found that 12 out of 17 isolates clustered with *B. cinerea*, while 4 out of 17 (IBx7, IBx9, IBx12 and IBx16) were closely related to *B. paeoniae* and one isolate (IBx15) showed to be related to *B. pseudocinerea*. Determination of the vegetative-incompatibility allele (*Bc-hcb*) confirmed the identification of *B. cinerea* and *B. pseudocinerea*. Therefore, three different *Botrytis* species are involved in gray mold of peony in Chile.

The isolates were characterized through genotyping based on PCR determination of transposons *Boty* and *Flipper*. Among the 12 isolates identified as *B. cinerea*, 7 were typed as *flipper* (isolates containing just the *flipper* transposon) which is a genotype scarcely reported in others plant host, while 4 isolates were identified as *transposa* (containing both transposons), a genotype quite common in other well studies crops such as grapes, and just one isolate was genotyped as *boty* (containing just the *Boty* transposon). This result suggests that *B. cinerea* isolates present in peony have a particular population structure. Regarding the four *B. paeoniae* isolates, 3 were *transposa* and one *boty*. The *B. pseudocinerea* isolate was identified as *flipper*. No *vacuma* isolate (containing neither transposon) was detected to be present in the studied isolates.

Finally, with the purpose to establish a specific, fast and reliable molecular identification of *B. paeoniae* the necrosis and ethylene-inducing protein gene (NEP1) was tested in order to develop a PCR assay. A pair of primers denoted as BpNP1-F41 and BpNP1-R378 designed on the NEP1 sequence showed to amplify a 470 pb band specific to *B. paeoniae* isolates, thus allowing an easy identification of the phytopathogen.



## P.2.3 Genetic and phenotypic study of *Botrytis* spp. isolates from Blueberry in Chile

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The Chilean blueberry production for export is affected by *Botrytis cinerea* (*Bc*). Losses associated to post-harvest *Bc* rot can be over 20%. According to previous studies, the most susceptible phenological stages in pre harvest period are: blossom, early and late harvest. In order to learn more about the *Bc* blueberry dynamic population and sensitivity levels of the pathogen to some fungicides (fenhexamid, iprodione, cyprodinil & fludioxonil and fludioxonil), during the 2011/2012 season, flowers or fruits were collected in blossom and early and late harvest, respectively, from two varieties (Legacy and Brigitta) in orchards located in the three major blueberry growing zones of Chile (Central Zone [Maule], Central – South Zone [Bío-Bío] and South Zone [de Los Ríos]). Sixty three *Bc* isolates were analysed for each variety, phenological state and growing Zone (total<sub>n</sub>=189). The detection of transposable elements (*boty/flipper*) was based on polymerase chain reaction with duplex-PCR and specific primers. Distinction between group I and group II *Bc* isolates was based on PCR-restriction fragment length polymorphism (RFLP) method. The sensitivity to fungicides was determinate by EC<sub>50</sub> values (myceliar growth) in Sisler or PDA medium, depending of the fungicide tested.

In the two varieties and three zones *vacuma* genotype was predominant (57.4%); *flipper* was detected occasionally during blossom (4%); and in general *transposa* and *boty* had low frequency (14.6 and 11.9%, respectively). *Boty* genotype was not detected in the Bío-Bío and de los Ríos Zones. 83.5% of the isolates corresponded to group II (*Botrytis cinerea sensu stricto*), independent from the variety, phenological stage and the growing Zone, with predominance of *vacuma* genotype (51.2%). On group I (*Botrytis pseudocinerea*, 16.5%), the highest proportion corresponded to the *vacuma* genotype (71.4%) and the remaining percentage was only *boty*.

Regarding the fungicide sensitivity assessment, results indicate that in both varieties (Legacy and Brigitta) and in the three growing Zones, the *Bc* populations behaved as slightly to moderately resistant to fenhexamid (EC<sub>50</sub>=0.485 µg·mL<sup>-1</sup>), slightly resistant to iprodione (EC<sub>50</sub>=1.198 µg·mL<sup>-1</sup>) and highly sensitive to cyprodinil & fludioxonil (EC<sub>50</sub>=0.045 µg·mL<sup>-1</sup>). In the Central – South and South Zones were detected few isolates slightly resistant to cyprodinil & fludioxonil. But all the isolates analysed behaved as highly sensitive to fludioxonil (EC<sub>50</sub>: 0.021 µg·mL<sup>-1</sup>).

These results are an important contribution to be considered in order to design a more efficient blueberry *Botrytis* rot control programs.

Supported by U. de Chile – Blueberry Committee, ASOEX A.G., 2011-2012 / Conicyt –Post-doctorate Fellowship 3120001.

## P.2.4 Genetic and phenotypic characterization of *Botrytis cinerea* Pers. in *Actinidia deliciosa* in the Chilean Central Valley

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Currently, Chile is the third kiwifruit exporting country at worldwide level. Due to the ongoing growth of the export production and in order to search better market opportunities, the volume of fruit stored at the origin has increased, hence the losses associated to *Botrytis cinerea* (*Bc*) rot has increased. *Bc* has a high intraspecific variation levels in the pathogenesis mechanisms.

In order to learn more about the *Bc* population dynamics in kiwifruit in Chile, a representative amount of isolates recovered from symptomatic flowers and fruits collected pre and post harvest in the Central Valley orchards were characterized. 279 single spore isolates were randomly selected and thus genotype correspondence was determined through the use of specific primers (*boty*/*flipper*). Then, 10 isolates of each identified genotype were characterized by: 1) morphologic appearance (myceliar / sclerotial), 2) sclerotial forming capacity, and 3) sporulation level at 20°C (Malt Agar medium). In addition, 4) the virulence of the isolates was evaluated in: a) cv. Thompson Seedless berries (16.5° Brix), wounded and unwounded (20°C for 96 hours) and b) ‘Hayward’ kiwifruits (7-8° Brix) only wounded inoculated and incubated at 20°C and 0°C for 17 and 60 days, respectively.

*Vacuma* (*boty*-, *flipper*-) was the predominant genotype during blossom (36%) and post-harvest (69.2%); *transposa* (*boty*+, *flipper*+) had low frequency during blossom (20%) and, increased at harvest (84%) but only 7.7% at post-harvest. *Flipper* (*boty*-, *flipper*+) isolates had a low frequency in all the analysed periods, except for the pea size stage (24%), and the *boty* (*boty*+, *flipper*-) isolates were only recovered in a high proportion during blossom (32%), but not detected at post-harvest. These results show that the *Bc* population composition in kiwifruit is notoriously different from the one described on Chilean table grape, where *transposa* is predominant in all phenological stages and *vacuma* is only seen at a lower proportion in early bloom.

The isolates phenotype behaviours results (1, 2, 3, and 4) showed that only difference ( $p < 0.05$ ) in the disease severity levels. In table grapes wounded berries, *flipper* isolates were the most severe ( $p \leq 0.05$ ), but not in unwounded berries ( $p > 0.05$ ). In kiwifruit incubated at 20°C, *vacuma* genotype isolates always were more virulent, although was no statistical differences; in inoculated fruit incubated at 0°C, *vacuma* and *boty* isolates were significantly more aggressive than *transposa* and *flipper* genotypes ( $p < 0.05$ ).

These results suggest a possible association between the genotype composition of *Bc* population present in kiwifruit and the rot damage level in post-harvest.

\*Agronomical Engineer Diploma Thesis, Facultad de Ciencias Agronómicas, Universidad de Chile; \*\*Conicyt –Post-doctorate Fellowship 3120001.

## P.2.5 Cultural and morphological characteristics of kazakhstani apple and pear fruits *Botrytis* isolates

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In present research agriculturally important for Kazakhstan fruits (apple and pear) were surveyed to identify the *Botrytis* fungi. The *Botrytis* species complex was successfully isolated from apple, pear, and peach fruits during last 3 years Grey mold isolates were also obtained from tomato, soya, strawberry, wine grapes and ornamental crops cultivated in Almaty region located in south-eastern Kazakhstan. Several pome (apple, pear) and stone (peach) fruits are believed to have originated from the area on the border of Kazakhstan and China. For this reason strains isolated from apple and pear fruits during winter storage time were most important.

All isolated strains were identified as three distinct species, namely *B. cinerea*, *B. mali* and *Botrytis* sp. a/v. Morphological characteristics were observed in 4 and 10 d old cultures. *B. cinerea* colony surface is gray, colony reverse looks pale with dark scattered discrete sclerotia of different size and shape. *B. mali* colony surface is grayish with brownish patches, colony reverse looks pale with dark concentric sclerotia. *Botrytis* sp. a/v appearance is characterized as white radial mycelium with rare aggregated sclerotia and abundant about 2-3 mm grayish mounds.

Under the light microscope *B. mali* strains demonstrate a multiple clustered conidiophores located at the same parental hyphae with botryose clusters of conidia. Mostly unicellular, hyaline to pale gray or brown, ovoid conidia are mean 6-8 x 6-18  $\mu\text{m}$ . Some hyphae are organized in chains consisting of syringe like structures with dark cell wall. These unusual structures supposedly serve as an additional mechanism to penetrate into host surface cells. Moreover, intact bacterial cells inside the fungal hyphae were observed regularly under the electron microscope observations. Molecular identification procedures and additional studies under the electron microscope of all strains and their variants are in progress.

## P.2.6 Genetic diversity of *Botrytis* in New Zealand vineyards

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Genetic diversity of *Botrytis* in New Zealand vineyards was surveyed over the period 2008 to 2012 from five wine growing regions. Isolates were gathered from symptomless flower buds immediately prior to flowering and, from the same vines, from diseased fruit at harvest.

Two species were found, *B. cinerea* and *B. pseudocinerea*. All of the *B. pseudocinerea* isolates detected were fenhexamid resistant. The distribution of *B. pseudocinerea* was structured geographically, common in the two Auckland vineyards sampled, infrequent elsewhere. However, even in the Auckland vineyards, it was rarely isolated from diseased fruit.

The presence of the Boty and Flipper transposons was assessed using a PCR-based method. Isolates with all four transposon states (Boty only, Flipper only, both Boty and Flipper (transposa isolates), no transposons (vacuma isolates) were found for both species. Both of the vineyards sampled in the Auckland region had relatively high numbers of Flipper-only isolates in samples taken at flowering; both of the vineyards from the Waipara region sampled had relatively high numbers of Boty-only isolates in samples taken at flowering. The large majority of isolates collected from diseased fruit at harvest contained both transposons.

The isolates collected in spring represent the total genetic diversity of *Botrytis* associated with fruit in the vineyard, whereas the isolates collected in autumn comprise only that part of the *Botrytis* population that causes disease at harvest. Our results suggest that *B. pseudocinerea*, and isolates with one or both of the Flipper and Boty transposons missing, are less capable of causing disease than *B. cinerea* or of isolates with both transposons present. Pilot scale pathogenicity testing using detached grapes in the laboratory, appear to confirm that transposa isolates are more pathogenic. *Botrytis* is known to form both latent and truly endophytic infections on some hosts and it is likely that the part of the population not associated with diseased fruit at harvest remains present in the vineyard, on fruit in symptomless infections.

Two distinct clades were resolved within *B. pseudocinerea*. Isolates in both clades share the fenhexamid-resistant phenotype, both have a similar geographic and regional distribution within New Zealand, and we accept both as *B. pseudocinerea*. Only one of these clades has been reported from Europe from grape. However, based on matching hsp60 sequences, the second New Zealand *B. pseudocinerea* clade could be the same fungus reported as an endophyte of *Centaurea* from Europe.

Phylogenetic diversity within *B. cinerea* in New Zealand was similar to that known from Europe, including the occurrence of isolates that appear to match genetically the recently reported *Botrytis* 'Group S'.

The taxonomic implications of this genetic diversity will be discussed.

## P.2.7 A new qPCR method for detection and quantification of *Botrytis pseudocinerea* in gray mold populations.

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The *Botrytis* species complex, including two characteristic species, *Botrytis cinerea* and *Botrytis pseudocinerea*, causes gray mold disease. These two species differ in their sensitivity to fungicides, especially to the sterol biosynthesis inhibitor fenhexamid, a hydroxyanilide. *B. cinerea* is naturally sensitive to fenhexamid (HydS), while, *B. pseudocinerea* is naturally resistant (HydR1). Despite fenhexamid pressure, *B. pseudocinerea* represents at maximum 10% of *Botrytis* populations. Current biological tests to quantify *B. pseudocinerea* in gray mold populations are laborious and time consuming. In order to get a better insight into the epidemic behavior of *B. pseudocinerea* (season and/or host preference), our objective was to develop a qPCR method to detect and quantify the *B. pseudocinerea* species.

For this new quantification method, we developed two couples of specific primers and Taqman probes, one to quantify the *Botrytis* species complex, the other to quantify specifically *B. pseudocinerea* within the same sample. As candidate genes we selected the gene encoding G3PDH to detect all *Botrytis* species and the gene *ms547* (RNA helicase) to specifically detect *B. pseudocinerea* based on a four nucleotide polymorphisms in four codons. The couples (primers + probes) were tested on DNA of pure *Botrytis* strains to validate specificity and amplification efficiency. Then, we applied both assays on mixtures of DNA with serial dilutions of *B. pseudocinerea*. Preliminary results show precise quantification and sensitivity until 2% of *B. pseudocinerea* in the mixture. Multiplex assays are underway to finalize the method, which will then be tested on spore mixtures prior to its utilization on natural gray mold populations.

# Biology and Genetics

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## KN.3.1 Molecular tools and approaches to understand *Botrytis cinerea* biology

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Since more than 100 years researchers are studying the biology of the grey mould fungus, first by precise observations and detailed descriptions of its appearance and distribution, extended by “classical” genetic approaches, and nowadays by using a set of highly developed molecular tools. An important aspect of molecular genetics in *B. cinerea* is the high efficiency of targeted gene inactivation which allows a rapid functional analysis of interesting genes; RNA-mediated gene silencing represents an important alternative if deletions are not feasible. The publication of the genome sequence has facilitated large-scale transcriptomic and proteomic approaches, and resulted furthermore in the rapid expansion of the number of functionally characterized genes.

Live-cell imaging techniques using fluorescent proteins (GFP, mCherry) for studying gene expression, protein localization and protein-protein interactions have been enabled recently by implementation of an expression system basing on the use of codon-optimized reporter genes (Leroch *et al.*, 2011) and the targeted integration of the constructs at defined gene loci (Schumacher, 2012).

Most of the genes analysed so far encoding e.g. components of signal transduction pathways, lytic enzymes, or enzymes involved in secondary metabolism, were chosen based upon a priori knowledge of their putative functions. Their targeted mutations were intended to illuminate their role in virulence and other phenotypes (“reverse genetics”). However, a “forward genetics” approach that comprises the screening of a mutant population for the phenotype of interest followed by the identification of the mutated gene is an excellent method for identifying genes that function in particular processes. Several years ago, Tudzynski and co-workers generated a mutant library by *Agrobacterium tumefaciens*-mediated transformation (ATMT) containing 2,367 mutants carrying random insertions of the T-DNA. A first virulence screen on detached tomato leaves resulted in the identification of 560 less virulent strains (Giesbert *et al.*, 2012). Recently, a gene (*bcltf1*) encoding a GATA-type transcription factor was identified as a new virulence-associated gene by this approach. BcLTF1 additionally exerts extraordinary functions in regulating light tolerance, light-dependent differentiation, the equilibrium between production and scavenging of reactive oxygen species (ROS), and secondary metabolism (*manuscript in prep*).

However, artificial mutagenesis in the laboratory is not necessarily required as *B. cinerea* field populations are characterized by high levels of variability in morphology and preferred mode of reproduction, virulence, spectra of produced (phytotoxic) compounds, and fungicide resistance. Thus, *VELVET* (*bcevt1*) was identified by combining classical and modern genetics approaches as the gene making the difference between *B. cinerea* isolates B05.10, T4 and 1750 (Schumacher *et al.*, 2012, 2013).

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## KN.3.2 Novel RNA mycoviruses in *Botrytis cinerea* and *B. porri*

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Two hypovirulent strains (BerBc-1 and CanBc-1) of *Botrytis cinerea* and one hypovirulent strain (GarlicBc-72) of *B. porri* were isolated and identified in China. Mycoviruses in these three strains were investigated.

Strain BerBc-1 was detected to harbor a RNA mycovirus, designated as *Botrytis cinerea* RNA virus 1 (BcRV1). The genome of BcRV1 is 8952 bp long. It was deduced to contain two overlapped open reading frames (ORFs), ORF I encoding a 1314-aa polypeptide and ORF II encoding 1338-aa polypeptide containing the RNA-dependent RNA polymerase (RdRp) with eight conserved motifs. The RdRp sequence of BcRV1 was found to be closely related to a few unassigned dsRNA mycoviruses (SsNsV-L, FgV3, FvV1, FvV2, PgV2, DsRV1 and PiRV3).

Strain CanBc-1 was detected to harbor a RNA mycovirus designated as *Botrytis cinerea* mitovirus 1 (BcMV1). The genome of BcMV1 was 2804 nt long and AU-rich (66.8%). It shared 95% nucleotide sequence identity with *Ophiostoma novo-ulmi* mitovirus 3b (OnuMV3b). However, BcMV1 appeared 472 nt longer than OnuMV3b. Mitochondrial codon usage revealed that BcMV1 contains one ORF encoding RdRp, which is 96% identical to the RdRp of OnuMV3b. BcMV1 infection resulted in generation of malformed mitochondria in hyphal cells of *B. cinerea*.

Strain GarliBc-72 was detected to harbor a bipartite mycovirus designated as *Botrytis porri* RNA virus 1 (BpRV1). The genome of BpRV1 comprises two dsRNAs, dsRNA-1 (6,215 bp) and dsRNA-2 (5,879 bp), which shared sequence identity of 62 and 95% at the 3'- and 5'-terminal regions, respectively. Two ORFs, ORF I (dsRNA-1) and ORF II (dsRNA-2), were detected. The protein encoded by the 3'-proximal coding region of ORF I showed sequence identity by 19–23% with RdRp encoded by mycoviruses in the families *Totiviridae*, *Chrysoviridae* and *Megabirnaviridae*. However, the proteins encoded by the 5'-proximal coding region of ORF I and by the entire ORF II lack sequence similarities to any reported virus proteins. Phylogenetic analysis showed that BpRV1 belongs to a separate clade distinct from those of other known RNA mycoviruses. Purified virions of ~35 nm in diameter encompass dsRNA-1 and dsRNA-2, and three structural proteins with 70, 80 and 85 kDa. Introduction of BpRV1 virions into the virulent strain GarlicBc-38 of *B. porri* generated a derivative strain 38T with reduced mycelial growth and hypovirulence. All these results suggest that BpRV1 is a novel bipartite dsRNA virus belonging to a new virus family.

### O.3.1 Characterization of phenotypic traits in *Botryotinia fuckeliana* according to membership into *vacuma* or *transposa* sub-populations

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*Botryotinia fuckeliana* (de Bary) Whetz. (an. *Botrytis cinerea* Pers.) has a great genetic variability and shows adaptation to various environmental conditions. Studies have been carried out to understand whether genetic characters are associated with phenotypic traits, but these studies are not yet conclusive. In this work, the association between genetic and phenotypic variability was investigated in strains distinguished by the presence of two transposable elements (TEs), namely Boty and Flipper, thus belonging to different sub-populations (TE types). The *transposa* sub-population (Boty+Flipper+) has both TEs, *vacuma* lacks both of them (Boty-Flipper-), and the two other TE types are Boty+Flipper- and Boty-Flipper+. Differently from the previous studies, the focus was on measuring many different phenotypic traits of few representative strains rather than considering few traits of a larger sample of strains.

The effects of temperature, relative humidity and water activity on colony growth on different media, production and germination of conidia were investigated using four *vacuma* strains, four *transposa* strains, one strain with only the Boty TE and one strain with only the Flipper TE. The strains had different geographic origin, and were obtained from the culture collections of the University of Bari, Italy, and from INRA (UMR-Save) in Villenave d'Ornon, France. The strain ability to cause infection on grapevine at different growth stages, from inflorescence emergence to berry ripening, was also tested by inoculating with either conidia or mycelium at different temperatures, wetness durations and relative humidity regimes.

The data were analyzed using multivariate analysis in order to study the similarity level of the different strains for the complex of the 53 phenotypic traits considered and how the grouping of strains, based on these phenotypic traits, matches their genotypic characterization. In a first step, the analysis was carried out considering colony growth, sporulation, germination of conidia, and infection efficacy separately; afterwards, all phenotypic traits were combined to characterize each strain by a multiplicity of traits.

Data showed very high variability between the strains for most of the phenotypic traits considered, including infection efficiency. The data analysis further investigates the putative relationships between the grouping of the strains, based on either single or combined phenotypic traits, and the TE type as well as the strain geographical origin. Based on these analyses, among the complex of the phenotypic traits, the results of berry infection confirmed the trend of a differential virulence related to the membership into the *transposa* or *vacuma* sub-population.

## O.3.2 Nitric oxide modulates gene expression and affects germination in *Botrytis cinerea*

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*Botrytis cinerea*, as a necrotroph, grows preferentially on senescent or dead tissues. However, it also infects healthy tissues and experimental evidence accumulate indicating that the fungus is able to modulate the plant defense responses and that it even benefits from the host programmed cell death occurring during the activation of the plant hypersensitive response. Reactive oxygen species (ROS) and Reactive Nitrogen Species (RNS) produced by the host are major key players orchestrating the complex set of plant defense responses. They create a hostile environment in which *B. cinerea*, however, survives. Functional analysis of fungal components involved in the response to high levels of ROS, and considered to be important for pathogenicity, suggest the fungus does not experience a strong oxidative stress *in planta*. Remarkably, it has been demonstrated that the fungus itself produces ROS that might contribute to the oxidative status *in planta*. In addition to their participation in pathogenicity processes, a more basic role related to the regulation of differentiation and virulence processes has been proposed for fungal produced ROS. Similarly, recent work carried out in our laboratory has demonstrated that *B. cinerea* possesses a nitric oxide (NO) detoxification system based on a single flavohemoglobin (BCFHG1). Although it represents the main, if not the only one, NO detoxification system in *B. cinerea*, and the mutants are more sensitive to NO, its elimination does not affect pathogenicity. The production of NO by the fungus both during saprophytic growth and during the interaction with the host has also been demonstrated, being this production regulated by the development stage of the fungus, the exposure to exogenous NO and the host. This regulated production of NO suggests that, although the enzyme detoxifies efficiently exogenous NO, 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.

In order to unravel the physiological processes in which NO participates in *B. cinerea* we have initiated a wide pharmacological, expression and functional analysis in response to exogenously added and to endogenously produced NO. Utilization of NO donors and of NO scavengers provided evidences indicating that NO affects germination. Transcriptomic analysis in response to exogenous NO shows that it modulates the expression of numerous genes, being the number of genes responding in growing mycelium larger than in germinating conidia. Approximately 10% of the genes encoding transcription factors (TFs) in the *B. cinerea* genome appear to respond to NO, with members of all the main groups of TFs being represented. One TF coding gene specifically induced in response to NO in mycelium, one responding specifically in germinating conidia and one induced when endogenous NO is removed, have been selected for functional analysis. In addition to genes encoding TFs, functional categories related to "Redox genes", "Stress response" and "Mycelium development" appear to be enriched in the NO up or down regulated genes. Representative genes have also been selected for functional analysis. Transcriptomic analysis of a  $\Delta Bcfhg1$  mutant made it possible to identify a number of genes responding both to exogenous and to endogenous NO, among them, interestingly, genes involved in polyamine biosynthesis. Taking together, the experimental data generated are indicative of NO modulating differentiation in *B. cinerea*, at least in some developmental stages. Information about genetic factors mediating these processes will be presented.

