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The **Società Italiana di Patologia Vegetale, SIPaV, (Italian Society for Plant Pathology)** was established in 1992 following the dissolution of the Italian Society for Crop Protection (SIF) and the Italian Phytopathological Association (AFI). Its main aims are to promote research into different branches of plant pathology, to disseminate knowledge about plant diseases and their aetiological agents and to promote cooperation among experts working in the field of plant pathology, and partnership in fundamental and applied reasearch. The Society organizes meetings, gathers and distributes information about plant diseases, and maintains cooperation with other national and international scientific organizations and with national and local administrative authorities on problems involving plant health management.

The Society publishes a journal (Journal of Plant Pathology), which hosts articles by members and external contributors, a bulletin and other bibliographic material to exchange information among members.

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EDITORIAL PREFACE

This special issue of the Journal of Plant Pathology was edited on the occasion of the second PATHOLUX Conference "Impact of Plant Pathogens on the Quality of Crops and Wine (Patholux - Grapelux) - Plant Pathology in Relation to Food Safety and Food Quality", which took place in Mondorf-les-Bains in Luxembourg on October 22 and 23, 2012, gathering participants from 15 countries. It contains abstracts from oral presentations and posters, as well as full contributions.

The Conference was organized by members of the working group "Plants, food and nutrition" of the Public Research Center Gabriel Lippmann located in Belvaux, Luxembourg.

The objective of the 2-day Conference was to discuss the impact of plant pathogens on food quality aspects, including wine. A wide number of topics were addressed, covering the control and detection of fungi, viruses, and bacteria, their metabolites, the mechanisms of plant-pathogen interactions and the impact of pathogens on quantitative and qualitative properties of crops and resulting food products.

More specifically, the following topics were addressed on day 1:

- Secondary metabolites: origin, regulation and their role in organism interactions
- Understanding toxin production and plant pathogens and their detection for improving food safety
- Pathogen control: what matters?

The topics covered on day 2 included:

- Challenges for grape protection under future climate conditions
- Innovative approaches in pesticide reduction
- Impact of pathogens on wine quality

Full contributions were peer reviewed by at least one external reviewer and one member of the scientific committee.

We are grateful for the scientific exchange and insightful results gained during this Conference, and would especially like to extend our gratitude to all authors for their contributions, as well as to the reviewers of the full manuscripts.

We further wish to thank Elisabeth Clot, Alexandra Dobrowolski, Boris Untereiner, Aude Corvisy, Marie-Caroline Jonville, Nicole Baron and Servane Contal for technical support and Robert Ley and André Mehlen for their availability as Chairmen.

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IMPACT OF PLANT PATHOGENS ON THE QUALITY OF CROPS AND WINE (PATHOLUX - GRAPELUX) - PLANT PATHOLOGY IN RELATION TO FOOD SAFETY AND FOOD QUALITY

DAY 1

22 OCTOBER 2012

FACTORS AFFECTING TAN SPOT ON WINTER WHEAT IN THE GRAND-DUCHY OF LUXEMBOURG

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SUMMARY

Tan spot of wheat caused by *Drechslera tritici-repentis* was identified for the first time in the Grand-Duchy of Luxembourg (GDL) in 1999 on the basis of morphological characters. In order to optimize disease control measures in this country, tillage methods, cultivar resistance, and fungicide effects were investigated during 1999-2009 in four sites. Over this period, only three years (i.e. 1999, 2000, and 2009) with epidemic outbreak were recorded. Field experiments showed a significant difference in disease severity between sites ($P < 0.001$), cultivars ($P < 0.0001$) and years ($P < 0.001$). In years with epidemic outbreaks, the interaction of cultivars with non-inversion tillage, intensive winter wheat production, and favorable weather conditions caused an early outbreak of the disease and a significant severity at growth stage 83 (early dough). Non-inversion tillage was found to be a major factor increasing tan spot severity compared with conventional tillage. Furthermore, the analysis revealed that disease severity was related to the cultivar's susceptibility. For cultivars with similar phenology, disease severity beetween the most and the least susceptible cultivars differed by a factor of two to four. The study also showed that no fungicide (mix of triazoles and strobilurins) effect was observed in the epidemic years, except in 2000.

Key words: *Drechslera tritici-repentis*, cultivar, tillage methods, disease control.

INTRODUCTION

Winter wheat (*Triticum aestivum* L.) is an economically important crop in the Grand-Duchy of Luxembourg (GDL): 12,969 ha with an annual production of *ca.* 86,040 tons in 2009 (El Jarroudi *et al.*, 2011). *Pyrenophora tritici-repentis* (*Ptr*) (Died.) Drechs (anamorph, *Drechslera tritici-repentis*) (Died.) Shoem., causal agent of wheat tan spot (WTS), is an economically important pathogen in many wheat-growing regions worldwide (Ali and Francl, 2002; Ali *et al.*, 1999; Fernandez *et al.*, 2002; Perelló and Dal Bello, 2011; Tadesse *et al.*, 2006; Wakulinski *et al.*, 2003). The fungus can cause considerable yield losses to winter wheat when epidemiological conditions are favorable (Fernandez *et al.*, 2002; Hosford, 1982; Hosford *et al.*, 1987; Rees and Platz, 1983; Tadesse *et al.*, 2006; Waltkins *et al.*, 1978). Numerous studies showed that the yield losses can reach up to 50% depending on cultivar susceptibility and climate (Tadesse *et al.*, 2006). In the GDL, *Ptr* was identified for the first time in 1999 and was characterized through lens-shaped necrotic lesions with a chlorotic halo on susceptible cultivars (El Jarroudi *et al.*, 2004).

The increase in the severity of WTS has been associated with changes in the spectrum of the varieties grown, the expansion of the area where low and no-tillage practices dominate, which allows the build-up of inoculum on wheat stubble over time (Perelló *et al.*, 2003). An integrated control approach including the use of resistant cultivars, fungicides and appropriate cultural practices is currently recommended for WTS management (Perelló and Dal Bello, 2011). However, the level of resistance in commercial wheat cultivars is relatively low.

Various studies have demonstrated the efficacy of fungicides in controlling foliar diseases of wheat and increasing grain yield. In Kansas, over a period of 6 years, applications of propiconazole significantly increased the yield of winter wheat (Kelley, 2001). Other authors reported that fungicides containing tebuconazole, tebuconazole plus prothioconazole, and pyraclostrobin were very effective in reducing leaf spots in winter wheat in North Dakota (Ransom and McMullen, 2008). Some fungicides were reported to become less effective in controlling WTS because of the development of resistance in *Ptr* populations (Jørgensen and Olsen, 2007). Furthermore, in the last 10-15 years, fungicide chemistry evolved considerably and new fungicides became available on the market.

The objectives of the present study were: (i) to evaluate the occurrence, severity and the damage potential of WTS in the GDL and (ii) to identify factors that affect the severity of WTS in this country.

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MATERIALS AND METHODS

Crop management. Four locations close to the Luxembourgish villages Burmerange [49°29'N, 6°19'E], Christnach [49°47'N, 6°16'E], Everlange [49°46'N, 5°57'E], and Reuler [50°03'N, 6°02'E] were selected to carry out field experiments between 1999 and 2009 (Fig. 1). Crop management data for these experiments are given in Table 1. Experimental fields were typically sown around mid-October. The sowing and harvest methods and crop practices used reflected the usual wheat production practices in the GDL. In each location and for each cropping season, winter wheat cultivars were sown in a randomized block design with four replicates. Susceptible (Bussard, Biscay, Drifter, Ritmo, Vivant), semi-susceptible (Aron, Akteur, Batis, Boomer, Privileg, Shamane, Dekan, Sponsor, Tommi), and weakly-susceptible cultivars (Achat, Cubus, Dream, Flair, Rosario, Parador, Urban) (Anonymous, 2002, 2008) were used in these trials. Individual plots measured 8.0x1.5 m. Plant growth stages (GS) were assessed according to a decimal scale (Zadoks *et al*., 1974). All plots received 40-70 kg N/ha (ammonium nitrate) at GS 25, followed by 60-70 kg N/ha at GS 32, and a final application of 65-95 kg N/ha at GS 59. Weeds were controlled by two herbicide applications [pre-emergence: IP flow, 1.0 l/ha (Isoproturon, Bayer Crop Science Germany); post-emergence: Javelin, 2.0 l/ha (Didlufenican + Isoproturon, Bayer Crop Science, Germany]. Throughout the paper, reference will be made to the specific leaf positions on the wheat stem. These leaves are numbered with reference to the uppermost flag leaf, or L1 (for Leaf 1), with the leaf immediately below designated as L2, followed by L3, and so on (Shaner and Buechley, 1995).

The fungicide treatment was always a mix of strobilurin(s) and triazole(s). Over the 1999-2006 period, only two fungicide treatments were tested at each site. Between 2006 and 2009, three fungicide treatments were

Table 1. Agronomic data of the trials at the four experimental sites over the 1999-2009 period in the Grand-Duchy of Luxembourg.

Site	Year	Cultivar	Previous crop	Tillage system	Sowing date	Nitrogen (N/ha)
Burmerange	2000	Bussard, Dream, Flair, Ritmo	Maize	No tillage	16 Oct. 1999	200
	2001	Bussard, Dream, Flair, Ritmo	Maize	Tillage	30 Oct 2000	n.a. [a]
	2003	Dekan	Oilseed rape	Tillage	4 Oct 2002	185
	2004	Cubus	Oilseed rape	No tillage	1 Oct 2003	185
	2005	Cubus	Oilseed rape	No tillage	13 Oct 2004	185
	2006	Cubus	Oilseed rape	No tillage	30 Sep. 2005	192
	2007	Cubus	Oilseed rape	No tillage	11 Oct 2006	192
	2008	Cubus	Oilseed rape	No tillage	06 Oct 2007	228
	2009	Cubus	Oilseed rape	No tillage	06 Oct 2008	228
Christnach	2000	Bussard, Dream, Flair, Ritmo	Maize	No tillage	18 Oct 1999	200
	2001	Bussard, Dream, Flair, Ritmo	Maize	Tillage	28 Oct 2000	n.a.
	2003	Flair	Oilseed rape	Tillage	2 Oct 2002	200
	2004	Flair	Oilseed rape	Tillage	13 Oct 2003	200
	2005	Rosario	Maize	Tillage	27 Oct 2004	200
	2006	Flair	Maize	Tillage	12 Oct 2005	200
	2007	Tommi	Maize	Tillage	12 Oct 2006	200
	2008	Flair	Maize	Tillage	23 Oct 2007	200
	2009	Boomer	Maize	Tillage	23 Oct 2008	200
Everlange	1999	Batis, Flair, Ritmo, Sponsor, Vivant	Oilseed rape	No tillage	09 Oct1998	190
	2000	Bussard, Dream, Flair, Ritmo	Oilseed rape	No tillage	15 Oct 1999	195
	2001	Bussard, Dream, Flair, Ritmo	Oilseed rape	No tillage	18 Oct 2000	230
	2002	Achat, Biscay, Drifter	Pea	Tillage	12 Oct 2001	175
	2003	Achat	Oilseed rape	Tillage	4 Oct 2003	165
	2004	Achat, Urban, Aron	Oilseed rape	Tillage	14 Oct 2003	195
	2005	Achat (2x), Parador, Akteur	wheat, oilseed rape	Tillage	22 Oct 2004	190
	2006	Akteur, Flair	Fallow	Tillage	10 Oct 2005	225
	2007	Achat, Akteur	Pea	Tillage	10 Oct 2006	195
	2008	Rosario	Fallow	Tillage	09 Oct 2007	195
	2009	Achat, Privileg	Oilseed rape	No tillage	13 Oct 2008	195
Reuler	2003	Bussard	Oilseed rape	Tillage	5 Nov.2002	213
	2004	Bussard	Oilseed rape	Tillage	16 Oct 2003	200
	2005	Flair	Oilseed rape	Tillage	5 Oct 2004	200
	2006	Dekan	Maize	Tillage	13 Oct 2005	200
	2007	Akteur	Maize	Tillage	07 Oct 2006	200
	2008	Schamane	Oilseed rape	Tillage	10 Oct 2007	200
	2009	Schamane	Oilseed rape	Tillage	10 Oct 2008	200

[a] n.a.: not available.

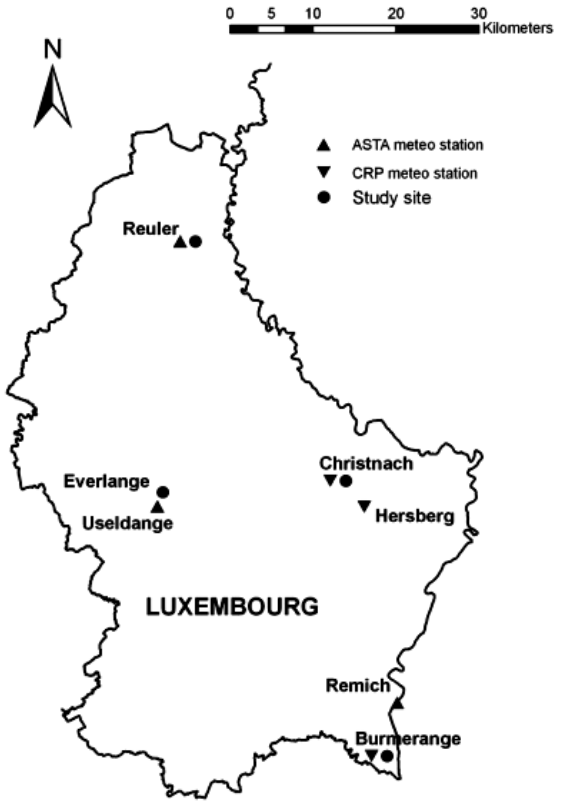

Fig. 1. Location of the experimental sites (filled circles) and meteorological stations (triangles). CRP: Centre de Recherche Publique-Gabriel Lippmann. ASTA: Administration des Services Techniques de l'Agriculture.

tested at each site (Table 2). Patterns of fungicide treatment were associated with wheat GS, and the products used were commercially available (Table 2). The products Allegro, Opus team, Opera, Input pro set, Bravo, Opus team, Stereo, and Swing gold contained the active ingredients Epoxiconazole (125 g/l) and Kresoxim-methyl (125g/l), Epoxiconazole (84 g/l) and Fenpropimorphe (250 g/l), Epoxiconazole (50 g/l)+Pyraclostrobin (133 g/l), Prothioconazole (250 g/l) and Spiroxamine (500 g/l); Chlorothalonil (500 g/l); Epoxiconazole (84 g/l) and Fenpropimorph (250 g/l); Cyprodinil (250 g/l) and Propiconazole (62.5 g/l), and Epoxiconazole (50 g/l) and Dimoxystrobine (133 g/l), respectively. Fungicide treatments were aimed at controlling leaf and ear diseases (Table 2).

Crop growth monitoring. At the beginning of GS 30 up to GS 87, plant samples from the field were inspected for tan spot occurrence. The percentage of infected leaf area was recorded using the standard area diagrams for tan spot and other cereal diseases, which were available to evaluators, who assessed the percentage of infection in the field prior to making observations (James, 1971; Tomerlin and Howell, 1988).

Laboratory analyses and characterization of the fungus. To recover *Ptr* from field samples, infected leaves were collected from both fungicide-treated and untreated plots at Everlange in 2000. Leaves were cut into 2-cm long fragments from symptomatic tissues and 10 leaf pieces per plot (40 leaves in total per treatment) were plated in plastic 9 cm Petri dishes containing three layers of dampened Whatman no. 1 filter paper. To dampen filter papers, 3 ml of sterilized distilled water were added to each plate. Leaves were incubated in an alternating cycle of 24 h light at 22°C and 24 h in dark at 16°C for 96 h to induce conidiophore and conidia formation. Incubated leaf pieces were examined under a stereoscope, and conidia were picked individually with a flamed steel needle that had been cooled in a plate containing V8PDA (150 ml of V8 juice, 10 g of Difco potato dextrose agar, 3 g of $CaCO_3$, 10 g of Bacto agar, and 850 ml of distilled water) (Lamari and Bernier, 1989). This procedure produces a sticky needle tip for picking of a single conidium which was then transferred onto an individual V8PDA plate for 7 days.

The mycelium produced by fungal isolates (coming from fungicide treated and untreated leaves) recovered from the sampled leaves was flattened with the bottom of a test tube to induce the formation of conidiophores (continuous light for 24 h) and conidia (18-24 h dark at 16°C). Conidia were photographed with a Hamamatsu digital charge-coupled-device camera (Hamamatsu, Japan) and a Zeiss Axioskop II microscope.

Statistical analyses. Statistical analyses were carried out using the SAS System (version 9.01, SAS Institute, USA). Comparison of tan spot between different areas, years, cultivars and tillage system was carried out in linear mixed model using the percentage of infected leaf area of L3, L2 and L1 as dependant variables, and the field site, cultivars and treatment as independent variables. Post-hoc tests (Tukey's test) were carried out following the Fisher-F test. P-values below 0.05 (2-sided) were considered as significant.

RESULTS

The mean comparison by linear mixed models showed that the cultivars, sites, year, treatment and the interaction between cultivars x sites x year x treatment were highly significant ($P < 0.001$) (Table 3)

1998-1999 cropping season. Severe damage caused by WTS occurred in the 1998-1999 cropping season, but no symptoms were seen before late April/early May. There were significant differences ($P < 0.01$) in the level of disease severity among cultivars assessed at GS 85 (Fig. 2). Commonly grown cultivars, e.g. Ritmo, Batis and Vivant, revealed a high degree of susceptibility to

Table 2. Dates, growth stages of the different fungicide sprays and disease assessments at each studied site in the Grand-Duchy of Luxembourg.

Sites	Year	First assessment	GS[a]	Last assessment	GS	Experimental Code	Stages of fungicide application	Fungicide treatment	Growth stage	Date
Everlange	1999	16/06/1999	65	16/07/1999	85	T0	Control	No fungicide application		
						T1	GS39	1l/ha Allegro	39	24/05/1999
						T2	GS31	1l/ha Allegro	33	12/05/1999
							GS59	1.5l/ha Opus team	55	5/06/1999
	2000	16/04/2000	30	12/07/2000	87	T0	Control	No fungicide application		
						T1	GS59	1l/ha Allegro	55	2/06/2000
						T2	GS31	1.5l/ha Opus team	31	4/05/2000
							GS59	1l/ha Allegro	59	2/06/2000
	2001	9/04/2001	24	19/07/2001	90	T0	Control	No fungicide application		
						T1	GS37	1l/ha Allegro	37	19/05/2001
						T1	GS45	1l/ha Allegro	49	31/05/2001
						T1	GS59	1l/ha Allegro	61	12/06/2001
						T2	GS31	1.5l/ha Opus team	32	9/05/2001
							GS59	1l/ha Allegro	65	14/06/2001
	2009	16/04/2009	30	22/07/2009	90	T0	Control	No fungicide application		
						T1	GS31	1.6l/ha Input®pro set + 1l/ha Bravo	31	29/04/2009
						T1	GS37	1.6l/ha Input®pro set + 1l/ha Bravo	37	13/05/2009
						T1	GS59	1.6l/ha Input®pro set + 1l/ha Bravo	59	3/06/2009
						T2	GS31	0.5l/ha Opera + 0.5 l/ha Input®pro set + 1l/ha Bravo	31	29/04/2009
							GS59	1.5 l/ha Swing gold + 1l/ha Bravo	59	3/06/2009
						T3	GS31	0.7 l/ha Stereo + 1l/ha Bravo	31	29/04/2009
							GS37	0.5l/ha Opera + 0.5 l/ha Input®pro set + 1l/ha Bravo	37	13/05/2009
							GS59	1.5 l/ha Swing gold + 1l/ha Bravo	59	3/06/2009
Christnach	2000	20/04/2000	30	12/07/2000.	85	T0	Control	No fungicide application		
						T2	GS31	1.5l/ha Opus team	39	23/05/2000
							GS59	1l/ha Allegro	59	2/06/2000
	2001	9/04/2001	24	19/07/2001	87	T0	Control	No fungicide application		
						T2	GS31	1.5l/ha Opus team	32	9/05/2001
							GS59	1l/ha Allegro	57	9/06/2001
Burmerange	2000	20/04/2000	30	12/07/2000	89	T0	Control	No fungicide application		
						T2	GS31	1.5l/ha Opus team	39	23/05/2000
							GS59	1l/ha Allegro	59	2/06/2000
	2001	9/04/2001	30	9/07/2001	89	T0	Control	No fungicide application		
						T2	GS31	1.5l/ha Opus team	32	9/05/2001
							GS59	1l/ha Allegro	67	9/06/2001

[a] GS: growth stage (Zadoks *et al*., 1974); T1, T2, and T3: One, two and triple fungicide treatment, respectively.

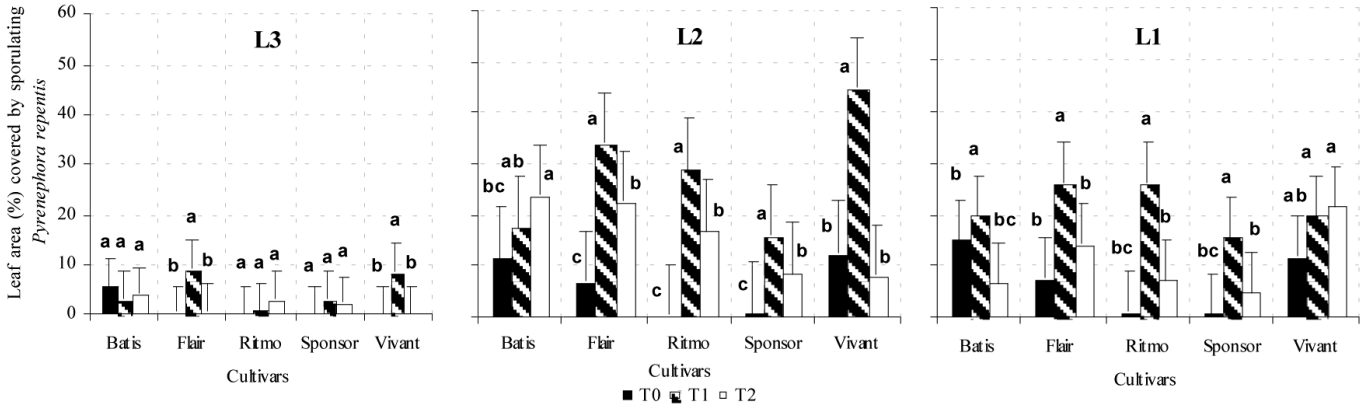

Fig. 2. Variation of the tan spot severity on the upper leaves at GS 85 (July 14, 1999) at Everlange according to the fungicide treatment. L3, L2, and L1: leaf 3, 2 and 1 (flag leaf), respectively; T0: Control; T1 and T2: One and two fungicide treatments respectively. Significant different variations among treatments are indicated by different letters ($P < 0.05$).