### O.3.3 The role of hydrophobins in sexual development of *Botrytis cinerea*

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Hydrophobins are small secreted proteins that play a role in a broad range of developmental processes in filamentous fungi, e.g. in the formation of aerial structures. Hydrophobins allow fungi to escape their aqueous environment and confer hydrophobicity to fungal surfaces. In *Botrytis cinerea* (teleomorph *Botryotinia fuckeliana*), one class I and two class II hydrophobin genes have been identified, as well as a number of hydrophobin-like genes. Previous studies showed that hydrophobins are not required for conferring surface hydrophobicity to conidia and aerial hyphae. We investigated the role of hydrophobins in sclerotium and apothecium development.

Nine hydrophobin mutants with MAT1-1 background, as well as a wild type MAT1-1, and wild type strain SAS405 (MAT1-2 background) were used in this study. To study the sclerotia development, sclerotia were cold treated in darkness for one month. For analyzing apothecial development, a reciprocal crossing scheme was set up.

RNAseq analysis of gene expression during different stages of apothecium development revealed high expression of the Bhp1 (class I hydrophobin) gene and of the Bhl1 (hydrophobin-like) gene in certain stages, whereas Bhp2 and Bhp3 (class II hydrophobins) were always expressed at very low levels. We characterized different hydrophobin mutants: four single gene knockouts, three double knockouts as well as a triple knockout. Sclerotia of  $\Delta$ Bhp1/ $\Delta$ Bhp3 (double knock out) and  $\Delta$ Bhp1/ $\Delta$ Bhp2/ $\Delta$ Bhp3 (triple knock out) mutants showed easily wettable phenotypes.

In apothecia development, morphological aberrations were observed in crosses with some hydrophobin mutants. When the  $\Delta$ Bhp1/ $\Delta$ Bhp2 (double knock out) and  $\Delta$ Bhp1/ $\Delta$ Bhp2/ $\Delta$ Bhp3 (triple knockout) mutants bearing a MAT1-1 mating type were used as maternal parents (sclerotia), and fertilized with microconidia of a wild type MAT1-2 strain, the resulting apothecia were swollen, dark brown in colour and had a blotted surface. Instead of growing upwards, the apothecia in some cases fell down. This aberrant apothecial development was not observed in the reciprocal cross, when the same mutants were used as paternal parent (microconidia).

In conclusion, our results indicate that hydrophobins Bhp1 and Bhp3 are important for normal development of sclerotia, whereas the presence of hydrophobins Bhp1 and Bhp2 in maternal tissue is important for normal development of apothecia.

### O.3.4 Noble rot in Hungary: genetic characterization of *Botryotinia fuckeliana* isolates from Tokaj and Eger wine-growing region

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The filamentous ascomycetous fungus *Botryotinia fuckeliana* (de Bary) Whetz. (an. *Botrytis cinerea* Pers.) is the causal agent of both the destructive grey mould (*pourriture grise*) as well as the noble rot (*pourriture noble*), an infection that results in highly prized, sweet, smooth, full-bodied, special quality wines with a most pleasant bouquet, a style of wine which has a long history in Hungary.

The development and evolution of noble rot in grapevine have several key conditions: (i) intact berries, (ii) adequate weather conditions (wet weather have to expose the fruit in full maturation, wet period following by dry, warm weather), (iii) appropriate grape variety with suitable properties. The increasing concentration of sugars and other organic compounds in the matured and infected berries will increase the osmotic pressure. These circumstances restrict the fungal growth and affect its metabolism with changing the activity of extracellular enzymes. Ideally hyphae grow only in the subcutaneous cell layers and there is just a minimal aerial mycelium on the surface of the berry or completely missing. The total weight of the noble rotted berries is half-fifths comparing to the healthy ones, and the amount of the must is minimal, but these entails with a very high quality improving. The characteristic aroma and flavors are the main values of the botrytised grapes, in addition to the increased sugar content.

In the course of our work we have investigated the genetic properties of *B. cinerea* populations isolated from noble rotted berries through three sequential years in two remarkable Hungarian wine growing region Eger and Tokaj. In the initial stage, 100 isolates of grapevine berry-growing *B. cinerea* from various locations of Eger wine region and 97 isolates of grapevine berry-growing *B. cinerea* from various locations of Tokaj were collected. Individual strains were obtained by single-spore isolation. Characterization of their genotype was done by analyzing MSB1 minisatellite sequences, which is located in the intron of the ATP synthase and  $\beta$ -tubulin. Data and sequences was analysed by phylogenetic methods.

In general, sequence analysis revealed a high degree of genetic diversity. The analysis of MSB1 and  $\beta$ -tubulin sequences indicated different patterns. The MSB1 pattern of Tokaj isolates was identical to those isolated from the Eger wine region, but were clearly different from the sequences detected by other authors. During the examination of  $\beta$ -tubulin sequences we found several alleles in either region.

A corresponding analysis of the fixation index revealed that there was differentiation between territories and we could also detect some differentiation between the different vintages and geographically isolated populations.

This work was supported by NKFP-A2-2006/0017 grant.

### O.3.5 Towards a better understanding of *Botrytis cinerea* population genetics: how agricultural environments shape population structure and consequences for disease management

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An understanding of the causes of genetic subdivision in pathogens is essential for forecasting disease emergence and implementing sound disease management strategies. We investigated population subdivision in the multi-host fungus *Botrytis cinerea*, by comprehensive multiyear sampling on different hosts (*Solanum*, *Vitis*, *Rubus*) in different cropping systems (indoor, outdoor) in three French regions and under two chemical control regimes in vineyards.

Population structure analyses revealed a clear differentiation between populations from indoor and outdoor environments, indicating that cropping system is a potentially powerful isolating factor in pathogens. Indoor and outdoor populations were significantly differentiated across sampling dates, with a quite higher magnitude than subdivision due to host plant, and to a lesser extent, to geography, in outdoor populations. The significant association between genetic differentiation and host of origin is consistent with host adaptation, but the distribution of inferred genetic clusters and the frequency of admixed individuals indicated a lack of strict host-specificity. Three genetic clusters coexisted on the same plant, *Vitis*. Linkage disequilibrium analysis indicated that recombination was not synchronous in the different clusters, suggesting a possible role of temporal isolation in genetic differentiation.

Among anthropic selective pressure applied in agricultural environments, fungicides constitute one of the most powerful determinants of population adaptation acting in a short time span. We found that fungicides treatments had no or little effect on population subdivision at neutral loci, as well as on diversity or reproduction mode. Nevertheless, we found evidence of stronger genetic drift in some treated plots, consistent with the regular application of fungicides. Moreover, we observed spatial structure in resistance frequency for two loci under contemporary selective pressure, as reflected by cline patterns. At last, using a modelling approach, we estimated fitness costs of resistance to fungicides, responsible for resistance frequency decay during winter. Further work is needed to estimate parameters of positive selection and migration exerted on *B. cinerea* populations, and to disentangle the relative effect of the evolutionary forces at work.

Our findings open up new perspectives for disease control by managing plant debris in outdoor conditions, prophylactic measures in greenhouses and by developing chemical strategies with limited effect on resistance evolution.

### **P.3.1 Vegetative compatibility and Botrytis virus X transmission during conidial anastomosis in *Botryotinia fuckeliana* (*Botrytis cinerea*)**

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Control of *Botryotinia fuckeliana* (de Bary) Whetz. (an. *Botrytis cinerea* Pers.) relies heavily on the use of fungicides since there are few host crops where resistant cultivars are available. There are several concerns with the use of fungicides including the emergence of fungicide resistant strains and fungicide residue on the edible part of the crop which is often consumed without further processing. These factors are making biological control agents an attractive candidate to add to the *B. cinerea* control tool-box.

Botrytis virus X (BVX) has some potential as a biocontrol agent for *B. cinerea* as it has been shown to reduce pathogenicity under certain circumstances. The only known mode of horizontal transmission of mycoviruses is via hyphal anastomosis which we demonstrated for BVX in *B. cinerea* by generating heterokaryons through anastomosis between infected and uninfected complementary nitrogen auxotrophic mutants. The progeny of the heterokaryon was then tested for BVX using RT-PCR. The newly infected host showed statistically significant reduction in sporulation, radial growth rate, and sclerotia formation when compared to the un-infected parent. However, *B. cinerea* has many vegetative incompatibility (VI) groups which are a potentially barrier to virus transmission, although this is suppressed in some fungi during conidial anastomosis.

To determine whether this is the case in *B. cinerea* a conidial anastomosis assay, optimized for imaging with fluorescence microscopy, was developed in order to study anastomosis of incompatible strains expressing different fluorescent proteins on a cellular level. We are using an available vector system with multiple antibiotic resistances and fluorescent protein markers which we have adapted for nuclear localization. These will enable us to differentiate between strains during the imaging of the fusion as well as identify heterokaryon formation. They will also enable us to easily select for the progeny of heterokaryons.

Results of conidial anastomosis between incompatible strains will be discussed.

### P.3.2 Functional analysis of genes in the mating type locus of *Botrytis cinerea*

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*Botrytis cinerea* is a heterothallic ascomycete with two mating type loci, *MAT1-1* and *MAT1-2*, each containing two genes. Besides the archetypal genes encoding the *MAT1-1-1* (alpha-domain) protein and the *MAT1-2-1* (HMG-box) protein, each idiomorph contains one additional gene, designated *MAT1-1-5* and *MAT1-2-4*, respectively. Homologs of these genes are only found in closely related taxa, and their function is as yet unknown.

Knockout mutants were generated in all four genes in the *B. cinerea* *MAT1* locus, either in the *MAT1-1* strain SAS56 or in the *MAT1-2* strain SAS405. Mutants were crossed with a strain of the opposite mating type, either the wild type or a knockout mutant, in all possible combinations.

Knockout mutants in the *MAT1-1-1* gene and the *MAT1-2-1* gene fail to show any sign of primordial outgrowth and are entirely sterile. This confirms the essential role of the alpha-domain protein and the HMG-box protein in the mating process. By contrast, mutants in the *MAT1-1-5* gene and the *MAT1-2-4* gene do produce stipes, but these fail to develop further into an apothecial disk. The *MAT1-1-5* and *MAT1-2-4* mutants show identical phenotypes, suggesting that these two genes jointly control the transition from stipe to disk development.

RNAseq data were obtained from a cross between two wild type strains and from a cross involving a *MAT1-1-5* knockout mutant, from tissue at the stage of transition from stipe to disk. Differential gene expression analysis was performed to identify genes that are possibly involved in development of the apothecial disk.

In conclusion, our study revealed that the *MAT1-1-1* and *MAT1-2-1* are required for sexual reproduction of the heterothallic ascomycete *B. cinerea*. We were able to demonstrate that *MAT1-1-5* and *MAT1-2-4* play a key role in the apothecium disk development.



### **P.3.3 Molecular and phenotypic characterization of noble rotted *Botrytis cinerea* isolates from Eger wine-growing region**

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*Botrytis cinerea* Pers.:Fr., the anamorph of *Botryotinia fuckeliana*, causes grey mould on a high number of crop plants in the temperate zones worldwide including grapes. In vineyards, the frequent occurrence of *B. cinerea* prior to harvest results in serious losses of bunches and deterioration of wine quality. *Botrytis* infection requires moist conditions and if the weather stays wet, the malevolent form, “grey rot” can destroy grapes yields.

Grapes typically become infected with *Botrytis* when they are ripe, but when then exposed to dry conditions become partially shrivelled up 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. A clue to these strategies lies in the understanding of the genetic structure and dynamics of fungal populations.

In the course of our work we have investigated the genetic and phenotypic properties of grape pathogenic populations of *B. cinerea* in two small vineyard of Szomolya village which is located in the wine-growing region of Eger, Hungary. Eger, the region of Bull’s Blood is well known from its dry wines and not for their botrytised wines, which can be made here as well in a very high quality. About 200 noble rotted isolates were collected from these two vineyards during the year 2011 and 96 were examined. Individual strains were obtained by single-spore isolation. Characterization of their genotype was done by analysing of the  $\beta$ -tubulin and MSB1 minisatellite sequences. Data and sequences was analysed by phylogenetic methods. As a phenotypic property, the sclerotia formation of isolates was examined.

A moderate variability was found among the investigated sequences. The minisatellite allele map contrast strikingly, but the variability of alleles were moderate as well. We could not detect differentiation between the isolations form territories and grape varieties, but there was variability between the sample collection time. The majority of isolates produced sclerotia and a different pattern of sclerotia formation was found depending on sample collection time as well. In summary, our data underline a significant specialization of populations from this territory and that difference could arise by using a dissimilar, botrytis saving grape protection technology.

This work is connected to NKFP-A2-2006/0017 grant.

### **P.3.4 Genetic variation in *Botryotinia fuckeliana* (*Botrytis cinerea*) populations on greenhouse vegetable crops in Lebanon**

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*Botryotinia fuckeliana* (de Bary) Wetzell (*Botrytis cinerea* Pers.), the causal agent of grey mould on several host plants worldwide, is well known for its extraordinary variability and its capability to acquire resistance to fungicides used in crop protection.

One hundred and forty-two representative isolates of *B. fuckeliana* populations infecting strawberry, tomato, cucumber and eggplant in nine different Lebanese districts were characterized for resistance to fungicides (benzimidazoles, *N*-phenylcarbamates, dicarboximides and anilinopyrimidines), transposable elements, vegetative compatibility and double-stranded RNAs.

Wild-type fungicide-sensitive phenotype was shown by few isolates (<10%), while more than 90% of them were resistant to at least one fungicide. High resistance to benomyl (BenHR) associated to hypersensitivity to *N*-phenylcarbamates was very common (90.1%). Dicarboximide-resistant isolates were commonly found on strawberry and tomato (55%). Anilinopyrimidine-resistant mutants (7.7%) were detected only on strawberry in the Choueifat district despite these fungicides have been intensively used in almost all the monitored fields. No mutants resistant to phenylpyrroles or hydroxyanilides were detected.

More than 95% of the analysed isolates were classified as “transposa”, because characterized by the presence of at least one of the two transposable elements *Boty* and *Flipper*. In particular, they included 80% of *Boty*<sup>+</sup>*Flipper*<sup>+</sup>; 19.3% *Boty*<sup>+</sup>*Flipper*<sup>-</sup>, mostly collected from strawberry, and only 0.7% *Boty*<sup>-</sup>*Flipper*<sup>+</sup>. Generally, “transposa” isolates were resistant to fungicides whereas the “vacua” ones, lacking the two transposons, were sensitive.

Twenty-two vegetative compatibility groups (VCGs), including an average of 1.8 isolates per group, were recovered into a single population present in a strawberry field. The high level of genetic diversity was confirmed by the polymorphism of mycoviral dsRNAs that were detected in 67% of the tested isolates.

### **P.3.5 Reconstitution of epicuticular waxes from table grapes: effect on growth, germination and gene expression of *Botrytis cinerea***

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*Botrytis cinerea* is a phytopathogenic fungus that attacks diverse plant species. Table grape is the most important Chilean fresh fruit for export. Gray mold caused by *B. cinerea* is a serious problem, which provokes substantial economic losses in different species, particularly table grape berries. Physical and chemical properties of the fruit surface could be recognized for *B. cinerea*, affecting processes such as germination of conidia and hyphae differentiation, closely linked to penetration into the host tissue. In order to evaluate the effect of epicuticular waxes from table grapes on growth, germination and gene expression of *B. cinerea*, we reconstituted the major components of the wax of table grape on an inert surface and analyzed the effect of this matrix on conidia from different *B. cinerea* isolates.

*B. cinerea* isolated from table grape (B05.10), grew approximately 25% more on a matrix of table grapes alkanes, in comparison to an isolate from apricot (D). In germination assays of 2.5 hours, B05.10 germinated 15% and 30% more in the presence of table grape alkanes, compared to conditions without alkanes or with blueberries alkanes, respectively. In similar experiments, *B. cinerea* isolated from blueberries (A) germinated 25% more on blueberries alkanes than on table grape alkanes.

The main components of table grape wax are oleanolic acid (OA) and n-fatty alcohols of different carbon chain length. Different *B. cinerea* isolates increased the germination rate in presence of a mixture of n-fatty alcohols (C22, C24 and C28) and this effect was more marked in early stages of germination. Interestingly, in absence of OA a significant increase of B05.10 germination was observed, until 4 hours post treatment.

Finally, the effect of n-fatty alcohols on expression of *Bctub*, *Bcbtr* and *Bcbex* genes was analyzed. In the presence of a mixture of C22, C24 and C28, *Bctub* slightly increased its expression only during the first hour of treatment. In contrast, expression of *Bcbtr* and *Bcbex* showed a significant increase in the first hour after contact with the n-fatty alcohols, which was maintained until 4 hours post treatment, suggesting that the presence of these compounds have a specific effect at the beginning of germination. The results indicated that *B. cinerea* isolates exhibit selectivity for certain components of the host waxes, mainly n-fatty alcohols, activated early expression of genes related with germination of *B. cinerea*.

Grants UNAB DI-130-12/R; DI-23-12/R and Posdoctoral Fellowship 3120001 FONDECYT.

## Session 4

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# Disease Management (Chemical Control)

*Chairperson:*

**Sabine Fillinger**

*UR 1290 BIOGER CPP, INRA Thiverval-Grignon, France*

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## **KN.4. 1 Novel fungicides in crop protection: a journey from discovery to the market**

**Gabriel Scalliet**

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World population is predicted to increase to over 8 billion by 2030 which means that each hectare of cultivated land will have to feed more than 5 people by this time. We estimate that already around ~40% of current world food would not exist without crop protection. For the same crop, yields vary widely around the globe reflecting sub-optimal agricultural practices mainly in developing countries, where the potential for yield increase is the greatest. Our current research is focusing towards more integrated solutions in order to bring the full potential of plants to life.

Plant diseases account for significant yield losses. In this lecture we examine current approaches to the discovery and development of new active ingredients for the control of plant pathogens. Some practical examples will be given to illustrate the challenges presented and how they can be overcome, with an emphasis on new technologies and trends in discovery.

## KN.4.2 Fungicide resistance in *Botrytis cinerea*: back and forth between field aspects and molecular mechanisms

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Resistance to fungicides is an evolutionary process resulting from the selection of advantageous genotypes in naturally diverse populations. Seven fungicide modes of action are authorized to control grey mould caused by *Botrytis cinerea* on grapevine in France, and five of them have encountered specific resistance, with variable frequencies in populations and possible consequences for field fungicide efficacy. Moreover, multidrug resistance is caused by fungicide efflux and allows a weak resistance towards six unrelated modes of action.

Here, a review is given of the fungicide resistance status of *B. cinerea* in France, particularly in the vineyards of Champagne, which is the most affected. Recently developed resistance and recent findings concerning the associated resistance mechanisms are focused upon. More particularly, we will characterize the diversity of fenhexamid resistance mechanisms in *B. cinerea* and *B. pseudocinerea*. We will also demonstrate how the various field mutations affecting the *sdh* genes are responsible for specific resistance towards SDHIs and affect differentially the activity of the targeted succinate deshydrogenase and the fitness of isogenic strains. Finally, anti-resistance strategies will be presented and examples of managed resistance will be discussed in a more general manner, with the aim of extending this knowledge to other crops and countries undergoing similar resistance problems.

## O.4.1 The phytopathogenic fungus *Botrytis pseudocinerea* is resistant to the fungicide fenhexamid due to detoxification by a cytochrome P450 monooxygenase Cyp684

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The *Botrytis* species complex responsible for grey mould disease on grapevine is composed of two species: *Botrytis cinerea* the major one (about 90%) and *Botrytis pseudocinerea*. Despite their genetic polymorphism, these species cannot be morphologically distinguished. However, they do differ in their response to several fungicides, especially to the sterol biosynthesis inhibitor fenhexamid. While *B. cinerea* is sensitive to this hydroxyanilide fungicide, *B. pseudocinerea* is naturally resistant. Because a strong synergism was found on *B. pseudocinerea* between fenhexamid and sterol 14  $\alpha$ -demethylation inhibitors (DMIs) known to inhibit Cyp51, a cytochrome P450 monooxygenase, it was thus hypothesized that detoxification of fenhexamid by a cytochrome P450 monooxygenase similar to Cyp51 is involved in *B. pseudocinerea*'s resistance. To test this, we sought the gene overexpressed in the presence of fenhexamid with the highest similarity to Cyp51. Taking into account the CypP450 classification based on homology and phylogenetic criteria, this gene whose function is unknown belongs to the Cyp684 family. It was then deleted in a *B. pseudocinerea* strain. Cyp684 knock out mutants exhibit loss of fenhexamid resistance and synergism between DMIs and fenhexamid, showing that the Cyp684, cytochrome P450 protein is responsible for *B. pseudocinerea*'s natural resistance to fenhexamid and involved in fenhexamid detoxification. Although Cyp684 is also present in *B. cinerea*, which is sensitive to fenhexamid, a polymorphism was observed in *B. pseudocinerea* the Cyp684 promoter shows a deletion of 25 bp. We are currently establishing the *Cyp684* expression profiles in both species in order to analyze the impact of the promoter deletion on its expression. In parallel, metabolization studies are conducted to identify metabolites to know the Cyp684 enzymatic functions and determine sensitivity of *Botrytis* sp. to the metabolites.



## O.4.2 General characteristics of fenpyrazamine, a novel fungicidal compound for controlling gray mold

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Fenpyrazamine (PROLECTUS®) is a novel fungicidal compound discovered and developed by Sumitomo Chemical Co., Ltd.

Fenpyrazamine has strong antifungal activities against *Botrytis* spp., *Sclerotinia* spp., and *Monilia* spp..

Preventive efficacies, translaminar activities and inhibition on lesion development were assessed against *Botrytis cinerea* on cucumber leaves. Moreover, its rainfastness was assessed on field grapevines. Fenpyrazamine showed not only excellent preventive efficacies but also translaminar, inhibition on lesion development and rainfastness. It is expected fenpyrazamine shows high efficacy under various conditions. Accordingly, fenpyrazamine showed excellent control of *B. cinerea* on field crops.

No germination of conidia of *B. cinerea* was affected by fenpyrazamine. However, germ tube elongation was strongly inhibited even at low concentration and treated germ tubes showed morphological change such as swelling, which is similar to that treated with ergosterol biosynthesis inhibitors. For sterole-related metabolite profiling, unsaponified metabolites of *B. cinerea* were analyzed by GC-MS. Fenpyrazamine-treated *B. cinerea* had lower ergosterol and higher 3-keto sterol concentrations. To estimate the inhibitory activity of fenpyrazamine against *B. cinerea*-derived 3-keto sterol reductase (3KR), *in vitro* 3KR assay was established by using recombinant 3KR from *B. cinerea*. This assay revealed that fenpyrazamine directly inhibits 3KR (IC<sub>50</sub> = 0.15 μM).

Furthermore, fenpyrazamine showed good toxicological profiles and low adverse effect on various beneficials. Fenpyrazamine will be a new interesting tool to preserve the quality of fruits and vegetables when it will be used in a program.

### O.4.3 Multiple fungicide resistance profile, *sdhB* mutation frequency and population structure of *Botrytis cinerea* from strawberries and greenhouse-grown tomatoes in Greece

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*Botrytis cinerea* is a pathogen with high genetic variability that has also shown high risk for fungicide resistance development. In the past, development of resistance to target-site inhibitors has been reported throughout the world. The study was conducted to: i) investigate the fungicide resistance profile of pathogen isolates obtained from strawberries and tomatoes in 2 and 3 distinct geographic regions of Greece, respectively, ii) identify mutations associated with SDHI resistance and iii) determine the structure of the tested populations based on a novel genetic discrimination of pathogen groups.

In total 1,153 single-spore isolates were tested for sensitivity to the SDHI fungicide boscalid, the QoI pyraclostrobin, the anilinopyrimidine cyprodinil, the hydroxyanilide fenhexamid, the phenylpyrrole fludioxonil, and the benzimidazole carbendazim, using an agar plate technique with discriminatory doses. In the populations obtained from strawberry a high frequency of isolates with multiple resistance to anilinopyrimidines, benzimidazoles, QoIs and SDHIs was found, in both sampled regions. In fungal populations obtained from tomatoes, the predominant phenotype was that of dual resistance to benzimidazoles and anilinopyrimidines in the Crete island (49.3%), of single resistance to benzimidazoles in the region of Preveza (57.0%), while in the region of Kyparissia the sensitive isolates were the dominant (53.1%). None of the tested isolates was found to be resistant to either hydroxyanilides or phenylpyrroles. The correlation of these results with the fungicide spray schedules applied in each region will be discussed.

A marked difference was observed in SDHIs resistance frequency among populations from strawberries and tomatoes despite similar numbers of SDHI spray applications in the two crops. Results showed wide-spread occurrence of boscalid-resistant phenotypes in the strawberry populations ranging from 46.7% to 76.8%, while boscalid resistance frequency in the tomato population was ranging from only 0.8 to 13.2%. Identification of *sdhB* mutations in the boscalid-resistant fractions of the populations using a PIRA-PCR technique, showed that the *sdhB* H272R mutation was the predominant in all the sampled populations, while other *sdhB* mutations such as H272Y/L, P225F and N230I were found in low or very low frequencies.

A recent study has shown that within *B. cinerea* populations from German strawberry fields, a novel clade exists (provisionally called *Botrytis* “group S”) that is genetically distinct from *B. cinerea sensu stricto*. Discrimination was based on the presence of a 21 bp insertion in the transcription factor *mrr1* gene. PCR-based detection of this 21 bp indel, indicated that *Botrytis* “group S” was predominant within the tomato populations obtained from all the 3 sampled regions with frequencies ranging from 62 to 75% of the isolates, while within strawberry populations, *B. cinerea* was predominant with frequencies of 75-95%. The predominance of *B. cinerea* in the strawberry populations may be due to fungicide-driven selection since none of the SDHI-resistant isolates was found to be *Botrytis* “group S”.

## O.4.4 Selection, fitness, and control of *Botrytis cinerea* isolates with variable levels of resistance to fenhexamid

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Although fenhexamid has been used to control gray mold on multiple crops internationally for over a decade, and *B. cinerea* isolates resistant to it have been detected in nature, documented control failures resulting from a proliferation of such isolates (“practical resistance”) are rare. One reason may be reduced fitness of these isolates, which has been shown for laboratory-induced mutants although there are few studies with naturally-occurring resistant types. Furthermore, the definition of resistance to fenhexamid is inconsistent, with some investigators using a threshold EC<sub>50</sub> value of 0.1 µg ml<sup>-1</sup>; others defining it as ≥0.4 µg ml<sup>-1</sup>; and still others using ≥50 or even 100 µg ml<sup>-1</sup>. This confusion is multiplied by the lack of data relating sensitivity *in vitro* to the level of disease control provided by typical fungicide doses *in vivo*. This study was designed to examine such issues.

Of 683 isolates collected across 3 years from vines in a fungicide test block, 31 were classified as putatively resistant (EC<sub>50</sub> ≥0.1 µg ml<sup>-1</sup>). Of these, only 16 and 15 survived and sporulated in culture long enough to further test for mycelial growth and conidial germination frequency, respectively. For them, mean mycelial growth *in vitro* was 57% of the mean value for 30 representative baseline isolates (EC<sub>50</sub> = 0.03 µg ml<sup>-1</sup>), with a range of 1.8 to 105% relative to the baseline mean for individual isolates; differences versus the baseline mean were significant ( $P = 0.01$ ) for 10 of the 16. Conidial germination frequencies for six of the 15 resistant isolates tested were significantly lower than baseline.

Four isolates representing a range of fenhexamid sensitivities (EC<sub>50</sub> = 0.03, 0.15, 0.32, and 62.5 µg ml<sup>-1</sup> for isolates A, B, C, and D, respectively) were assayed for their ability to cause disease on grape berries treated either preventively (1 or 5 days pre-inoculation) or curatively (1 or 3 days post-inoculation) with 150-600 mg L<sup>-1</sup> fenhexamid, corresponding to 25-100% of the recommended field rate. In both of the preventive assays, all fenhexamid concentrations significantly ( $P=0.01$  to  $P<0.0001$ ) delayed the onset and progress of the epidemic relative to untreated berries inoculated with the baseline isolate A and the putatively resistant isolate B, with a clear rate response but no significant differences between the two isolates. In contrast, none of the concentrations provided significant control of the two most resistant isolates ( $P = 0.44$  to  $0.87$ ) in either assay. Similarly, mean AUDPC values for the baseline isolate A were reduced by 79, 88, and 94% relative to the control following treatment 1 day post-inoculation with concentrations of 0.15, 0.3, and 0.6 mg L<sup>-1</sup>, respectively; for isolate B, these respective reductions were a similar 84, 89, and 95%. However, as in the protective assays, all three rates provided little to no control of the more resistant isolates C and D. In a duplex qPCR test, the ratio of pathogen/host DNA nearly doubled between 3 to 14 days inoculated with isolate A post-inoculation after inoculation of untreated berries, whereas it declined during this period in berries treated curatively with 0.6 mg L<sup>-1</sup> at 1 dpi.

In a 2-yr field trial, gray mold control was affected by fenhexamid rate and the number and timing of spray applications, but these differences did not appear to result from variable control of *B. cinerea* isolates with EC<sub>50</sub> ≥0.1 µg ml<sup>-1</sup>, as such phenotypes were recovered from 4 to 6% of diseased berries in all treatments, including the check.

## O.4.5 Integrate grey mould control using biocontrol agents and fungicides on table grape

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In Southern Italy, grey mould (*Botryotinia fuckeliana* = *Botrytis cinerea*) can cause heavy yield losses, especially in table-grape vineyards covered with plastic films to delay the harvest until December. Under such conditions, several fungicide sprays are often needed, but they can leave high residue concentration in grapes and have a significant environmental impact. Furthermore, acquired resistance to almost all allowed fungicides has often been experienced in *B. fuckeliana* on such crop.

During the period 2010-2012, three field trials were carried out in table-grape vineyards to assess the efficacy of protection schedules based on three biocontrol agents (BCAs), *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Aureobasidium pullulans*, in controlling grey mould with the main aim of reducing the use of chemicals and the risk of acquired resistance to fungicides.

Preliminarily, the sensitivity of *B. subtilis* and *B. amyloliquefaciens* to the fungicides commonly used on grape against grey mould was ascertained through an *in vitro* test; only copper derivatives and fluazinam inhibited the growth of the two BCAs. On the ground of these results, the two BCAs were applied in the schedules alone, in alternation and/or in mixture with iprodione, boscalid, pyrimethanil, fenhexamid and the mixture cyprodinil+fludioxonil. Spray schedules were compared to the untreated check. Prevalence (percentage of infected bunches) and McKinney's index were used as parameters to estimate the incidence of grey mould infections. Three-eight days after the last spray, samples of infected berries were collected and the percentages of fungicide-resistant conidia were assessed in the laboratory, by using fungicide-amended media.

The incidence of grey mould symptoms was assessed from October to November and the mean values of prevalence on the untreated check were 90.3% (2010), 16.7% (2011), and 74.4% (2012). The results showed that the tested BCAs are effective and useful in reducing the use of fungicides in appropriate IPM strategies, in particular when applied at highest label rate.

High frequencies of anilinopyrimidine-resistant conidia (65%-94%) were always detected on all the theses, and no significant differences were appreciated among the schedules by the use of the BCAs. A significant percentage of boscalid-resistant conidia (23%) was observed only in the thesis treated exclusively (6 sprays) with this new fungicide, whereas the percentages were lower when it was used in mixture (7%-15%) or alternations (6%) with one of the BCAs.

Further evaluations need to be conducted to confirm the results so far obtained and to get deeper knowledge on the most suitable application modalities of BCAs in IPM strategies aimed at reducing the use of chemicals and the risk of acquired fungicide resistance in *B. fuckeliana*.

## P.4.1 Molecular bases of the fungistatic effects of a plant natural berberine-like molecule on *Botrytis cinerea*

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The use of plants as source of natural compounds with antifungal properties in substitution of synthetic molecules has intensified in recent years. Here we report the results on the effect of a berberine-like molecule, extracted from the bark of *Zanthoxylum rhoifolium*, on the expression of a set of genes (TOR, PDR5, P450, CYP51 and PLK1), previously reported to be involved in developing fungi resistance in *Botrytis cinerea*.

Agar plugs of actively growing cultures of *B. cinerea*, isolated from lettuce, were transferred to PDA medium amended with i) the chloroform/methanol plant fractions, containing the berberine-like molecules, ii) iprodione, a fungicide, able to inhibit spore germination and mycelia growth, but against which fungi have raised resistance mediated by down-regulating the PDR5 gene, coding for an efflux pump involved in the cellular detoxification; iii) solvent chloroform/methanol (control). The plant fractions or iprodione were added to the medium at a concentration corresponding to IC<sub>50</sub> value. Plates were maintained at 24°C in permanent dark conditions. Total RNA, extracted from untreated and treated mycelia of *B. cinerea* grown for 7 and 10 days, was reverse-transcribed and the levels of gene expression of the above mentioned genes were determined by quantitative Real-Time PCR, using specific sets of primers.

After 7 days of incubation with the active fractions of *Z. rhoifolium* or iprodione, the expression of all the analyzed genes resulted inhibited, compared to control, with the following percentage, respectively: TOR (72 and 47%), PDR5 (83 and 58%), P450 (85 and 66%), CYP51 (40 and 76%) and PLK1 (74 and 70%).

Interestingly, extending for three days the incubation time in the presence of the active plant fractions, the level expression of most the target genes was partially rescued, while the expression of PDR5 gene decreased further. These data indicate that the active fractions of *Z. rhoifolium* might be beneficial in avoiding the onset of the already described PDR5-mediated resistance in *B. cinerea*. These results also suggest a potential practical use of this plant natural compound, in combination with fungicide-like compounds, such as iprodione, with the final aim to increase the efficiency of antifungal treatments.

## P.4.2 Effect of structurally related essential oil components on growth of *Botrytis cinerea*

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Essential oils are used as an alternative to chemical fungicides mostly to control post-harvest diseases, included grape grey mould. Main components of some essential oils are structurally related but may differ in fungicide activity. The major aim of this work was to compare the fungicidal activity against *Botrytis cinerea* Pers. of some related components of essential oils.

The compounds, compared by a plate growth assay, showed the following order of decreasing activity: eugenol, thymol, cinnamaldehyde, isoeugenol, carvacrol, vanillin and guaiacol. The EC50 for eugenol was about 100 µg/ml for the strain B05.10 but was even less against the PM10 strain.

Treatment with eugenol affected also conidia germination, in particular 500 µg/ml completely inhibited spore germination (MIC) of the PM10 strain also at 60 hpi.

The same eugenol concentration was used in a leaf assay and was effective in preventing the infection of both strains of *B. cinerea* for the entire duration of experiment (10 days). Treatment of grape berries was also performed and confirmed the effectiveness of eugenol in delaying *B. cinerea* infection. However, the effect was influenced by the grapevine cultivar; in fact, treated cv. Cabernet Sauvignon was more sensitive to *B. cinerea* infection than cv. Merlot, although both cultivars were similarly infected in the untreated controls.

Although eugenol is known to be an antioxidant compound and increases bacterial membrane permeabilization, and it is reported as a strong inhibitor of soybean lipoxygenase activity, the mechanism by which it affects *B. cinerea* spore germination and growth is not yet known.

In order to better understand the targets of eugenol and the fungal response to this phenolic compound, transcriptomic analysis of *B. cinerea* mycelium grown for 72 h and then treated for 6 and 12 hours with eugenol by using EC50 and EC80 concentrations is currently under investigation.

### P.4.3 Antifungal activity and detoxification of compound derived from 8,8-dimethyl-3-[(R-phenyl) amino] -1,4,5 (8H)-naftalentriones against *Botrytis cinerea*

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It has been reported that derivatives of anthraquinones and anthrahydroquinones exert a toxic effect on *Botrytis cinerea*. The difference in the antifungal activity of these compounds is related to structural differences in the molecules as well as to the amount and type of substituents. The aim of this study was to evaluate the fungitoxic activity and the detoxification mechanism of different compounds derived from 8,8-dimethyl-3-[phenylamino] -1,4,5 (8H)-naftalentriones (Fig 1). For this, a total of 13 compounds, which differ in the type, position and number of substituents on the aromatic ring were analyzed.

The antifungal activity on solid medium was evaluated on the *B. cinerea* strain B05.10. It was found that the antifungal effect depends on the substitution in the aromatic ring. The most active compounds were the 7 (p-Br) and 9 (p-CH<sub>3</sub>) (IC<sub>50</sub> 4,1µg/mL). On the other hand, it was shown that the compound 7 at the concentrations tested did not inhibit conidia germination.

To study the detoxification mechanism of compound 7 by *B. cinerea* B05.10, the biotransformation reactions of this compound and the effect of the ATP binding cassette (ABC) expulsion pump were evaluated. The results of biotransformation of compound 7 showed the presence of possible modified metabolites of lower polarity at 96 h of incubation. The effect of ABC expulsion pump in the detoxification of this compound was determined by comparing the effect of the compound on mycelia growth of *B. cinerea* B05.10 wild type and a mutant that does not express the ABC expulsion pump (*BcatrB*).

The results showed that the ABC expulsion pumps were not involved in the detoxification process of this compound.

Finally, it was evaluated the effect of compound 7 on cellular respiration, by measuring oxygen consumption. The results showed that this compound did not cause an effect on the electron transport chain of the oxidative phosphorylation.

## P.4.4 Genetic and phenotypic characterization of *Botrytis cinerea* isolates from Chile with different levels of strobilurin sensitivity

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*Botrytis cinerea* (*Bc*) control is based on the use of fungicides applied during the critical infection periods (blossom and véraison to pre-harvest). Due to their dual effect (oidicide and botryticide action) strobilurins are interesting alternatives to use against *Bc*, particularly during the pre and post bloom periods. Recently, in some regions of Europe, North America and Chile, the beginning of loss of sensitivity of *Botrytis* to QoIs has been detected. Fungal resistance to this fungicide group it is associated to three types of mutations in cytochrome b, being the most important the replacement of Glycine for Alanine in codon 143 (G143A).

The aim of this study was to determine the potential relationship existing between the loss of sensitivity to QoIs, pathogen behaviour and presence of some of the 3 associated mutations. With this purpose, twenty *Bc* isolates were recovered from grapes and selected based on previous data on sensitivity to azoxystrobin (10 sensitive and 10 resistant isolates). These isolates were genetically characterized through allele-specific PCR using the three pairs of primers already described in the literature (BcAR, Cytb-Bc and Cytb-129) in order to detect the presence of mutations associated to resistance to QoIs. In addition, the genotype correspondence of the *Bc* isolates was determined by means of duplex PCR and through the use of specific primers to detect the *boty* and *flipper* transposable sequences.

The phenotypic characterization considered the assessment of the following parameters: mycelial appearance, sporulation, sclerotia-forming capacity (MA medium at 20°C), mycelial growth rate (MA medium at 15, 20 and 25° C), and virulence on wounded and unwounded cv. Thompson Seedless berries, under incubation at 0 and 20°C.

Ten isolates initially considered as resistant showed the mutation G143A. Two structures of cytochrome b of the pathogen were detected by means of specific allele PCR using the Cytb-Bc primer pair followed by sequencing. The first contained three introns and it was present in the ten resistant and in five of the sensitive isolates. The second, presented an additional 1205 pb intron, and it was detected in the 5 remaining sensitive isolates. This constitutes the first detection of an additional intron in Chilean *Bc* isolates.

The twenty isolates corresponded to the *transposa* genotype. They behaved equally in terms of their mycelial appearance, sporulation, sclerotia-forming capacity and virulence ( $P>0.05$ ). Differences were only detected in mycelial growth at 20°C, at which temperature the resistant isolates grew significantly less than the sensitive ones ( $P<0.05$ ).

\* Thesis for a Master's Degree in Agricultural and Livestock Sciences, Major in Plant Protection, Facultad de Ciencias Agronómicas, Universidad de Chile



## P.4.5 From enzyme to fungal development or how *sdhB* mutations impact respiration, fungicide resistance and fitness in the grey mold agent *Botrytis cinerea*

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Respiration inhibitor fungicides are widely used to control fungal diseases on multiple crops. The succinate dehydrogenase inhibitors (SDHIs) are among the latest introduced molecules against the grey mould agent *Botrytis cinerea* on grapevine. We have recently isolated and characterized *B. cinerea* field strains resistant to the SDHIs. Most of the strains harbour one single mutation in the succinate dehydrogenase subunit gene, *sdhB* (Leroux *et al.*, 2010) affecting the ubiquinone-binding pocket. In this study we have introduced these mutations into a *B. cinerea* wild-type strain (B05.10) in order to evaluate the impact of each mutation on SDH and respiratory activity and inhibition by SDHIs from different chemistries. We also analysed several parameters of *B. cinerea*'s life cycle to assess the fitness cost associated with the resistance mutation.

Our results show a strict correlation between the *sdhB* mutation and the resistance spectra to SDHIs. These resistances can be fully explained by the affinities of the SDHIs to its modified target enzyme. Four out of the seven *sdhB* alleles led to significantly reduced SDH activity and, in three cases (H272L, N230I, P225L), to reduced respiration rates. Concerning the fungal biology, we tested mycelial growth and sclerotia production on different media and temperatures, conidia production and germination, resistance to oxidative stress and ROS production, as well as pathogenicity on tomato and bean leaves. All mutants were affected for at least one parameter. However, fitness parameters of mutants *sdhB*<sup>H272R</sup> and *sdhB*<sup>P225L</sup> showed the strongest modifications among all strains, e.g., reduced pathogenicity, strongly reduced conidia- and sclerotia-production (H272R). A clear correlation between fitness and respiration on one hand, fitness and allele frequency among natural populations on the other, is not yet obvious. However, our results in terms of resistance spectra and fitness parameters should help defining more efficient treatment strategies against grey mould.

## P.4.6 Distribution of sensitivity to common botryticides in Lombardy

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On grapevine, grey mould caused by *Botrytis cinerea* Pers. induces serious yield losses and unpleasant changes in the qualitative characteristics of berry content. Fungicides are usually applied in order to control *B. cinerea* but their repeated use in vineyard can select resistant strains of the pathogen.

Aim of the present work is not only to monitor the sensitivity distribution of the pathogen strains to the botryticides most commonly used in vineyard, but also the possible correlation between the sensitivity level and both the mating type and the presence of transposons *Boty* and *Flipper*.

During 2011, 20 strains were randomly isolated from symptomatic samples collected in 4 vineyards selected in each of the main viticultural provinces in Lombardy, Brescia, Mantova, Pavia and Sondrio. An untreated vineyard was included in the survey. The 320 strains isolated in Lombardy were tested for their sensitivity to boscalid, fenhexamid, cyprodinil and fludioxonil, their mating type and the presence of transposons. The Resistance Factor (RF =  $EC_{50}$  of the selected strain/average  $EC_{50}$  in the untreated vineyard) was calculated for the strains showing high  $EC_{50}$ . RFs > 10 indicate a reduced sensitivity.

The average  $EC_{50}$  observed in Lombardy for boscalid, fenhexamid and fludioxonil were similar to those calculated for *B. cinerea* in Europe, while higher values of this parameter were obtained for cyprodinil. No remarkable differences were observed in the  $EC_{50}$  distribution in the 4 provinces, apart from the higher values for cyprodinil and fludioxonil recorded in Pavia. RFs > 10 characterized 7, 12 and 5 strains respectively for boscalid, cyprodinil and fludioxonil and 5 isolated showed a reduced multi-drug sensitivity. The presence of *Boty* either alone or together with *Flipper* seems to be associated with higher RFs concerning all the tested fungicides. Association between mating type and fungicide sensitivity is less defined. From these data, *B. cinerea* populations in Lombardy seem to be characterized by an adequate sensitivity to botryticides. The strains showing a reduced sensitivity need to be further characterized, in particular for their fitness.

## P.4.7 The new SDHI fungicides boscalid and fluopyram in the control of grey mold: efficacy and resistance

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The control of grey mould on various crops is still largely based on the use of fungicides, and is difficult because the disease is particularly severe in time close to the harvest, when fungicide sprays may leave residues on edible products. In Southern Italy, grey mould can cause heavy yield losses, especially in table-grape vineyards covered with plastic films to delay the harvest until December.

*Botryotinia fuckeliana* (de Bary) Whetz. (an. *Botrytis cinerea* Pers.) is recognized by FRAC among the pathogens at highest risk of resistance and fungicide resistance in practice has been indeed experienced with almost all the fungicides used against grey mould on various crops and in numerous countries. The SDHIs (Succinate DeHydrogenase Inhibitors) are new fungicides effective against grey mould that can help the growers to improve the efficacy of IPM strategies.

In the seasons 2010-2012, four trials were carried out in arbour vineyards (“tendone”) of table grape cv. Italia in South Italy covered in the middle of August with plastic sheets to delay harvesting. The 12 vines of each plot in four-randomized blocks were sprayed by means of a motorised knapsack sprayer, with an equivalent volume of 1,000 l/ha. The two SDHIs, boscalid and fluopyram, were used alone or in alternation with fungicides commonly used against grey mould, such as the anilinopyrimidine pyrimethanil, the mixture cyprodinil+fludioxonil and the hydroxyanilide fenhexamid. Spray schedules were based on the first application at bunch closure (berries touch), a second spray at véraison (change of colour of berries) and further sprays (1-4) 21 days after the previous one. According to the season, the first grey mould symptoms were observed from the first half of September to the first half of October. Under such conditions, all the protection schedules reduced grey mould symptoms as compared to the untreated check, especially when based on the usage of fluopyram in late sprays. The schedules based on fluopyram were generally more effective than those based on boscalid.

The presence of SDHI-resistant isolates was ascertained collecting conidia from infected berries at harvesting. Generally, SDHI-resistant isolates were recovered in the fungal populations submitted to the selection pressure exerted by fungicide sprays. Isolates resistant to boscalid and sensitive to fluopyram (0-32%) were more frequent than the mutants resistant to both the fungicides (up to 6.5%). Isolates resistant only to fluopyram were never detected.

Further evaluations need to be conducted to confirm these findings and to get deeper knowledge on the best IPM strategies against grey mould especially when *B. fuckeliana* is particularly exposed to risk of acquiring resistance to fungicides.

## P.4.8 Current status of fungicide resistance in *Botryotinia fuckeliana* (*Botrytis cinerea*) on strawberry in South Italy

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*Botryotinia fuckeliana* (de Bary) Whetzel (*Botrytis cinerea* Pers.) is included among phytopathogenic fungi at higher risk of development of resistance to fungicides posing a major threat to their field performance. Monitoring of pathogen's populations is of crucial importance to evaluate the resistance risk and to suggest the prompt adoption of appropriate anti-resistance strategies.