WTS ($P < 0.001$). The disease was observed at rather high severity on the leaves treated lately (T1 and T2) as compared to untreated leaves (T0) (Fig. 2). Fungicide treatments were ineffective 40 days (GS 85) after the last application (Fig. 2). The fungicide used in this case was Allegro (Kresoxim-methyl, Epoxiconazole) for T1 and Opus team (Fenpropimorph, Epoxiconazole) and Allegro (Kresoxim-methyl, Epoxiconazole) for T2 (Table 2). The DMI (demethylation inhibitors) fungicides Epoxiconazole and the strobilurin Kresoxim-methyl less efficient and did not stop the disease (Fig. 2).

1999-2000 cropping season. In 2000, WTS was detected in all experimental fields, its severity being significantly different between locations ($P < 0.05$). The highest severity was found at Everlange (West), followed by Burmerange (South) and Christnach (Center). Differences among cultivars were highly significant ($P < 0.001$) within each field as well as between fields (Fig. 3).

The treatment effect was highly significant ($P < 0.01$) at GS 75 on L1 (flag leaf), but this treatment did not show any effect at GS 85. Cultivars Bussard and Ritmo appeared as the most sensitive to WTS ($P < 0.0001$) at GS 75. While no significant difference (P=0.73) in disease severity was observed between cvs Flair and Dream at the same GS. The range of fungicide products used in our tests provided some protection against the disease at GS 75. However, these products did not stop a very important outbreak of the disease at GS 85. Indeed, disease severity reached 50% (cvs Bussard and Ritmo) at the beginning of July (July 10) for T1 and T2 treatments 38 days after the last fungicide spray. Laboratory characterization of the fungus from treated and untreated leaves identified two strains (Fig. 4) denoted: (i) strain "LB2038-1" for treated leaves, and (ii) strain "LB2038-3" for untreated leaves.

At Burmerange, WTS severity was important at GS 85 (Fig. 3). T0 showed the highest disease severity on L1 with 15% for cv. Bussard and 25% for cv. Ritmo, whereas the lowest severity (< 1%) was observed for T2.

At Christnach, cv. Ritmo differed significantly from the three other cultivars ($P < 0.004$) only for L2, whereas for L1 no significant difference between cultivars was observed. However, for this leave, the disease severity was much more important in T2 than T0 (Fig. 3).

Table 3. Analyses of variances (ANOVAs) for the effects of year, site, cultivars, and treatment and the interaction between year, site, cultivars, and treatment.

Source	Type III Sum of Squares	Mean Square	F	P^a
Year	18186.09	18186.09	351.28	***
Site	5596.08	2798.04	54.05	***
Cultivars	10616.02	3538.67	68.35	***
Treatment	2567.63	2567.63	49.60	***
Year*Site*Cultivars*Treatment	72322.63	1808.07	34.92	***

[a] Significance levels: *= $P<0.05$; **= $P < 0.01$; *** = $P < 0.001$; and NS= $P>0.05$.

2001-2008 cropping season. At Everlange, WTS was of minor importance throughout this period. In 2001, WTS severity at GS 85 was very low in all sites compared to the previous cropping seasons (Table 4) and the difference was significant between T1 at each stage, T2 and T0 depending on the cultivar ($P < 0.001$) (Table 4). On cv. Bussard, plots with the GS 49-fungicide treatment showed the highest disease severity on L2 (Table 4). On cv. Flair, T2 showed the highest disease severity. At Burmerange, no disease was observed; while at Christnach the disease occurred in a much lower percentage (Table 4).

Nevertheless, in 2004 the behaviour of cv. Achat towards WTS severity differed depending on the previous crop (i.e. wheat and oilseed rape) for infections occured at an early stage (GS 37) when the previous crop was wheat. In 2007, the disease was observed on L4 between April 30 and May 7 on cvs Achat (2%), Akteur (< 1%) and Flair (5%).

At Burmerange no WTS was detected in 2001 (Table 4) and at Christnach, it occurred with a much lower percentage than in 2000 (Table 4). The difference between cultivars and treatments was not significant ($P > 0.05$). At these sites, WTS was negligible over the 2002-2008 period, even though in 2007 it was observed in an early stage (GS 30-31) with symptoms only on the lower leaves. At Reuler, symptoms were observed only in 2007 on L5 at the beginning of the season (GS 30, mid-April).

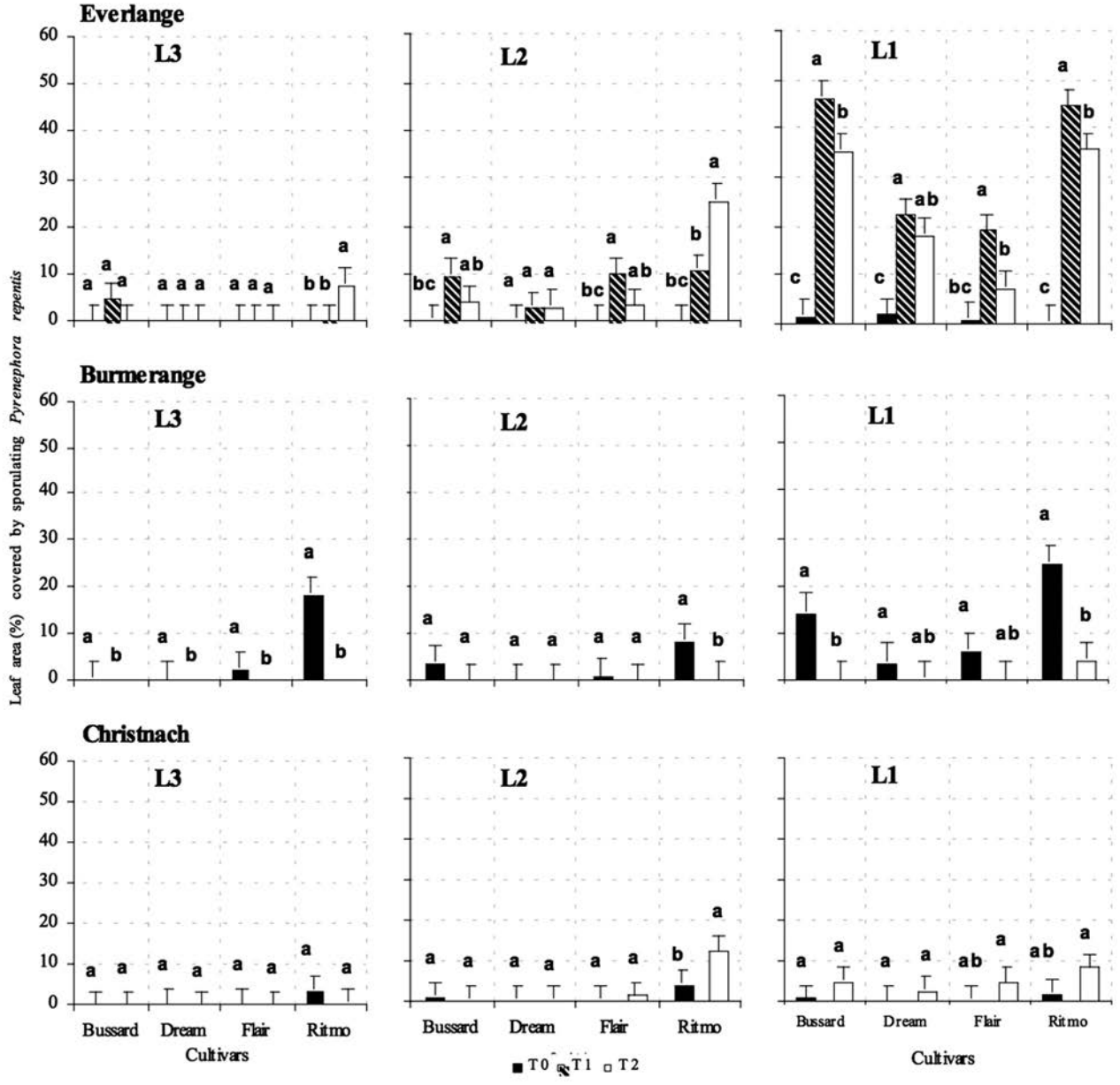

Fig. 3. Variation of the tan spot severity on the upper leaves at GS 85 (July 10, 2000) at three studied sites as affected by the fungicide treatment. L3, L2, and L1: leaf 3, 2 and 1 (flag leaf), respectively; T0: Control; T1 and T2: One and two fungicide treatments respectively. Significant different variations among treatments are indicated by different letters ($P < 0.05$).

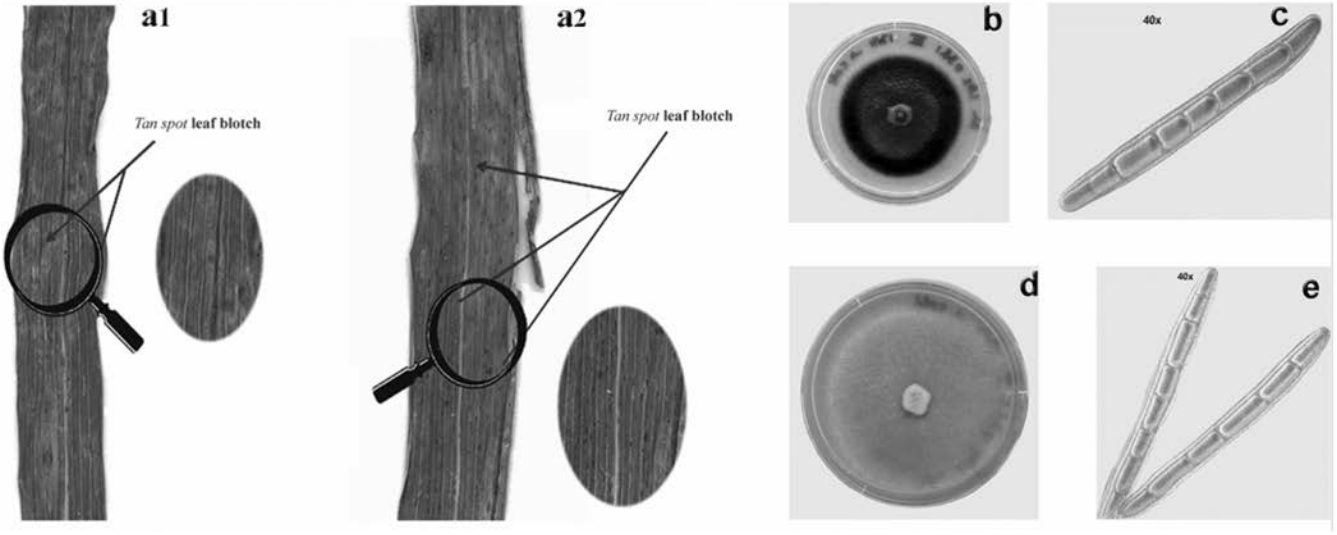

Fig. 4. Tan spot blights of winter wheat in the Grand-Duchy of Luxembourg. a1, L3 from cv. Bussard at Everlange (sample harvested on July 10, 2000). a2, L3 from cv. Bussard treated at Everlange (sample harvested on July 10, 2000). b, strain LB2038-1 of treated L3 pushed on PDA. c, conidia of LB2038-1 of treated L3. d, strain LB2037-3 of control L3 pushed on PDA. e, conidia of LB2037-3 of control L3.

2008-2009 cropping season. WTS was observed only at Everlange. The difference between the cultivars and the treatments was highly significant ($P < 0.01$) (Table 5). Disease severity was very low on cv. Achat for the two upper leaves (L1 and L2) while it was very high on the cv. Privileg. For this last cultivar, the comparison between the treatments (T1 and T2) and T0 revealed that fungicide-treated plots showed the highest disease severity. The highest severity (26%) on L1 occurred in plots with T1 performed at GS 39 (Table 5). T2 and T3 plots showed a severity of 11% and 10%, respectively.

The comparison of the number of days between the first appearance of WTS and the latest fungicide application for each treatment (Fig. 5) showed that the disease appeared before the end of the product protective activity. WTS was observed in T2 and T3 plots while the number of days since the last application was only 19 days (Fig. 5).

DISCUSSION

Wheat crops grown repeatedly in sequence (monocropping) can suffer from various soil and stubble-borne disease, although the range and aggressive-

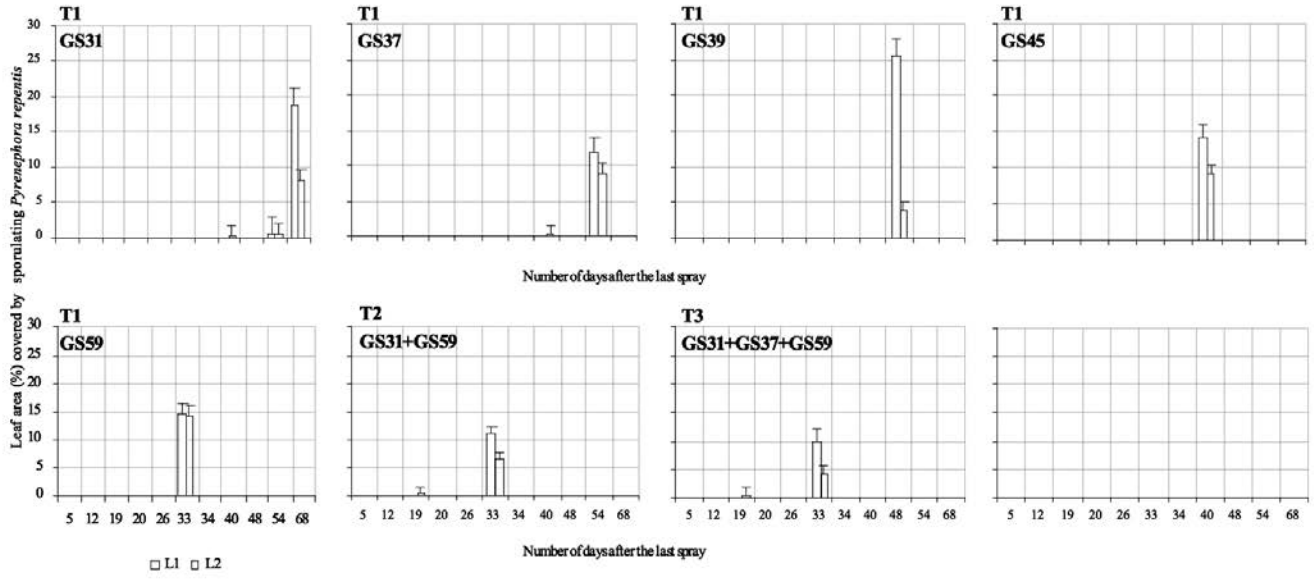

Fig. 5. Variation of tan spot severity on the two upper leaves of cvs Privileg at Everlange in 2009 after the last fungicide spray. T0, control; T1, T2, and T3, one, two, and triple fungicide treatment, respectively; L1 and L2, leaf 1 and leaf 2.

Table 4. Wheat tan spot severity (% leaf area covered by sporulating *Pyrenophora tritici-repentis*) in three experimental sites at GS 85 (July 6, 2001) during the 2000-2001 cropping season.

Cultivars	Experimental code	Time of spray	Disease severity (mean ± SD)					
			Burmerange		Christnach		Everlange	
			L1[b]	L2[b]	L1	L2	L1	L2
Bussard	T0[a]	Control (no spray)	0.0 ± 0.0	0.0 ± 0.0	1.5 ± 1.8	1.0 ± 1.4	0.2 ± 1.1	0.2 ± 1.1
	T1[a]	GS37					2.2 ± 3.1	5.0 ± 6.0
		GS49					1.2 ± 2.1	11.2 ± 12.1
		GS61					1.0 ± 1.6	5.5 ± 6.4
	T2[a]	GS31+GS59	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 2.3	2.0 ± 3.0	3.4 ± 4.3
Dream	T0	Control	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.8	0.0 ± 0.0	0.2 ± 1.1	0.8 ± 1.7
	T1	GS37					0.0 ± 0.0	0.6 ± 1.5
		GS49					0.1 ± 1.0	0.5 ± 1.4
		GS61					0.0 ± 0.0	0.5 ± 1.4
	T2	GS31+GS59	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.8	0.0 ± 0.0	1.1 ± 2.0
Flair	T0	Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.4	0.0 ± 0.0	0.6 ± 1.5
	T1	GS37					0.3 ± 1.2	1.0 ± 1.7
		GS49					1.1 ± 2.0	1.0 ± 1.7
		GS61					0.0 ± 0.0	1.1 ± 2.0
	T2	GS31+GS59	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 2.0	7.3 ± 8.2
Ritmo	T0	Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 2.6	2.6 ± 3.5
	T1	GS37					1.0 ± 1.9	1.8 ± 2.7
		GS49					0.5 ± 1.4	1.2 ± 2.1
		GS61					0.0 ± 0.0	1.2 ± 2.1
	T2	GS31+GS59	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 1.3	0.2 ± 1.1	2.0 ± 2.9

[a] T0: Control; T1 and T2: One and two fungicide treatments, respectively.
[b] L1 and L2: Leaf 1 and leaf 2.

Table 5. Wheat tan spot severity (% leaf area covered by sporulating *Pyrenophora tritici-repentis*) at Everlange during the 2008-2009 cropping season in cvs Achat and Privileg.

Treatment	Time of fungicide spray	Disease severity (mean ± SD)			
		Achat		Privileg	
		L1	L2	L1	L2
T0[a]	Control (no spray)	0.0 ± 0.0	0.0 ± 0.0	5.0 ± 4.7	1.8 ± 1.9
T1[a]	GS31	0.0 ± 0.0	0.1 ± 0.3	18.1 ± 11.5	8.1 ± 5.7
	GS37	0.0 ± 0.0	0.0 ± 0.0	12.0 ± 9.5	8.9 ± 7.0
	GS39	1.6 ± 2.7	1.0 ± 1.4	25.5 ± 11.7	4.0 ± 4.3
	GS45	0.0 ± 0.0	0.0 ± 0.0	14.2 ± 9.9	8.8 ± 7.8
	GS59	1.9 ± 3.3	0.8 ± 1.6	14.6 ± 8.5	14.0 ± 9.4
T2[a]	GS31+GS59	0.3 ± 0.5	0.1 ± 0.3	10.9 ± 7.2	6.6 ± 5.0
T3[a]	GS31+GS37+GS59	0.0 ± 0.0	0.3 ± 0.7	10.0 ± 10.9	4.2 ± 6.5
ANOVA[b] crossing the effects of treatment and cultivars					
Treatment		***			
Cultivars		***			

[a] T0: Control; T1, T2 and T3: One, two, and triple fungicide treatment, respectively.
[b] A multifactorial ANOVA evidences significant differences. Significance levels: *= P<0.05; **= P < 0.01; *** = P < 0.001; and NS= P > 0.05.

ness of particular pathogens vary widely both regionally and seasonally. For some diseases, crop tolerance (e.g. crown rot caused by *Fusarium pseudograminearum*), resistance (e.g. cereal cyst nematode, *Heterodera avenae*) or seed dressings and fungicides (e.g. eyespot caused by *Tapesia yallundae* and Tan spot by *Ptr* in Europe) form part of the control strategy (Kirkegaard *et al.*, 2008). Prior to 1999, WTS was regarded as rare or absent in winter wheat crops grown in GDL. However, over the 1999-2009 period, observations based on visual assessments in four experimental sites indicated that WTS occurred frequently at Everlange. These observations also showed that the most severe outbreaks of WTS occurred in fields where the previous crop was winter wheat and where non-inversion tillage was practised. Farmers began to abandon crop rotation after pesticides and nitrogen fertilizer became available, as many believed that they could replace the benefits of crop rotations (Karlen *et al.*, 1994). Some authors indicate that crops grown in short rotations or monoculture often suffer from yield decline compared to those grown in longer rotations or for the first time (Bennett *et al.*, 2011). It should be noted that between late 1999 and early 2000, the Luxembourgish farmers began to practice non-inversion tillage and intensified the wheat-after-wheat cropping practice. In 2000, WTS was observed in all experimental sites. In these sites and for this year, the non-inversion tillage was practised. In the same year, the disease was also found in several GDL neighbouring countries, mainly France and Germany (Jørgensen and Olsen, 2007; Moreno and Perelló, 2010; Reimann and Deising, 2005). WTS introduction coincided with the spread of non-inversion tillage and wheat-after-wheat cropping systems (Sutton and Vyn, 1990). Under the climatic conditions of Belgium and Germany the pathogen's pseudothecia were detected on straw and stubbles during winter and ascospores were found to mature and disperse at mid-April. This led to the primary attack in late April or early May (Maraite *et al.*, 1992; Wolf and Hoffmann, 1995).