*B. fuckeliana* resistance to six classes of fungicides was monitored in 2013 in 20 commercial greenhouses of strawberry located in South Italy ('Metaponto' area). *In vitro* assays were carried out on conidia samples collected at harvesting time from naturally infected fruits and inoculated on appropriate medium amended with each fungicide [vinclozolin (5 mg l<sup>-1</sup>), pyrimethanil (1 mg l<sup>-1</sup>), fludioxonil (0.3 mg l<sup>-1</sup>), fenhexamid (0.4 and 4 mg l<sup>-1</sup>), boscalid (10 mg l<sup>-1</sup>), fluopyram (10 mg l<sup>-1</sup>) or trifloxystrobin (1 mg l<sup>-1</sup>)] at concentrations discriminating between wild-type sensitive and resistant phenotypes. *B. fuckeliana* genotypes carrying mutations in target genes responsible for resistance to fenhexamid (*Erg27*), SDHI (*SdhB*) and QoI (*cytb*) fungicides were detected and quantified through Allele-Specific(AS)-PCR or real time-PCR assays using DNA extracted from conidia.

Resistance to the old dicarboximide fungicides, still used on strawberry against grey mould, and to anilinopyrimidines (APs) were always detected at frequencies generally ranging from 70 to 100% and from 75 to 100%, respectively, in fields exposed to intensive spray schedules with the fungicides, and relative low (up to 35%) in untreated fields. The frequencies of conidia resistant to the phenylpyrrole (PP) fludioxonil (used in mixture with the AP-fungicide cyprodinil) was usually low (0-29%), with only few exceptions.

Conidia showing resistance to fenhexamid were detected at variable frequencies ranging from less than 1 to 100%. Variants of the *Erg27* gene at codon in position 412 (F412S/V and less frequently F412I), conferring high level of resistance to the fungicide, were detected in all the analysed samples.

Resistance to the SDHI boscalid, applied in mixture with the QoI pyraclostrobin, were detected in almost all the monitored fields at variable frequencies (3-93%) whereas resistance to the new fungicide fluopyram was extremely rare (up to 4%). Molecular assay showed the common occurrence in commercial fields of *SdhB* mutants carrying the H272R/Y substitutions responsible for resistance to boscalid and sensitivity or hypersensitivity to fluopyram. Mutants carrying different substitutions (H272V, P225L/F and N230I) conferring resistance to both SDHI fungicides were also detected.

QoI-resistant mutants associated to the G143A mutation in *cytb* gene were detected in all conventional strawberry fields at frequencies usually higher than 72%.

## P.4.9 Analysis of the resistance to azoxystrobin and the cytochrome *b* gene diversity of Hungarian *Botrytis cinerea*

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In a cell there are several mitochondria. The phenomenon when the genomes of all mitochondria in the cell are identical is called homoplasmy. If in the cell there is wild and mutant mitochondrial DNA this is called heteroplasmy. The genes being in the mitochondrial DNA primarily encode the enzymes of cellular respiration. Fungicides belonging to the family of quinon oxidase inhibitors (QoIs) play an important role in the protection against several plant diseases caused by fungi. These fungicides bind to the cytochrome *bc1* complex so they block electron transport between cytochrome *b* and cytochrome *c1*. This way these fungicides inhibit the ATP synthesis. The QoI resistance has at least two mechanisms. One of them is the point mutation of the cytochrome *b* gene (*cyt b*), e.g. the substitution of a single glycine by alanine at position 143 results in high-resistance. The other is the cyanide-resistant alternative respiration sustained by the alternative oxidase. Whether the mutation in the mitochondria causes phenotypic diversity or not depends on the dose, i.e. it depends on the percentage of the changed mitochondria.

We investigated QoI resistance and related mitochondrial genotypes in Hungarian group I and II *B. cinerea* populations. Allele-specific PCR reaction and PCR-RFLP method were used to confirm the presence of the G143A mutation. The development of the QoI resistance was induced by using mediums with increasing concentrations of azoxystrobin. Using real time PCR we developed a method to track heteroplasmy. In the studies single copy gene located in the mitochondrial genome (*cox1*) was used as a standard. The existence of an alternative group I-type intron in the *cyt b* gene immediately after the codon 143 was suggested by PCR fragment length analysis.

All isolates, where the G143A mutation could be detected with both PCR-RFLP and allele-specific PCR, showed high resistance to azoxystrobin. In a few cases, the mutation could only be detected with allele-specific PCR, but not with PCR-RFLP, and these strains were sensitive to azoxystrobin. This would indicate marginal presence of the resistance-conferring mutated mtDNA and these strains may well develop resistance rapidly when faced with QoIs in the field. *In vitro* we managed to induce the development of the resistance to azoxystrobin. Using the developed real time PCR method we proved that in the presence of azoxystrobin the ratio of the mitochondrial genome which carries the resistance is increased compared to the sensitive sequence. We related the change in the ratio of the mitochondrial DNA containing the G143A point mutation compared to the sensitive, "wild" sequence. The alternative group I intron was present in a considerable part (>32%) of the Hungarian *B. cinerea* field isolate collection. Some group II isolates gave rise to both fragments (~1750 bp and ~560 bp), which could imply *cyt b* heteroplasmy in monosporic isolates.

*B. cinerea* is a pathogen at high risk of resistance to single-site fungicides, like QoIs. The reason for this is its extreme genetic variability and short life cycle. In most cases the high resistance to QoIs comes along with one particular G-to-C point mutation in the mitochondrial *cyt b* gene. Many Hungarian *B. cinerea* field isolates carried the G143A mutation.

## Session 5

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# Disease Management (Biological Control)

*Chairperson:*

**Antonio Ippolito**

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## KN.5.1 Bio-based control strategies for *Botrytis*: utilising combinations and mixtures

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*Botrytis cinerea* causes significant economic losses on many crop plants including strawberry, grape, tomato, lettuce and kiwifruit. For many years, control relied solely on the use of synthetic chemicals but this situation has since changed due to problems with fungicide resistance and an increasing consumer demand for non-pesticide based control measures. Many biologically based products are now available commercially but the level of disease control provided is often highly variable. This paper outlines the development and use of two bio-based products for *Botrytis cinerea* control with different modes of action: Sentinel<sup>®</sup>, based on a *Trichoderma atroviride* active ingredient that acts through competition, and Regalia<sup>®</sup>, a *Reynoutria*-based plant extract that acts as an elicitor of induced systemic resistance (ISR) in the plant.

*Trichoderma atroviride* LU132 has been shown to be effective (56-85% disease control) against *B. cinerea* on strawberries, tomatoes and grapes in New Zealand in a series of glasshouse and field studies conducted over the period 2004-2010. The main mechanism of action is competition for nutrients whereby the fungus protects the plant tissue from pathogen colonization by competitive exclusion and suppression of secondary inoculum production. LU132 has been commercialized as Sentinel<sup>®</sup> for control of grey mould of grapes with label claim extensions for tomato, strawberry and berryfruit. The product is formulated as a wettable powder and applied to foliage at 14-21 day intervals at a rate of 40-60 g/100 L water depending on the crop. The product can be applied up until harvest with no withholding period. A protoplast variant of LU132 has recently been developed that is low temperature tolerant and fungicide resistant. This improved strain will provide a wider spectrum of use for the product.

Regalia<sup>®</sup> is an extract from giant knotweed (*Reynoutria sachalinensis*) that protects plants from attack by a range of plant pathogens, including *B. cinerea*, by stimulating an ISR response. The eliciting compounds have been identified as the anthraquinones, emodin and physcion. In addition to stimulating the accumulation of plant defense related compounds and strengthening the plant cell walls by lignification, the active ingredients can directly inhibit pathogen spore germination. The product is a micro-emulsion concentrate containing 5% active ingredient and is applied to foliage at 1-4 quarts per acre in a minimum of 5 gallons water. The product has been extensively trialed against Botrytis diseases in crops in US, Europe and Brazil and shown good efficacy when compared to standard fungicide treatments. Regalia<sup>®</sup> has the added advantage that it is also active against powdery mildew thus providing a dual spectrum of activity on many crops.

Previous research has revealed synergistic activity when either of these bio-based products have been combined with low rates of chemicals as tank mixes and our most recent data has shown that *Trichoderma* bioactivity can be enhanced when the fungus is combined with Regalia. The potential for the development of a pre-mix product based on *Trichoderma* and *Reynoutria* that will provide more effective and sustainable control will be discussed.



## O.5.1 Induced resistance, competition and antibiosis - a tripartite mode of action of *Pseudozyma aphidis* against *Botrytis cinerea*

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Plant pathogens challenge our efforts to maximize crop production due to their ability to rapidly develop resistance to pesticides. This can result in immense yield losses on an annual basis. One of the main research goals of this century involves the development of new tools to control pathogens.

Fungal biocontrol agents have become an important alternative to the use of chemicals due to environmental concerns.

Biological control can be achieved by one or a combination of mechanisms that hinder pathogen growth and development, thereby reducing disease. The complex mode of action of biocontrol agents reduces the likelihood that pathogens will develop resistance to them. We recently isolated a unique, biologically active isolate of the epiphytic fungus *Pseudozyma aphidis*. Our data demonstrated that *P. aphidis* proliferates on infected plant leaves, suggesting it may compete for space and nutrients with plant pathogens. Furthermore *P. aphidis* secretes extracellular metabolites, which inhibit several fungal and bacterial pathogens *in vitro*.

Application of *P. aphidis* spores to greenhouse-grown tomato or *Arabidopsis* plants activated their local and systemic defense responses and significantly reduced *B. cinerea* infection. In addition, application of spores on tomato or cucumber plants in the greenhouse significantly reduced bacterial wilt and canker and powdery mildew infection. Taken together, our results strengthen the hypothesis that we can use *P. aphidis* for the development of a new and efficient biocontrol agent that could contribute to reducing the amount of chemicals required for pathogen control.

## O.5.2 Integration of *Aureobasidium pullulans* in grey mould control in soft fruit

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Fruit decay is one of the most serious diseases in plants and causes especially in commercial soft fruit production enormous economic losses. *Botrytis cinerea* causing grey mould is the main pathogen in strawberries and starts infections already at the beginning of the blooming period. The occurrence of infections on ripe fruit depends very much on weather conditions during harvest. Because the pathogen can only be combated protectively, up to five fungicide treatments have to be done during bloom to protect all open blossoms. Distinctive for the chemical active ingredients nowadays used in *Botrytis* control is the specific mode of action (single site inhibitors). The repeated use of these substances led to a selection of resistant strains in the pathogen population.

One opportunity to reduce the risk for resistance is the reduced use of specific fungicides and the application of products with a different mode of action like antagonistic yeasts.

Boni Protect forte, based on the yeast like fungus *Aureobasidium pullulans*, has antagonistic potential against several pathogens in fruit-growing. In numerous trials *A. pullulans* showed its high efficacy. Therefore the strategic use of Boni Protect forte could be a helpful manner to prevent resistances in *B. cinerea* against chemical fungicides.

Field trials were done in strawberries at two locations in Germany in 2011 and 2012, and in a raspberry field 2011 in Poland.

In 2011 *Botrytis* incidence was low in Buchholz at BBCH 85 evaluation. All treatments showed significant disease reduction with efficiencies of more than 59%. At a higher infection pressure resulting in an incidence of 180 infection sites per 28 plants in 2012, again all three treatments reduced the disease incidence significantly. The stand-alone treatment with Boni Protect forte as well as the spray strategy using chemical fungicides in alternation with Boni Protect forte was comparable to the chemical standard in both years. In Oberkirch *Botrytis* incidences in the control were comparable in both years. In 2011 the chemical standard as well as Boni Protect forte reduced *Botrytis* incidence by 73%. In 2012 efficiency of 57% and 63% was reached with the chemical standard and Boni Protect forte, respectively.

*Botrytis* incidence in the raspberry trial was 29% in untreated control. All three treatments showed significant reduction of *B. cinerea* incidence. The spray strategy (eff. 86%) and the chemical standard (eff. 89%) were significantly better than the stand alone treatment with Boni Protect forte (eff. 69%).

In all five trials Boni Protect forte reduced the *B. cinerea* incidence significantly compared to untreated control. Spray strategies of Boni Protect forte and chemical fungicides showed also significant effects and tended to be better than the stand-alone treatment with Boni Protect forte.

## P.5.1 Screening epiphytic yeasts for control of *Botrytis cinerea* on strawberry and tomato

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A total of 1151 yeast strains were isolated from healthy leaves of strawberry. Through plate dual-culture screening and *in vitro* leaf and fruit screening, two strains (C410 and YCXT3) were selected, identified and tested to suppress *Botrytis cinerea* on strawberry and/or tomato.

Strains C410 and YCXT3 was identified as was identified as *Candida intermedia* and *Sporidiobolus pararoseus*, respectively, based on morphological and physiological characteristics and by analysis of the DNA sequence of the internal transcribed spacer (ITS) region (ITS1-5.8S rDNA-ITS2) of ribosomal gene.

Both strains were tested to suppress *B. cinerea* on postharvest strawberry fruits. Results showed that inoculation of the yeast cells of strains C410 or YCXT3 on strawberry fruits significantly suppress infection by *B. cinerea*. Concentration of yeast cells of each strain was found to be the major factor affecting biocontrol efficacy of the two strains.

Production of volatile organic compounds (VOCs) and efficacy of the VOCs in suppression of *B. cinerea* were investigated. Results showed that both yeast strains were detected to produce antifungal VOCs. However, they differed in VOC composition. Strain C410 was found to produce 49 VOCs including esters, alcohols, alkenes, alkanes, alkynes, organic acids, ketones, aldehydes, benzenes and amines. Two VOCs, namely 1,3,5,7-cyclooctatetraene and 1-butanol, 3-methyl-, were the most abundant. Synthetic chemicals of 1,3,5,7-cyclooctatetraene; 1-butanol, 3-methyl-; 2-nonanone; pentanoic acid, 4-methyl-, ethyl ester; 1-butanol, 3-methyl-, acetate; acetic acid, pentyl ester and hexanoic acid, ethyl ester could inhibit conidial germination and mycelial growth of *B. cinerea*. Strain YCXT was found to produce 39 VOCs inhibitory to conidial germination and mycelial growth of *B. cinerea*. Synthetic 2-ethyl-1-hexanol (one of the VOC components produced by strain YCXT) was detected to have strong anti-fungal activity against *B. cinerea*. Results also showed that incidence and severity of Botrytis fruit rot of strawberry was significantly ( $P < 0.01$ ) reduced by exposure of the strawberry fruits to the VOCs from cultures of strains C410, YCXT3, C410- or YCXT3-infested strawberry fruits.

Strain C410 was tested to control *B. cinerea* on tomato grown in plastic films-protected fields in 2011 and 2012. Results showed that spray of the yeast cell suspension of strain C410 ( $10^7$  cells ml<sup>-1</sup>) at the flowering stage provided effective control of *B. cinerea* with the disease incidence being reduced by about 50% compared to control (water spray).

These results suggest that strains C410 and YCXT3 are promising biocontrol agents against *B. cinerea*.

## **P.5.2 Evaluation of the antifungal activity of organic and inorganic salts against *Botrytis cinerea*: the causal agent of tomato grey mold**

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The aim of this work was to find an alternative to synthetic fungicides used in the control of the devastating fungus *Botrytis cinerea*, the causal agent of grey mould disease of tomato. *In vitro* trials were conducted to evaluate the effect of several inorganic and organic salt compounds on mycelial growth, spore germination and germ tube elongation of *B. cinerea*. Among 36 tested compounds, copper sulfate, EDTA and sodium metabisulfite completely inhibited mycelial growth of *B. cinerea* at only 0.02 M. Other compounds, such sodium bicarbonate, sodium carbonate, sodium phosphate dibasic, sodium nitrite, sodium salicylate and potassium carbonate, completely inhibited the mycelial growth of the pathogen at 0.2 M. The Minimum Inhibitory Concentration (MIC) and the Minimum Fungicidal Concentration (MFC) were determined for the most active salt compounds. The effect on spore germination and germ tube elongation were determined for the salts having inhibited mycelial growth by more than 50%. Results from this study provide an important basis for further *in vivo* study into the use of salt compounds for the control of grey mold of tomato.

### **P.5.3 New molecular approaches to quantify the populations of *Bacillus subtilis* and *Bacillus amyloliquefaciens* used to control grey mould**

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*Botryotinia fuckeliana* (de Bary) Whetz. (an. *Botrytis cinerea* Pers.) is well known for its adaptability to the selective pressure exerted by sprays with the onset of resistance to fungicides. Nowadays, resistance to the most commonly used fungicides is quite common in the populations of the pathogen on several crops. Moreover, consumers require fruits and vegetables with no or low amount of residues of plant protection products. These are the reasons underlying an increasing attention to the chance to use biocontrol agents (BCAs) in crop protection.

*Bacillus subtilis* and *Bacillus amyloliquefaciens* are currently applied against many plant pathogens, including *B. fuckeliana*, on several crops, in greenhouse as well as in the field. Commonly, farmers use both BCAs with the same approach used for chemicals. Data on the survival of the two BCAs in table-grape vineyards and under hot and dry climatic conditions, such as those of Southern Italy, are lacking.

Quantitative nested real-time PCR (qPCR) detection method was set up and applied to quantify the populations of the two BCAs. Specie-specific primers and TaqMan probes were designed on nucleotide sequences of the *B. subtilis* strain QST 713 (Serenade Max, Basf Italia) and the *B. amyloliquefaciens* strain D747 (Amylo-X, Biogard-CBC) and used in qPCR. The molecular assays proved to be highly specific and sensitive, enabling detection and quantification from  $10^9$  to  $10^2$  bacterial cells of both BCAs.

Both BCAs were used in field trials carried out in table-grape vineyards. The spray schedules were based on 2 to 8 applications of BCAs. Berries were sampled after different times and population density of BCAs evaluated through qPCR. Both BCAs were detected on berries until one month after the last spray.

The molecular assays presented herein will be useful to investigate the most properly and timely usage of both BCAs in IPM strategies aimed at limiting the number of fungicide sprays, reducing the chemical residues on crops and the risk of resistance to fungicides in *B. fuckeliana*.

## Session 6

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# Disease Management (Postharvest)

*Chairperson:*

**Samir Droby**

*Department of Postharvest Science ARO, The Volcani Center, Bet Dagan, Israel*

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## KN.6.1 Stress responses and the induction of post-harvest tolerance to *Botrytis cinerea* in apple.

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Apples are rarely consumed directly at harvest but are preferentially stored for periods of up to 8 months postharvest to ensure a steady, year-round supply of high-quality fruits. The major losses during this period result from postharvest storage disorders and from infections with postharvest pathogens, of which *Botrytis cinerea* is one of the most important. Susceptibility to *B. cinerea* differs substantially between apple cultivars, but little is currently understood about the molecular/cellular basis for these differences. As previous results from our lab have indicated a correlation between cultivars containing higher antioxidant content and improved tolerance to post-harvest infection with *B. cinerea*, the aim of this work was to determine the possible roles of host antioxidant metabolism in explaining differences in postharvest tolerance to *B. cinerea*.

As expected, the susceptibility of apple cultivars to *B. cinerea* infection increased as a function of postharvest storage time. It was also found to be strongly cultivar dependent, and in general, the red-side of the fruit, which contains elevated vitamin C and antioxidant contents, was found to be more tolerant to artificial infection with *B. cinerea* than the green side. Again however, the extent of these differences varied between cultivars and from year to year, indicating a role for preharvest environmental factors on susceptibility. We also studied in detail the response of antioxidant metabolism to artificial infection with *B. cinerea* in the cultivar 'Braeburn' and results show that susceptibility could be modified by treatment with various reactive oxygen species (ROS) prior to infection. Finally, a transcriptomics analysis to compare gene expression levels in tissues from the red and the green side of 'Braeburn' fruit indicate that there were relatively few differences in constitutive gene expression levels, but from a gene ontology classification, it was found that the majority of the differentially-expressed genes were involved in biotic and abiotic stress responses.

Our results suggest that preharvest exposure to abiotic stresses, such as high light and elevated temperature, can modulate the postharvest susceptibility of apple fruit to postharvest *B. cinerea* infection. Possible involved mechanisms include induced cross-tolerance and an altered host ROS signaling capability that influences host defense responses.



## KN.6.2 Innovative control strategies for *Botrytis cinerea* in different postharvest fruit systems

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*Botrytis cinerea* is the causal agent of gray mold, which can be considered the most important agent of postharvest decay of fresh fruit. In several fruits, *B. cinerea* is the main postharvest pathogen (e.g. strawberry, table grapes, kaki, pomegranate), while for others it can induce considerable damage (e.g. sweet cherry, apricot, peach, apple, pear, citrus fruit). *B. cinerea* can survive in the field as a saprophyte, where it colonizes flower residues, dead leaves, or other non-living plant portions. After harvest, the pathogen can take advantage of the higher relative humidity, and it can infect the fruit through wounds or intact surfaces, and from one fruit to another (nesting). *B. cinerea* can progress with its infections also at storage temperatures (usually 0-5°C), which is also when the fruit resistance is decreased.

The main way to control postharvest gray mold is through the application of synthetic fungicides in the field. However, after harvest the residues of these fungicides in on fruit decline and so decay can develop during storage. Moreover, the use of fungicides after harvest is most of generally banned by national regulations. Treatments with antifungal agents used as adjuvants during storage (e.g. SO<sub>2</sub>) have negative effects on humans and are even more strictly regulated (e.g. SO<sub>2</sub> use is not permitted for organic grapes).

There is thus a strong need for alternative technologies to the use of synthetic fungicides to prolong the storage of fruit, and to keep it as best as possible at its harvest quality. Four categories of treatments can be used: i) biocontrol agents; ii) natural antimicrobials; iii) decontaminating agents; and iv) physical means. The research on biocontrol agents is active, with some formulations able to control of *B. cinerea* that have recently appeared on the market (Shemer, Candifruit), although it is often particularly difficult to move from the discovery of an effective antagonist to the development of a commercial product. Several natural antimicrobials of plant (essential oils, extracts, gels) and animal (chitosan) origin have the potential to be used on a large scale, although these need to be registered as plant protection products and not, as often occurs, as adjuvants. Decontaminating agents (ethanol, acetic acid, electrolyzed oxidizing water) have uses for fruit surface sterilization, mainly when the process of washing is included in fruit cooling or movement in the packing houses. The use of physical means (UV-C irradiation, ozone, CA/MA, hypobaric or hyperbaric treatments) has the advantage that they avoid direct contact with the fruit, although often their effects last only as long as they are applied. A way to further improve the effectiveness of alternatives to fungicide residues is the integration of different approaches. However, once a treatment is considered effective, it is necessary to carefully check its potential introduction in the packinghouse, transport and market chain.

Most of these treatments has direct effects on *B. cinerea* and induce host defenses, with a double mechanism in decay control. An issue for the control of postharvest decay is the slowing down of fruit respiration and ripening, which make the tissues less reactive and more prone to *B. cinerea* infection. Knowledge of the plant genomes will be helpful to better understand the changes that occur in the host plants following treatments, and then to optimize the application to get the greatest benefits from the minimum number/time/concentration of their application.