Moisture and temperature are primary determinants of WTS infection. On susceptible cultivars, a minimum wet period of 6 h is necessary for conidia to infect leaves (Hosford *et al.*, 1990) and disease severity on susceptible cultivars increases with longer wet periods (Lamari *et al.*, 1992). Thus, temperatures around 20°C and 24 h wetness are suitable conditions for a successful infection (Lamari *et al.*, 1992). In the GDL, a stochastic model was developed to predict WTS (El Jarroudi *et al.*, 2004). The inputs are hourly data (i.e. temperature, relative humidity, and rainfall) provided by a network of automatic weather stations and used to calculate periods with high probability of infection by *Ptr*. WTS development in the experimental field sites requires a period of at least 24 consecutive hours with temperatures between 4 and 25°C and a relative humidity greater than 70%, with optimal values varying between 8 and 16°C and relative humidity greater than 90% (El Jarroudi *et al.*, 2004). Statistical validation using regression analysis also showed a strong correlation between the number of hours with these specific meteorological conditions and the percentage leaf area covered by WTS lesions on the upper leaves.

WTS control is complex and fits well an integrated disease management system, i.e. a combination of chemical, cultural, genetic and, sometimes, biological methods (Bockus *et al.*, 1992; Duczek and Jones-Flory, 1994; Hosford and Busch, 1974; Perelló *et al.*, 2003, 2006; Simón *et al.*, 2011).

Cultivar susceptibility is one of the important elements to take into consideration for disease occurrence (Gurung *et al.*, 2012). Among farmers practising non-inversion tillage and growing wheat-after-wheat there is a need to grow cultivars with good resistance to WTS since this cropping system facilitates disease establishment. In our study, the difference between the cultivars in terms of susceptibility was highly significant ($P < 0.001$). Cultivars Bussard, Ritmo and Privileg were the most susceptible to WTS, whereas cv. Achat was among the most tolerant thus representing a good option to reduce disease risk. Some variation in the ranking of the cultivars was seen over the years, but the overall trend between years was similar for the most resistant and the most susceptible cultivars.

Regarding treatments, our study showed that for years with major WTS oubreaks, and during the early crop development (GS75), the disease severity was well controlled by single (T1) and double (T2) fungicide treatments. This was consistently observed in 1999, 2000 and 2009. However, at later growth stages (GS85), the contrary occurred for the highest disease severity was observed in the plots treated with a single or a double fungicide treatment (T2 and T1). It is probable that the lower sensitivity of *Ptr* populations to strobilurins (Kresoxim-methyl, Pyraclostrobin) and triazoles (Epoxiconazole) may have reduced the efficiency of these fungicides. Indeed, the fungicide used in the last spray for T2 and T3 during the 2008-2009 cropping season were Swing gold (Epoxiconazole+Dimoxystrobine) and Bravo (Chlorothalonil). For T1, the fungicide used was Input Pro set (Prothioconazole+Dimoxystrobine) and Bravo (Chlorothalonil). Strobilurin- (Kresoxim-methyl) and triazole-resistant (Epoxiconazole) *Ptr* isolates were found in 2000 and 2001 in Germany (Reimann and Deising, 2005).

In western Europe, WTS is considered to be a problem in Sweden, Germany, France, and Denmark (Jørgensen and Olsen, 2007). Since 2003, Denmark has seen strobilurin resistance in a number of trials (Jørgensen and Olsen, 2007) and strobilurin resistance has also appeared in Germany and Sweden (Jørgensen and Olsen, 2007). The development of strobilurin (Kresoxim-

methyl, Pyraclostrobin) and Epoxiconazole resistance means that the recommendations for WTS control must changed in favour of other active ingredients. It is probable that this resistance will be more frequent in the future and this should be monitored closely. Another hypothesis is that the fungicide treatments strongly affect the microbial consortia present on the wheat leaf surface. Among these microbes, yeast-like fungi, hyphal fungi, and bacteria that are known to play an important antagonistic role towards leaf surface pathogens (Bashi and Fokkema, 1977; Rodgers-Gray and Shaw, 2001; Wachowska, 2005), are very susceptible to some fungicides. The effect of a fungicide may differ according to the microbial consortia involved. For example, the reducing effect of chlorothalonil on fungal numbers (i.e. *Ascomycetes*) was often compensated for by an increase of bacterial numbers (Rodgers-Gray and Shaw, 2001). It is highly possible that under fungicide pressure, useful antagonistic organisms on wheat leaf surface are inhibited, leaving an open space for colonisation by pathogenic fungi. Very little is known about naturally occurring organisms at the surface of wheat leaves and their role in protecting the plant against pathogenic fungi. This research area deserves particular attention when developing disease control strategies based on the use of fungicides.

The years 1999, 2000 and 2009 showed that WTS could be an acute problem for wheat production in the GDL. The increase in conservation tillage and the intensification of wheat production through shorter rotations and monoculture are favouring WTS and are unlikely to change. Successful management of WTS will depend on research efforts such as disease forecasting technology and monitoring of fungicide resistance, and research on the interaction between the microclimatic conditions and the structure of the population as well as its architecture.

Currently, susceptible cultivars are closely monitored in non-inversion tillage and wheat-after-wheat cropping systems for recommending the appropriate fungicide treatments. In all other situations, the severity of WTS was too low to justify special control in 3 out of 10 years during the monitoring period.

The trend in farming today is towards using more reduced and non-inversion tillage systems to optimizing the cost of production. In order to reduce the WTS risk and the use of fungicides, it is, however, important to couple non-inversion tillage with resistant cultivars and adequate crop rotation.

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BIOCONTROL OF FUSARIUM HEAD BLIGHT BY SPIKE APPLICATION OF *TRICHODERMA GAMSII*

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**S. Sarrocco and F. Matarese contributed equally to this work*

SUMMARY

Trichoderma gamsii 6085 is known for its features as antagonist, mycoparasite and competitor on natural substrates of mycotoxigenic *Fusarium graminearum* and *Fusarium culmorum*. This beneficial isolate is also able to significantly reduce deoxynivalenol (DON) production by these pathogens. In the present study, the ability of *T. gamsii* 6085 to grow in the presence of DON was investigated. On the basis of HPLC results showing no differences in the amount of DON in the growth medium after 72 h of incubation, the possible role of some PDR-ABC transporters in DON resistance by *T. gamsii* 6085 was investigated. *T. gamsii* 6085 was then tested as biocontrol agent under field conditions for two growing seasons (2011 and 2012), in order to evaluate its ability to reduce FHB symptoms on spikes and prevent mycotoxin production in kernels. The isolate was inoculated into soil before sowing or on spikes during anthesis. Spike application of *T. gamsii* 6085 reduced FHB severity by 57%. Colonization of spikes and spikelet components by the antagonist was assessed and discussed in connection with disease control. Results concerning spike colonization and the effects on the disease are encouraging. These preliminary results suggest that *T. gamsii* 6085 deserves further attention as a potential biocontrol agent of FHB.

Key words: Fusarium Head Blight, deoxynivalenol, *T. gamsii*, biological control, spike colonization.

INTRODUCTION

Fusarium head blight (FHB), also known as scab, is a significant destructive disease of small cereals reported throughout the world. Wheat is one of the most heavily FHB-affected crops and suffers the largest economic damage. The disease is caused by a complex of *Fusarium* spp., with *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schweinitz) Petch] and *F. culmorum* (W.G. Smith) as the most prevalent (Parry *et al.*, 1995). Other causal agents linked to FHB include *F. avenaceum*, *F. poae, F. equiseti*, *F. tricinctum*, *F. sporotrichioides, Microdochium nivale* and *M. majus*. Wheat growers, millers, bakers and consumers of wheat products may all be affected by the effects of the disease. FHB leads to significant yield reduction associated with reduced kernel size and weight, germination rate, protein content, baking quality of the flour and other technological parameters, due to the fungal colonization of the ear and the cutting off of nutrients supply to the upper spikelets (Parry *et al.*, 1995). This is coupled with quality reduction due to contamination of grains with mycotoxins that have adverse effects on human and animal health (Foroud and Eudes, 2009; Oerke *et al.*, 2010).

The prevailing mycotoxins associated with FHB are trichothecenes, with deoxynivalenol (DON) and its acetylated derivates, 3- and 15-acetyl-deoxynivalenol (3AcDON and 15AcDON), and nivalenol (NIV) as the most frequently encountered in FHB of wheat throughout Europe (Schothorst and van Egmond, 2004). DON causes loss of appetite, vomiting, diarrhoea and bleeding of the intestines at high doses and can lead to further effects such as impairment of immune function in both humans and animals (Dejardins, 2006; Eriksen and Pettersson, 2004). DON plays a role in *F. graminearum* virulence and disease development, as shown by several field and molecular studies: results of inoculation studies showed that although trichothecene-deficient mutants of *F. graminearum* were capable of inciting FHB, disease severity was generally higher on spikes inoculated with wild-type (toxin-producing) strains (Proctor *et al.*, 2002). Production of this compound may also be related to competition with other fungi and bacteria. Mycotoxins production increases by as much as 1000 times in co-inoculations of *F. graminearum* with other FHB causal agents, suggesting that competition results in greater production of trichothecene mycotoxins (Xu and Nicholson, 2009).

Fungal contamination has become a key concern in food and feed safety assessments and many countries have set legal limits for *Fusarium* toxin concentrations

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in food and feed. The early detection and control of trichothecene-producing *Fusarium* spp. is crucial to prevent toxins entering the food chains (Wagacha and Muthomi, 2007). Management of FHB and its associated mycotoxins has been based on strategies such as host resistance, agricultural practices as tillage or crop rotation and fungicides, but none of these methods alone is able to significantly reduce the disease. Therefore, many efforts have been initiated to identify FHB antagonists such as beneficial fungi to be used in biological control and/or in integrated management strategies (Dawson *et al.*, 2004; Luongo *et al.*, 2005; He *et al.*, 2009; Sarrocco *et al.*, 2012). As the main source of FHB inoculum are the infected plant debris in soil, on which the pathogens overwinter, whereas anthesis is the most susceptible growth stage of wheat (Parry *et al.*, 1995), biological control of FHB entails treatment of crop residues with antagonists to reduce pathogen inoculum on wheat heads at anthesis (Bujold *et al.*, 2001; Pirgozliev *et al.*, 2003; Tsitsigiannis *et al.*, 2012).

The aim of the present work was to investigate the ability of a *T. gamsii* isolate 6085 (*Tg*6085) to grow in the presence of DON and to evaluate the putative role of PDR-ABC transporters in resistance to DON. Since this isolate is well known as beneficial fungus against *F. graminearum* and *F. culmorum*, preliminary promising results concerning its efficacy as a biocontrol against FHB under field condition are reported in this paper.

MATERIALS AND METHODS

Fungal isolate. *Tg*6085 was isolated from uncultivated soil in Crimea and it is well known for its antagonistic and mycoparasitic activity against *F. graminearum* and *F. culmorum* mycotoxigenic isolates. This beneficial isolate is also able to compete on natural substrates against both pathogens, significantly reducing their DON production (Matarese *et al.*, 2012).

Growth of *Tg* 6085 in presence of DON and fate of mycotoxin in cultural filtrates

The ability of *Tg*6085 to grow in the presence of DON was assayed by microspectrophotometry in 96-well microplates (PbI International, Italy). Three wells were inoculated with 150 µl of a potato dextrose broth (PDB) (Difco, USA) spore suspension (10^6 conidia ml^{-1}) containing a final concentration of 50 ppm of DON (Sigma Aldrich, USA). DON was dissolved in 85% spectroscopic grade methanol and 15% HPLC water. Three wells with PDB added with methanol/water 85:15 solution or PDB alone were used as controls. Fungal growth was monitored for 72 h by measuring absorbance (OD) at 595 nm at intervals of 4 h in the day (12 h during the night) using a microplate reader 680 (Bio-Rad, USA). The whole experiment was repeated three times. The absorbance values were used to create growth curves in the presence/absence of DON by SIGMAPLOT 10 program (Sigmaplot Software, USA). Curves were subjected to analysis of variance of regression by GraphPad 5 (GraphPad Software, USA).

The fate of DON in the medium was analyzed by HPLC. After 72 h, cultural broth from each well was collected and filtered through a 0.2 µm Minisart-RC syringe filter (PbI International). Samples were kept in at -80°C until chromatographed. DON was quantified by HPLC, using a method adapted from Omurtag *et al.* (2007), according to Matarese (2010). Filtrates from cultures in PDB without DON were used as negative controls.

Involvement of PDR-ABC transporter genes in resistance to DON by *Tg*6085. In order to investigate a possible mechanism of *Tg*6085 in resistance to DON, expression analysis of some PDR-ABC transporter genes was performed. Firstly, *Tg*6085 was used in a confrontation assay against mycotoxigenic isolates of *F. graminearum* 124 and *F. culmorum* 627 on half strength Oatmeal agar at 25°C. The fungi were harvested when the mycelia were *ca.* 5 mm apart (before contact, BC), at contact (contact, C) and after the colonies were overgrown (5 mm) by *Trichoderma* (after contact, AC). *Trichoderma* inoculated against itself and alone were used as controls and harvested at the same time points. All combinations were made in triplicate. Peripheral hyphal zones were harvested from each confrontation stage by cutting agar pieces, were shock frozen in liquid nitrogen and stored at -80°C. Isolation, purification and quantification of total RNA and cDNA production were done according to Matarese *et al.* (2012).

For assessing the transcription of some PDR-ABC transporter genes, primers TRIAT131379 (Fw 5'-GCAGAGGTCAGCGTAATCG-3, Rv 5'-GGAAGCTGAGGATGAAGAGG-3', Tm 57°C), TRIAT88381 (Fw 5'-GCCATCTATCAAGCCAGCC-3', Rv 5'-CGCATTCTCTTGGCCTGTC-3', Tm 60°C) and tabc2 (Ruocco *et al.*, 2009) were used. TRIAT131379 and TRIAT88381 were designed for this study on *T. atroviride* sequences due to the lack of information about the *T. gamsii* genome. The *tef1* gene and the housekeeping *gpdh* genes were used as controls. The amplification program consisted of 1 min initial denaturation (94°C), 25 cycles of amplification (1 min at 94°C, 1 min at the primer-specific annealing temperature, 1 min at 72°C) and a final extension of 7 min at 72°C.

The expression of PDR-ABC transporter genes by *Tg*6085 was also assessed in a competition test on rice, against *F. graminearum* 124 and *F. culmorum* 627. These growth conditions are strongly conducive for DON production (Matarese *et al.*, 2012). Substrates were inoculated with a spore suspension (10^4 conidia ml^1) of *Tg*6085 alone and with a spore suspension of each

pathogen (10^4 conidia ml^{-1}) and incubated at 25°C at 12 h light/darkness. Non-inoculated substrates were used as control. After 7, 14 and 21 days incubation total RNA was extracted from 100 mg of ground material, purified and reverse transcribed in cDNA according to Matarese (2010). Gene expression was determined by real-time RT-PCR using a MJ Opticon thermocycler (Bio-Rad) and IQ SYBR Green Supermix (Bio-Rad). The *tef1* gene was used as 'housekeeping' to normalize the relative gene expression of the candidate genes. For ABC transporter genes and *tef1* the same above mentioned primers were used. PCR cycling conditions were: pre-incubation at 95°C for 3 min; amplification step of 40 cycles at 95°C for 5 sec and 60°C for 20 sec. Finally, a post PCR melt curve analysis was included, to detect non specific amplification in cDNA samples. All experiments were repeated three times.

Biological control of FHB by *Tg*6085 under field condition. Evaluation of FHB control by *Tg*6085 was conducted under field conditions near Grosseto (Italy) during two successive growing seasons, 2010/2011 and 2011/2012. The experimental design included three replicate plots (2.5 m x 2.5 m) for each growing season and for each of the following treatments: (i) soil inoculated with 1.5 kg of organic matter pre-fermented for one week at 24°C with 10^6 spore ml^{-1} of *Tg*6085; (ii) wheat plants growing in non-inoculated soil inoculated at anthesis with an aqueous spore suspension (10^6 spore ml^{-1}) of *Tg*6985 with Tween 80 (0.04%); (iii) non-inoculated control. During the second growing season, 7 and 14 days after application of *Tg*6085 on heads, four spikes were collected from each replicate of plots that had not been inoculated or inoculated during anthesis. Four pairs of central spikelets, numbered 1 to 4, were collected from each of the spikes and superficially sterilized with NaClO (1% active chlorine) in 50% ethanol. Four spikelets (one per each pair) were sterilized before and the remaining four after separation into their components (glumes, first flowers, second flowers and sterile flowers) (Figs. 1a and 1b). Spikelet components were plated on *Trichoderma* selective medium (P190) (Sarrocco *et al.*, 2009) in order to evaluate the ability of *Tg*6085 to colonize plant tissues. Percentages of spikelets and components colonized by the *Trichoderma* were subjected to analysis of variance (ANOVA) by Systat 11 (Systat Software, USA) after angular transformation. Treatment, time (days after *Tg*6085 application), spikelet position (1 to 4, see Fig. 1a), spikelet component (see Fig. 1b), sterilization method (before or after separation of components) and all possible interactions were considered source of variability.

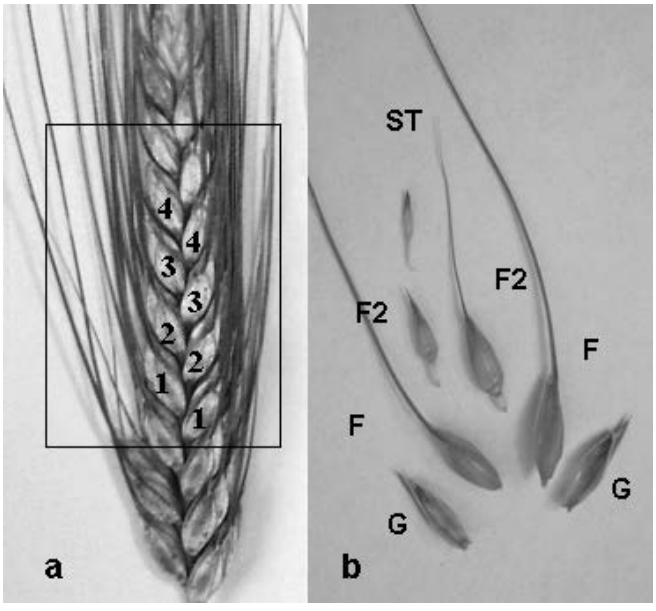

Fig. 1. Spikelets collected from each spike to evaluate *T. gamsii* 6085 colonization ability (a). Components of each spikelet: G= glumes, F= first flowers, F2= second flowers, ST= sterile flower (b).

To estimate FHB symptoms, 21 days after *Tg* 6085 inoculation of spikes, visual disease assessment was made by examining 100 heads randomly collected from each plot. The disease incidence [DI, percentage of infected heads] and disease severity [DS, percentage of infected spikelets in each ear (Mackinney index: Σ number of heads per evaluation class x evaluation class/total number of scored heads)] were calculated. Evaluation class was assessed by using a 13 class scale similar to that of Stack and McMullen (1995). All data were submitted to ANOVA after angular transformation, if required. Growing season, treatment and growing season x treatment interaction were considered as source of variability. The presence of the trichothecenes deoxynivalenol (DON), nivalenol (NIV), 3-acetyl-deoxynivalenol (3Ac-DON), zearalenone (ZON), neosolaniol (NEO), T-2 toxin (T2), HT-2 toxin (HT2), Fusarenon-*X* (FUS X) and diacetoxyscirpenol(DAS) was measured on wheat kernels harvested from control and inoculated samples according to Somma *et al.* (2010).

RESULTS

Growth of *Tg*6085 in the presence of DON and fate of mycotoxin in cultural filtrates. Growth rates of *Tg*6085, in the presence and absence of DON, were used to create growth curves that resulted in highly significant equations when subjected to regression analysis ($R^2 \geq 0.956$, $P<0.0001$). Growth curves (Fig. 2) show no significant differences of growth in the presence/absence of DON ($P_{slope}=0.7040$ and $P_{elevation}=0.3020$). When submitted to HPLC analysis, none of the cultural substrates containing DON showed a mycotoxin content significantly different from the initial concentration of 50 ppm. Moreover, analysis of liquid chromatograms

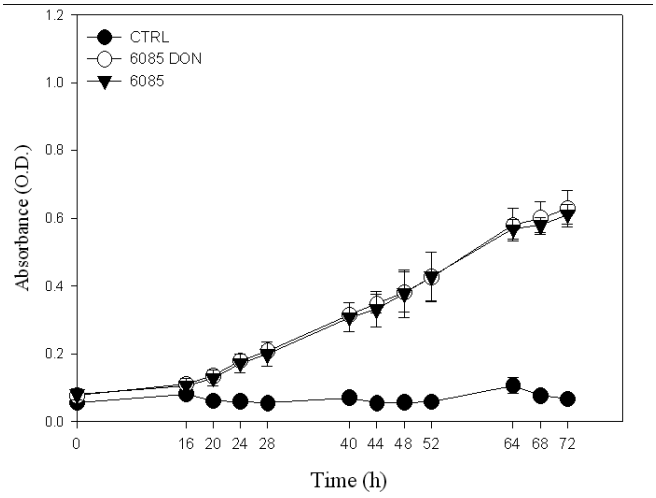

Fig. 2. Growth curves of *T. gamsii* 6085 in the presence (*open circle*) and absence (*filled triangle*) of DON at 50 ppm. Values are expressed as absorbance at 595 nm and represent the average of three independent replicates (*filled circle* represents non-inoculated PDB).

showed no others products such as acetylated forms possibly derived from DON degradation.

Involvement of PDR-ABC transporter genes in resistance to DON by *Tg* 6085. Analysis of transcription patterns in dual agar cultures showed no differences in the expression of TRIAT131379, TRIAT88381 and tabc2 genes by *Tg*6085 before the contact (BC) and at the contact (C) with both *F. graminearum* and *F. culmorum* and with itself. A very low, if any, expression was detected after the antagonist/pathogens and antagonist/antagonist contact (AC). When alone, all the genes, with TRIAT88381 to a lesser extent, were transcribed allowing us to hypothesize their constitutive expression (Fig. 3). Quantitative expression from rice plates obtained by qRT-PCR is reported in Fig. 4. In all combinations and when alone, *Tg*6085 showed a very low expression of both TRIAT88381 and tabc2, as shown by the relative expression values (Fig. 4, y axes values). As to TRIAT131379, after 7 days in the presence of *F. culmorum,* a higher expression was observed than when alone or in the presence of *F. graminearum*. After 14 days, almost no expression was detected in all the three combinations whereas after 21 days the same expression, higher than that shown in the presence of *F. graminearum*, was detected when alone and in the presence of *F. culmorum*.

Biological control of FHB by *Tg* under field condition: preliminary results. The analysis of variance of DI, performed on data collected 21 days post inoculation, indicated that the growing season was a significant source of variability (P=0.038), while treatment was not significant (P=0.107). The interaction growing season x treatment was significant (P=0.055), indicating that *Trichoderma* application had different effects on DI during the two growing seasons. The analysis of variance of DS data indicated that the growing season was not a significant source of variability (P=0.998) while treatment was significant (P=0.021). The interaction growing season x treatment was not significant (P=0.076), indicating that *Trichoderma* application had similar effects on DS during the two growing seasons. In details (Table 1), DI

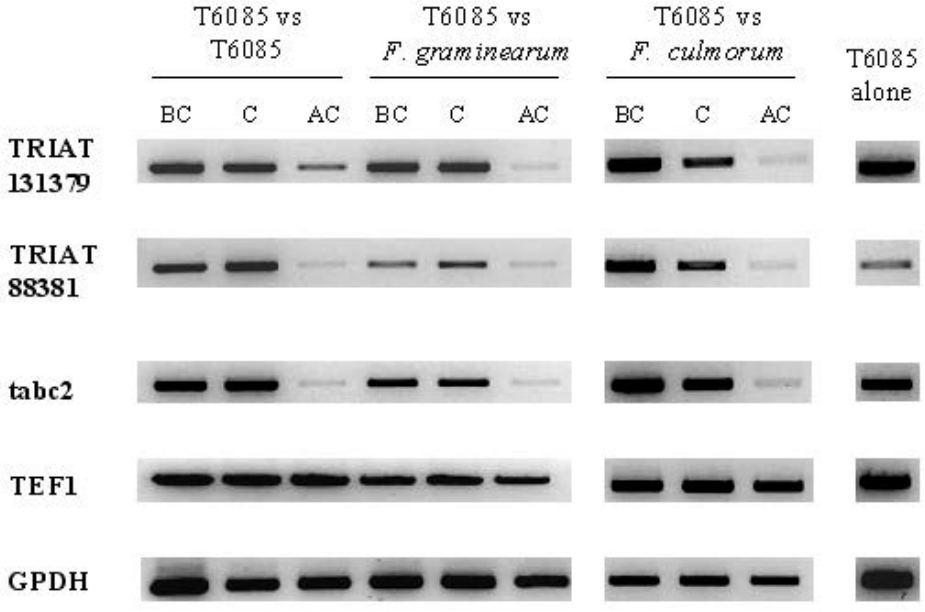

Fig. 3. Transcript patterns of ABC transporter genes in *T. gamsii* 6085 (T6085) vs *F. graminearum* and *F. culmorum*. Mycelia were harvested before contact (BC), at contact (C) and after contact (AC) of the mycelia. The *tef1* gene, encoding translation elongation factor 1-α, and *gpdh*, encoding glycerinaldehyde-3-phosphate-dehydrogenase, were used as control genes.

was significantly higher during 2011/2012, whereas no differences emerged for DS. No significant differences in DI resulted among treatments whereas applying *Tg*6085 on the spikes during anthesis a significant reduction of the percentage of infected spikelets (DS) was obtained. Anyway, the significance of the interaction between growing season x treatment, concerning DI (Fig. 5) tells that *Trichoderma* effects on DI are significantly affected by epidemiologically relevant factors that vary between the two growing seasons.

When kernels were submitted to mycotoxin analysis, DON values were up to 208 ppb in 2010/2011 and up to 25 ppb in 2011/2012, without differences among

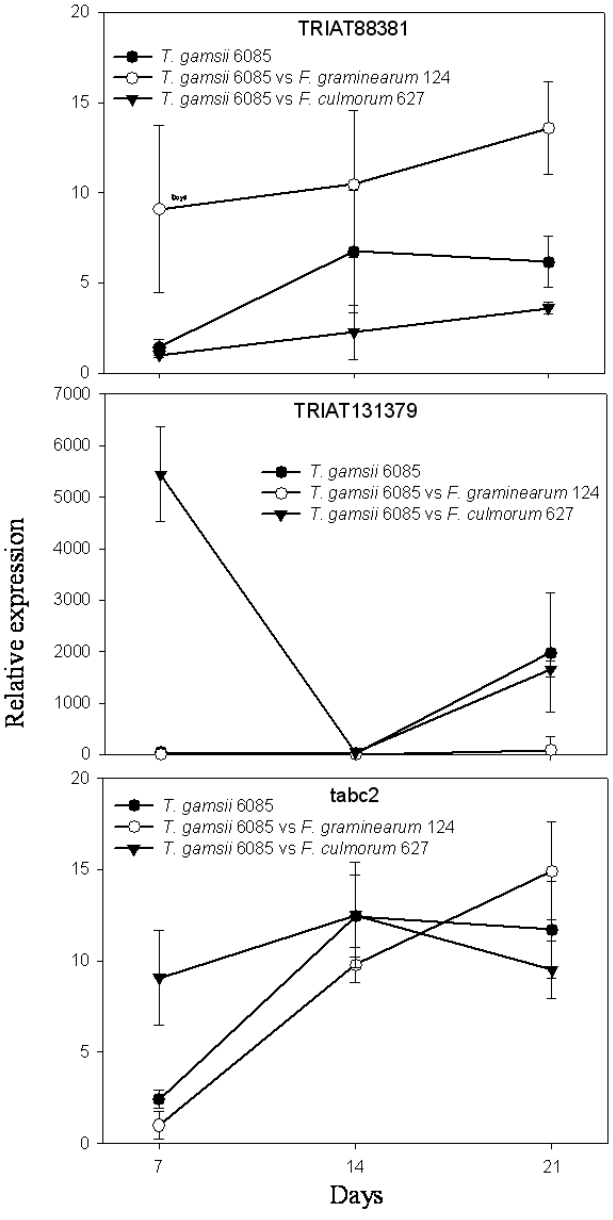

Fig. 4. Relative expression (normalized to *tef1* expression) of TRIAT88381, TRIAT31379 and tabc2 ABC transporters by *T. gamsii* 6085 on rice, in presence/absence of *F. graminearum* 124 and F. *culmorum* 627 at different time.

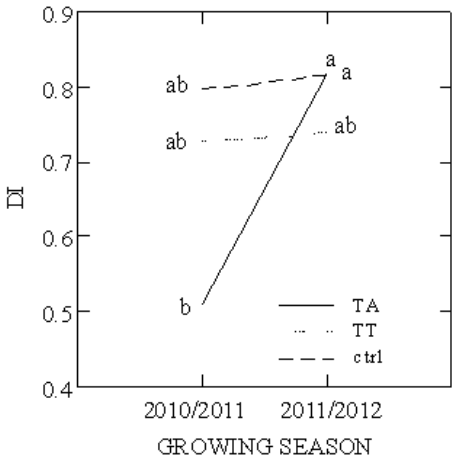

Fig. 5. Effect of the interaction growing season x treatment on disease incidence (DI). TA: *T. gamsii* 6085 inoculated on spikes during anthesis; TT: *T. gamsii* 6085 inoculated in soil before sowing; ctrl: non-inoculated control. Data points with the same letter are not significantly different for P= 0.05.

treatments. NIV values were up to 138 ppb in 2010/2011, without differences among treatments, whereas there was no detection in 2011/2012. Additionally, 3Ac-DON, ZON, NEO, T2, HT2, FUS X and DAS were below the limits of detection. The data obtained do not allow us to speculate on the ability of this beneficial isolate in preventing or controlling mycotoxin production in wheat, in addition to FHB control.

In 2011/2012 the fate of *Tg*6085 on the spikes was assessed. No *Trichoderma* was recovered from control spikes so all further analysis were performed on *Tg*6085-treated spikes. The analysis of variance of percentage of colonization of inoculated materials indicates that spikelet position (P=0.000), spikelet component (P=0.000) and sterilization method (P=0.013) were significant sources of variability, whereas time and all possible interactions were not significant. As time was not significant, the percentage of colonization was expressed as the average of values resulting from both 7 and 14 days samplings (six replicates). In detail (Tab. 2), spikelets at position 4 (see Fig. 1a) were significantly less colonized by *Tg*6085, whereas no differences emerged for the other positions. Sterile flowers (see Fig. 1b) were significantly less colonized by the beneficial isolate while percentages of colonization of the other three components were similar. When the effect of sterilization was evaluated, treatment on separate components resulted in a significant lower colonization by *Trichoderma* than when spikelets were sterilized entirely. Values shown in Table 2 revealed that *Tg*6085 was endophytic in almost 40% of the spikelets components plated.

Table 1. Evaluation of FHB on wheat in field experiments during 2010/2011 and 2011/2012 growing seasons.

Sources of variability	DI (%)[a]	DS (%)[b]
Growing Season[c]		
2010/2011	41.7 a	16.9 a
2011/2012	50.7 b	15.5 a
Treatment[d]		
Ctrl	52.5 a	20.5 a
T6085 in soil	44.9 a	15.5 ab
T6085 on spikes	41.6 a	11.8 b

[a]Disease incidence= % of infected ears; [b]Disease severity = % of infected spikelets within each ear (Mckinney index); [c]values represent the average of all treatments, three replicates for each thesis; [d]values represent the average of both growing seasons, three replicates for each thesis; [§]at different letters, within the same column and for each source of variability, correspond values statistically different.

Table 2. Colonization of spike tissues by *T. gamsii* 6085 after 7 and 14 days from head inoculation.

Sources of variability	% Colonization[§a]
Spikelet position	
1	70.3 a
2	70.3 a
3	68.8 a
4	9.4 b
Spikelet component	
F	35.6 a
G	31.5 ab
F2	25.8 ab
ST	23.8 b
Sterilization method[b]	
Entire	70.3 a
Separate	39.1 b

[§]values represent the average of six replicates, four spikes per replicate; [a]or each source of variability, at different letters correspond values statistically different. [b]Spikelets were surface sterilized before (Entire) or after (Separate) components separation.

DISCUSSION

Prevention of mycotoxin formation during pre- and/or post-harvest stages of the various susceptible crops is the preferred strategy for reducing concentration of mycotoxins in food and animals feed. The use of many of the available physical and chemical methods for the detoxification of mycotoxin-contaminated agricultural products is restricted due to problems concerning safety issues and possible losses of nutritional quality of treated commodities, coupled with limited efficacy and cost implications (Kohl *et al.*, 2011). The development of fungicide resistance as well as a rising public concern over risks associated with pesticides use has lead to significant interest in the development of alternative non-chemical and environmentally friendly methods of pest and disease control. This strategy is also fostered by the European Union (Directive 2009/128/CE). Several biological control agents have been tested in laboratory and field experiments to effectively reduce colonization and mycotoxin contamination in the Mediterranean basin, where occurrence of *Fusarium* mycotoxins, especially trichothecenes such as DON, associated with FHB on wheat is of great concern. Different fungal species have been examined for their ability to reduce the potential inoculum of *Fusarium* pathogens, mainly by reduction of biomass in plant residues or as competitors on heads (Tsitsigiannis *et al.*, 2012).

Trichoderma gamsii 6085, used in the present work, is well known for its interesting antagonistic and mycoparasitic abilities against mycotoxigenic strains of *F. culmorum* and *F. graminearum* (Matarese, 2010; Matarese *et al.*, 2012). Its ability to compete with *F. graminearum* and *F. culmorum* on rice, expressed as a significant reduction of pathogens' growth and DON production, has also been assessed (Matarese *et al.*, 2012). To deeply investigate this beneficial isolate, *Tg*6085 was studied for its ability to grow in the presence of DON due to the possible role of this compound in microbial competition (Sarrocco *et al.*, 2012). To choose the selective amount of DON to be used in our experiments we took into account that heads of wheat inoculated with *F. graminearum* were found to contain, on an average, DON concentrations of about 93 ppm in the rachis, 50 ppm in the chaff, 25 ppm in the kernels and 16 ppm in the peduncle (Sinha and Savard, 1997) and that barley seedlings infested with different strains of *F. culmorum* contained 0.2-50 ppm DON (with an average of 17 ppm) (Hestbjerg *et al.*, 2002). Here, 50 ppm was chosen, an amount that could be found in nature but much higher than the maximum level admitted by EC Regulation No 1881/2006 on unprocessed cereals placed on the market. From our results, *Tg*6085 was able to grow in the presence of DON, a not prevalent character with-

in *Trichoderma* spp. as our isolate belongs to a narrow group of strains (10 out of 100) showing a similar growth in the presence/absence of DON (data not shown). This apparently conflicts with the results by Lutz *et al.* (2003) where DON did not affect *Trichoderma* growth. The different strains tested and the different assessment methods, Lutz *et al.* (2003) measured *Trichoderma* radial growth on agar containing DON whereas we evaluated the effect of DON on conidia germination in liquid culture, make results hardly comparable.

In nature, filamentous fungi encounter numerous antibiotic compounds produced by other microorganisms. They also need to handle toxins of endogenous origin such as antibiotics and mycotoxins that provide the producing organism with a competitive advantage in its ecological habitat (Stergiopoulos *et al.*, 2002). In this context, we performed HPLC analysis for detecting residual DON in *Tg*6085 cultural filtrates in order to investigate the fate of the mycotoxin. DON content in culture filtrates was not modified by fungal growth and no others products, such as its acetylated forms, were found, enabling us to reject the hypothesis of DON degradation. Therefore, the possible involvement of PDR-ABC transporters was investigated. Various families of integral membrane proteins can mediate transport of natural toxic compounds to biological membranes in fungi. Two major classes of transporter proteins are ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters (Del Sorbo *et al.*, 2000). Adam *et al.*(2001) identified several mechanisms contributing to DON resistance in *Saccharomyces cerevisiae* such as active toxin efflux, detoxification and target modification. Mitterbauer and Adam (2002) reported that *S. cerevisiae* strains with a disrupted ABC transporter PDR5 "pleiotropic drug resistance" gene showed increased sensitivity to DON and other trichothecenes. With a BLAST search of *Trichoderma atroviride* genome, a species very close to *T. gamsii* whose genome sequence is not yet available, we found two genes, TRIAT88381 and TRIAT131379, with high homology with PDR10 and PDR5 of *S. cerevisiae* genes encoding ABC transporter proteins with specificity for the trichothecene deoxynivalenol (Abolmaali *et al*., 2008; Mitterbauer and Adam, 2002). We also investigated the ABC transporter tabc2, previously characterized in *T. atroviride* (Ruocco *et al.*, 2009). Tabc2 and ABC transporters, in general, are reported to help *T. atroviride* to successfully antagonize or colonize various plant pathogens in many different environmental conditions (Marra *et al.*, 1996). The data obtained allow us to hypothesize a constitutive expression of these genes by *Tg*6085 thus, their involvement in DON resistance which, however needs to be better defined. This subject deserves further investigations taking into account that we tested only three ABC-PDR transporter genes, whereas in *Thichoderma* spp. drug resistance derives from ABC transporters, pleiotrophic drug resistance (PDR) and multidrug resistance MDR-type genes (Kubicek *et al.*, 2011).

When used in field experiments as spike inoculant at anthesis, *Tg*6085 was able to colonize almost 70% of untreated and 40% of treated spikelet components, the latter behaviour demonstrating an endophytic lifestyle. Endophytism is a key character for a potential biocontrol isolate as it is strictly connected with its beneficial effects on host plants, especially under stress conditions (Lorito *et al.*, 2010). The different colonization abilities on different spikelets and different components within the same spikelet, with the upper part less colonized than the lower, could depend by different factors as the timing of flowering within the spikes where, at anthesis, the central spikelets are open, or by a dropping effect of applied spore suspension due to gravity. In this context, more than one application and the use of adhesive compounds have been scheduled during the next field experiment.

Within the same field trials, *Tg*6085 showed interesting results in terms of reduction of disease severity whereas no significant reduction of disease incidence occurred. This suggested that *T. gamsii* was not able to interfere with the arrival of the inoculum on spikes and on their infection but is able to reduce the colonization of infected spikes. *Trichoderma* is a well known beneficial fungus used against different plant pathogens on different crops. Anyway, very little information, in some cases with contrasting results, is available about the use of isolates belonging to this genus for the biocontrol of FHB (Musyimi *et al.*, 2012; Riungu *et al.*, 2008; Luongo *et al.* 2005) and this is the first report on the use of a *T. gamsii* isolate for the control of the disease in field.

From our preliminary results, no correlation between FHB symptoms and mycotoxin accumulation on kernels was detected. Grains collected at the end of the growing cycle showed a very low contamination of DON and NIV whereas other trichothecenes were under the limit of detection in spite of FHB symptoms. Correlation between visual disease and DON content is still controversial. Previous studies have shown that visual disease assessments and DON content may or may not be correlated with each other. This inconsistency might be due to variability in competitiveness (Miedaner *et al.*, 2000; Chandler *et al.*, 2003; Ludewig *et al.*, 2005; Xu *et al.*, 2009) between and within pathogen species in field samples. Correlation between FHB and DON is also significantly affected by the wheat type, study type and study location (Wegulo *et al.*, 2011) with environmental conditions differently affecting the infection, the colonization process and comparative abundance of different species associated with FHB (Xu and Nicholson, 2009). Recent surveys on the occurrence of DON in wheat collected in Italy indicated a widespread DON contamination in samples grown in northern and

central Italy with incidence and levels of contamination depending on the growing seasons (Haidukowski *et al.*, 2012). Variability among years affects the efficacy of our *T. gamsii* isolate in terms of pathogen spread but not in terms of disease severity, indicating that this isolate deserves attention as a possible active ingredient of a biopesticide to protect wheat from FHB.

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TRANSCRIPTIONAL CHANGES OF PUTATIVE *FUSARIUM GRAMINEARUM* TRANSPORTER SEQUENCES IN RESPONSE TO TRIFLOXYSTROBIN AND DEOXYNIVALENOL

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SUMMARY

Fusarium graminearum, the most important causal agent of head blight of cereals, produces mycotoxins like the trichothecene deoxynivalenol (DON). This fungus was resistant towards the fungicide trifloxystrobin and externally applied DON whereas it was sensitive towards the demethylation inhibitors epoxiconazole and tebuconazole. Forty-six expressed sequence tags (ESTs) were screened for induction after exposure of mycelium to sub-lethal concentrations of trifloxystrobin, epoxiconazole, tebuconazole, or DON. Four putative transporter genes were up-regulated more than 3-fold after treatment with trifloxystrobin, a fungicide derived from a natural toxin produced by fungi of the genus *Strobilurus*. Interestingly, the same four genes were also transcriptionally induced by externally applied DON. In contrast, no significant transcriptional induction of the four candidate genes was observed after exposure of the fungus to epoxiconzole and tebuconazole. Two of the up-regulated genes belong to the MFS-transporter family (FGSG_01584; FGSG_07802), one is a putative ABC-transporter (FGSG_17395), the fourth is a probable polypeptide-transporter (FGSG_07227).

Key words: ABC-transporter, efflux, fungicide, *Gibberella zeae*, MFS-transporter, mycotoxin.

INTRODUCTION

Fusarium graminearum (teleomorph *Gibberella zeae*) is a plant pathogenic fungus with saprophytic capabilities. The fungus can produce mycotoxins such as the trichothecene DON, a potent inhibitor of eukaryotic protein biosynthesis. When infecting plants, mycotoxins increase the aggressiveness of the fungus (Mesterházy, 2002) and contribute to overcoming plant defense mechanisms in a host-specific manner (Maier *et al.,* 2006). Previous publications (Hobden and Cundliffe, 1980; Iglesias and Ballesta, 1994) suggest that trichothecene-producing organisms are resistant towards trichothecenes due to an alteration of their ribosomal structure.

Antifungal plant defense compounds and conventional fungicides can be neutralized by efflux transporters such as ABC- (ATP-binding cassette) or MFS- (Major facilitator superfamily) transporters located in the outer membrane of fungi (Stergiopoulos *et al*., 2002). ABC transporters utilize adenosine-5'-triphosphate (ATP) as energy source to transport compounds across a membrane against a concentration gradient. They have 12 trans-membrane domains arranged in two groups of 6 trans-membrane domains each (Deising *et al*., 2008). Each ABC transporter possesses two nucleotide-binding domains. MFS transporters utilize the proton motive force as energy source. They have 12 trans-membrane domains arranged in only one battery. The MFS is a ubiquitous group of proteins that is involved in the transport of a wide range of molecules, including toxins (Law *et al*., 2008). In *Fusarium sporotrichioides*, a gene for a trichothecene efflux pump (*TRI12*) was identified with high homology to MFS-transporter (Alexander *et al*., 1999).

Fungicides containing triazoles as active ingredients can reduce DON levels by about 50% when applied to wheat stands during infection (Beyer *et al*., 2006), whereas complex II inhibitors are not effective against *F. graminearum* (Dubos *et al*., 2011). However, fungicides generally lose their efficacy more or less rapidly, because less sensitive individuals survive the treatments and form new populations. First evidence for a slow loss of efficacy was reported (Klix *et al*., 2007). Furthermore, a shift towards more toxigenic *F. graminearum* strains was reported (Ward *et al*., 2008). The mechanistic reason(s) for both effects is unknown, but efflux of fungicides and mycotoxins may well be involved, particularly because transmembrane transport proteins are known to export various substances, thereby conferring multi-drug resistance to the cells expressing the transport proteins (Kretschmer *et al*., 2009). The genome of *F. graminearum* was sequenced (Cuomo *et al*., 2007) and several putative transporter sequences were found. It was the objective of the present study to test whether efflux candidate sequences might be transcriptionally in-

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fluenced by fungicide or mycotoxin treatment.