## O.6.1 Postharvest control of grey mould (*Botrytis cinerea*) by in-season crop protection

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*Botrytis cinerea* (teleomorph: *Botryotinia fuckeliana*), the causal agent of grey mould, is one of the most frequent postharvest pathogen in the global food chain affecting the storage ability of a large number of economically important horticultural crops (e.g. table grapes, small berries, lettuce). Such postharvest losses caused by *Botrytis cinerea* are often attributed to non-symptomatic, latent infections established in floral organs of fruiting crops which ultimately infect and decay ripening fruits. However, fungal infection can also occur later in the growing season as *B. cinerea* infest fruits (e.g. grape berries) through stigmata, pedicels, wounds, or by direct penetration.

To preserve fruits and vegetables from problematic fungal diseases, fluopyram, a unique fungicide from a new chemical sub-class of succinate dehydrogenase inhibitors (complex II) has been developed. Fluopyram affects the fungal growth at all stages of development demonstrating beside an effective powdery mildew control as well an excellent activity against fungal pathogens described to cause latent infections such as *B. cinerea*, *Monilinia* spp. and *Sclerotinia sclerotiorum*.

Field experiments in strawberries and table grapes have been performed to investigate whether fluopyram-containing mixtures incorporated in commercial fungicide spray programs deliver a positive effect on the shelf-life of fruits. Level of disease incidence and number of marketable fruits were assessed at harvest and after storage. Solely marketable fruits or bunches appearing visibly disease-free at harvest time were transferred into storage.

In strawberries, pre-harvest treatments with fluopyram decreased postharvest disease development of *B. cinerea*, thereby increasing the percentage of marketable fruits at harvest and after storage by 40% and 66%, respectively. In table grapes, fluopyram-containing spray programs ensured the highest level of protection on fruits after a 3-weeks cold chamber storage period. The evolution of grey mould incidence in boxes with fluopyram-treated bunches was delayed significantly, compared to other spray programs.

The significant higher number of marketable fruits at harvest and after storage in multiple crops indicates the potential of fluopyram-containing solutions to reduce the formation and persistency of latent fungal infections taking place in orchards and fields. By controlling those infections of fungal pathogens, fluopyram enables fruit producers to enlarge the period of storage ability increasing the shelf-life of harvest produces.

## O.6.2 Impacts of preharvest treatments with chitosan or potassium sorbate on postharvest gray mold and quality of table grapes

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After harvest, grape berries are particularly sensitive to infections by gray mold, caused by *Botrytis cinerea*. The pathogen grows under cold storage temperatures and penetrates directly to the berry or spreads rapidly from infected to adjacent berries (nesting). To reduce postharvest decay in conventional agriculture, bunches are sprayed with fungicides during the season and stored in the presence of sulfur dioxide. The use of chitosan or salts, such as potassium sorbate, has been considered as valid alternative to reduce pathogen resistance to fungicides and fungicide residues in the fruit. The aim of this study was to assess the effectiveness in controlling postharvest decay of table grape by preharvest application of chitosan or potassium sorbate as compared to a fungicide strategy used in conventional agriculture. In addition, the effect of these treatments on berry quality parameters and the induction of resistance were evaluated.

A program of four fungicides (consisting of pyrimethanil, cyprodinil plus fludioxonil, pyraclostrobin plus boscalid and fenhexamid) or potassium sorbate were applied to clusters of ‘Thompson Seedless’ grape berries at berry set, pre-bunch closure, véraison, and 3 weeks before harvest, during the 2009 and 2010 seasons. In 2011, the experiment was repeated including three chitosan commercial formulations (OII-YS, Chito Plant, and Armour-Zen, all used at 1% chitosan). The incidence of natural rots that developed after cold storage on the treated bunches and the gray mold that developed on single detached berries after artificial inoculation with conidia of *B. cinerea* were evaluated. The effects of these treatments were evaluated on the quality of table grapes by the basic parameters (soluble solids, titratable acidity, pH, and berry size) as well as K content. Additional parameters associated with potential induction of berry resistance were chitinase activity, phenolic compounds and hydrogen peroxide content.

The natural incidence of postharvest gray mold was reduced by potassium sorbate, the fungicide program, or both mixed together, in 2009 and 2010. In 2011, chitosan or the fungicide treatments significantly reduced the natural incidence of postharvest rots. All the treatments reduced berry shattering, while only one chitosan formulation and the fungicide program decreased berry shrivel and improved rachis appearance. Berries harvested from vines treated by two of the chitosan formulations or the fungicide program had fewer infections after inoculation with *B. cinerea* conidia. None of the treatments harmed the basic parameters of berry quality while all treatments increased berry endochitinase activity. Chitosan decreased the berry content of hydrogen peroxide, which was localized by scanning electronic microscopy. One of the chitosan formulations increased quercetin, myricetin, and resveratrol content of berry skin. It is suggested that the treatments with chitosan and potassium sorbate reduced the postharvest decay of table grape through their antimicrobial activity and possibly by induction of resistance in the host tissues.

## P.6.1 Multiple mechanisms of biocontrol of a strain of *Pichia guilliermondii* against *Botrytis cinerea* on apple

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The strain M8 of *Pichia guilliermondii* isolated from the carposphere of apples cv. Golden Delicious showed a high efficacy in controlling grey mould, caused by *Botrytis cinerea*, on apples under semi-commercial conditions. After storage at 1°C for 120 days, M8 reduced grey mould incidence from 45.3% (control) to 20.0%.

Its possible modes of action were investigated both *in vitro* and *in vivo* experiments. In apple juice medium and in wound-inoculated apples, M8 at  $10^9$  and  $10^8$  cells ml<sup>-1</sup> inhibited the spore germination of *B. cinerea* and the grey mould development. When co-culturing *B. cinerea in vitro* or *in vivo* in the presence of the yeast, neither inactivated cells nor culture filtrate of the yeast had any effect on spore germination or germ tube elongation. In apple juice medium, the spore germination was significantly recovered by the addition of 1% glucose, sucrose and fructose, or 0.5% and 1% of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, phenylalanine and asparagine. Pre-treatment with M8 at  $10^8$  cells ml<sup>-1</sup> followed by washing, significantly reduced grey mould lesions, suggesting an induction of defence responses.

Light microscopy revealed that the yeast strongly adhered to the hyphae and spores of *B. cinerea*. M8 produced hydrolytic enzymes, including  $\beta$ -1,3-glucanase and chitinases in minimal salt media with different carbon sources. Moreover, M8 produced high amounts of active exo-1,3- $\beta$ -glucanase in Lilly-Barnett minimal salt medium with different carbon sources, which greatly inhibited *B. cinerea in vitro* and *in vivo* tests. Therefore, an exo- $\beta$ -1,3-glucanase gene, named as PgExg1 was cloned from the genomic DNA of the strain M8 by genome walking. The sequencing and the nucleotide BLAST analysis indicates that no introns are present inside the gene, which was confirmed by amplifying the full gene from cDNA of the yeast. An open reading frame of 1224 bp encoding a 408-amino acid protein with a calculated molecular weight of 46.9 kDa and an isoelectric point of 4.5 was characterized. Protein BLAST and phylogenetic tree analysis of the deduced amino acid sequences from the PgExg1 gene suggested that the glucanase produced by PgExg1 gene belongs to the Glycoside Hydrolase Family 5. Expression of PgExg1 in *Escherichia coli*, followed by identification with Western-blotting, purification with Ni-NTA and analysis with enzyme assay, yielded homogeneous recombinant PgExg1. At its optimal pH of 5.0 and its optimal temperature of 40°C, the recombinant enzyme protein showed the highest activity towards laminarin, while the highest stability was obtained when the enzyme was stored at pH of 7.0 and temperature of 4°C.

Direct attachment, competition for nitrogen and carbon sources, secretion of hydrolytic enzymes and induction of host resistance act synergistically in the biocontrol mechanism of *P. guilliermondii* M8 against *B. cinerea*.

## P.6.2 Resistance inducers as alternatives to synthetic fungicides to control postharvest strawberry rots

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Gray mold and Rhizopus rot are caused by *Botrytis cinerea* (Pers.) and *Rhizopus stolonifer* (Ehrenb.), respectively, and they are the main causes of postharvest decay of strawberry (*Fragaria x ananassa* Duch.). Infections from *Penicillium* spp. (blue mold) and *Mucor* spp. (Mucor rot) can also occur occasionally. These diseases are usually managed by fungicide treatments that are applied around flowering, and are repeated up to harvest. However, in organic agriculture and after harvest, the use of fungicides is not permitted, so there is a need for alternatives. Among these, the use of resistance inducers has been proposed as potential strategy for disease management, since they can increase plant defenses, and at times can also exploit their antimicrobial properties.

Within the European project EUBerry, the objective of this study was to compare the effectiveness in the control of postharvest diseases of strawberry: (i) of solutions obtained by dissolving practical grade chitosan in acetic, glutamic, formic and hydrochloric acids, and of a water-soluble commercial chitosan formulation; (ii) of different resistance inducers such as the commercial chitosan formulation, benzothiadiazole, oligosaccharides, soybean lecithin, calcium and organic acids, extracts of *Abies sibirica* and *Urtica dioica*.

Organically-grown strawberries cv. Camarosa were immersed for 10 s in solutions of the tested compounds. Fruits dipped in deionized water at pH 5.6 were used as control. After treatment the strawberry were stored for 7 days at  $0\pm 1^\circ\text{C}$  and then exposed to shelf-life at  $20\pm 1^\circ\text{C}$ , when the incidence and severity of postharvest strawberry rots were evaluated.

The treatments with chitosan acetate, chitosan chloride, chitosan formate, chitosan glutammate, and the commercial chitosan provided reductions of gray mold of 59, 44, 53, 51 and 53% respectively, and of Rhizopus rot of 85, 68, 72, 72 and 79% respectively, as compared to the control. However, no significant differences were observed between solutions from practical grade chitosan and commercial chitosan formulation. Respect to practical grade chitosan, commercial formulations have the advantage of more practical use, as viscosity is lower than that of the biopolymer dissolved in acid solutions.

Concerning the data obtained with the different resistance inducers, the reductions, as compared to the control, in gray mold were 53, 54, 60, 68, 63, 30 and 42% for the fruit treated with the *Abies* extract, oligosaccharides, benzothiadiazole, commercial chitosan, calcium with organic acids, *Urtica* extract, and soybean lecithin, respectively, and for blue mold were 50, 29, 78, 92, 50 and 50%, respectively. Only treatments with chitosan reduced the incidence of Rhizopus rot of 82% as compared to the control. Among the tested resistance inducers, chitosan gave the highest disease reduction. Indeed chitosan has itself antimicrobial properties, could induce plant defense, and when applied over vegetal surface could form an ideal coating, that confers a physical barrier to moisture loss, delaying dehydration and fruit shriveling.

The tested compounds reduced the postharvest rots of strawberry and their use in IPM could be proposed. However, further studies even through pre-harvest trials are needed. Investigation at molecular level could furnish new knowledge concerning the mechanisms of action of these resistance inducers.

### P.6.3 Early detection of postharvest grey mould of table grapes

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Gray mould caused by *Botrytis cinerea* Pers. is regarded as one of the most dangerous postharvest diseases of table grapes, since the pathogen can spread even at very low temperatures, shortening the duration of storage and marketing.

Different infection sites have been described for *B. cinerea*: (i) on flowers, through petals, stigmas, styles, or stamens; (ii) on berries, through stigmata, pedicels, wounds, or by direct penetration of the cuticle. However, following hyphal establishment, *B. cinerea* may remain inactive, maintaining grapes symptomless, until ripening and/or a conducive environmental conditions occur. Therefore, serious economic damages may arise from harvesting apparently healthy grape berries, which might arrive to distant markets partially or totally rotten. Moreover, the accurate detection of *B. cinerea* latent infections or inoculum size in the field is essential for applying effective control strategies.

Preliminary investigations were conducted by fruit plating and freezing on local table grape varieties. They identified the 'Red Globe' as the less contaminated one and confirmed the preferential localization of latent infections at the berry-pedicel attachment zone (calotte) and pathogen presence on stamens. However, these traditional detection techniques are laborious, time consuming and require skilled expertise. Therefore, a quantitative real-time PCR (qPCR) detection method, based on a probe designed on *B. cinerea* intergenic spacer (IGS) regions and a reported probe for *Vitis vinifera* L. as internal control, was set up to reveal pathogen presence on bunches and floral parts.

The system proved to be highly specific and sensitive, enabling quantification of as little as 10 fg of *B. cinerea* DNA and detection of single conidia in artificially inoculated grape berries; moreover, it allowed reliable detection of the pathogen in naturally infected asymptomatic tissues. In particular, it revealed the presence of *B. cinerea* in 80 and 65% of apparently healthy calottes and stamens, respectively, with efficiency higher than that obtained from freezing and plating techniques. Furthermore, significant correlations ( $R^2 = 0.89$  and  $0.94$ ) were found between qPCR results and the actual disease incidence on bunches from which calottes and stamens were sampled.

In conclusion the assay presented herein might be extremely useful to producers, not only for choosing the less contaminated packs to be long stored or shipped, but also to properly and timely apply the available control means. Moreover, when further adapted and tested on a semi-commercial scale, this assay could be also applied on non-perishable samples (stamens) that can be easily shipped to distant laboratories and processed with great economic benefits.



## Session 7

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# Cultural and Integrated Management of Diseases Caused by *Botrytis* spp.

*Chairperson:*

**Yigal Elad**

*Department of Plant Pathology and Weed Research, ARO, The Volcani Center, Bet Dagan, Israel*

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## KN.7.1 Cultural methods for the management of *Botrytis cinerea* grey mould

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Grey mould is an important disease of greenhouse crops. Sweet basil (*Ocimum basilicum*) is one of the crops affected by this disease and will serve as an example for presenting the potential of cultural means for grey mould control. Basil is harvested several times during the growing season and *B. cinerea* mainly infects the stem cuts, resulting in severe disease that quickly kills the entire plant. The use of chemical fungicides is restricted, thus there is a need to develop alternative systems for grey mould management. Heating the greenhouses in which the basil is grown was a popular strategy, but the cost of this heating has forced farmers to abandon this control strategy. This has left them with increased incidence and severity of the disease, while the susceptible organs of the crop plants remain wet for longer periods every day. The objectives of this project were to explore cultural means of grey mould control and to develop an integrated system of crop management that will yield high-quality produce with no chemical residue.

Field experiments were conducted repeatedly in commercial-like greenhouses over 5 years. In these experiments, basil was planted in 3-10 m<sup>2</sup> plots. There were five replicates of each treatment and the treatments were arranged in randomized blocks. Disease incidence was recorded periodically. Basil branches were sampled for post-harvest disease evaluation at optimal disease conditions of 20°C and high humidity (in a humidity chamber).

Several cultural methods were examined. Disease was suppressed in the field and in plant material sampled from the field plots in which various cultural methods were used and in which chemical fungicides were applied long before harvest. Effective cultural methods include planting at half of the original density, fertigation with increased potassium (and to a lesser extent, calcium), spray application of a potassium salt and passive solar heating of the greenhouse. Plant yield was not reduced and actually increased in several cases that involved high disease potential in the plot or increased temperature. Post-harvest tests revealed increased resistance to *B. cinerea* infection in the plant material collected from the cultural treatments, as compared the untreated plots.

A combination of treatments provided better grey mould suppression than the individual treatments, providing sufficient disease control with minimal use of chemical fungicides. In the past, we examined measures of disease control that included timing of harvest (avoiding harvesting on rainy days, to minimize the wetness of potential infection sites), the timing of fungicide applications (immediately after harvest, to protect the fresh harvest wounds), aeration and air circulation. However, due to cropping limitations, the new methods are more likely to assist with disease management. Interestingly, the suppression of disease caused by *Sclerotinia sclerotiorum* was also achieved in the plant-spacing and passive-heating treatments.

## O.7.1 Importance and control strategy of gray mold caused by *Botrytis cinerea* in raspberry plantations in Latvia

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Raspberry (*Rubus idaeus* L) is the third most common berry crop in Latvia. In 2007 and 2008 expeditions were organized to determine spectrum of pathogens causing raspberry diseases. Damages caused by *Botrytis cinerea* Pers. were found in all inspected plantations on flower buds, flowers and fruits, it was concluded that gray mold caused by *Botrytis cinerea* is one of the major limiting factors in raspberry production. In Latvia both raspberry types are grown, the summer-bearing and primocane raspberry, which bear fruits in the late summer and fall. Due to weather conditions in the late summer and autumn gray mould is especially important in primocane raspberry production. Losses of potential harvest can reach more than 50% during periods of rainy, wet weather and also a shelf life of raspberry production due to disease is shortened. Control of *B. cinerea* in raspberry is mainly based on the use of chemicals during flowering period. The only fungicide registered for the use in raspberry in Latvia is Switch 62.5 WG. Fungicide is allowed to use twice, the first time before flowering and the second time after. Such a strategy does not provide a sufficient disease control. Lack of fungicides and transition to integrated fruit growing promotes to seek environmentally friendly alternatives how to reduce an incidence level of the gray mold.

One of the alternatives could be raspberry growing in high tunnels. Investigations with raspberry growing in high tunnels for the first time were started in 2011 in collaboration with the Latvia State Institute of Fruit-Growing. Gray mold incidence level under high tunnel production conditions was compared to field production. After two years observations it was concluded that fungicide applications for gray mold control in summer-bearing raspberry in high tunnels are not needed, the amount of rotten fruits did not exceed 5%. In primocane raspberry the use of fungicides would be necessary also under a tunnel, especially during high relative humidity.

The second alternative would be the use of substances of natural origin as crop protectants, safe for both humans and the environment. The development of a new environmentally friendly plant protection products, spruce and pine biomass extracts, was started in 2010. During 2010-2012 several laboratory and field investigations were carried out to test the effectiveness of different spruce and pine biomass extracts against *B. cinerea*. The effects of formulations were tested *in vitro* on the mycelia growth of *B. cinerea* test cultures, applying fungal radial growth test. All formulations had inhibitory effect on mycelium growth (inhibition rate >50%), using the concentration 20 g L<sup>-1</sup> in medium. In field trials an effectiveness of spruce biomass extract at three different concentrations (1, 2 and 4%) was tested on primocane raspberry cultivar 'Polana'. In the treatment with spruce biomass extract at the highest concentration an amount of rotten fruits was significantly ( $p < 0.05$ ) reduced in comparison with the untreated control. None of tested spruce biomass extract concentrations showed higher effectiveness than fungicide Switch 62.5 WG as a standard product.

## O.7.2 Preliminary evaluation on the susceptibility of new table-grape cultivars to grey mould in the field and in postharvest

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In Southern Italy, grey mould (*Botryotinia fuckeliana* (de Bary) Whetz. (an. *Botrytis cinerea* Pers.) can cause heavy yield losses, especially in table-grape vineyards covered with plastic films to delay the harvest until December. Under these conditions, several fungicide sprays can be required. Nowadays, the production of table grape is difficult due to the pathogen that is at high risk of resistance to fungicides and the low numbers of residues of chemicals required by buyers. A help to the farmers can be the availability of new varieties tolerant/resistant to grey mould. New selections of table grapes obtained by breeders crossing *Vitis vinifera* L. and other species of the *Vitis* genus in Moldova (Tudor Cazac, Scientific-Practical Institute for Horticulture and Food Technologies, Chisinau), Hungary (Pal Kozma, Research Institute for Viticulture and Enology, Pécs) and Serbia (Nada Korać and Dragoslav Ivanišević, University of Novi Sad) were evaluated against *B. fuckeliana* in the field and in postharvest.

An experimental field with randomized blocks with 4 replicates including twenty-one new grapevine selections and five commercial cultivars was implanted in Ginosa (Taranto, Southern Italy) in 2010. At the beginning of August 2012, it was covered with plastic film in order to delay the harvest. During the season no fungicide sprays were done. At the end of October, all bunches in each plot were observed and grey mould prevalence, severity and McKinney's index (MKI) values were ascertained. At the time of the assessment, grey mould symptoms were observed on 43.6% of bunches of cv. Italia (selected as reference) with a MKI 18.7%. The Hungarian cv. Moldova, Palatine, Poloskei Muskotaly and Suzy proved more tolerant since the MKI values ranged between 8% (Poloskei Muskotaly) and 0.7% (Moldova).

The attitude to the cold-storage of 12 of the tested medium- late-ripening cultivars was assayed. About 3 Kg of healthy bunches were collected, packaged and maintained at  $4\pm 1^{\circ}\text{C}$  per 5 weeks. The incidence of grey mould was evaluated weekly observing singularly all bunches. After 35 day of cold-storage, *B. fuckeliana* infected till to 95% of bunches cv. Guzun. On the reference cv. Italia MKI value was 26.9%; while cv. Moldova, Palatina, Poleskei Muskotaly and Suzy confirmed to be the more tolerant to grey mould (MKI: 11-16%).

Further evaluations need to be conducted to confirm these findings and to get deeper knowledge on the mechanisms involved in the plant-pathogen interactions also aiming at improving tolerance to grey mould in well appreciated Italian cultivars.

Work supported by MiPAAF [National Research Program (PNR 2005-2007). OIGA - Project "Evaluation of new resistant germplasm for table-grape growing with low environmental impact."].

### O.7.3 Plant responses to biochar soil treatment and *Botrytis cinerea* infection

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Biochar, a solid product of biomass pyrolysis, induces systemic resistance to foliar pathogens in various plants when added to the soil in which they are growing. Various soil-applied biochars have been shown to improve plant performance. The objectives of this study were to explore the ability of biochar to induce systemic resistance to *Botrytis cinerea* in strawberry and tomato plants and to examine the effects of this treatment on plant defense mechanisms, at the molecular level.

Disease development was examined on plants grown on 1 or 3% biochar-amended potting mixture and the relative expression of five defense-related strawberry genes (*FaPR1*, *Faolp2*, *Fra a3*, *Falox*, and *FaWRKY1*) was quantified using real-time PCR. To dissect the induced resistance pathway mediated by biochar in the tomato – *B. cinerea* pathosystem, we studied the effect of genetic variations affecting salicylic acid (SA), ethylene or jasmonic acid (JA) on responses to biochar-mediated induced resistance in tomato mutant lines and their wild-type relatives. The variation in the early cellular response to H<sub>2</sub>O<sub>2</sub> burst was associated with biochar-mediated resistance and transcriptional changes in 13 defense-related genes were observed upon *B. cinerea* inoculation of leaflets that had been detached from plants grown in biochar-treated soil.

The addition of biochar to the potting media of strawberry plants suppressed grey mould, as well as anthracnose and powdery mildew. These diseases are induced by pathogens that have very different infection strategies. Biochar treatment stimulated a range of general defense pathways, as confirmed by qPCR analysis of defense-related gene expression. Additionally, a primed-state of defense-related gene expression was observed upon infection by *B. cinerea*. It appears that the ability of soil-applied biochar to promote transcriptional changes along different plant defense pathways probably contributes to its broad-spectrum disease suppression.

Amendment of the potting mix with biochar resulted in an approx. 50% reduction in disease severity in all of the tested tomato lines, except for the JA-deficient mutant, *def1*, which did not exhibit any biochar-mediated induced resistance to *B. cinerea*. Following *B. cinerea* inoculation of the different genotypes, we observed stronger and earlier H<sub>2</sub>O<sub>2</sub> accumulation in plants growing in biochar-treated potting mix. This effect was disrupted by the *def1* mutation. Finally, while transcriptional changes due to biochar amendment and infection varied among the tomato genotypes, the expression of the genes *Pti5* and *Pi2*, which are known to play important roles in resistance to *B. cinerea*, was generally up-regulated by the combination of biochar and pathogen infection more strongly or to the same extent as it was by infection alone. The results from the quantitative disease assays and the induction of gene expression strongly suggest that a JA-signaling pathway is important for biochar-mediated resistance to *B. cinerea* in tomato.