MATERIALS AND METHODS

Fungal material and bioassay. *Fusarium graminearum* (strain 74b) was isolated in 2004 from a blighted wheat head from a field at Ahrensfelde (northern Germany) (longitude E 10° 23'/latitude N 53° 49') and can be obtained from the authors on request. The isolate formed perithecia and ascospores typical for *Gibberella zeae* and its identity was confirmed by PCR using the species-specific primer pair Fg11 [f: CTC CGG ATA TGT TGC GTC AA, r: GGT AGG TAT CCG ACA TGG CAA (Doohan *et al*., 1998)].

Trifloxystrobin (Sigma-Aldrich, Buchs, Switzerland) and tebuconazole (Sigma-Aldrich Seelze, Germany) were solved in ethanol. Epoxiconzole (Sigma-Aldrich) was solved in ethanol:etlylacetat (9:1 v/v), and DON (Sigma-Aldrich) in methanol. Trifloxystrobin is a derivate of strobilurin A, which is a natural compound originally produced by the fungus *Strobilurus tenacellus*. The average concentration of the stock solution was 4 mg/ml. The exact concentration of each stock solution was determined by measuring the mass of the antibiotic on a microbalance before adding the solvent(s). The chemical purity of the compounds was ≥ 97%. Fungicides were transferred into the wells of sterile, clear, flat bottom 96 microtiter plates resulting in concentrations of 50, 25, 5, 2.5, 0.5, 0.05, 0.005, and 0% of the stock solution. Solvents were allowed to evaporate overnight. Subsequently, 100 µl of a spore suspension (treatment) or 100 µl water (control) and 100 µl of PDB medium were transferred into each well. All experiments were conducted with four replicates. Microtiter plates were sealed with Parafilm and incubated on an orbital shaker at 120 rpm at 22°C in the dark for 5 days. Afterwards, the optical density of the spore suspensions and the respective control liquids was determined using an absorbance reader (Tecan GENios, Austria). Optical densities were corrected for the absorbance of the microtiter plates, the medium, and the intrinsic colour of the fungicide. Data were expressed relative to the optical density of the untreated control. An optical density of 0% indicated a complete inhibition of fungal growth whereas an optical density of 100% indicated no difference from the untreated control. Medians of four replicates were calculated for each antibiotic and plotted against antibiotic concentration.

Antibiotic/fungicide treatments. Flasks containing 200 ml PDB liquid medium were prepared and inoculated with 500 µl of a macroconidial suspension. Flasks were incubated at room temperature on a shaker for 7 days. Flasks containing the fungal material were opened under a sterile workbench and antibiotic solutions were transferred into the inner side of the lids. Solvents were allowed to evaporate. Finally, lids were screwed on the flasks again, which were incubated upside down on a shaker for 6 h, so that the antibiotics on the inner side of the lids were in direct contact with the liquid medium and fungal material. Concentrations of the compounds were 10^{-5} mg ml^{-1} for epoxiconazole, tebuconazole and trifloxystrobin and $10^{-2,3}$ mg ml^{-1} for deoxynivalenol during the 6 h incubation.

RNA extraction, cDNA synthesis and realtime-PCR. Total RNA was isolated from fungal cultures 6 h after the treatment using TRIZOL reagent (Invitrogen, USA) according to the manufacturer's protocol and cDNA synthesis was performed using the SuperscriptIII Reverse Transcriptase (Invitrogen, USA). Putative transporter genes were selected from the MIPS-*Fusarium graminearum* database [http://mips.helmholtz-muenchen.de/genre/proj/FGDB/ (Ma *et al*., 2010)]. For 43 sequences, the designed primer pairs were suitable to produce a specific amplicon. cDNA-synthesis was performed using the Superscript III cDNA Synthesis kit (Invitrogen, USA) using Oligo dT-primer according to the manufacturers' guidelines. A first estimation of gene expression was conducted in a semi-quantitative PCR screening (data not shown). Thirteen of these 43 candidate sequences were subsequently tested in an RT-PCR experiment using UBC as housekeeping gene in three biological and two technical replications. Primer sequences are shown in Table 1. Real-time PCR was conducted on an ABI 7300 real-time PCR system using the MAXIMA SYBR green qPCR Mix with the following cycling parameters: 50°C for 2 min, 95°C for 10 min, [95°C for 20 sec, 60° for /45 sec] for 50 cycles. Calculation of relative gene expression was conducted following the method of Pfaffl (2001).

Southern analysis. Genomic DNA was extracted from *F. graminearum* mycelium as described by Rogers and Bendich (1985) and digested with the restriction endonucleases EcoRI, HindIII and XbaI. After restriction, genomic DNA was separated on 0.75% agarose gels and transferred onto Hybond-XL membrane (GE Healthcare, USA) by capillary diffusion blotting overnight, using 0.25 M NaOH/1.5 M NaCl as blotting solution. Southern blots were hybridized with ^{32}P-labelled (Hartmann analytics, Germany) DNA probes derived from amplified EST-products at 62°C and washed twice (0.5X SSC, 0.2% w/v SDS) for 30 min and exposed at -70°C for 72 h. As DNA-probes, PCR products amplified from *F. graminearum* cDNA with the already introduced gene-specific primers respectively used and labeled. PCR products were separated on 1% low melt agarose gels. Fragments were released from the gel with the gel extraction kit (Qiagen, Germany) ready for probe labeling.

Table 1. Designation of loci, putative function and sequences of primers used for amplifying putative nucleotide transporter sequences. *Primer for the housekeeping gene UBC according to Lysoe *et al.* (2006).

Locus	Putative function		Sequence
FGSG_03541	TRI12trichothecene efflux pump (Mfs)	forw	CGTCTTACGGATAGATTTGG
		rev	GACAGTTACAGCGAGGAG
FGSG_17395	related to ABC transporter	forw	TTCCACCGAACAACCTCC
		rev	GCCGTCCAAGAACAAGC
FGSG_07227	probable oligopeptid Transporter	forw	ACGAGAATGAGGAAGAAGAG
		rev	GAGCGATAAGAGGACCAATG
FGSG_01584	related to putative multidrug transporter Mfs1.1	forw	TGCCTTTCAAGCATCCAC
		rev	CCTTCTCCTCCAGACCATAC
FGSG_07802	related to putative multidrug transporter Mfs1.1	forw	ATCGCTTGTGGTTTGTTC
		rev	CTGAAATGTCGGATGCTG
FGSG_02580	related to maltose permease (MalP)	forw	CAAACCTCTGACTCAACTAC
		rev	CTACAATCTCGCAGACAATG
FGSG_12051	related to DHA14-like major facilitator efflux transporter	forw	CCAACTCAACGGACGAAG
		rev	CGCTGCTCACTATTACGG
FGSG_03107	related to high affinity methionine Permease	forw	ATCGTGCAATCTGTCGGAAG
		rev	GCGCAAACAAAGCATACTGG
FGSG_03646	related to nicotinamide mononucleotide Permease	forw	GGTCTTCCGAAAGGTTGATG
		rev	AGACACAGAGCATACCAAGG
FGSG_04580	probable ABC1 transport protein	forw	CAGGACATGAACGTCTTTGG
		rev	TGTAGTTGAAGTAGGTGCTG
FGSG_06331	related to zinc transporter	forw	CACTGCCACTACTGATGTTC
		rev	ACAGGCAGAAGATGAAGAAC
FGSG_01526	similar to peroxisomal ABC transporter	forw	GTACAACCCGTCTACTCGC
		rev	GCTTCTTTACTGGACCACC
FGSG_02025	probable iron inhibited ABC transporter 2	forw	GTGTCCTCGCTTCTACC
		rev	CGACTCGCATACGCCAAC
FGSG_10805	UBC housekeeping gene	forw*	TCCCCTTACTCTGGCGGTGTC
		rev*	TTGGGGTGGTAGATGCGTGT AG

RESULTS

Bioassays. Epoxiconzole or tebuconazole inhibited fungal growth of *F. graminearum* completely at concentrations ≥ 10^{-3} mg ml^{-1} (Fig. 1). Below this concentration, the fungus survived and inhibition increased with concentration. In the case of DON, no significant inhibitory effect was observed. For trifloxystrobin, a maximum inhibition of about 40% was found in comparison with the untreated control culture within the range of concentrations tested (Fig. 1). To assess the transcriptional response of the fungus towards the antibiotics in the experiments that followed, fungal cultures were grown in presence of 10^{-5} mg ml^{-1} epoxiconazole, tebu-

conazole, or trifloxystrobin, or $10^{-2.3}$ mg ml^{-1} DON, to allow for sufficient biomass production in the presence of the respective antibiotic.

Selection of candidate sequences and expression analysis. At present (FG3 assembly), 24 putative ABC (ATP binding cassette) transporters, 14 putative MFS (Major facilitator superfamily) transporters, and 20 putative efflux pumps can be found in the *F. graminearum* genome [http://mips.helmholtz-muenchen.de/genre/proj/FGDB/ (Ma *et al*., 2010)]. Sequence information was downloaded from the database and primers were designed. The position of forward and reverse primers was chosen in a distance of about 200 for optimal detection of amplicons during real-time PCR. Primers were tested on genomic DNA to assure specificity. After the specificity was verified by conventional PCR, a set of 43 primer pairs was selected for relative quantification of gene expression. First, a semiquantitative PCR approach was used to generate a general overview of transcriptional activity of the putative transporter genes. After this screening, 13 primer pairs were selected for the relative quantification of gene expression by real-time PCR, which was performed with three replicates using the UBC-gene as reference (Lysoe *et al*., 2006). Eight candidate genes (FGSG_01526, FGSG_02025, FGSG_03107, FGSG_03208, FGSG_03541, FGSG_03646, FGSG_04580, FGSG_12335) showed no significant changes in expression. A set of four putative transporter genes (FGSG_17395, FGSG_07227, FGSG_01584, FGSG_07802) were up-regulated by at least 3-fold in comparison with the untreated control in response to DON [3.54 (±0.8) to 13.46 (±1.28) (Fig. 2)] and trifloxystrobin [4.84 (±0.86) to 6.91 (±0.43) (Fig. 2)]. The transcriptional response of the same sequences towards epoxiconazole and tebuconazole was ≤ 3 fold [up to 2.75 (±0.77) (Fig. 2)]. FSGS_17395 showed the highest induction of gene expression after DON-treatment of 13.46 (±1.28) in comparison to the untreated control. In contrast, the trichothecene efflux pump FGSG_03541 was interestingly not significantly induced by DON at the transcriptional level [1.76 (±0.46)].

Sequence analysis of candidate genes. The four identified sequences (FGSG_17395, FGSG_07227, FGSG_01584, FGSG_07802) plus the previously described trichothecene efflux pump TRI12 (FGSG_03541) were selected for some additional molecular analysis. Genomic distribution of the genes was determined by Southern hybridization (Fig. 4). It confirmed that the ESTs selected for more detailed analyses were single copy sequences as expected according to *in silico* analysis of the genomic database. At the protein level, homologous domains could be found in the two candidates which belong to the MFS-family (FGSG_01584 and FGSG_07802) as well as the non-responsive trichothecene pump FGSG_03541 (Fig. 3). Despite these similarities, at the amino acid level the DNA sequences of the putative transporter genes were not closely related.

DISCUSSION

Here we report about four putative transporter genes in *F. graminearum*, which were significantly up-regulated after application of sublethal doses of DON or trifloxystrobin. Towards both compounds, a high level of resistance was observed. Treatments with the azole fungicides epoxiconazole or tebuconazole had no significant effect on the expression of the studied efflux transporter candidate genes and a high level of susceptibility towards the latter two compounds was observed. Liu *et al*. (2009) detected only one putative transporter (FGSG_09697) being induced by a treatment with tebuconazol in an expression profiling approach, suggesting that azoles hardy trigger the induction of efflux in *F. graminearum*. The four putative transporter genes responding to DON and trifloxystrobin in this work, belong to different transporter classes. Two of them (FGSG_01584 and FGSG_07802) are potential MFS-transporters, one of them (FGSG_17395) is a putative ABC-transporter and the last one (FGSG_07227) is an isp4-protein. ABC- and MFS-transporter were reported to be redundant within microbial genomes and often exhibit overlapping substrate specificity and operate as toxin transporters which participate in the avoidance of accumulation of mycotoxins, produced by the fungus itself (Stergiopoulos *et al*., 2002; Deising *et al*., 2008; Roohparvar *et al*., 2008).

The gene FGSG_07227 shares homology with an isp4 transporter. Originally, the isp4-transporter was identified in *Schizosaccharomyces pombe* (Lubkowitz *et al*., 1998). It shares a high level of similarity with the oligopeptide transporter gene *OPT1* of *Candida albicans*, but lacks the typical signature motif of ABC- and peptide transporters. Therefore, the gene was classified as a member of the OPT-transporter family, a group that facilitates the transport of small peptides across the outer cell membrane. Isp4 is up-regulated during the induction of meiosis and its expression level is increased in response to nitrogen deficiency. It shares a high level of homology with known glutathione transporters, but it does not seem to be the primary glutathione transporter (Lubkowitz *et al*., 1998; Dworeck, 2009). Toxic compounds are bound either directly to glutathione, or glutathione-S-transferase catalyzes an enzymatic binding. In fungi and plants, glutathione is a substrate in the synthesis of chelatins and thus involved in the cellular detoxification of heavy metals. Furthermore, it is involved into the neutralization of reactive oxygen (Grant, 2001).

Efflux as a detoxification mechanism in phytopathogenic fungi was intensively studied in *Zymoseptoria triti-*

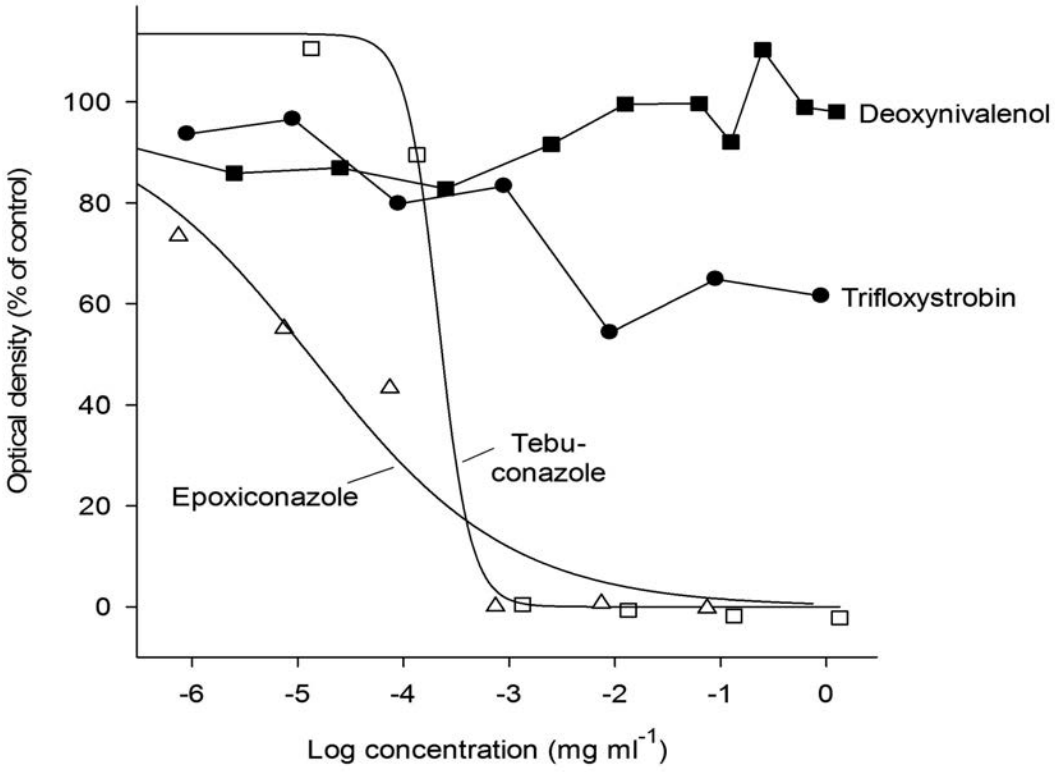

Fig. 1. Effect of different concentrations of deoxynivalenol (′), epoxiconazole (ρ), tebuconazole (≤), or trifloxystrobin (□) on the growth of *Fusarium graminearum* (isolate 74b) in PDB medium. The optical densities of the fungal cultures were evaluated after incubation for 5d at 22°C and 120 rpm and expressed as percent of the optical density of non-treated control cultures (100 = normal growth; 0 = total inhibition of growth). Plot symbols represent medians of 4 replicates.

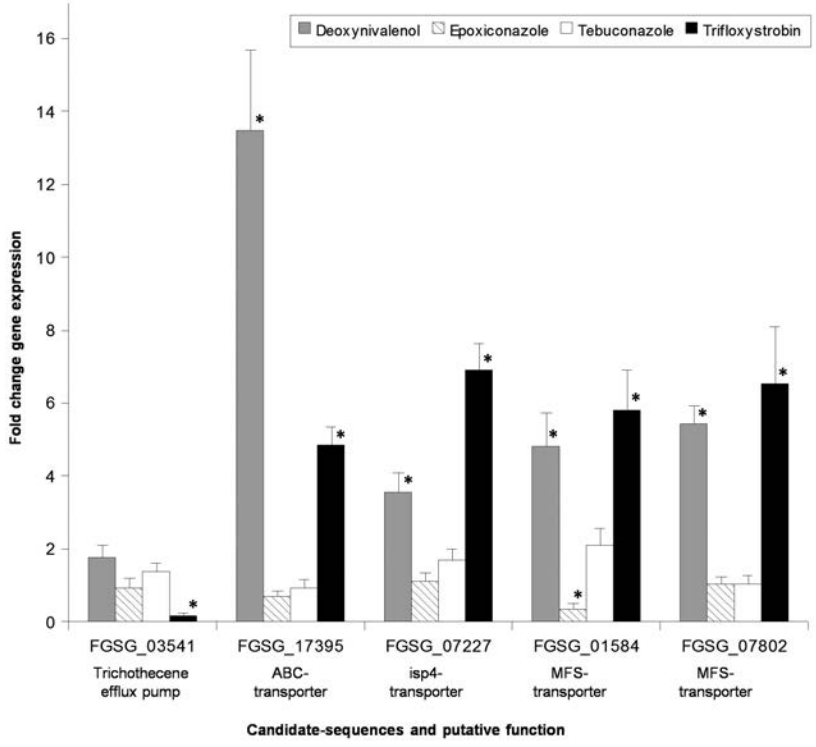

Fig. 2. Relative quantification of gene expression of the selected *Fusarium graminearum* candidate sequences after treatment with deoxynivalenol, tebuconazole, epoxiconazole, or trifloxystrobin determined by real-time-PCR. Expression of untreated control = 1. Expression level changes marked with "*" were significantly different (one-sample-t-test, SPSS version 16) from 1.

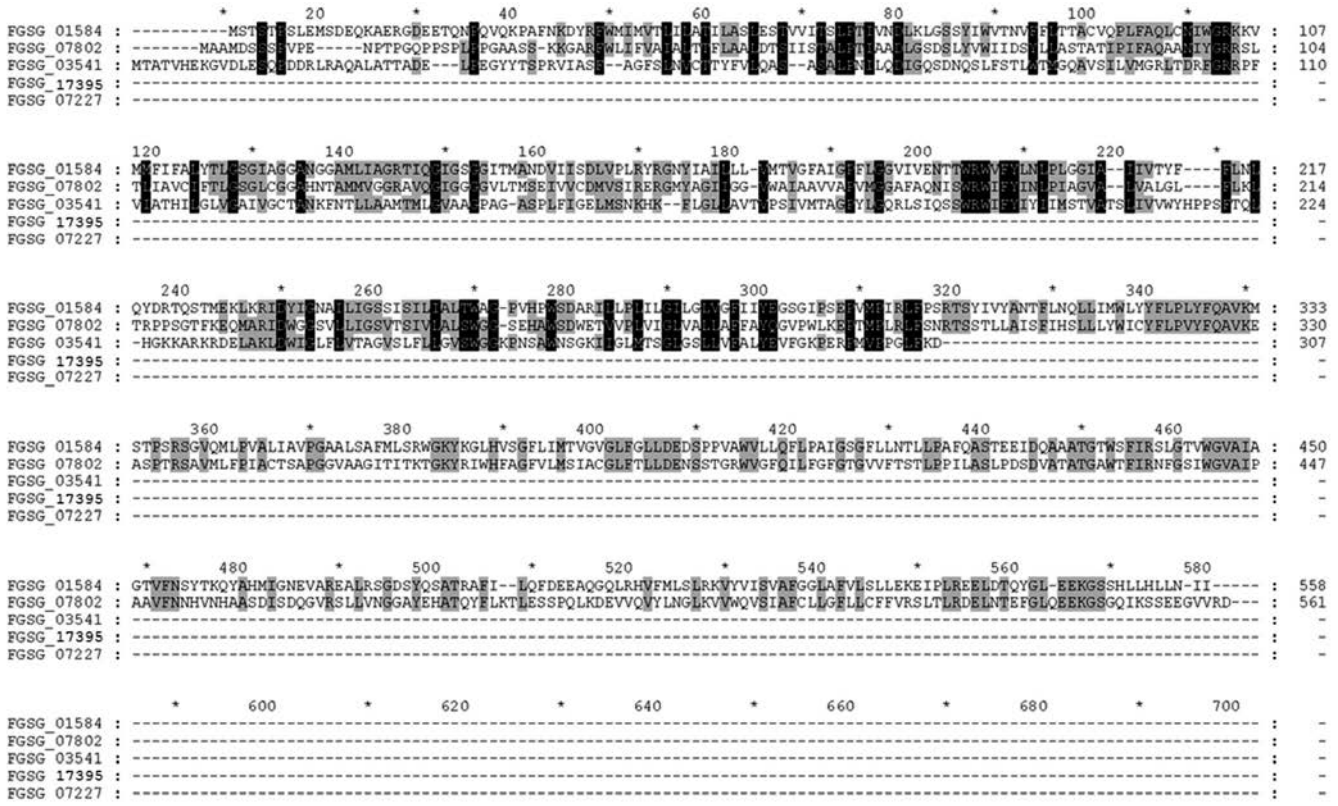

Fig. 3. Alignment of amino acid sequence of the 5 analyzed potential transporter. The alignment was performed by using the GENEDOC software (Nicholas *et al*., 1997). Homologous amino acids are shadowed.