This research was funded by the Autonomous Province of Trento (Italy), Call for Proposal Major Projects 2006, Project ENVIROCHANGE and by the Chief Scientist of the Israel Ministry of Agriculture Fund.

## P.7.1 Aerobiology of tomato grey mould under greenhouse conditions: relationship between airborne *Botrytis cinerea* conidia concentration and flower and stem infections

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Grey mold of tomato, caused by *Botrytis cinerea*, is an important constrain to production in greenhouses. Most experiments on the influence of temperature, relative humidity and inoculum concentration were conducted using aqueous inoculum, however, in greenhouse inoculum consists mostly in dry conidia. A better knowledge of the effect of airborne conidium concentration (ACC) on disease development is needed to design efficient management methods. Therefore, the influence of ACC on both flower and stem wound infections was studied. The experiment was conducted in a greenhouse maintained at temperature of 15, 20, or 25°C using diseased tomato leaves as source of dry inoculum. ACC was monitored using spore samplers and a previously developed qPCR assay to quantify *B. cinerea* conidia. Infection was measured as the proportion of diseased flowers (PIF) and diseased stem wounds (DSW) on 10 plants.

The mean and maximum inoculum concentration was 182.86 and 2134.93, 230.83 and 2635.72, and 277.26 and 3189.22 conidia/m<sup>3</sup> for the trials conducted at 15, 20, and 25°C, respectively. The mean proportion of infected flowers over the three trials was 0.21 with a mean of 0.18, 0.22, and 0.23, for the experiment conducted at 15, 20, and 25°C, respectively. The mean proportion of infected stem wound over the three trials was 0.03 with a mean of 0.02, 0.03, 0.02, for the experiment conducted at 15, 20, and 25°C, respectively. For the flower infection, this relation between ACC and PIF was not linear and was best explained by a sigmoid model of the form  $PIF = 1.093 / (1 + \exp(-(x - 2.412) / 0.474))$  with a  $R^2 = 0.97$ . The proportion of infected flowers remained low when ACC was below 10 conidia/m<sup>3</sup>. Above this concentration, flower infections increased with increasing ACC. For the stem infections, no infected wound was observed when ACC was below 100 conidia/m<sup>3</sup>, above which infection increased linearly with increasing *B. cinerea* ACC.

The results suggest that effective control can be achieved through integrated management which should include maintaining low relative humidity and warm temperatures; using optimal spacing and adequate pruning; promoting ventilation; careful handling of the plants especially during de-leafing to prevent wounding; and by removing inoculum sources through adequate plant sanitation. Periodically and closely monitor for disease symptoms, especially in the spring and fall seasons for early symptoms combined with monitoring airborne *B. cinerea* inoculum could help ensuring good sanitation practices and in timing de-leafing operations.



# Botrytis "-omics" and post-genomics

*Chairperson:*

**Jan A.L. van Kan**

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## KN.8.1 Status and perspectives of genome sequencing of *Botrytis* species

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Genome sequencing has revolutionised biological research in general, and fungal biology in particular. Of all the eukaryote genomes that have currently been determined and made public, the vast majority of organisms sequenced are fungal species. Costs of sequencing have dropped dramatically and for most fungal species, it is currently feasible to spend less than € 1000/isolate for obtaining raw genome sequence data that are good enough for genome assembly. For gene expression analysis, RNA sequencing can be performed for roughly € 500/sample. The costs are expected to drop further in the near future. The obvious challenge for a scientist is to handle these data and make biological sense out of it, by performing a structural and functional annotation. Analysing the annotated genome may generate novel hypotheses that can be experimentally validated. In this presentation, I will give an overview of the current status of the genome sequencing of *Botrytis cinerea* and other *Botrytis* species, and I will illustrate how genome sequence information has helped to obtain information on particular genes (and sets of genes), which have increased our understanding of the lifestyle of *B. cinerea* and the mechanisms that it exploits to infect host plants.

It is well established that *B. cinerea* displays an enormous genetic diversity in the field. The availability of a high quality reference genome will enable to study genetic diversity among isolates at a genome-wide level, especially by identifying single nucleotide substitutions and small insertions/deletions or isolate-specific sequence regions, which may be relevant for the biological properties of the isolate(s). Moreover, comparative genome analyses will help resolving the complex genetic structure of the 'dicot-specific' clade of the genus *Botrytis* and of the species complex, which might comprise *B. cinerea*, *B. pseudocinerea*, as well as *B. fabae* and a recently identified group, designated as '*Botrytis* group S'.

## KN.8.2 Global analysis of the *Botrytis cinerea* signalling network; exploring the fungal phosphoproteome

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The mechanism developed by *B. cinerea* to infect different plants and tissues is a very complex process, being cellular signalling cascades the bridge between environmental conditions and the cellular machinery of the fungus, and therefore responsible for regulating fungus development as well as the infective stages. This process is based in the transport and transduction of signals from the exterior to the cytosol and nucleus, which is mediated by one or more ligands and its receptors, or by post-translational modifications of proteins involved in a signalling cascade. Among these modifications, the addition of phosphate groups is one of the most relevant post translational modifications of proteins. Despite to their importance in the metabolism control, and the fact that the some of the *B. cinerea* pathogenicity factors has been described as phosphoproteins (<http://www.phibase.org/>), to date, there is no a global description of these proteins during the infective cycle of phytopathogenic fungi. In this sense, proteomics approaches followed by MS analysis had proved its relevance to unravel complex biological processes from a global point of view, without the necessity to select a set of previous candidate genes as is usual in other molecular approaches. According to our previous experience, we are currently developing a global study the mechanism of activation/regulation of the infective cycle of *B. cinerea* from a proteomic differential approach, focusing on post-translational modifications (PTMs), specifically in phosphorylation, considering that, due to its participation in signalling transduction process, this subproteome plays a key role in setting the infection strategy developed by fungus at each specific stage.

In order to establish an efficient strategy for large scale phosphoproteomic study in *B. cinerea*, we are working on an efficient method for the characterization of phosphorylated proteins that are expressed by the fungi. Our strategy is based in a two-step protocol, a phosphopeptide enrichment method based on the use of a first ERLIC step to isolated phosphopeptides selectively from tryptic digests and separate it in different fractions with high resolution. Secondly, a supplementary enrichment step by TiO<sub>2</sub> stagetips column protocol. These fractions are them analyze by nanoflow HPLC/electrospray ionization mass spectrometry, and obtained results analysed by using TPP–Transproteomic Pipeline and X! Tandem search engine). Using this method, we are comparing *B. cinerea* phosphopeptides under different pathogenicity induction conditions to reveal the role of this PTM in the infection cycle. Following this approach, we will study the modifications in the membrane proteins to obtain a proteomic global network of the signalling cascade events occurring during plant infection.

## O.8.1 Microarray analysis of the infection cushion of *Botrytis cinerea*

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*Botrytis cinerea* is an ascomycete responsible for gray mould on hundreds of dicot plants. This necrotrophic fungus is thought to enter the host mainly by producing degrading enzymes and causing an oxidative burst rather than using physical pressure. Appressorium-like structures depicted as swollen tips growing from a germinated conidiospore have been found frequently. These primary infection structures are not highly melanized and not always separated from the germ tube by a septum, which would be necessary for the generation of an osmotic pressure. On the other hand, when plants are infected with mycelium, a different form of penetration has been observed, whereby hyphae growing on the plant surface heavily ramify into short bulbous cell aggregates, similar to the melanized “infection cushion” (IC) of the closely related fungus *Sclerotinia sclerotiorum*. The importance of IC in fungal penetration has not yet been molecularly investigated in *B. cinerea*.

A microarray analysis was conducted to identify genes that may putatively function as key determinants for the differentiation and development of infection cushions in *B. cinerea*. The formation of IC was obtained from conidiospores germinated in the presence of exogenous nutrient on the surface of a cellophane membrane and hybridizations of cDNAs were performed on a Nimblegen chip. Using a p-value of 0.05, our analysis revealed 932 genes up-regulated greater than 2-fold and 1045 genes down-regulated greater than 2-fold during IC development relative to undifferentiated hyphae. Transcriptome profiling uncovered metabolic and regulatory processes specifically dedicated to the plant penetration.

## O.8.2 Preliminary results of whole transcriptome RNA-Seq analysis to study morphological and sexual differentiation in *B. fuckeliana*

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The availability of the complete genome of *Botryotinia fuckeliana* has opened new and intriguing perspectives on the genetic and molecular mechanisms involved in crucial aspects of the fungal biology.

In its life cycle, *B. fuckeliana* produces vegetative mycelium and conidia, the primary source of infections, sclerotia for survival under adverse conditions, and as a basis for sexual development, microconidia functioning as male gametes, and apothecia as result of the sexual process. *B. fuckeliana* usually exhibits a heterothallic bipolar mating system. Sexual compatibility is controlled by a single *MAT1* locus with two alternative idiomorphic sequences (*MAT1-1* or *MAT1-2*) conferring sexual identity. Homothallic behavior (*MAT1-1/2*) was occasionally observed in isolates of the fungus.

Next Generation Sequencing (Illumina technology) was applied to gain insight into gene expression profiles associated with different development stages and identify key genes involved in regulation of morphogenesis and sexual differentiation in *B. fuckeliana*. Global transcriptome was analysed in two near-isogenic *B. fuckeliana* reference strains of opposite mating type at the following stages: i) actively growing mycelium; ii) mature sclerotia; iii) carpogenesis-induced sclerotia; iv) spermatized sclerotia; v) apothecial primordial; vi) fully developed apothecia. Total RNA from each sample was submitted to cDNA library construction using standard protocols for RNA-Seq experiments. About 16 million of short sequencing reads (50 bases in length) generated from each libraries were aligned to both the available *B. fuckeliana* reference genomes (strain T4, <http://urgi.versailles.inra.fr/Species/Botrytis> and strain B05.10, [http://www.broadinstitute.org/annotation/genome/botrytis\\_cinerea/MultiHome.html](http://www.broadinstitute.org/annotation/genome/botrytis_cinerea/MultiHome.html)) and analyzed to measure transcript levels. A wide range of variations in the number of regulated genes and their patterns of regulation was recorded.

Differentially expressed genes were divided into groups based on their expression modulation, containing genes positively or negatively modulated along the whole development progress, or specifically induced at each stage. Genes showing great differences in transcription levels ( $FC > 20$ ) in the two strains of opposite mating types included: mating-type pheromone receptors and other membrane proteins, *bet* domain-containing proteins, transcription factors and signal transducers, and several proteins of unknown function.

Tag sequence density resulted adequate for quantitative analysis even for genes weakly transcribed, and led to ascertain constitutive transcription of the idiomorph-specific genes at *MAT1* locus (*MAT1-1-1* or *MAT1-2-1* genes, encoding transcription factors, and *MAT1-1-5* and *MAT1-2-4* genes, encoding proteins of unknown function), in *MAT1-1* and *MAT1-2* strains, at each of the analysed development stages. Constitutive transcription of *MAT1*-specific genes was confirmed by RT-PCR experiments that also revealed, for the *MAT1-2-1* gene, two transcript isomorphs resulting from antisense gene transcription and alternative splicing.

## **P.8.1 Transcriptional study of the ABC transporters encoding genes during plant infection and in response to fungicides treatment in the phytopathogenic fungus *Botrytis cinerea***

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The grey mould fungus *Botrytis cinerea* causes losses of commercially important fruits, vegetables and ornamentals worldwide. Various fungicides with different modes of action are effective against this pathogen, but isolates with multiple fungicide resistance phenotypes (Multi Drug Resistance, MDR) have been observed with increasing frequencies. In fungi, ATP binding cassette (ABC) transporters participate to drug efflux and their activity has been correlated with their gene transcription level. Here we report on the parallel transcriptional study of all predicted 53 ABC transporter encoding genes in *B. cinerea*. Following fungal exposure to two different fungicides *in vitro*, fast transcriptional induction of a large number of these genes was recorded. Common genes in the two responses and specific expression profiles allowed the demonstration of links between these responses and the finding of putative regulatory DNA motifs in these genes promoters. Expression studies during plant infection revealed large induction of the ABC encoding genes, individual differences between expression in conidia and *in vitro* grown mycelium, and overall distinct transcriptional behavior of families E and F. Lastly, expression profiling during infection of treated plants revealed over-expression of specific genes, weak induction levels and differences with expressions recorded *in vitro*.

## **P.8.2 Identification of complex, polygenic disease networks in *Botrytis cinerea* and *Arabidopsis thaliana* using natural variation**

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While there are numerous examples of large effect, single gene resistance mechanisms in plant pathology, most plant-pathogen interactions involve many genes in both the plant and pathogen. To identify these polygenic networks controlling disease resistance, we used the natural variation within the *Botrytis/Arabidopsis* model pathosystem to conduct a genome wide association study (GWAS) in the host and identify co-expression networks associated with disease resistance among the natural accessions of the host *Arabidopsis thaliana*. Infected leaf tissue for 96 accessions of *Arabidopsis* was monitored for 86 visual and 45 chemical phenotypes measured by automated image analysis and HPLC assays at 72 hpi with either a control or one of four genomically and phenotypically diverse *Botrytis cinerea* isolates (Apple517, B05.10, Supersteak, or UKRazz). A multivariate ANOVA analysis showed that the genetic background of the host affected 69 visual (79.3%) and 44 chemical (97.8%) phenotypes while the genetic background of the pathogen affected 50 visual (57.5%) and 41 chemical (91.1%) phenotypes. More importantly, 29 visual (33.3%) and 38 chemical (84.4%) phenotypes showed a significant interaction between the host and pathogen genotypes. For all phenotypes showing a significant effect from the host, a heteroscedastic effects model was applied to a collection of 115,301 single nucleotide polymorphisms present in at least 20% of the *Arabidopsis* accessions for each individual isolate and the resulting genes were used to find co-expression networks in the ATTEDII database. For lesion area, 3,354 genes were identified with only 477 genes (14.2%) found in three or more isolates, indicating that the *Botrytis* resistance networks for lesion area are largely isolate specific. A total of 246 co-expression networks were identified with 20 networks having more than 10 nodes. Genes associated with resistance to more than three isolates were dispersed evenly across many networks suggesting that there is no central co-expression network for *Botrytis* resistance. Instead, it appears that there is variation in numerous networks that have diverse putative functions, including specialized metabolism, cell wall biosynthesis, vesicle trafficking, transcriptional regulation and kinase signaling cascades. Results from this experiment have direct implications for developing quantitative experiments to elucidate complex disease interactions in pre-domesticated species, understanding mechanisms of disease resistance in natural plant populations, and determining novel strategies for combating infection.

### **P.8.3 Functional characterisation of two signal transduction genes identified by phosphoproteomics in *Botrytis cinerea***

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Protein phosphorylation and dephosphorylation are cellular processes rapidly induced by external *stimuli* adapting regulatory circuits and enzymatic functions to changing environmental conditions. Signal transduction (ST) pathways necessary for signal perception and phosphorylation cascades are involved in many physiological processes such as development, stress adaptation, virulence etc.. We established a gel-free phosphoproteomic approach for systematic identification of phosphorylated peptides in *Botrytis cinerea*.

The gel-free phosphoproteomic approach combines two sequential steps after trypsin digestion: i) SCX chromatography (strong cation exchange); ii) IMAC (Immobilized metal affinity chromatography) for the enrichment of phosphopeptides prior to LC-MS/MS analysis. We identified approximately 1650 different phosphopeptides from fungal cultures grown with or without the fungicide fludioxonil. However, only 94 ( $p < 0.05$ ) differentially phosphorylated proteins were identified by comparative phosphoproteomics of both types of extracts with 15% of ST proteins. We started the functional characterisation of two candidate genes through gene inactivation. A protein-kinase homologous to the cell cycle kinase Pom1 de *Schizosaccharomyces pombe* is particularly important for *B. cinerea* infection development. The second target gene encoding a phosphatase-like protein, homologous to the *Aspergillus nidulans* PhnA protein, involved in the G $\beta$  $\gamma$ -protein complex, seems important for asexual development.

We are going to analyze the role of phosphorylation in both proteins as well as their interaction with known ST cascades.



## P.8.4 Modifications in membrane proteins profile of *B. cinerea* under pathogenicity induction. Analysis of fungal membranome

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*B. cinerea* possess a wide set of weapons to infect plant tissues. The fungus uses these factors to penetrate into the plant, degrade the plant biomass, overcome plant defences, etc.. These fungal tools has been described one by one through molecular approaches and its role in the infection cycle established by molecular approaches based on site specific mutations procedures describing the mutant phenotype. The obtained collection of genes is known as collection of virulence/pathogenicity factors. Most of these genes belongs to this collection are listed in available web resources as PHI (Pathogen host interaction database). Nowadays, the relevance of high throughput molecular technologies may improve the detection of new proteins involved in the fungal pathogenicity cycle. Between others available techniques, proteomics approaches has showed its potency to unravel complex biological processes, proving its advantages as an alternative against other “-omics” methods. Recent data show the differences observed between mRNA and proteins abundance in several organism, showing that the proteome is the relevant set of analysis. Moreover, data from obtained peptides may help in the functional annotation of sequenced genome.

In order to search for proteins components involved in the pathogenicity of *B. cinerea*, we initiated the analysis of the membrane proteins components (membranome), due to those proteins must be the bridge between environmental conditions and intracellular molecular processes that trigger fungal pathogenicity machinery. As initial step, we used two-dimensional gel electrophoresis (2-DE) to compare the different profiles of membrane proteins expressed by *B. cinerea* under different pathogenicity induction obtained by cultivating *B. cinerea* 2100 (CECT) on MSM supplemented with 1% of (i) glucose, as constitutive state and (ii) tomato deproteinized cell wall (TCW) as an inducer of pathogenicity. Membrane protein extraction and 2-DE was optimized showing, in both stages, a subproteome located between 4 and 8 of *pI* with a molecular weight from 116 to 14 kDa. Analysis of these profiles showed a differential expression of membrane proteins following the induction of pathogenicity with TCW, showing a higher number of "spots". We detect common and specific spots in each assayed carbon source, which has been analysed by MALDI TOF/TOF. Most of these proteins may have a role in the infection process. However, these subproteome present problems during 2DE probably due to the hydrophobic nature of these proteins based in its transmembrane domains, showing low number of spots in the Sypro Ruby stained gels. For this reason we have to change to gel-free system based on LC MS/MS analysis to increase the number of detected peptides and identified proteins. This study may help us understand the role of these proteins as pathogenic factors, revealing new components of the signalling network.

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# Host-Pathogen Interactions (Fungal Virulence Factors)

*Chairpersons:*

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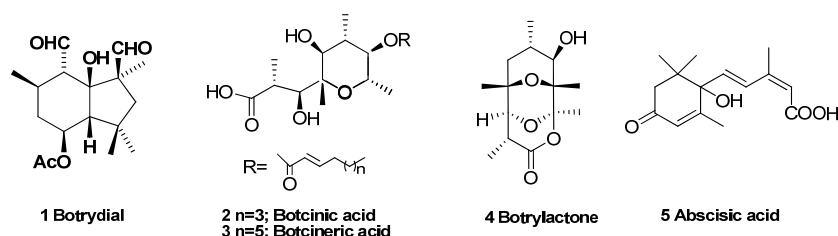
## KN.9.1 From genes to molecules. Induction of silent biosynthetic pathways for the expansion of the metabolome in *Botrytis cinerea*

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*Botrytis cinerea* is a well known pathogen of a number of commercial crops and produces many structurally diverse metabolites. Among them, two series of phytotoxic metabolites were identified: a family of characteristic sesquiterpene metabolites which contain the basic botryane skeleton, principally botrydial (**1**) and two polyketide lactones types i.e. botcinins (**2**, **3**) and botrylactone (**4**). Additionally, abscisic acid (**5**), a sesquiterpene known as a phytohormone, has been isolated and reported.

The recent availability of the *B. cinerea* genome at the Genoscope (<http://www.genoscope.cns.fr/>) and at the Broad Institute (<http://www.broad.mit.edu/annotation/cgi/>) provided the opportunity to investigate secondary metabolism gene clusters including those putatively involved in sesquiterpene biosynthesis. Although only two families of toxins have been isolated and reported, the sequencing of the genomes of the B05-10 and T4 strains revealed an abundance of novel biosynthetic gene clusters. Genomic data revealed that *B. cinerea* has 43 secondary metabolism key enzymes; some of them are specific of this phytopathogen. Within the genome, in addition to 22 polyketide synthases (PKSs), 6 genes coding for putative sesquiterpene cyclases (STCs) and 3 genes coding for putative diterpene cyclases (DTCs) have been identified.



To study them, we have undertaken different approaches: chemical- and molecular-based studies including comparative genomics, gene inactivation and overexpression. As a result new molecules with novel structures were found. The isolation and characterization of these molecules are under study and their structures and biosynthetic pathways, using deuterated acetate and labelled glucose experiments, are shedding light on new biological targets to control *Botrytis* infection. Furthermore, new hybrid molecules have been designed and some of them have displayed potent antifungal activity.

## **KN.9.2 Virulence factors in *Botrytis cinerea*: an overview**

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Several decades of research about the molecular mechanisms underlying the extremely successful pathogenic ability of *B. cinerea* have greatly increased our understanding of its infection strategy, but have revealed less virulence factors than initially expected. We now know that key features of this strategy include the secretion of a vast array of enzymes aimed at the degradation/softening of the plant defensive barriers, the generation of an oxidative burst of fungal origin which adds to the plant-derived oxidative burst, the induction of PCD (Programmed Cell Death) in the host, and the secretion of phytotoxins. The genes/proteins responsible for this armament seem to act synergistically in what has been defined as an overkill strategy, while the individual players making a significant individual contribution to the infection process, i.e. virulence factors, are scarce. The nature of these factors will be reviewed, with special emphasis on secreted proteins.

## KN.9.3 Ca<sup>2+</sup> / Calcineurin-mediated signal transduction in *Botrytis cinerea*

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Eukaryotic cells respond and adapt to external stress by a network of signal transduction pathways, which are essential for survival. Many cellular processes are mediated through transient increases of the cytosolic Ca<sup>2+</sup> concentration. Those arise from internal stores like the vacuole or the ER but can also derive from influx via the plasma membrane.

In *S. cerevisiae* two channel proteins are described to be the major players in maintaining the intracellular Ca<sup>2+</sup> level: Mid1 a putative stretch-activated Ca<sup>2+</sup> channel, and the voltage-gated high-affinity Ca<sup>2+</sup> channel Cch1. The cellular impact of external Ca<sup>2+</sup> was investigated by functional characterization of deletion mutants of the corresponding homologs in *B. cinerea*<sup>1</sup>. Both deletion mutants (*bcmid1* and *bccch1*) and the double knockout mutant exhibited similar phenotypes, as they displayed reduced growth under low-calcium conditions. All mutant strains were not able to grow at all when they were cultured in the presence of the Ca<sup>2+</sup>-chelating agent BAPTA, whereas supplementation with external CaCl<sub>2</sub> rescued the mutants' phenotype. Localization studies with the Mid1 protein fused to GFP indicated that BcMid1 is an intracellular channel protein, probably located in the ER.

The Ca<sup>2+</sup>/calmodulin-dependent phosphatase calcineurin (CN) is a conserved protein that plays a critical role in calcium signaling and stress response in eukaryotic cells. CN activity itself is regulated by a class of conserved proteins termed Calcipressins. Deletion mutants of *bccnA*, encoding the catalytic subunit of CN, exhibit severe growth and development defects and form small, compact colonies<sup>2</sup>. In comparison, deletion of the only calcipressin homologous gene, *bcrn1*, affects vegetative growth in a similar way. While *bccnA* deletion mutants are completely avirulent, virulence of *bcrn1* deletion mutants on bean plants appears to be only reduced. Studies with the CN-inhibitor Cyclosporine A (CsA) showed that the expression of several genes, e.g. those involved in phytotoxin biosynthesis, is regulated by CN and the CN-responsive transcription factor BcCrz1 in *B. cinerea*. Interestingly, the same set of genes is down-regulated in  $\Delta bcrn1$  mutants indicating that BcRcn1 functions as an activator of CN. In addition, the transcript levels of *bcrn1* and *bccr1* itself are increased after activation of the CN-dependent signaling pathway through stimulation with external Ca<sup>2+</sup>. Motifs in the BcRcn1 protein sequence were identified which are probably essential for the activation of this regulator and for binding to CN.

<sup>1</sup> Harren K. and Tudzynski B., 2013. Cch1 and Mid1 are functionally required for vegetative growth under low-calcium conditions in the phytopathogenic ascomycete *Botrytis cinerea*. *Eukaryotic Cell*, doi:10.1128/EC.00338-12.

<sup>2</sup> Harren K., Schumacher J. and Tudzynski B., 2012. The Ca<sup>2+</sup>/calcineurin-dependent signaling pathway in the gray mold *Botrytis cinerea*: The role of calcipressin in modulating calcineurin activity. *PLoS ONE*, doi:10.1371/journal.pone.0041761.

## O.9.1 The NADPH Oxidase Complexes in *Botrytis cinerea*

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Reactive oxygen species (ROS) are generated in all aerobic environments and therefore play a major role for many organisms depending on oxygen. For example they act as messenger molecules for intercellular signaling or play a role during defense mechanisms against pathogens (Heller and Tudzynski, 2011). One good example is the oxidative burst; plants rapidly produce large amounts of ROS as the first defense reaction towards pathogen attacks. NADPH oxidases (Nox) are the most common enzymatic system to produce these ROS. Nox are enzyme complexes, which transport electrons through biological membranes and therewith reduce oxygen to superoxide. In fungi they are shown to be involved in differentiation processes and pathogenicity and are therewith in our focus to gain insights into plant - fungi interactions.

In the phytopathogenic fungus *Botrytis cinerea* two NADPH oxidases (BcNoxA and BcNoxB) as well as their putative regulator (BcNoxR) were previously identified (Segmueller *et al.*, 2008). Besides their involvement in pathogenicity and sclerotia production, deletion studies have revealed that BcNoxA and BcNoxR are also involved in hyphal germling fusions (Roca and Weichert *et al.*, 2012).

Recent analyses show a localization of the catalytical subunits BcNoxA and BcNoxB to the ER and partly to the plasma membrane of hyphae, while the regulator BcNoxR is localized in vesicles and at the hyphal tips (Siegmund *et al.*, 2013).

Nox are multi-enzyme complexes, whose regulatory process and the participating proteins are well described in mammals. However, in fungi not all components have been identified, yet. For *B. cinerea* interaction studies with potential candidates identified the small GTPase Rac, the GEF BcCdc24, the scaffold protein BcBem1 and the PAKs BcCla4 and BcSte20 as interacting proteins within the BcNox complex.

Heller J. and Tudzynski P., 2011. Reactive oxygen species in phytopathogenic fungi: Signaling, development, and disease. *Annu. Rev. Phytopathol.*, 49, 369–390

Roca M.G., Weichert M. *et al.*, 2012. *FungalBiol.*, 116(3), 379-387.

Segmueller N. *et al.*, 2008. *Mol. Plant Microbe Interact.*, 21, 808-819.

Siegmund U. *et al.*, 2013. The NADPH oxidase complex in *Botrytis cinerea*: evidence for a close association with the ER and the tetraspanin Pls1. *PLoS One*, 8:e55879.

## O.9.2 Functional characterization of *Bclae1* gene in *Botrytis cinerea*

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The heterotrimeric Velvet complex comprises a highly conserved fungal specific group of at least three proteins, VeA, VeB, and LaeA. This complex was first studied in *A. nidulans* and it was shown to be involved in fungal development and secondary metabolism. Last year, the homologue of VeA, BcVEL1, was also identified in *Botrytis cinerea*, and it was shown to be involved in the regulation of differentiation processes (sporulation and sclerotia formation) and the production of oxalic acid and melanin (Schumacher *et al.*, 2012). In addition, BcVEL1 is essential for full virulence on different hosts, but the production of the phytotoxic secondary metabolites botrydial and botcinic acid is not affected by the *bcvell1* deletion.

Later on, we identified the gene *BclaeA*, encoding a histone methyltransferase, which was shown to be a component of the Velvet complex in several fungi. The *A. nidulans laeA* mutant is unable produce sterigmatocystin, lovastatin and penicillin. Similarly, LaeA homologues are involved in the control of secondary metabolism also in other fungi. Thus, it is required for gibberellin production in the phytopathogenic fungus *F. fujikuroi* (Wiemann *et al.*, 2010) and penicillin formation in *Penicillium chrysogenum* (Hoff *et al.*, 2010). Besides, it has also key functions in fungal development, e.g. in conidiation and sexual development (Bayram *et al.*, 2012).

In this work we identified the *Botrytis cinerea Bclae1* gene and characterized it functionally by the generation of knock out mutants. These  $\Delta laeA$  deletion strains have a similar phenotype as the velvet mutant: they cannot produce sclerotia in the dark, but instead produce conidia in a light-independent manner. They also produce more melanin and are impaired in oxalic acid production (*bcoahA* transcript levels were lower than the wild type). They are also impaired in pathogenicity, however not as severe as the velvet mutant.

Beside the similarities to the *bcvell1* mutant, there are some features which are unique to the *laeA* mutant: they show reduced levels of botrydial gene expression detected by qPCR, suggesting a reduced level of botrydial production. Comparative microarray analyses with the wild type and the *bcvell1* and *bclaeA* mutants are currently under investigation.



### **O.9.3 *Botrytis cinerea* protein O-mannosyltransferases play critical roles in morphogenesis, growth, and virulence.**

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Protein O-glycosylation is crucial in determining the structure and function of numerous secreted and membrane-bound proteins. In fungi, this process begins with the addition of a mannose residue by protein O-mannosyltransferases (PMTs) in the lumen side of the ER membrane. We have generated mutants of the three *Botrytis cinerea* *pmt* genes to study their role in the virulence of this wide-range plant pathogen. *B. cinerea* PMTs, especially PMT2, are critical for the stability of the cell wall and are necessary for sporulation and for the generation of the extracellular matrix. PMTs are also individually required for full virulence in a variety of hosts, with a special role in the penetration of intact plant leaves. Furthermore, PMT2 also contributes significantly to fungal adherence on grapevine and tobacco leaves. Analysis of extracellular proteins showed significant changes in the pattern of protein secretion and glycosylation by the *pmt* mutants, and allowed the identification of new protein substrates putatively glycosylated by specific PMTs. Since plants do not possess these enzymes, PMTs constitute a promising target in the development of novel control strategies against *B. cinerea*.

## O.9.4 The phytotoxic activity the *B. cinerea* cerato-platanin BcSpl1 resides in a two-peptide epitope in the protein surface

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BcSpl1, a cerato-platanin family protein abundantly secreted by *B. cinerea*, was previously reported to contribute to virulence and to cause necrosis when infiltrated in tomato, tobacco, and *Arabidopsis* leaves, an effect that is caused by the induction of the hypersensitive response in the treated area. Besides, BcSpl1 has also been shown to induce SAR (Systemic Acquired Resistance) in the treated plants. The necrosis-inducing activity of BcSpl1 on *Arabidopsis* is partially dependent on the signaling protein BAK-1, suggesting that this protein is being recognized as a PAMP (Pathogen Associated Molecular Pattern) by membrane-associated pattern recognition receptors of the plant immune system. These receptors usually bind to evolutionary-conserved regions in protein surfaces, and several of these were identified in BcSpl1. Serial deletion studies with the *bcspl1* gene, and the expression and purification of the resulting truncated proteins, pointed to two of these conserved short regions forming a single epitope in the protein surface as putatively involved in the necrotizing activity. Two chemically-synthesized peptides with these two sequences showed that the two have necrotizing activity by themselves, but they show the highest activity when mixed. The expression of a chimeric protein with the two peptides in the same orientation on a different, unrelated, protein backbone further confirmed that the two comprise the necrotizing-active epitope of BcSpl1. Moreover, transformation of the *B. cinerea bcspl1* mutant with this chimeric protein showed that this two-peptide epitope suffices for the BcSpl1 contribution to virulence. On the other hand, preliminary results on the search for a putative BcSpl1 receptor in tobacco and *Arabidopsis* will be presented.

## O.9.5 D-galacturonic acid utilization by *Botrytis cinerea*

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The plant cell wall is the first barrier to pathogen invasion. The fungal plant pathogen *Botrytis cinerea* produces a spectrum of cell wall degrading enzymes for the decomposition of host cell wall polysaccharides and the consumption of the monosaccharides that are released. Especially pectin is an important cell wall component, and the decomposition of pectin is required for full virulence of *B. cinerea*. An effective concerted action of the appropriate pectin depolymerising enzymes, monosaccharide transporters and catabolic enzymes is important for complete pectin utilization by *B. cinerea*.

In this study, RNA sequencing was performed to compare genome-wide transcriptional profiles in *B. cinerea* grown in media containing glucose and pectate as sole carbon sources. Transcript levels of 32 genes that are induced by pectate were further examined in cultures containing 6 different monosaccharides by quantitative RT-PCR. We identified 8 genes that are specifically induced by D-galacturonic acid, including *Bcgar2* and *Bcga1* (encoding two key enzymes in the D-galacturonic acid catabolic pathway) and two putative monosaccharide transporter genes. In addition, conserved motifs were identified in the promoters of genes involved in pectate decomposition and D-galacturonic acid utilization. The role of these motifs in regulating D-galacturonic acid-induced expression was functionally analysed in the promoter of *Bcga1* gene. We will present the data on the functional characterization of the transporter genes and the identification of motifs required for D-galacturonic acid induction.

## P.9.1 The thioredoxin system in *Botrytis cinerea*- not just a redox system but a key player in fungal development?!

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In the course of infection the pathogenic grey mould fungus *Botrytis cinerea* triggers an oxidative burst as early plant defense reaction. This leads to an active release of reactive oxygen species (ROS), which are on the one hand known to be responsible for molecular damages of biological molecules, but on the other hand they are also involved in cell signalling pathways. We are particularly interested in the influence of ROS on pathogen-host interaction and development. In order to investigate the maintenance of the fungal intracellular redox state, we focused on the thioredoxin system, which is composed of two enzymes, the thioredoxin (BcTrx) and the thioredoxin reductase (BcTrr).

Knock-out and complementation approaches of *bctrx1* and *bctrr1* revealed a severe impact on pathogenicity. The mutants were able to penetrate, but only caused small necrotic lesions that were not able to spread. Furthermore,  $\Delta bctrr1$  and  $\Delta bctrx1$  showed a strong sensitivity to oxidative stress; in addition an enhanced H<sub>2</sub>O<sub>2</sub> production of  $\Delta bctrr1$  and generally retarded growth compared to  $\Delta bctrx1$  and the wild-type was striking. Northern analyses showed that oxidative stress response genes were constitutively expressed in the  $\Delta bctrr1$  mutant, while the transcriptional level of these genes was not altered in the  $\Delta bctrx1$  mutant. Consequently, the thioredoxin system seems to be essential for the detoxification of ROS, fungal pathogenesis and the development of *B. cinerea*.

Currently we are analyzing a putative BcTrx2 and created a double deletion of both thioredoxins ( $\Delta\Delta bctrx1/2$ ). Furthermore, consequences to the redox status of the mutants by applying oxidative stress will be followed with the help of a redox sensitive GFP (roGFP; Heller *et al.*, 2012). Moreover, the connection to other signalling components, especially those involved in oxidative stress response, like Bap1 and Skn7, is under investigation.

Heller J., Meyer A.J. and Tudzynski P., 2012. Redox-sensitive GFP2: use of the genetically encoded biosensor of the redox status in the filamentous fungus *Botrytis cinerea*. *Mol. Plant Pathol.*, 13(8), 935–947.

## **P.9.2 *In planta* early expressed genes by the phytopathogenic fungus *Botrytis cinerea***

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The recent annotation of the *Botrytis cinerea* genome (Amselem *et al.*, 2011) has revealed a large number of putative genes which might be expressed during different stages of the fungal life cycle.

Especially interesting are those genes expressed during plant infection. They could be responsible for the aggressiveness of the fungus, having an important role during the “gray mold” disease it causes.

Conidia germination and plant surface penetration are two crucial points at the early infection process, and it is when the fungus has to be able to establish itself on the plant surface and prepare to spread across the plant. At these early stages, a set of significant genes might be highly expressed.

Trying to understand the molecular mechanisms during host-pathogen interactions, we focused on the identification of genes highly expressed at these very early stages. Once we have good candidates, they could be used as targets to develop new tools for effective treatments against this organism.

Using microarrays, we have identified more than 150 genes which are expressed during the early stages of infection, but not in conidia. Most of these genes codify for proteins with unknown function. By means of bioinformatic analyses, transmembrane domains and signal peptides were identified in some of these proteins, suggesting a possible role in signaling pathways or as effectors in the interaction with the plant.

We are currently focusing on 18 of these genes: 9 single genes and 9 genes located in clusters. We validated their high expression by real time PCR. Some of them showed an expression at 12 hours post inoculation even 10,000-fold compared to the expression in conidia. In order to elucidate their possible role, we have generated knock-out mutants. Pathogenicity studies as well as further characterization of the different deletion mutants have been performed.

### **P.9.3 The role of MAP kinase signaling in gene expression, appressoria formation and pathogenesis in *Botrytis cinerea***

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The infection cycle of *Botrytis cinerea* starts with the germination of the conidia which is induced by physical and chemical stimuli. The filamentous growth MAP kinase (MAPK) cascade, consisting of Ste11 (MAP3K), Ste7 (MAP2K), BMP1 (MAPK), and the Ste50 scaffold protein, serves as a central signalling complex that is involved in germination and essential for infectious growth. *B. cinerea* deletion mutants in any of the MAPK components are unable to form appressoria and have lost pathogenicity. We also identified a putative surface sensor protein, the signalling mucin Msb2, which seems to act upstream of the BMP1 MAPK cascade. *Msb2* deletion mutants are defective in appressoria formation and show reduced BMP1 phosphorylation levels during germination on hard surfaces. In contrast, another putative sensor protein, Sho1, was not involved in surface sensing, but found to be essential for secondary lesion formation.

Microarray transcriptome analysis of conidia germinated on apple-wax coated surfaces was performed to identify genes that are involved in appressorium function and early host invasion. Many genes showing peak expression during the first few hours encoded secreted proteins, indicating that *B. cinerea* conidia show a peak of secretory activity during germination. Expression of many genes encoding secreted proteins was dependent on both Msb2 and BMP1, which was interpreted as further evidence for a regulatory link between Msb2 and BMP1.

To identify new proteins interacting with BMP1 MAPK pathway components, the split ubiquitin yeast two-hybrid system was used. This resulted in the identification of two new BMP1 interacting proteins, Bic5 and Bic8. While Bic5 is a membrane protein with a possible function in glycosylation, Bic8 is a predicted cytosolic protein possibly involved in nitrogen metabolism. The function of these two proteins will be analysed by knockout analysis. Furthermore, a yeast two-hybrid cDNA screen will be performed to identify more components of the Msb2 - BMP1 signalling pathway.

Leroch *et al.*, 2013. *Eukaryot. Cell*, 12, 614-626.

## P.9.4 Characterizing a regulator of G protein signaling up-regulated in germling and unicellular appressorium of *Botrytis cinerea*

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A microarray analysis was conducted to identify genes that may putatively function as key determinants for the differentiation and development of unicellular appressorium in *Botrytis cinerea*. The formation of appressoria was obtained from conidiospores germinated in the presence of exogenous nutrient on the surface of a teflon membrane and hybridizations of cDNAs were performed on a Nimblegen chip. Among the genes that were up-regulated at both the germination and the unicellular appressorium stages, one coding a Regulator of G protein Signalling (RGS) was identified. Over-expression of this RGS encoding-gene was also observed during the early infection stage of grape berries.

In fungi, G-protein-mediated signalling pathways are involved in the regulation of a variety of cellular functions related to vegetative growth, mating, conidiation, secondary metabolism, infection structure differentiation, and pathogenicity. RGS proteins make up a diverse and multifunctional protein family that plays a critical role in controlling heterotrimeric G protein signalling. All RGS proteins possess a conserved 130 amino acids domain (RGS box) that interacts with an activated guanosine triphosphate (GTP)-G alpha subunit. This interaction greatly accelerates the intrinsic GTPase activity of that subunit, and rapidly turns off activated G protein signals, thereby acting as negative regulators by switching off G protein-coupled signaling pathways. However, in addition to directly suppressing G protein signalling through GTPase-accelerating protein (GAP) activity, RGS proteins can also enhance the activation of G-protein pathways by acting as effectors, effector antagonists, and scaffolding proteins for receptors, G proteins, or other regulatory molecules (Li *et al.*, 2007).

The protein encoded by the gene identified in this study contains the RGS box but also an additional PAS protein domain probably acting as a sensor. PAS domains are important signalling modules that monitor changes in light, redox potential or overall energy level of a cell in bacteria or fungi. In order to investigate the biological role of this RGS-protein in *B. cinerea*, a replacement mutant was constructed. The preliminary phenotypic analysis of the mutant confirms the implication for this RGS-protein in the virulence of *B. cinerea*.

This work is funded by the SafeGrape ANR project and by the Comité National des Interprofessions des Vins d'appellation d'origine (CNIV).

Li *et al.*, 2007. Heterotrimeric G Protein Signaling in Filamentous Fungi. *Annu. Rev. Microbiol.*, 61, 423-452.

## P.9.5 Expression profiles of Sesquiterpene Cyclase gene family in the phytopathogenic fungus *Botrytis cinerea*

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The phytopathogenic fungus *Botrytis cinerea* shows a wide variety of mechanisms for infecting host plants. *B. cinerea* can infect more than two hundreds plant species and it uses different mechanisms depending on host plant species and environmental conditions among other factors. It is known, that *B. cinerea* can secrete a broad range of cell wall-degrading enzymes and toxins<sup>1</sup>. During the last years, two families of toxins have been identified, and their biosynthesis reported in collaboration between several research groups, including genomic and chemical studies, i.e. the sesquiterpene botrytidal and related compounds and botcinic acid and its derivated<sup>1,2,3</sup>.

In the biosynthesis pathway of toxin botrydial, five genes are co-regulated in a physical cluster, being one of them a sesquiterpene synthase (BcBOT2). Inactivation of this gene leads to abolish botrytidial biosynthesis and to overproduce of botcinic acids<sup>4</sup>. The recent publication of the complete genome sequence of this fungus, and its subsequently annotation, have allowed to find other genes encoding key enzymes such as terpene synthases. In total, six putative sesquiterpene cyclase genes have been identified along the genome sequence of *B. cinerea*<sup>1</sup>, but not all of them have been totally characterized yet, and they could play an important role in the fungal cycle life.

This study presents the analysis of expression profiles shown by quantitative reverse transcription-PCR of the six genes encoding sesquiterpene cyclase enzymes in *B. cinerea*. A culture medium supplemented with plant material was used for stimulating the expression of these genes involved in the secondary metabolisms of the fungus. The study described the evolution of the gene expression since first hours of germination until the fifth day of fermentation, in which the level of toxins production reaches the highest amount described until date<sup>2</sup>.

<sup>1</sup> Amselem J., Cuomo C.A., van Kan J.A.L., Viaud M., Benito E.P., *et al.*, 2011. Genomic Analysis of the Necrotrophic Fungal Pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. *PLoS Genet*, 7(8), e1002230. doi:10.1371/journal.pgen.1002230.

<sup>2</sup> Collado I.G., Macias Sánchez A.J., Hanson J.R., 2007. Fungal terpene metabolites: biosynthetic relationships and the control of the phytopathogenic fungus *Botrytis cinerea*. *Natural Product Report*, 24(4), 674-686.

<sup>3</sup> Massaroli M., Moraga J., Bastos Borges K., Ramirez-Fernández J., Viaud M., Collado I.G., Durán-Patrón R., Hernández-Galán R., 2013. A Shared Biosynthetic Pathway for Botcinins and Botrylactones Revealed through Gene Deletion. *ChemBioChem*, 14(1), 132-136.

<sup>4</sup> Pinedo C., Wang C.M., Pradier J.M., Dalmais B., Choquer M., Le Pêcheur P., Morgant G., Collado I.G., Cane D.E., Viaud M., 2008. Sesquiterpene synthase from the botrydial biosynthetic gene cluster of the phytopathogen *Botrytis cinerea*. *ACS Chemical Biology*, 3(12), 791-801.





## Session 10

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# Host-Pathogen Interactions (Plant defence)

*Chairpersons:*

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## O.10.1 A surprising defense phenotype is observed when a grapevine polygalacturonase-inhibiting protein is overexpressed in transgenic grapevine and infected with *Botrytis cinerea*

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Grapevine is a fruit crop of great economic importance. The widely cultivated grapevine species, *Vitis vinifera*, is unfortunately highly susceptible to fungal pathogens such as *Botrytis cinerea* leading to compromised grape quality and reduction in yields. North American *Vitis* species show low susceptibility to fungal infections compared to *V. vinifera*. Plants utilise a family of proteins called polygalacturonase-inhibiting proteins (PGIPs) in an attempt to counteract and suppress the activity of pathogen-derived cell wall degrading enzymes called endopolygalacturonases (ePGs) during infection. We have previously isolated and functionally characterised PGIPs from *V. vinifera* (VvPGIP1)<sup>1</sup>, as well as North American *Vitis* species (non-*vinifera* PGIPs)<sup>2</sup>. When overexpressed in tobacco, all the PGIPs decreased the susceptibility against *B. cinerea* dramatically, but the non-*vinifera* PGIP lines showed even lower susceptibility to the fungus compared to the plant lines overexpressing VvPGIP1<sup>3</sup>. This promising result prompted a study where two of the non-*vinifera* PGIPs was overexpressed in *V. vinifera* cultivars under the control of a strong constitutive promoter. The transgenes were therefore expressed in all organs at high levels, in contrast to the endogenous *Vvpgip1* gene, which has been shown to have a tissue-specific and developmentally controlled expression pattern<sup>4</sup>. Transgene presence, expression, intergration and phenotypic analyses of the transgenic populations confirmed the stable integration of the transgenes and production of active PGIPs. These plant lines were subsequently infected with *B. cinerea* in whole plant infection assays. Four leaves per plant were infected on the adaxial side with three infection spots per leaf, and a total of 1000 spores per infection spot. The infection assay was carried out in high humidity perspex chambers maintained at room temperature under a 16/8 hour light/dark cycle. The non-*vinifera* PGIP overexpressing lines were found to be hyper-susceptible to the pathogen. These results, as well as the progress made to explain this surprising phenotype will be reported. It is clear that the hyper-susceptibility phenotype is not due to gene-silencing of the transgene, or the endogenous *Vvpgip1* gene. The population of transgenics and the controls are currently being used as important plant resources to study the grapevine-*Botrytis* interactome.