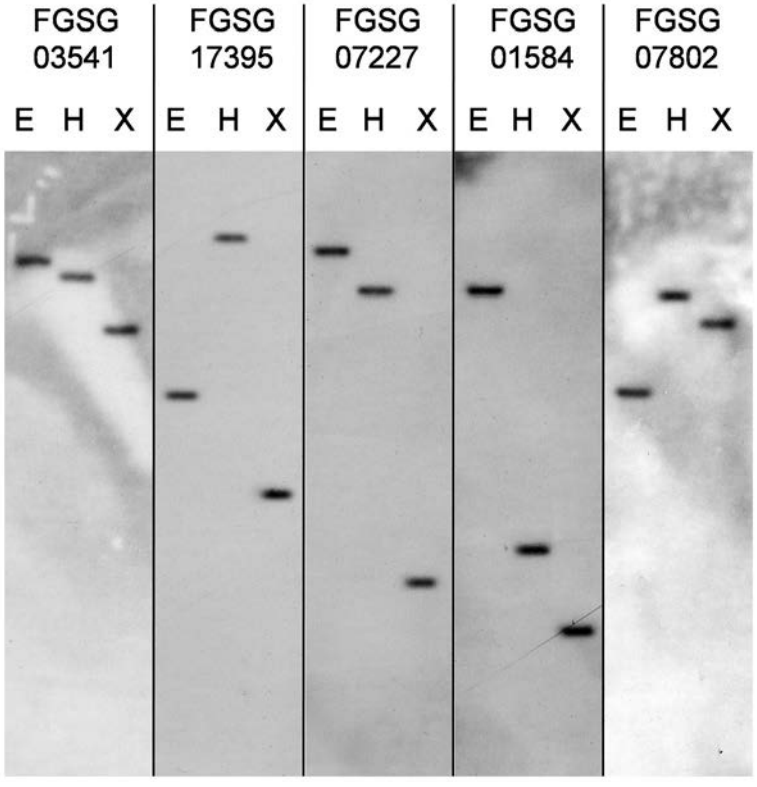

Fig. 4. Southern hybridization of the responsive sequences FGSG_17395, FGSG_07227, FGSG_01584, FGSG_07802 and the non-responsive trichothecene efflux pump FGSG_03541. E=*Eco*RI, H=*Hind*III and X= *Xba*I.

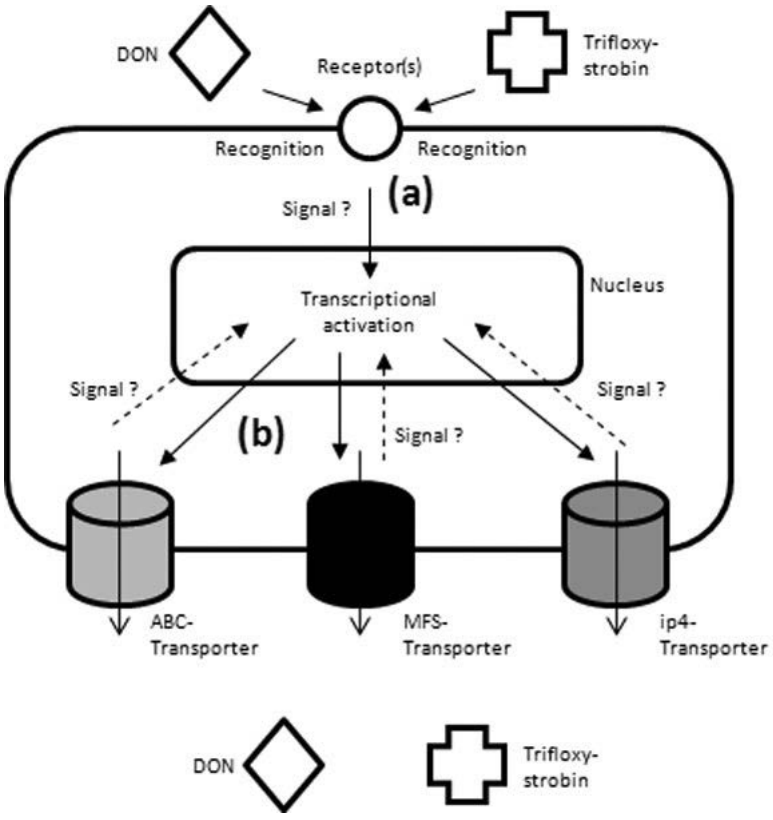

Fig. 5. Hypothetical functional model of signal transduction after external application of mycotoxin DON and trifloxystrobin. External application of DON or trifloxystrobin leads to a transcriptional activation of diverse transporter genes through (a) recognition by a receptor and a signal cascade to the nucleus, or (b) as a self-induction process by the transporter proteins leading to an enhanced transcriptional activation.

ci. All of the studied strobilurin-insensitive *Z. tritici* isolates show a modification of the target gene, the G143A point mutation within the cytochrome b gene (Gisi *et al*., 2002). Nevertheless in the resistant isolates, *MgMfs1*, a strong strobilurin transporter, was transcriptionaly upregulated after application of a sublethal dose of strobilurin. The relevance of this second detoxification mechanism is still unknown (Roohparvar *et al*., 2008). Maybe this is part of a rudimental broad detoxification apparatus that has developed during evolution. Reimann and Deising (2005) studied the role of ABC-transporters as part of a detoxification mechanism against natural and synthetic toxins. *Pyrenophora tritici-repentis* adapted to fungicides at sublethal concentrations, developed a quantitative resistance against a broad spectrum of fungicides, including strobilurins. This isolate became susceptible against fungicides again after treatment with an inhibitor of the energy-dependent efflux transport (hydroxyflavonderivate).

Here we showed that four putative transmembrane transport proteins are up-regulated in response to externally applied DON and trifloxystrobin. This is clear evidence that *F. graminearum* is capable of recognizing a high external toxin concentration and reacts to it, probably by increasing efflux capacities. Concerning the target gene of strobilurins, Kaneo and Ishii (2009) reported that none of the mutations conferring resistance towards strobilurins in other fungi (Gisi *et al*., 2002) were found in the cytochrome b gene of two Japanese *F. graminearum* isolates. Provided that there are no other still unknown mutations in the *F. graminearum* cytochrome b gene conferring resistance towards trifloxystrobin, efflux could be a key factor in this resistance.

The lack of response of the trichothecene efflux pump at the transcription level towards externally applied DON suggests that the transcription of the gene is triggered by factors other than the external DON level. Since DON was reported to be involved in the pathogenic process (Maier *et al*., 2006), plant or microbial competitor factors might be needed.

Prior to gene activation, recognition of antibiotics including the own mycotoxin by one or more fungal receptors is required. The signal that activates the transcription of the putative transporters is still unknown. Following the results of this study, the signals of DON and trifloxystrobin lead to a common process of gene activation (Fig. 5a). Alternatively, the transporter genes could be part of a self-activating mechanism that induces gene expression in the presence of natural toxins (Fig. 5b). It has still to be determined whether other

kinds of stress are able to activate the same set of transporter genes. Likewise, how the regulation takes place remains unclear. In *Botrytis cinerea* a transcriptional regulator of transporter genes was identified that is involved in multi-drug resistance (Kretschmer *et al.*, 2009) and its transcriptional activation was described as a part of decreasing sensitivity against fungicides. It might be speculated that *F. graminearum* has developed a common protection mechanism against toxic compounds of natural origin based on the activation of a heterogeneous set of efflux transporters that is activated after exposure to antimicrobial molecules.

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EMERGING FUNGAL (AND OOMYCETE) THREATS TO PLANT AND ECOSYSTEM HEALTH. S.J. Gurr. *Department of Plant Sciences, University of Oxford, UK. E-mail: sarah.gurr@plants.ox.ac.uk*

Fungal diseases have been increasing in severity and scale since the mid-20th Century and now pose a serious challenge to global food security and ecosystem health (Gurr *et al.*, 2011). Indeed, we have demonstrated recently that the threat to plants of fungal infection has now reached a level that outstrips that posed by bacterial and viral diseases combined, as evidenced by a major review of ProMed and HealthMap databases (1994-2011, 83,545 alerts) and by review of the literature (Web of Science) on emerging infectious disease (Fisher *et al.*, 2012, highlighted in *Science*, 2012 337, 636-638). This presentation will highlight some of the more notable persistent fungal and oomycete plant diseases of our times. It will draw attention to the emergence of new pathotypes affecting crop yields and to fungi and oomycetes decimating our natural and managed landscapes. Here, we have calculated that the losses due to persistent disease (that is, non-epidemic) caused, for example, by rice blast, wheat stem rust, corn smut, soybean rust and potato late blight, if mitigated, would be sufficient to feed 8.5% of the global population (based on 2000 calories per day for 1 year). In terms of cost, losses for rice, wheat and maize due to infection by the pathogens listed, accounts for some 60 billion US$ of agricultural revenue *per annum*. Moreover, tree losses due to fungal and oomycete diseases such as dutch elm, chestnut blight, sudden oak death, jarrah die-back and pine beetle/blue stain fungus, thus far, have been estimated to account for CO_2 sequestration losses of between 230 and 580 megatonnes (Fisher *et al.,* 2012). The spread of such organisms around the world is facilitated primarily by trade and transportation, but there is increasing concern that climate change may allow their establishment in regions hitherto deemed unsuitable. Increasing latitudinal ranges are anticipated under rising temperatures, due to enhanced growth and reproductive success, and elevated overwintering survival rates. However, the interactions between climate change, crops, and natural enemies are complex, and the extent to which crop pests and pathogens have altered their latitudinal ranges in response to global warming is largely unknown. We can demonstrate, from 26,776 observations of hundreds of pests and pathogens (612), their shift polewards since 1950, with a more rapid shift since 1990 (Bebber *et al.*, 2012). This latitudinal shift, of some 26.6 km per decade, is seen in both the Northern and Southern hemispheres. Moreover, the rate of movement since 1950 is identical to that predicted by global climate data. This observed trend cannot be explained by latitudinal variation in technical capacity to detect and report pest incidences. I shall invoke use of the basic disease triangle concept to highlight the "missing" data, with regards to pathogen and host biology and to the various environmental parameters which dictate disease spread. Given these data "voids" I shall comment on the implementation of policy and conclude with a series of recommendations for improved disease surveillance and reporting, the need for greater public awareness of these issues and a call for greater funding for fungal research. I shall conclude by discussing current and futuristic plant disease control strategies.

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BIODIVERSITY OF TOXIGENIC *FUSARIUM* SPECIES IN PLANT PATHOLOGY AND FOOD SAFETY. A. Logrieco, A. Susca, S. Somma, F. Fanelli, G. Mulè and A. Moretti. *Istituto di Scienze per le Produzioni Alimentari, CNR, Bari, Italy. E-mail: antonio.logrieco@ispa.cnr.it*

The genus *Fusarium* comprises most of the main mycotoxigenic fungi responsible for various plant, animal and human diseases. This genus includes complexes of pathogenic and opportunistic species able to produce a large number of toxic secondary metabolites (mycotoxins) in infected plant tissue. Environmental conditions that exist in the various agro-eco-systems in which plants are grown, are of particular interest in biodiversity studies because such conditions can influence fungal populations associated with these crops, fungal-plant interactions, and production of biologically active secondary metabolites, including mycotoxins. Wheat and maize are two examples of important plants colonized by a complex of mycotoxigenic *Fusarium* species which can produce different classes of mycotoxins, and induce disease under different environmental conditions. Such plant colonization is an example of mutual aggressiveness of microorganisms towards host species as well as humans and animals that eat food or feed derived from infected and multimycotoxin-contaminated plants. To know the mutual benefit of mycotoxigenic fungi and how they follow each other is of great importance both for risk assessment and for control/reduction strategies. In this context, MYCORED, a large collaborative project funded by the European FP7-"Food, Agriculture and Biotechnologies" Work Programmes (www.mycored.eu), is promoting a network of cooperation-interaction with the whole scientific community in order to optimize and rationalize the efforts for studying mycotoxigenic fungi at the global level and developing strategic solutions for reducing mycotoxin contamination in major crops.

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HPLC-MS/MS-ANALYSIS OF MYCOTOXINS: CURRENT APPLICATIONS AND FUTURE PERSPECTIVES. H.-U. Humpf and B. Cramer. *Institute of Food Chemistry, Westfälische Wilhelms-Universität Münster, Germany. E-mail: humpf@wwu.de*

Mycotoxins are toxic fungal metabolites that can be found in a broad variety of different food products as well as animal feed. Depending on the growth, production and storage conditions, several mycotoxins can occur making the quality control a very challenging and laborious task. Therefore, since several years attempts are made to detect most regulated mycotoxins simultaneously. Despite the fact that several of these methods are published, there is still a need for an official analytical method suitable to determine all required mycotoxin limits. As several new developments in the field of mass spectrometry and chromatography have been made recently, the impact of these techniques on mycotoxin analysis will be discussed. As examples, the use of additional mass spectrometric experiments and techniques such as MRM3 and ion mobility as well as high resolution mass spectrometry (LTQ-Orbitrap) will be presented.

FINE-TUNED REGULATION OF TRICHOTHECENE BIOSYNTHESIS BY THE "CEREAL KILLER" *FUSARIUM GRAMINEARUM*. C. Barreau, M. Montibus, J. Merhej, E. Zehraoui and F. Richard-Forget. *INRA UR1264 MycSA, 33883 Cedex Villenave d'Ornon, France. E-mail: cbarreau@bordeaux.inra.fr*

The filamentous fungus *Fusarium graminearum* is one of the main causal agents of "Fusarium head blight" and "Maize ear rot" responsible for severe epidemics with disastrous yield losses (Kazan *et al.*, 2012). However, the main problem is that during infection, this fungus produces deoxynivalenol (DON), a toxic secondary metabolite belonging to the trichothecenes family, which accumulates in the grains. This mycotoxin represents a threat for human and animal consumers and is now strictly regulated for the cereals commercialized in Europe (European Commission Regulation No. 1126/2007). The biosynthetic pathway involving specific *Tri* genes responsible for DON biosynthesis has been described: the biosynthesis of DON in *F. graminearum* is assumed to be done by 15 genes which are grouped in the Tri5 cluster on chromosome 2 and two transcriptional regulators have been identified on this cluster (Alexander *et al.*, 2011). However, the control of the Tri5 cluster by other non-cluster regulators was not clearly demonstrated until very recently (Merhej *et al.*, 2011a). This motivated the studies conducted in our laboratory to better understand how biosynthesis of DON is genetically controlled by general regulators and how the interaction with the host plant components interferes with toxin accumulation. In this presentation, the potential role of various general transcription factors is hihglighted. Particularly, we previously showed that pH is a determinant for induction of deoxynivalenol biosynthesis and demonstrated that FgPac1, the general regulator of pH homeostasis, is a strong repressor of *Tri* gene expression (Merhej *et al.*, 2011b). More recently, we demonstrated the implication of two other general regulators in regulation of trichothecene biosynthesis: FgVe1, the central component of the velvet complex in fungi (Merhej *et al.*, 2012) and FgLae1 (unpublished information), a general regulator of secondary metabolites in fungi. A few years ago, we also showed that oxidative stress was an enhancing factor for deoxynivalenol accumulation (Ponts *et al.*, 2006, 2007), meanwhile plant antioxidants such as cinnamic acid derived phenolic acids were potent inhibitors of *Tri* gene expression and strongly reduced accumulation of the toxin *in vitro* (Boutigny *et al.*, 2010). We recently demonstrated that FgAP1, the *F. graminearum* homologue of the AP1 general regulator of oxidative stress in yeast (Yap1) and in other fungi, is implicated in the regulation of DON production. Different authors have reported that other transcriptional regulators also determine the induction of thrichothecene biosynthesis. As a general conclusion, it will be schematized how theses different regulation circuits are finely tuned and specially adapted for optimal toxin production by the fungus during the process of grain infection and then, how they contribute to the accumulation of DON in the crop in response to environmental conditions. These basic researches may open new perspectives for a better control of *Fusarium* development and mycotoxin accumulation in cereals.

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MONITORING OF *PHYTOPHTHORA INFESTANS* POPULATIONS OF THE EUROPEAN PART OF RUSSIA IN 2008-2011: A PHENOTYPIC AND GENOTYPIC STUDY.

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A total of 510 isolates of *Phytophthora infestans* from six Russian regions (Moscow, Leningrad, Smolensk, Kostroma, Astrakhan, Nizhni Novgorod) and the Mariy El Republic, almost all from commercial potato and tomato fields, were collected in 2008-2011. Phenotypic diversity was assessed using mating type and metalaxyl resistance; genotypic diversity was assessed using two allozyme loci of peptidase (Pep1 and Pep2) and the mitochondrial DNA haplotype. In addition, virulence patterns were determined for all studied populations. All but one population were represented by isolates that were susceptible or moderately resistant to metalaxyl. Both A1 and A2 mating types were present in the examined isolates at different ratios, depending on the region and the host plant; in addition, a small number of A1A2 isolates was found in two regions. In all populations, the 100/100 genotype at the Pep1 locus was predominant; five populations also contained the 92/100 genotype, and the 92/92 genotype was identified in one "potato" population (Moscow region), which was the only population, containing all three observed genotypes. The variability at the Pep2 locus was higher, though the 100/100 genotype dominated in the majority of populations; five populations contained all three observed variants (100/100, 100/112, and 112/112). Two mtDNA haplotypes, Ia and IIa, were detected; haplotype Ia was the most common in almost all studied populations. The majority of the studied populations contained virulence gene 9, which had not been observed in Russia during the last 15 years, and were represented mainly by complex races, containing 5-11 virulence genes. The most complex race, which included 11 virulence genes, was revealed in the "potato" population from the Moscow region. Relatively simple races were observed only in two regions, Astrakhan (race 3.4) and Kostroma (race 4.10). Virulence genes 5, 6, and 9 were the most rare in "potato" populations and absent in "tomato" populations (except for the "tomato" population from the Mariy El Republic, in which the frequency of the gene 9 was 0.7%). Atypical virulence patterns were observed for the Astrakhan "tomato" and Smolensk "potato" populations, characterized by low values of the virulence factor. In general, the maximum diversity level was observed in the Moscow region, followed by the Kostroma region and the Mariy El Republic. This finding can can be explained by the existence of a well-developed potato production industry in these regions, providing an active import of seed potato and, therefore, the possible appearance of new pathogen strains with

infected seed tubers. The comparison of data, obtained for "potato" and "tomato" *P. infestans* populations showed that the first ones have a higher diversity concerning such parameters as the mtDNA haplotype and Pep1/Pep2 loci and contain a higher number of virulence genes.

MAKING NEW MOLECULES - EVOLUTION OF PATHWAYS FOR NOVEL METABOLITES IN PLANTS. A. Osbourn. *John Innes Centre, Norwich, UK. E-mail: anne.osbourn @jic.ac.uk*

Plants produce a huge array of natural products, many of which are specialised metabolites associated with particular species. These secondary metabolites often have important ecological roles, facilitating pollination and seed dispersal and/or providing protection against attack by pests and pathogens. Although the ability of plants to perform *in vivo* combinatorial chemistry by mixing, matching and evolving the genes required for different secondary metabolite biosynthetic pathways is likely to have been critical for survival and diversification of the Plant Kingdom we know very little about the mechanisms underpinning this process. A recent and exciting development in plant biology has been the discovery that genes for the synthesis of multiple major classes of secondary metabolites are organised in clusters in plant genomes. These clustered pathways determine important crop traits such as pest and disease resistance and also the production of important drugs such as alkaloids in poppies. More such clusters are emerging as the body of sequence information available for plants continues to grow, accelerated by advances in high-throughput sequencing technology. Investigation of these clusters is yielding important new insights into mechanisms of metabolic diversification in plants and into plant genome organisation and dynamics more widely. This talk will focus on plant natural product function and synthesis, the origins of metabolic diversity and potential for metabolic engineering, drawing on our research on terpene synthesis in crop and model plants.

REGULATION OF ELICITOR-TRIGGERED PRODUCTION OF PHYTOALEXINS IN *ARABIDOPSIS*. S. Ferrari. *Dipartimento di Biologia e Biotecnologie "Charles Darwin", Sapienza Università di Roma, Roma, Italy. E-mail: Simone.Ferrari@uniroma1.it*

Production of secondary metabolites in plants is a typical response to pathogen infection. The expression of gene encoding enzymes involved in different metabolic pathways responsible for the biosynthesis of compounds with antimicrobial activity is often triggered by microbe- or host-derived signalling molecules, called elicitors. Treatment of whole plants or cell cultures with raw or purified elicitors is therefore a widely used method to boost the production of metabolites of interest, thus understanding the molecular mechanisms that regulate the expression of secondary metabolite genes is of extreme importance to improve the accumulation of pharmaceutics or nutraceutics. The model plant *Arabidopsis thaliana* has been extensively investigated to identify genes involved in the perception and trandsuction of elicitors and downstream components of the signalling pathways regulating secondary metabolism. The most important phytoalexin in *Arabidopsis* is camalexin, an indole compound important for resistance to necrotrophic fungi. We have previously shown that, in *Arabidopsis*, biotic stress strongly induces the expression of genes in the camalexin biosynthetic pathway. In particular, expression of PHYTOALEXIN DEFICIENT 3 (PAD3), encoding a P450 cytochrome catalyzing the last step of camalexin biosynthesis, is strongly induced by oligogalacturonides, flagellin or after inoculation with the fungal pathogen *Botrytis cinerea*. Moreover, PAD3 is required for basal and elicitor-induced resistance against *B. cinerea*. We have now identified a receptor-like kinase (RLK) that negatively regulates basal levels of expression of PAD3. Null mutants for this RLK show enhanced expression of PAD3 in absence of stimuli, and are more resistant to *B. cinerea*, compared to wild type plants. Notably, this RLK is positively regulated by abscisic acid (ABA) which is known to negatively regulate defense responses against *B. cinerea* infection. We propose a model to explain elicitor-ABA negative cross-talk in plant cells, which may be useful also to increase the production of secondary metabolites important for human use.

PHOTODYNAMIC TOXINS PRODUCED BY THE NECROTROPHIC FUNGUS *RAMULARIA COLLO-CYGNI* AND THEIR RELATION TO QUANTITY AND QUALITY OF MALTING BARLEY PRODUCTION. M. Hess, M. Nyman, S. Weigand and H. Schempp. *Lehrstuhl für Phytopathologie, Technische Universität München, Germany. E-mail: m.hess @lrz.tum.de*

Leaf spotting is a common barley symptom observed in particular since the late 1980s. The phenomenon is frequently associated with a physiologic reaction of the host plant to abiotic stresses like changes in temperature and radiation involving reactive oxygen species (ROS). Its frequent occurrence in past years seems related to climate changes. At the same time the fungus *Ramularia collo-cygni* was identified as the causal agent of the *Ramularia* leaf spot disease. A peculiarity of this fungus is the production of photodynamic toxins, anthraquinone derivates named Rubellins. After activation through light, Rubellins produce ROS which were shown to be able to cause symptom development through chlorophyll bleaching and lipid peroxidation, leading to cell wall degradation and formation of necrotic spots. It was hypothesized that Rubellins play a role as virulence factor in the host-parasite interaction. This is further supported by the correlation of symptom development and epidemics with the breakdown of the antioxidative system during plant senescence. Testing of archive samples by PCR have shown that the fungus was present on seeds long before becoming a major disease for barley and the detection during the growing season has discovered a high latency and the possibility of seed transfer. The current investigations quantify Rubellins through HPLC and compare it to the occurrence of symptoms and DNA content of *R. collo-cygni* in the plant to find out where and when Rubellins are produced and to further elucidate the role of ROS and abiotic stresses in epidemics. Yield analysis revealed a strong effect of the disease complex not only on the quantity but on several parameters of the malting quality.

ANTIFUNGAL AGENTS BASED ON BENEFICIAL FUNGI AND THEIR BIOACTIVE METABOLITES: FROM GENOMICS TO THE FIELD. M. Lorito. *Università di Napoli Federico II, Italy and Istituto di Protezione delle Piante del CNR, UOS Portici, Italy. E-mail: lorito@unina.it*

Beneficial microbes are used to sustain agriculture yields and reduce environment impact. The basic technology has been substantially modified and improved by using a variety of 'omics' techniques, including proteomics and metabolomics. Last generation bio-products have a strong scientific base, derived from de-

tailed studies of the multiplayer interactions involved (plant-pathogen-biocontrol agent), and are much more effective and reliable. Many novel formulations are now applied as mixtures of living microbes and bioactive molecules, showing activity on the entire plant and being compatible with other bio-products and commonly used pesticides. Knowledge obtained by studying the genome and the mechanism of action of worldwide used antagonistic fungi and bacteria has allowed the development of ready-to-use technology packages to be implemented directly in medium-to-large farms for control of fungi, bacteria, viruses, nematodes and detrimental effects of abiotic stresses. The new technology, mainly destined to developing countries, has produced a substantial reduction of the use of agrochemicals and has permitted the commercialization of new lines of horticultural products labelled as "zero-residue" without organic farming. From 'omics to the field" projects have been successfully carried out in Honduras, Costa Rica, Brasil, Perù, Cina, Libya, Venezuela, etc. against diseases of melon, pineapple, strawberry and tomato. Finally, new plant stimulating molecules, including some fungal hydrophobins, have been identified, which are able to activate ISR, promote growth and root development, increase resistance to drought and lack of nutrients, and kill directly several fungal pathogens.

THE FUNGICIDE RESISTANCE PROFILE OF *FUSARIUM GRAMINEARUM*: A CHALLENGE FOR INTEGRATED PEST MANAGEMENT

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Fusarium graminearum (*Gibberella zeae*) is the most common species complex among the Fusarium head blight pathogens of cereals. It forms toxic secondary metabolites that pose a threat to the health of humans and animals, and facilitate the infection of plants. Chemical control of *F. graminearum* in cereal crops is greatly limited by its natural resistance towards respiration inhibitors (Dubos *et al.*, 2011, 2013). The reason for the natural resistance is at present poorly understood. Evidence and potential causes for declines in sensitivity of *F. graminearum* towards demethylase inhibitors is presented (Klix *et al.*, 2007). Due to an increase in Central Europe of the acreage of maize, the *Fusarium* host, and the tendency to abandon the removal of infested plant residues from the soil surface by ploughing, an increased *Fusarium* inoculum can be expected in the near future. Options for *Fusarium* and mycotoxin control by traditional (Beyer *et al.*, 2006; Klix *et al.*, 2008) and the remaining chemical methods are discussed.

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Dubos T., Pasquali M., Pogoda F., Hoffmann L., Beyer M., 2011. Evidence for natural resistance towards trifloxystrobin in *Fusarium graminearum*. *European Journal of Plant Pathology* **130**: 239-248.

Dubos T., Pasquali M., Pogoda F., Casanova A., Hoffmann L., Beyer M., 2013. Differences between the succinate dehydrogenase sequences of isopyrazam resistant *Fusarium graminearum* and sensitive *Zymoseptoria tritici* strains. *Pesticide Biochemistry and Physiology* **105**: 28-35.

Klix M.B., Verreet J.-A., Beyer M., 2007. Comparison of the declining triazole sensitivity of *Gibberella zeae* and increased sensitivity achieved by advances in triazole fungicide development. *Crop Protection* **26**: 683-690.

Klix M.B., Beyer M., Verreet J.-A., 2008. Effects of cultivar, agronomic practices, geographic location, and meteorological conditions on the composition of selected *Fusarium* species on wheat heads. *Canadian Journal of Plant Pathology* **30**: 46-57.

IMPACT OF FUNGICIDE TIMING ON THE COMPOSITION OF THE FUSARIUM HEAD BLIGHT DISEASE COMPLEX AND THE PRESENCE OF DEOXYNIVALENOL (DON) IN WHEAT.

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It is known that commercial fungicides efficiently control Fusarium head blight (FHB) when applied at normal rate under optimal conditions. However, operating in the field often implies working under suboptimal conditions, and problems regarding FHB presence and concomitant mycotoxins might occur. In the present study, field trials demonstrated that triazole application generally results in a shift in the FHB population in favour of *F. poae.* At the level of symptom development, all tested triazole fungicides when optimally applied at GS55 resulted in similar disease symptoms reduction. When varying the timing of application, it was obvious that triazole application at GS65 came too late to efficiently reduce FHB symptoms. On the contrary, fungicide spraying at GS39 resulted in a significant reduction of disease symptoms. A combined application at GS39+GS65 resulted in a synergistic effect of the treatments. With regard to deoxynivalenol (DON), several lines of evidence corroborate a role for timing of fungicide application. The efficiency of fungicides to reduce DON-levels seems to be limited and different from the disease symptom reduction. A maximum DON reduction of 50% was obtained compared to the untreated control fields when the DON concentration exceeded a level of 2 mg/kg. Interestingly, while application of triazoles did not affect disease symptoms when applied at GS65, a reduction of DON levels was observed. Contrary to observations on the symptom level, no additional effect was observed when performing a combined application of triazoles at GS39+GS65 compared to single applications at GS39 or GS65. In conclusion, this study peeled away several layers of complexity in the chemical control of FHB in wheat and the concomitant DON contamination.

THE COS-OGA OLIGOSACCHARIDIC ELICITOR - FROM BASIC RESEARCH TO PLANT PROTECTION.

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The oligosaccharidic complex COS-OGA developed at Namur University is a specific elicitor that combines both non self-molecules, chitosan oligomers (COS), and self-molecules, pectin-derived oligogalacturonides (OGA). Its composition was inspired by the observation of the natural plant-fungus interactions. Indeed, upon plant penetration, fungi deacetylate their wall chitin into positively charged chitosan to escape plant chitin receptors. Chitosan fragmentation by plant enzymes yields COS, a polycationic molecule that has been shown to bind and stabilize the so-called egg box conformation of polyanionic oligopectates (Cabr-

era *et al.*, 2010). The resulting molecule is a stabilized complex comprising calcium, OGA and COS that adopts a particular supramolecular conformation. The study of the proteome and the transcriptome on the model *Arabidopsis thaliana* cell suspension showed that the combination of COS and OGA stimulated plant defense in a synergistic way. The effects of COS-OGA complex spraying on tomato plant leaves were also investigated. Proteomic study revealed a strong upregulation of pathogenesis-related (PR) proteins, heat shock proteins and proteins involved in DNA/RNA remodeling. Transcripts quantification by quantitative Real-time PCR (qRT-PCR) was performed on systemic acquired resistance (SAR)- and induced systemic resistance (ISR)-associated genes of tomato. In agreement with the observed PR proteins upregulation, qRT-PCR showed that SAR-linked genes were significantly upregulated unlike ISR genes whose transcripts levels were not modified by COS-OGA foliar treatment. At this stage of the study, COS-OGA looks as a promising biological plant protection product and its activity was confirmed thanks to both greenhouse- and field-grown crops trials. A high protection was conferred against pathogens of the grapevine, apple, tomato, cucumber and others.

Cabrera J.-C., Boland A., Cambier P., Frettinger P., van Cutsem P., 2010. Egg Box Conformation of Oligogalacturonides. Chitosan oligosaccharides strongly modulate the supramolecular conformation and the biological activity of OGA in *Arabidopsis*. *Glycobiology* **20**: 775-786.

DIVERSITY OF THE *FUSARIUM* COMPLEX ON FRENCH MAIZE.

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Ear rot caused by *Fusarium* species is a major threat to maize production worldwide, causing yield reduction and poor grain quality. In addition, various species of the genus *Fusarium* can produce mycotoxins, which accumulate in the grain. The occurrence of mycotoxins in maize kernels is of great concern worldwide, because their presence in feeds and foods is often associated with chronic or acute mycotoxicoses in livestock and, to a lesser extent, in humans. The aim of this work was to investigate the diversity of the *Fusarium* species and in particular, the diversity of the *Fusarium graminearum* species complex (FGSC) on French maize. A total of 444 isolates were recovered from 20 maize samples collected at various locations in France in 2011. *F. graminearum* sensu lato (57%) was the predominant species isolated from maize, followed by *F. proliferatum* (19.4%), *F. poae* (10.6%), *F. temperatum* (6.1%) and *F. subglutinans* (3.2%). The other *Fusarium* species were represented by *F. equiseti* (1.4%), *F. verticillioides* (1.1%), *F. sporotrichioides* (0.9%), *F. andiyazi* (0.2%) and *F. oxysporum* (0.2%). In addition, 80 strains of the FGSC were characterized more in depth in order to assign them to a species and to determine their trichothecene chemotype, using a microsphere-based multilocus genotyping assay. These results constitute a first report on *Fusarium* diversity and on the FGSC diversity on French maize. In addition, we report here for the first time the presence of *F. temperatum* in France, a species that has been recently described on maize in Belgium (Scauflaire *et al.*, 2011). These results will contribute to a better understanding of the distribution and epidemiology of *Fusarium* species in France, and will help to predict mycotoxin contamination risks and to implement suitable disease management strategies.

Scauflaire J., Gourgue M., Munaut F., 2011. *Fusarium temperatum* sp. nov. from maize, an emergent species closely related to *Fusarium subglutinans*. *Mycologia* **103**: 586-597.

INFLUENCE OF PLANT DEVELOPMENT ON THE DIVERSITY OF *FUSARIUM* SPECIES ON BARLEY.

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Fusarium head blight is caused by a complex of several *Fusarium* species and known as a destructive disease in all cereal growing areas of the world. As in other small grain cereals, the infection of barley implies yield loss, quality reduction and mycotoxin contamination of grain. Investigations on distribution pathways and the impact of plant development, especially in regard to timing and duration of flowering, on infestation with different *Fusarium* species were subject of the present work. Plants of four winter barley (cvs Cartel, Esterel, Malwinta, Wintmalt) and two spring barley cultivars (Marthe, Quench) were observed and sampled throughout the season in a weekly frequency. The spring barley varieties were sown at two different dates, four weeks apart from each other. Plants were divided into heads, leaves and stems, and analyzed by semiquantitative and quantitative PCR for determining the *Fusarium* species, their allocation and development on different plant organs. Moreover, wind distribution and its contribution to head infection were evaluated. This was performed through sampling of air-borne *Fusarium* inoculum with a 7-day Burkhard spore sampler followed by quantitative PCR measurements. Standard PCR showed the predominance of *F. langsethiae*, *F. poae*, *F. avenaceum* and *F. tricinctum* in harvested grains, whereas *F graminearum*, *F. culmorum* and *F. sporotrichioides* were less present. Quantitative PCR measurements quantified DNA content of *F. avenaceum*, *F. culmorum* and *F. langsethiae* on different plant organs. Investigations of flowering plants indicated that *F. avenaceum* and *F. culmorum* distribution patterns on the leaves followed negative gradients. Higher leaves contained more fungal DNA than leaves of lower levels. Moreover, *F. langsethiae* was dominant in heads, whereas *F. avenaceum* and *F. culmorum* were less present in this organ. Results reflecting the whole vegetation period of plants, showed fluctuating *Fusarium* DNA contents on different plant parts, but a strong increase in heads towards harvesting. Those observations gave indication that different distribution pathways are more or less important for various *Fusarium* species. Sampling of air-borne inoculum and quantitative PCR measurements detected DNA of *F. graminearum*, *F. avenaceum*, *F. culmorum* and *F. langsethiae*, indicating that these species are distributed by wind. *F. avenaceum* was dominant, followed by *F. graminearum*, *F. culmorum* and *F. langsethiae*. Results showed specific peaks of inoculum densities. Peaks of *F. culmorum* could be identified early in the vegetation period, *F. langsethiae* was rarely observed. Air-borne inoculum of *F. avenaceum* and *F. graminearum* increased throughout the season.

A FIELD-SPECIFIC WEB TOOL FOR THE PREDICTION OF FUSARIUM HEAD BLIGHT AND DON CONTENT IN BELGIUM.

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Fusarium head blight (FHB) is a fungal disease of wheat. Besides reduced quality and yield loss, most *Fusarium* species produce mycotoxins (e.g. deoxynivalenol or DON) that can be hazardous to the health of humans and animals. Given the severe consequences, there is an urgent need to know the level of contamination in advance. Models for predicting FHB incidence and DON content in wheat provide farmers with a tool for preventing yield loss and mycotoxin contamination. Therefore, the main objective of this research was to develop and implement a web-based forecasting system to predict FHB incidence and DON content in winter wheat in Belgium. Growers may use the predictions to underpin decision making on the application of fungicides. For the food and feed industry the predictions are interesting to make marketing decisions. At the end of the growing season the predictions are helpful to identify regions with a higher disease pressure and thus improve sampling efficiency. Model development was based on data from wheat fields on 18 Belgian locations from 2002 until 2012, which resulted in 4,663 entries in the database. As a pre-processing step, a thorough exploration of this database was performed. In this study the influence of meteorological and agronomic variables on DON content and FHB incidence was studied. Evidence was brought forward demonstrating the effect of species interactions in wheat ears on DON content and the effect of weather conditions during the vegetative growth stage. Per month, three to fourteen variables that were mutually sufficiently uncorrelated (correlation less than 0.7) and contributed most in explaining the variation in the DON content were considered for further analyses. In a next step, various state-of-the-art machine learning techniques (ridge regression, support vector regression, boosting, etc.) were compared to predict the disease index (DI, a measure of the FHB incidence) and DON content. Since in general the performance of the metric regression techniques was unsatisfactory to implement in practice, we shifted to ordinal regression. The thresholds used in the ordinal model to predict DON content were based on the European legislation for mycotoxins. The thresholds for the DI model could not be based on any legislation and were therefore empirically determined. The predictive performance obtained for the ordinal regression models indicated that a substantial portion of the variance in the response variable can be explained by means of the predictor variables. Furthermore, we concluded that four-class penalized proportional odds models were the best choice to use for the web tool. In order to deliver the models to the growers and industry, a web tool was developed in collaboration with the Soil Service of Belgium. With this tool growers can obtain a field-specific prediction for DON content and the DI. The web tool provides a graphical representation of the predictions together with recommendations. This tool will lead to an integrated pest management and a more sustainable agriculture. It will also have an economic impact by reducing the input of fungicides and a more efficient sampling strategy to guarantee food and feed safety. The tool is developed for Belgium, so the predictions will only be accurate in regions similar as Belgium. However, the techniques and the framework used can be inspiring to generate tools for other regions and diseases. Additionally, the model parameters can always be updated to provide accurate predictions in the future.

SUSCEPTIBILITY OF SOME PLUM CULTIVARS TO *POLYSTIGMA RUBRUM* IN MONTENEGRO. J. Latinovic, D. Bozovic, V. Jacimovic and N. Latinovic. *University of Montenegro, Biotechnical Faculty, 81000, Podgorica, Montenegro. E-mail: jelenalat@ac.me*

Plum is a major fruit crop in northern parts of Montenegro. Pozegaca and other autochthonous cultivars are mainly grown for brandy production but due to traditional tree cultivation with inadequate agrotechnical measures yield is very low (5.8-6.2 kg/tree) and alternating. Therefore, in last decade, several new cultivars were introduced in an attempt to improve and intensify plum production in Montenegro. One of the most common and harmful plum diseases in Montenegro is red leaf spot caused by the fungus *Polystigma rubrum* (Person) de Candolle. The objective of the study was to identify plum cultivars with sufficient resistance to the disease that could have potential for use in integrated pest management of plum in Montenegro. Fifteen plum cultivars were studied for their susceptibility to *P. rubrum* during the period 2009-2011. At least five trees per cultivar were included in the study and in each tree 100 leaves were evaluated for symptoms. The estimations of the susceptibility were done in July and in September of each year. Assessed leaves were classified into six groups according to their infection degree (0-5 scale) and categories were defined based on the area of the infected leaf surface (Hobs *et al.*, 2010). According to the obtained results, the relatively resistant cvs Stanley, California Blue, Valerija and Angeleno could be grown under our conditions without taking control measures against *P. rubrum*. Control measures against *P. rubrum* in cultivars with weak susceptibility such as Anna Späth, Cacanska Rodna, Valjevka, Cacanska Rana, Cacanski Secer and Turgonja should be implemented only in the years when ecological conditions favour the development of *P. rubrum*. In susceptible cvs Cacanska Najbolja, Zlatka and Mildora one treatment per year would be enough to control the disease while in highly susceptible cvs Pozegaca and 'Cacanska Lepotica if the conditions favour the disease, two sprays are recommended to protect the tree canopy. Cultivar resistance to red leaf spot as an economically very harmful disease would have a great importance in decreasing the dependence on chemicals in integrated production of plum orchards in Montenegro.

Hobs I.J., Soltész M., Nyéki J., Szabó Z., 2010. Incidence of fungal diseases on three stone fruits cultivars in Hungary. *International Journal of Horticultural Science* **16**: 107-109.

***IN VITRO* INFLUENCE OF SOME FUNGICIDES ON THE MYCELIUM OF *ALTERNARIA* spp. ISOLATED FROM FIG FRUITS. N. Latinovic[1], P. Vuksa[2] and J. Latinovic[1].** *[1]University of Montenegro, Biotechnical Faculty, 81000 Podgorica, Montenegro. [2]University of Belgrade, Faculty of Agriculture, 11080 Beograd-Zemun, Serbia. E-mail: nlatin@ac.me*

In July 2012 in a fig orchard with 1,200 trees in the Podgorica region, wet depressed lesions followed by rotting appeared on *ca.* 15% of the fruits, a condition that had never seen previously in Montenegro. Since this disease was economically important, had an unknown nature and scarce literature data in general, attempts were made to isolate a possible pathogen from rotten fruit on potato dextrose agar (PDA), which consistently yielded a fungus belonging in the genus *Alternaria*. Chemical treatments of fig trees against diseases (including this one) have never been conducted in Montenegro before. Therefore, the susceptibility of the isolated fungus to the conventional fungicides Signum (boscalid + pyraclostrobin), Switch (cyprodinil + fludioxonil), Teldor (fenhexamid) and Nordox (copper oxide) was checked. Fungicides with different mode of action and in several concentrations were incorporated in PDA. A fungal mycelium fragment was transfered to the centre of Petri dishes with PDA with the fungicide and incubated at 25°C. Petri dishes with PDA but without fungicide served as a control. Five days later, when the fungal colony in controls covered more then

2/3 of Petri dishes, the fungus development was assessed based on colony diameter. Mycelial growth was significantly inhibited by all fungicides added to PDA compared to the control. Switch showed the highest inhibition, followed by Signum and Teldor, while Nordox had the slightest inhibitory effect. Data were processed by probit analysis and it was established that Signum possessed the lowest effective concentration, inhibiting 50% (EC_{50}) at amounts of 5.1 mg/kg, followed by Switch with 7.1 mg/kg. Teldor and Nordox expressed significantly higher EC_{50}, i.e. 229.2 mg/kg and 499.9 mg/kg, respectively. Coefficient of relative sensitivity of inhibition (b) of *Alternaria* spp. to the tested fungicides was the highest in Teldor (1.40), then in Nordox (0.59), followed by Signum (0.51) and Switch (0.40). These preliminary results can be important for further studies aimed at ascertaining the fungicide effectivness in the field. The studies will be continued considering the importance of fig cultivation in Montenegro.