<sup>1</sup> De Ascensao A., 2001. Isolation and characterisation of a polygalacturonase-inhibiting protein (PGIP) and its encoding gene from *Vitis vinifera* L.. PhD Thesis. Stellenbosch University, Stellenbosch, Republic of South Africa.

<sup>2</sup> Wentzel L., 2005. The endopolygalacturonases from *Botrytis cinerea* and their interaction with an inhibitor from grapevine. MSc Thesis, Stellenbosch University.

<sup>3</sup> Venter A., 2010. The functional analysis of *Vitaceae* polygalacturonase-inhibiting protein (PGIP) encoding genes overexpressed in tobacco. MSc thesis. Stellenbosch University, Stellenbosch, Republic of South Africa.

<sup>4</sup> Joubert D.A. *et al.*, 2013. Regulation of the grapevine polygalacturonase-inhibiting protein encoding gene: expression pattern, induction profile and promoter analysis. *J. Plant Res.*, 126, 267-281.

## O.10.2 Systems biology of tomato fruit cell wall disassembly during infection by *Botrytis cinerea*

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Ripening of fleshy fruit is an example of a developmental program that coincides with increased susceptibility to necrotrophic pathogens, including *Botrytis cinerea*. Ripening relies on a complex network of regulatory and signaling pathways that lead to significant physiological and biochemical changes to fruit. The increased susceptibility of ripe fruit to pathogens may require all ripening processes and events, or, alternatively, may be a consequence of only some of them. We have previously shown that the cell wall (CW) disassembly that underlies the fruit's gradual loss of firmness is crucial for fungal susceptibility.

Glycome profiling of CW extracts (i.e., ELISA-based screening using CW glycan-directed monoclonal antibodies) prepared from *B. cinerea*-infected (3 days post inoculation, dpi) and healthy tomato fruit at two ripening stages identified infection-induced changes in fruit CW composition and structure that are associated with resistant (unripe) or susceptible (ripe) fruit. Specific classes of tomato fruit CW polysaccharides that are depolymerized by *B. cinerea* during infection include arabinogalactan (AG-1 epitopes) and pectic arabinogalactan side chains of rhamnogalacturonan-I (RG-1/AG epitopes) within the pectin network. Remarkably, the most evident modifications in pectins occur during infection of unripe fruit. These results suggest that *B. cinerea* either secretes a large repertoire of pectin-modifying enzymes or triggers the premature expression of ripening-related CW-modifying enzymes to loosen the unripe fruit wall in an effort to penetrate the tissue, utilize the polysaccharides as a carbon source and eventually colonize the fruit. In contrast, *B. cinerea* infections seem to have minor effects on the ripe fruit CW, which had been disassembled by endogenous proteins during ripening, thus supporting fungal growth.

To identify plant and fungal genes that might be involved in the changes to the CW observed in the glycome profiling, a high-throughput transcriptomic analysis (i.e., RNAseq) of both tomato fruit (unripe and ripe) and *B. cinerea* genes was performed and then validated by qRT-PCR. At 3 dpi, expression of tomato pectin modifying genes (e.g., polygalacturonases (PGs) and pectin methylesterases (PMEs)) was observed in both unripe and ripe fruit; with more genes (17 in ripe and 5 in unripe fruit) expressed at higher levels in infected ripe fruit. In contrast, we observed significant up-regulation of *B. cinerea* CW-modifying proteins during infection of unripe fruit (3 dpi), including 5 PGs, 7 pectin/pectate lyases, a rhamnogalacturonase and a PME; while at 3 dpi up-regulation of the *B. cinerea* genes was not as pronounced in ripe fruit. These results establish that in unripe fruit, *B. cinerea* depends largely on its own enzymatic machinery to accelerate fruit CW disassembly, to eventually promote susceptibility, whereas plant enzymes may have a greater influence in fruit susceptibility when the ripening process has started.

Investigations on interactions between tomato transgenic lines with suppressed activity of fruit-specific CW modifying proteins (e.g., *LePG2A*, *LePME1-2*) and *B. cinerea* mutants (e.g.,  $\Delta BcPG1$ ,  $\Delta BcPG1\Delta BcPME1$ ,  $\Delta BcPME1\Delta BcPME2$ ) lacking enzymes relevant in CW degradation may reveal the roles of CW modifying enzymes in fruit susceptibility and serve as a framework for the future development of resistant commodities.

### **O.10.3 Tomato transcriptome and mutant analyses suggest plant stress hormones have a role in the interaction between fruit and *Botrytis cinerea***

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Fruit-pathogen interactions provide a valuable biological system to study plant development in the transition from resistant to susceptible. In general, unripe fruit are resistant to infection but become increasingly more susceptible as they ripen. During ripening, regulatory and signaling networks involving plant stress hormones coordinate numerous physiological and biochemical changes. The role of the plant stress hormones ethylene (ET), salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA) in the activation of defense responses to pathogens has been characterized mostly for vegetative tissues. In infections of fruit, however, our understanding of the functions of these hormones is limited. The roles of these hormones in the pathways that activate defense responses during fruit ripening need to be addressed.

In order to identify and characterize ET, SA, JA and ABA-related genes that are regulated by *Botrytis cinerea* infections of unripe (mature green, MG) and ripe (red ripe, RR) fruit and/or by normal ripening of uninfected fruit, we analyzed hybridization-based microarray data obtained from tomato fruit infected with *B. cinerea* B05.10. We focused on the patterns of expression of genes involved in hormone biosynthesis or signaling to infer the relevance of ET, SA, JA and ABA hormones in fruit-pathogen interactions. The expression profiles of 20 key hormone-related genes were validated by qRT-PCR using infected and healthy fruit tissues at the MG and RR stages. We integrated the gene expression results with the susceptibility phenotypes of fruit compromised in hormone synthesis and perception, in order to hypothesize how ET, SA, JA and ABA influence the resistance or susceptibility of unripe and ripe tomato fruit to *B. cinerea*.

Our results showed that high levels of ET and ABA, which stimulate senescence and ripening, may facilitate fruit colonization by *B. cinerea*. The balance between SA and JA responses seems to be crucial for resistance in MG fruit, while ABA production is associated with the susceptibility of RR fruit. ET, at an appropriate concentration, may contribute to the resistance in fruit by activating JA/ET responses and possibly by blocking the antagonistic effects of SA on JA signaling, but at higher concentrations, ET may promote fruit ripening and, thereby, facilitate susceptibility. Our results provide evidence that transcriptional reprogramming of multiple plant hormone networks occurs during the interaction between tomato fruit and *B. cinerea* and during normal uninfected fruit ripening.

In general, plant hormones can promote resistance or susceptibility in fruit depending on hormone concentration, timing of the synthesis and perception of the hormones, the competence of the host tissue to respond to active forms of the hormones, the localization of the plant's response to the hormones and the pathogen's infection strategy, including its own production of hormones. Our investigation of plant hormone synthesis and signaling in fruit takes us closer to the discovery of applications that promote resistance during particular stages of fruit ripening, perhaps by alterations to plant hormone signaling pathways.

## O.10.4 Resistance to *Botrytis cinerea* in PGI<sub>P</sub>-expressing tobacco: A shift in timing of hormone signals and defence responses

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The plant cell wall is a key line of defence against pathogens. One of the first cell wall degrading enzymes to be secreted by plant pathogens is polygalacturonase (PG). Pectin hydrolysis is one of the key infection strategies of *Botrytis cinerea*. This necrotrophic fungal pathogen possesses six PG isoforms, with BcPG1 and BcPG2 established as virulence factors. Plants have in turn evolved PG-inhibiting proteins (PGIPs) to protect against cell wall degradation. Constitutive expression of PGIPs successfully enhanced disease resistance in a number of plant hosts against a range of plant pathogens. Tobacco plants constitutively expressing *Vitis vinifera* PGI<sub>P</sub>1 (VvPGIP1) were resistant to *B. cinerea* in a whole-plant infection assay. *In vitro*, VvPGIP1 inhibited the activity of BcPG1, BcPG3, BcPG4 and BcPG6, but not BcPG2<sup>1</sup>, however BcPG2 was inhibited *in vivo* using transient expression into *Nicotiana benthamiana*<sup>2</sup>. Transcriptional analysis of transgenic lines prior to infection showed changes in gene expression, cell wall metabolism and lignin deposition, suggesting that VvPGIP1 also has functions unrelated to its inhibitory activity<sup>3</sup>.

In order to investigate the mechanism of resistance to *B. cinerea*, transgenic lines were generated, expressing VvPGIP1 under the control of the constitutive 35S promoter. Whole-plant infection assays were conducted and a significant decrease in lesion diameter was observed for transgenic lines. To elucidate the effect of VvPGIP1 expression on susceptibility to *B. cinerea*, the transcriptional changes during a whole-plant infection were studied. Eight week old VvPGIP1 line 37 (Vvpgip1-37) and the wild-type (WT) *Nicotiana tabacum* cv. Havana Petit SR1 plants were inoculated with *B. cinerea* at three leaf positions. Leaf position three was used to analyse the local response to infection at four time-points using the Agilent Tobacco Gene Expression Microarray. A fold change of 1.5 and significance of  $p < 0.05$  was used to select the probes significantly different from uninfected Vvpgip1-37 and WT. GO enrichment was used to functionally group these probes.

A subset of eight GO terms was selected to represent functional components of the response to *Botrytis* infection. In Vvpgip1-37, the GO terms “photosynthesis”, “defence response” and “response to osmotic stress” were enriched in upregulated probes prior to infection. Striking differences during the course of infection was observed for the GO terms “photosynthesis”, “carbohydrate metabolism”, “amino acid metabolism”, “cell death” and “hormone metabolism”. Closer inspection of the probes included in the GO terms showed changes in the timing of transcript induction, particularly with relation to jasmonate metabolism and signalling. These data support the hypothesis that resistance to *Botrytis* involves a potential signalling function of VvPGIP1, possibly mediating downstream effects via jasmonate signalling.

<sup>1</sup> Joubert D.A. *et al.*, 2006. *Transgenic Research*, 15, 687–702.

<sup>2</sup> Joubert D.A. *et al.*, 2007. *Molecular Plant-Microbe Interactions*, 20(4), 392–402.

<sup>3</sup> Alexandersson E. *et al.*, 2011. *BMC Research Notes*, 4(1), 493.

## O.10.5 Ripening and rotting: susceptibility of ripening tomato fruit to *Botrytis cinerea*

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Developmental programs influence interactions between plant hosts and pathogens. In fruit, dynamic development and ripening processes impact infections by *Botrytis cinerea* and lead to the significant losses due to rotting of harvested products. In tomato (*Solanum lycopersicum*), early fruit development, controlled by hormones and transcription factors, establishes structures and metabolism within the organ and leads to the accumulation of substrates that are reconfigured later during maturation and ripening. As fruit ripen around mature seeds, physiological and metabolic characteristics impact interactions with pathogens and are evident as traits of the terminally developed organ. Ripening is a highly regulated developmental process with significant impacts on susceptibility, particularly to a necrotroph, such as *B. cinerea*. Unripe tomato fruit are largely resistant and ripe fruit, typically, are susceptible to *B. cinerea*. The resistance of unripe fruit can be influenced by the developmental events in the fruit as well as by the infection strategy of the pathogen. In addition to alternative infection and growth strategies of *B. cinerea*, the increased susceptibility of ripe fruit may be a default outcome of the entire syndrome of ripening or susceptibility may require only a subset of ripening processes. By identifying regulators of fruit ripening and processes that are crucial for the susceptibility to *B. cinerea*, acceptably mature fruit with reduced rotting may yield improved fresh and stored products.

Tomato lines with mutations in transcription factors that affect development and maturation provide evidence for the roles of early fruit metabolism and subsequent ripening in susceptibility to *B. cinerea*. Tomato fruit ripening is regulated by hormones, particularly ethylene, and a network transcription factors, including *RIPENING INHIBITOR (RIN)*, *NON-RIPENING (NOR)* and *COLORLESS NON-RIPENING (CNR)*. Mutations in *rin* and *nor* reveal that some of the processes controlled by these ripening regulators are required for susceptibility but others are dispensable for infections by *B. cinerea*. The lack of resistance of *Cnr* mutant fruit suggests that selected ripening events can be activated and, thereby, promote susceptibility but not overall ripening. Early tomato fruit development is influenced by a *Golden 2-like* transcriptional factor, *SIGLK2*, which specifies the extent of chloroplast elaboration in fruit prior to ripening. Chloroplast development is controlled in fruit differently than in leaves, perhaps in anticipation of the remodeling that occurs as chloroplasts are converted into chromoplasts during ripening. The accumulation of metabolites, such as sugars, carotenoids and plant hormone precursors, in chloroplasts before ripening can influence *B. cinerea* metabolism and infection outcomes in the ripened fruit.

How susceptibility or resistance to *B. cinerea* is deployed or restricted is influenced by events inherent in the host as well as by the pathogen infection strategies. Analysis of tomato fruit infected with *B. cinerea* has identified host and pathogen processes occurring concurrently that influence disease outcomes. The plasticity and efficacy of defense responses in fruit against *B. cinerea* appear to be under developmental control and, therefore, the characterization of key regulators and their downstream pathways should provide a framework to identify targets to improve resistance of ripened fruit to rotting pathogens.



## O.10.6 Manipulating defense pathways in grapevine using plant defensins and polygalacturonase-inhibiting proteins: Does it work for *Botrytis*?

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Commercial viticulture relies on cultivars of the European grapevine (*Vitis vinifera* L.) due to their excellent fruit and wine quality. Grapevines' domestication history and propagation practices have contributed significantly to the high susceptibility of this species against pests and pathogens<sup>1</sup>. Analyses of the grapevine genome show that most cultivars are surprisingly closely related, but that there are high levels of unexploited genetic diversity in *Vitis*<sup>1</sup>. Understanding the defense pathways in grapevine is important to evaluate strategies to address the pathogen susceptibility in commercial cultivars. The most important fungal pathogens of grapevine include *Botrytis cinerea*, causing grey mould on grapes, as well as the powdery and downy mildew pathogens.

We have previously isolated, characterised and functionally confirmed the antifungal properties of a range of grapevine genes, including defensins<sup>2</sup> and polygalacturonase-inhibiting proteins (PGIPs) against *B. cinerea* in *in vitro* tests<sup>3,4</sup>, as well as analyses in model plant species. A selection of these genes has been overexpressed in commercial cultivars of grapevine and the transgenic populations subjected to genetical and phenotypical analyses. Here we report the evaluation of these transgenic populations in terms of their defense phenotypes against the grapevine fungal pathogens, and in particular to whole plant infections by *B. cinerea*. Our results confirm the discrepancies often seen between *in vitro* and *in planta* analyses as well as model versus crop plant results. It also provides insight into the mode of actions of the transgenes as well as the defense mechanisms activated in the transgenics and their effectiveness against the necrotrophic *B. cinerea*.

<sup>1</sup> Myles S. *et al.*, 2011. Genetic structure and domestication history of the grape. *PNAS*, 108(9), 3457-3458.

<sup>2</sup> De Beer A. and Vivier M.A., 2008. Vv-AMP1, a ripening induced peptide from *Vitis vinifera* shows strong antifungal activity. *BMC Plant Biology*, 8, 75-90.

<sup>3</sup> Joubert D.A. *et al.*, 2006. The grapevine polygalacturonase-inhibiting protein (VvPGIP1) reduces *Botrytis cinerea* susceptibility in transgenic tobacco and differentially inhibits fungal polygalacturonases. *Transgenic Research*, 15, 687-702.

<sup>4</sup> Joubert D.A. *et al.*, 2013. Regulation of the grapevine polygalacturonase-inhibiting protein encoding gene: expression pattern, induction profile and promoter analysis. *J. Plant Res.*, 126, 267-281.

## P.10.1 Towards an inhibition-interaction model for *Botrytis cinerea* endopolygalacturonases (BcPGs) and grapevine polygalacturonase-inhibiting proteins (PGIPs)

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During host plant infection, *Botrytis cinerea* releases endopolygalacturonases, known as BcPGs, which break down the pectin component of the plant's cell wall. *B. cinerea* possesses at least six isoforms of BcPGs (BcPG1, BcPG2, BcPG3, BcPG4, BcPG5 and BcPG6) with BcPG1 and BcPG2 being important virulence factors. The macerating action of the BcPGs on the plant's cell wall provides an entry route for the fungus, thus potentially leading to colonisation of the plant tissue. A group of plant-derived proteins called polygalacturonase-inhibiting proteins (PGIPs) counteract and suppress the activity of the BcPGs. The interaction of the plant-derived PGIPs and the fungal derived BcPGs plays a crucial role in determining the extent of damage that *Botrytis* can inflict on the host plant. We have previously characterised the inhibition interaction of BcPGs and a PGIP from *Vitis vinifera* (VvPGIP1)<sup>1</sup>, the commercial grape variety. In this study we are extending this analysis to PGIPs isolated from North American grapevine species. The dynamics of the interaction of BcPGs with the plant host's PGIPs during infection is being studied through *in-vitro* and *in-planta* analysis on *Vitis vinifera* plant lines overexpressing PGIP encoding genes from North American *Vitis* species (non-*vinifera* PGIPs). These transgenic plant lines exhibited high susceptibility to *B. cinerea* compared to the untransformed control plants when challenged with the fungus in whole plant infection assays, under conditions which are optimal for *B. cinerea* growth.

Purified BcPGs were used to analyse the necrotising/lesion formation ability on grapevine leaves from the transgenic and control lines. BcPG1 and 6 caused primary lesion formation whilst BcPG2 caused secondary necrotic lesions in all lines tested including the control line. No lesion formation was observed for BcPG3 and 4. The purified BcPGs were also used in *in-vitro* interaction assays against crude PGIP protein extracts from the transgenic and control lines. The crude PGIP extracts showed inhibition against BcPG1 and 6, but not against the rest of the BcPGs in this *in vitro* assay. The *in-planta* interaction of BcPGs and the non-*vinifera* PGIPs was also studied through agro-infiltration co-expression studies according to an established method<sup>2</sup>. Co-expressing the individual BcPGs with each of the non-*vinifera* PGIPs gives an indication of the *in-planta* inhibition profile of the non-*vinifera* PGIPs against specific BcPGs. This data is very useful to develop an inhibition interaction model for grapevine PGIPs and *B. cinerea* PGs.

<sup>1</sup> Joubert D.A., Slaughter A.R., Kemp G., Becker J.V.W., Krooshoof G.H., Bergmann C., Benen J., Pretorius I.S., Vivier M.A. 2006. The grapevine polygalacturonase inhibiting protein (VvPGIP1) reduces *Botrytis cinerea* susceptibility in transgenic tobacco and differentially inhibits fungal polygalacturonases. *Transgenic Res.*, 15, 687-702.

<sup>2</sup> Joubert D.A., Kars I., Wagemakers L., Bergmann C., Kemp G., Vivier M.A., Kan J.A.L. 2007. A Polygalacturonase-Inhibiting Protein from Grapevine Reduces the Symptoms of the Endopolygalacturonase BcPG2 from *Botrytis cinerea* in *Nicotiana benthamiana* Leaves Without Any Evidence for *In Vitro* Interaction. *MPMI*, 4, 392-402.

## **P.10.2 Hexanoic acid protects tomato plants against *Botrytis cinerea* priming enhanced resistance defenses and reducing the oxidative stress**

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Treatment with the resistance inducer hexanoic acid (Hx) protects tomato plants from the necrotrophic pathogen *Botrytis cinerea*. We demonstrated that this treatment can activate defense responses against this necrotroph (Vicedo *et al.*, 2009).

To determine the molecular mechanisms underlying the hexanoic acid-induced resistance (Hx-IR) an expression profile of treated plants upon *B. cinerea* inoculation was studied and compared to those of treated and *B. cinerea*-inoculated tomato plants at 24 hours after inoculation. The microarray analysis showed that Hx mostly primed for *B. cinerea*-induced genes. Among them, highlights the up-regulation of genes encoding for proteinase inhibitors, DNA-binding factors, enzymes involved in plant hormone signaling and synthesis and genes related with the oxidative stress response. In addition, Hx was found to promote genes not early induced by the fungus at 24 hpi that could be part of its priming mechanism.

The effect of Hx on the plant redox status was studied by measuring the accumulation of oxidative stress-related compounds and enzymatic activities. Primed plants inoculated with *Botrytis* showed a more restricted ROS accumulation around the infection site than untreated plants as shown by specific staining. The Hx treatment was associated to higher levels of reduced ascorbate and glutathione, along with antioxidant activities such as glutathione reductase and catalase closer to levels of non-infected plants.

These results shed more light on the complex mode of action of hexanoic acid demonstrating that protects tomato plants from *B. cinerea* by priming botrytis-specific and non-specific defense and signaling genes and preventing the harmful effects of the oxidative stress occurring during infection.

Vicedo B., Flors V., Leyva M.O., Finiti I., Kravchuk Z., Real M.D., García-Agustín P. and González-Bosch, 2009. Hexanoic acid induced resistance against *B. cinerea* in tomato plants. *Molecular Plant-Microbe Interactions*, 22, 1455-1465.

### **P.10.3 Transcriptomic data analysis of tobacco infected with *Botrytis*: establishing a framework using phylogenomic comparisons with other plant genomes**

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Tobacco (*Nicotiana tabacum*) was used as a heterologous host for the constitutive expression of *Vitis vinifera* polygalacturonase 1 (VvPGIP1). Initial characterization of the transgenic lines revealed an increased resistance to *Botrytis cinerea* during whole-plant infection assays. In order to evaluate the mechanisms of resistance in the transgenic lines, gene expression was monitored using the Agilent Tobacco Gene Expression Microarray. Expression profiles were generated for five time points in the control *N. tabacum* cv. Havana Petit SR1 and a resistant transgenic line (Vvpgip1-37).

Tobacco is an established model system for plant pathogen interactions; however it is not well described on the molecular level compared to other model plants such as *Arabidopsis*. The nuclear genome of tobacco has been published to Genbank, but annotation of it is not yet complete. The Agilent Gene Expression Microarray is an EST-based microarray, with ESTs sourced from TIGR (Release 3) from Sept 2006, IGR Plant Transcript Assemblies (Release 5) from Jun 2007 and UniGene (Build 11) from July 2008. The annotation supplied by Agilent provided gene descriptors for less than a fifth of the probes. Nearly half the probes are designated by the undescribed source mRNAs, while the rest are annotated as “unknown”.

The Gene Ontology (GO) annotations and gene descriptors of the microarray probes were updated by assigning the corresponding ESTs to orthologous clusters generated with 9 or 26 sequenced plant genomes using a phylogenomic comparison. Using 9 genomes provided GO annotations for 10,965 probes and gene descriptors for ~44% of the probes. A second re-annotation using 26 plant genomes in parallel Ortho-MCL provided GO annotations for 14,964 probes. The microarray probes were also mapped onto the *Arabidopsis* metabolic pathways, as represented in AraCyc for metabolic contextualization, and onto the *Arabidopsis* Interactome, in order to identify protein complexes of interest that were affected differentially during the course of this experiment.

As computing power increases and more plant genomes are sequenced the Agilent Tobacco Gene Expression Microarray may be increasingly better described through this approach. A number of the genes that have been identified as being of interest in this whole-plant infection assay were not well annotated in the original release documentation. Therefore, the re-annotation and contextualization has already provided an improved framework to interpret transcriptional changes during the infection of tobacco with *B. cinerea*.



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