YIELD DECREASE AND QUALITY CHANGE BY BROWN ROT FUNGI IN POME AND STONE FRUITS. N. Aitkhozhina. *Ministry of Education and Science, Institute of Microbiology and Virology, Almaty, Kazakhstan. E-mail: nag_aitkhozhina@yahoo.com*

Brown rot is one of the most devastating plant pathogen for pome and stone fruits. In the last ast decade, brown rot incidence has increased in south-east Kazakhstan, where apple, pear, apricot, and peach orchards are located. The disease also extended to small fruit-growing fields. Resistant varieties of apple and apricot have not yet been developed. Brown rot, caused by *Monilia fructigena* Honey ex Whetzel, is a common pre- and postharvest disease of pome and stone fruits. *M. fructigena* isolates were obtained from orchards and packinghouses and tested for their cultural and biochemical characteristics. Until 2007, brown rot in Kazakhstani peach and plum orchards was caused by *M. fructigena*, and in last years also *M. laxa* Aderh. et Ruhland has been detected. The primary inoculum sources of *M. fructigena* and *M. laxa* are mycelia overwintering on mummified apple and other fruits. During the vegetation season, the spores of these fungi are spread by wind, birds, rain and insects. Our results showed that the disease caused by both pathogens decreased apple yield and other fruits independently on the infection rates. Losses of up to 40% occur prior to harvest as a result of premature fruit drop in orchards and postharvest losses can also range up to 40%. Pome and stone fruit from the same tree, e.g. looking healthy and those having symptoms of brown rot differed considerably from one another in various fruit quality parameters. Generally, in infected fruits decreases in water, firmness, malic acid and sugar content and changes in fruit color were observed. In several orchards, apple and apricot fruits with multifungal disease symptoms were found. Besides *Monilia* species, fungi belonging to the genera *Penicillium, Alternaria, Botrytis, Gloesporium*, and *Aspergillus* were isolated from fruits, and stems and leaves sampled at different times during the growing season. One of the isolates was identified as an *Aspergillus flavus* strain that forms sclerotia. Aflatoxin contamination is a serious food problem throughout the world. Our results suggest that fruit trees should be monitored carefully during the vegetation season to avoid fruit spoilage during storage time. Additionally, biological control means to manage brown rot fungi are under progress. Our 3-year laboratory and field experiments have revealed the potent biocide action of *Brassicaceae* plants on the health and crops yield.

VALIDATION OF A QUICK CHEMOTYPE AND SPECIES DETERMINATION BY SEQUENCING PROTOCOL IN *FUSARIUM*. M. Pallez, M. Pasquali, L. Hoffmann, T. Bohn and M. Beyer. *Centre de Recherche Public-Gabriel Lippmann, Department of Environment and Agrobiotechnology, 4422 Belvaux, Luxembourg. E-mail: marine.pallez@yahoo.fr.*

PCR-based methods are routinely used in plant pathology. Several protocols for fungal DNA extraction have been reported, but most of them are very time consuming and expensive. We have established a quick culturing-extraction method for fungal DNA, allowing the reliable use for PCR processing and sequencing. Fungal genomic DNA was efficiently amplified using simple and multiplex PCR. Primers targeting the Elongation Factor 1α and genes of the TRI12 complex were used to characterize *Fusarium* species and their mycotoxin profile. The quality of direct sequencing was not statistically different from the quality achieved with a membrane-based DNA extraction kit. The newly developed method, therefore, is less expensive due to reduction of consumables and to overall time reduction of the procedure. The method was also applied with success on *Zymoseptoria tritici.* The procedure has potentials for screening large set of isolates avoiding time consuming extraction steps, and is currently in use within the framework of a project for the characterization of *Fusarium* chemotypes within Europe.

MYCOLOGICAL CHARACTERIZATION OF SOYBEAN SEEDS. M. Pasquali[1], F. Giraud[1], F. Caloni[2], C. Cortinovis[2], F. Pizzo[2], L. Hoffmann[1], T. Bohn[1] and A.C. Gutleb[1]. *[1]Centre de Recherche Public-Gabriel Lippmann, Belvaux, Luxembourg. [2]Department of Veterinary Sciences and Technologies for Food Safety, University of Milano, Italy. E-mail: pasquali@lippmann.lu*

In an effort to understand the fungal colonization of soybean, samples sold in Italy between 2008 and 2010 were analysed. Six seed batches were selected and the fungal population was determined at the genus level by morphological and microscopic observations. *Aspergillus*, *Mucor*, *Fusarium*, *Pennicilium*, *Microdochium* plus other non classified genera were detected. The majority of soybean seeds was contaminated by potential toxigenic species such as *Aspergillus* and *Fusarium*. A molecular investigation on *Fusarium* isolates carried out by sequencing the elongation factor 1α revealed that soybean seeds were contaminated by *Fusarium verticillioides*. It is suggested that fumonisin contamination is a possible risk to be monitored in soybean sold for animal feeding. Studies on soybean infection by *F. verticillioides* are needed, to clarify where and when colonization may occur. Moreover, the co-contamination of seed by multiple fungi able to produce a diverse array of toxins should draw attention towards the risk of co-occurrence to better reflect the real toxigenic risk of the feed.

HYDROGEN PEROXIDE INDUCES ACQUIRED RESISTANCE OF TOMATO PLANTS TO *FUSARIUM OXYSPORUM*. N.L. Pshybytko[1], N.B. Zhavoronkova[1], L.A. Zenevich[1], L.F. Kabashnikova[1] and E.A. Lysenko[2]. *[1]Institute of Biophysics and Cell Engineering, National Academy of Sciences of Belarus, 220072 Minsk, Belarus. [2]Institute of Plant Physiology, Russian Academy of Sciences, 127276 Moscow, Russia. E-mail: pshybytko@ibp.org.by*

The main physiological processes in tomato plants during the pathogenesis caused by *Fusarium oxysporum* under normal and high temperature conditions were studied. Four-month-old toma-

to plants were inoculated via the root system. Pathogenesis was monitored for about 30-40 days and drying up of tomato plants was observed after yellowing. Symptoms were caused by toxins of *Fusarium oxysporum*, which suppressed physiological processes in tomato plants such as the biosynthesis of main cell components. Moreover, the fungi proliferated actively in roots and enlarged and corked phloem was observed in response to mycelial growth. The decrease of xylem flow resulted in dehydration of plant tissue. The activation of destructive processes (lipid peroxidation and generation of reactive oxygen) was obtained following inoculation. Some peaks of reactive oxygen species (ROS) generation were detected after *F. oxysporum* inoculation. The first peak was identified half an hour after infection, but the physiological processes in tomato plants were not affected. Repeated increase in ROS generation was after 5-7 days. In this case destructive processes were accelerated and some physiological functions (photosynthesis, respiration) were limited. A PR3 protein (chitinase) accumulation was detected after 24 h. Thus, we surmise that the first ROS burst is signal for activation of protective mechanisms while the second ROS accumulation is a destructive process. For the purpose of inducing acquired resistance to pathogens the pretreatment of plants growing in hydroponics by exogenous hydrogen peroxide was tested. Exogenous hydrogen peroxide induced a fourfold increase in endogenous ROS content 3 h after treatment. Then, ROS levels decreased but it remained at a high level over a long period of time, during which proteins synthesis accelerated and pretreated plants had high level of chitinase. When infection by infection plant pathogens followed, they could not penetrate defence barriers or their development was suppressed. The mechanisms of plant-fungi interaction, signal transduction and the role of hydrogen peroxide in plant protection are discussed.

Study supported by a grant of the Belarusian Republican Foundation for Fundamental Research.

FUSARIUM GRAMINEARUM AND ASSOCIATED MYCOTOXINS ON MAIZE IN THE UNITED KINGDOM. R. Basler[1,2], S. Edwards[2] and J. Thomas[1]. *[1]National Institute of Agricultural Botany, Cambridge CB3 0LE, UK. [2]Harper Adams University College, Newport, UK. E-mail: ryan.basler@niab.com*

Fusarium graminearum clade species (Fg) are important maize pathogens that are capable of producing trichothecene mycotoxin deoxynivalenol (3AcDON and 15AcDON), nivalenol (NIV) as well as other toxigenetic compounds. An incidence study of *Fusarium* contamination of forage maize in stalk and kernels in the United Kingdom was undertaken in 2011 and 2012. There were 286 Fg isolates identified from 10 UK sites in 2011 by morphological characteristics and confirmed with species-specific molecular assays. Four of the sites from 2011 and one additional site will be sampled in 2012 to determine the incidence of *Fusarium* contamination in forage maize. To compare the major UK crop of wheat, kernel samples from 98 UK sites are being sampled for Fg contamination. The Fg isolates from 2011-2012 UK maize and 2012 UK wheat will be characterized with molecular assays for trichothecene chemotypes and potential genetic diversity in the population using variable number tandem repeats and simple sequence repeats. High performance liquid chromatography will be used to identify and quantify a subset of the Fg isolates for 3AcDON, 15AcDON and NIV. The population structure will be compared within the Fg maize population and with the Fg wheat population for correlations for climate and host interactions.

UPDATE ON THE ESTABLISHMENT OF A EUROPEAN MAP OF _FUSARIUM GRAMINEARUM_ AND _F. CULMORUM_ CHEMOTYPES IN WHEAT. S. Vogelgsang[1], A. Logrieco[2] and M. Pasquali[3]. *[1]Agroscope Reckenholz-Taenikon Research Station ART, Zurich, Switzerland. [2]Istituto di Scienze delle Produzioni Alimentari del CNR, 70126 Bari, Italy. [3]Centre de Recherche Public-Gabriel Lippmann, Department Environment and Agro-Biotechnologies, Belvaux, Luxembourg. E-mail: susanne.vogelgsang @art.admin.ch*

Among the most crucial factors limiting crop yield are losses caused by fungi, for example those from the genus *Fusarium,* causing Fusarium head blight (FHB). In addition, *Fusarium* spp. can produce a variety of mycotoxins, including deoxynivalenol (DON), nivalenol (NIV), T-2 toxin, and zearalenone (ZON), which frequently cause a decline in food quality (e.g. flattened bread), but also acute nausea, and with prolonged intake, interior organ damage, infertility, and cancer. European regulation for trichothecene type B in grains focuses on the deoxynivalenol (DON) content in grains despite chemotypes of *F. graminearum* and *F. culmorum* producing NIV are present. NIV is usually found less frequently in the field but it is more than 10 times as toxic as DON. Hence, there is a need to investigate which *Fusarium* populations are present in order to better predict the risk of DON and or NIV contamination.

As of to date, data on the European distribution of chemotypes are scattered in time and space and a comprehensive dataset on the toxigenic potential of *Fusarium* populations in Europe is missing. Associated with the European project MycoRed (Novel integrated strategies for worldwide mycotoxin reduction in food and feed chains), we initiated a concerted effort to assemble data on chemotypes of *F. graminearum* and *F. culmorum* strains isolated from wheat of various European geographic areas in order to establish a distribution map. In addition, this comprehensive dataset can be used for epidemiological studies of *Fusarium* populations in Europe and the obtained information may contribute to develop toxin specific preventative measures. Given also the interest in maize chemotype determination, a parallel effort will also include maize isolates collected in Europe. *Fusarium* researchers working with strains from Europe are invited to participate in the project by sharing strains and data on species that will be assembled in a joint publication on *Fusarium* chemotypes in Europe. As of now, we received information and strains from eleven European countries (BE, CH, DE, DK, FI, FR, IT, LU, PL, RS, TR). Preliminary results will be presented and discussed.

STUDYING INTERSPECIFIC AND INTRASPECIFIC VARIABILITY OF FUNGI OF THE GENUS _FUSARIUM_ IN RELATION TO PATHOGENICITY AND PHYTOTOXICITY. N.S. Zhemchuzhina, M.I. Kiseleva, A.A. Makarov and V.P. Dubovoy. *All-Russian Research Institute of Phytopathology, RAAS, 143050 B.Vyazemy Moscow region, Russia. E-mail: zhemch@mail.ru*

Fungi of the genus *Fusarium* are widespread in nature and under certain conditions most of them are capable of parasitizing various agricultural plants, including cereals. During the infection process, accumulation of toxic metabolites (mycotoxins), hazardous for human and animal health, takes place in the grain. Such grain cannot be used for food and feed. Because of the wide distribution of fusarial infections, their injuriousness, the long persistence on plants, the wide host range, and the toxicity of *Fusarium* strains, it is necessary to implement adequate protective measures. Protection of cultivated plants against diseases by means of chemicals has a number of shortcomings as, in particular, it leads to pollution of agricultural raw materials and en-

vironment. Cultivation of resistant cultivars is an ecologically safe and effective method for harvest preservation. In this context, the selection of cultivars that resist fusarioses is a task of paramount relevance. The knowledge of the pathogenic and toxigenic features of fusaria can help to cope with this problem. Thus, the our research was aimed at studying the interspecific and intraspecific variability of fungi of the genus *Fusarium* with reference to pathogenicity and phytotoxicity properties with the purpose of using certain strains in the selection of resistant cultivars. The pathogenicity and phytotoxicity of 22 strains of four species [*F. sporotrichioides (F. sporotrichioides var. poae), F. culmorum, F. poae,* and *F. oxysporum*] isolated from different crops and weed plants has been studied. The assessement of the pathogenicity and toxicity of the above species was carried out on the susceptible wheat cv. Mironovsky 808 using a biological test on seeds. Pathogenicity was determined as the effect of spore suspensions on wheat seedlings, whereas toxicity was determined based on the phytotoxicity of culture liquids. With respect to pathogenicity and toxicity, *Fusarium* strains were differentiated into four groups: (i) non-pathogenic and non-toxic (inhibition of seedling growth by 0-30%); (ii) weakly pathogenic and toxic (inhibition of seedling growth by 31-50 %); (iii) moderately pathogenic and toxic (inhibition of seedlings growth by 51-70%); (iiv) pathogenic and toxic (inhibition of seedling growth over 70%). Among strains of each species, intraspecific distinctions on pathogenicity and toxicity were observed. *F. oxysporum* strains possessed the widest parasitic activity. This species was isolated from roots of wheat, buckwheat, clover, vetch, rape, lupine, mustard, peas, potatoes, cucumber, bindweed. Strains from these crops were toxic to seedlings of the susceptible wheat cultivar used. Pathogenic properties of *F. oxysporum* strains were weaker. Only two strains were toxic and moderately pathogenic. Strains of *F. culmorum* were found only on cereals possibly due to narrow specialization of this pathogen. *F. culmorum* strains showed high pathogenicity and toxicity for the length of roots of wheat seedlings under the influence of spore suspensions and filtrates of culture liquids of *F. culmorum* strains was only 1.9-8.6% compared to controls. Also, a strong inhibition of seeds germination was noted. Strains of *F. sporotrichioides* from wheat and clover were toxic to wheat seedlings but their pathogenicity differed strongly. The strain of *F. poae* from oat roots was pathogenic and toxic to wheat seedlings. In conclusion, *F. culmorum* was highly pathogenic for wheat seedlings, whereas a high toxicity level was shown by the liquid culture filtrates of the strains of all tested species (*F. culmorum, F. oxysporum, F. poae, F. sporotrichioides*).

IMPACT OF PLANT PATHOGENS ON THE QUALITY OF CROPS AND WINE (PATHOLUX - GRAPELUX) - PLANT PATHOLOGY IN RELATION TO FOOD SAFETY AND FOOD QUALITY

DAY 2

23 OCTOBER 2012

OCCURRENCE AND EVOLUTIONARY RELATIONSHIPS OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3* ISOLATES IN HUNGARY

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SUMMARY

A survey of the frequency of *Grapevine leafroll associated virus -1,-2* and *-3* infections was done in Hungarian grape plantations. The 102 symptomatic samples analysed originated from different geographical areas: North-West, Middle-West, South-West and Middle-East parts of Hungary (Koszeg, Balatonboglár, Badacsonytomaj, Cserszegtomaj, Kecskemét, Villány, Nagyrada and Pécs). Of these, 16 tested positive using serological methods (DAS-ELISA). Four GLRaV-3 positive samples originated in the North- West region were confirmed by molecular methods (RT-PCR) and partial sequences of the 70 kDa heat shock protein homolog gene. Isolates 2.2, 3.5 and 4.2 were estimated to belong to group II. Isolate 1.4, coming from the same vineyard as 2.2, varied in sequence belonging to IV variant group together with two South African, two Austrian and one Syrah isolate. According to the phylogenetic analysis two variant groups occurred in Hungary.

Key words: Grapevine leafroll-associated virus 3, Hungary, RT-PCR, phylogenetic analysis.

The occurrence of grapevine virus and virus-like diseases in Hungary has been determined on the basis of the most characteristic symptoms: (i) degeneration, (ii) leafroll, (iii) fleck, (iv) rugose wood, (v) yellow mottle, (vi) line pattern, (vii) enation, (viii) vein necrosis or ix) vein mosaic (Cseh *et al.*, 2012). From 2008 to 2011 Hungarian wine growing regions were surveyed for virus-induced symptoms and virus infection. The most commonly observed symptoms were yellowing, reddening and rolling of the leaves (Cseh *et al.*, 2011a, 2011b), which can be induced by a complex of viruses, belonging to the family *Closteroviridae*. Nowadays, five serologically distinct viruses, designed *Grapevine leafroll-associated virus-1* to *7* (GLRaV 1-7), have been associated with the disease complex (Martelli *et al.*, 2012). In Hungary the GLRaV 1-5 have been found (Lázár *et al.*, 1995a).

The disease cause yield losses of as much as 20 to 40% (Routh *et al.*, 1998), abnormal discoloration of the fully expanded leaves and downward rolling of the leaves (Little and Rezaian, 2006). The symptoms appear mainly at the end of the growing season (Meng *et al.*, 2005). Virions of the family *Closteroviridae*, including the genera *Closterovirus* and *Ampelovirus*, are filamentous particles (c. 2000 nm), and contain a positive-sense RNA genome (Brunt *et al.*, 1996). *Grapevine leafroll-associated virus 3* (GLRaV-3) spreads via propagation material and grafting and is transmitted by mealybugs and soft-scale insects (Komínek *et al.*, 2005).

The aim of our study was to survey the frequency of GLRaV-3 infections in Hungarian grape plantations and to perform a molecular analysis of the pathogen. Grapevine leaves showing either abnormal discoloration as yellowing and reddening, or downward rolling were collected in autumn 2008 and 2009. These samples originated from geographical located in the North-West, Middle-West, South-West and Middle-East part of Hungary (Fig. 1).

Samples were stored at 4°C in plastic bags prior to virus checking by DAS ELlSA (Clark and Adams, 1977) using a commercial kit (Bioreba, Switzerland) to GLRaV-3. In addition, four GLRaV-3-positive samples from North-West Hungary were tested by PCR. Total RNA was extracted and purified from leaf tissues using the SPEKTRUM plant total RNA kit (Sigma-Aldrich, Germany). The specific primer pair: LC1F 5'-CGCTAGGGCTGTGGAAGTATT-3', LC2R 5'-GTTGTCCCGGGTACCAGATAT-3', was designed on the basis of the nucleic acid sequence of isolates GLRaV-3 NY1 (accession No. AF037268) to amplify fragment of 546 bp from the HSP70h gene. PCR conditions were as follows: denaturation at 94ºC for 3 min, followed by 40 cycles at 94ºC for 75 sec, 52ºC for 30 sec and 72ºC for 1 min. The final elongation step was done at 72ºC for10 min. Aliquots of PCR products were run on 1.5% agarose gels. Amplicons were purified using the Roche high pure purification kit, cloned into pGEM-T Easy cloning vector (Sambrook *et al.*, 1989) and sequenced by BAY-GEN (Hungary). Sequences were deposited in GenBank under the accession No. HE794022,

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Fig. 1. Locations and number of samples where collection took place.

HE794023, HE794024 and HE794025).

The phylogenetic studies were performed using multiple alignments of the amplified 500 bp sequence of the sequenced gene. Construction of the evolutionary model was performed using the CLC sequence viewer 6.5.1. (CLC bio, Denmark). A phylogenetic tree was obtained with the CLC sequence Viewer 6.5.1. using the UPGMA method and 1000 bootstrap iterations as a confidence test. In order to assess the relationship of the four Hungarian GLRaV-3 isolates, HSP70h gene sequences from NCBI/EMBL database were included in a phylogenetic analysis.

Among the 102 symptom-bearing samples collected, in 33 cases the serological tests gave positive results. RT-PCR amplified four samples respectively from the varieties Blue Frankish (1.4, 2.2) originated from Koszeg, Pinot noir (3.5) from Badacsonytomaj and Italian Riesling (4.2) from Cserszegtomaj.

By analyzing the 500 bp fragments of HSP70 gene of GLRaV-3 from twenty five isolates from different countries, included four Hungarian ones, five clusters were obtained (Fig. 2).The largest second group contained fifteen isolates: one (AJ748524) from Israel; two Chinese (AJ748514, DQ780887); and one (AJ748517) from Syria. Near-east isolates were different from the far-east ones. Other members of this group were from America (North and South), two from USA (DQ780891, AF037268) and one (EU344893) from Chile. South Africa was represented by one isolate (GQ352631). Some sequence data of GLRaV-3 from Europe showed high homology with the second group, as one (AJ748521) Italian; two Austrian (AJ748512, AJ748511); one Tunisian (AJ748522) and three Hungarian (3.5 from Badacsonytomaj; 2.2 from Koszeg and 4.2 from Cserszegtomaj).

Six isolates, from Austria (AJ748513, AJ748510) South Africa (GQ352632, EU259806), Syria (AJ748516) and one Hungarian isolate 1.4 from Koszeg formed the fourth, numerous group. Isolate 1.4 showed the highest homology with the South African isolates.

Phylogenetic analysis of the sequence of HSP70h gene confirmed the previously reported clustering into five groups (Fuchs *et al.*, 2009; Mekuria *et al.*, 2009; Jooste *et al.*, 2010). Hungarian isolates fitted into the two largest variant groups, some being similar to those from neighbouring Austria. Our results confirm the suggestion of other authors (Fuchs *et al.*, 2009; Mekuria *et al.*, 2009; Jooste *et al.*, 2010), that transmission by propagative material has the most important role in the distribution of GLRaV-3. Certification program should be effective in controlling it.

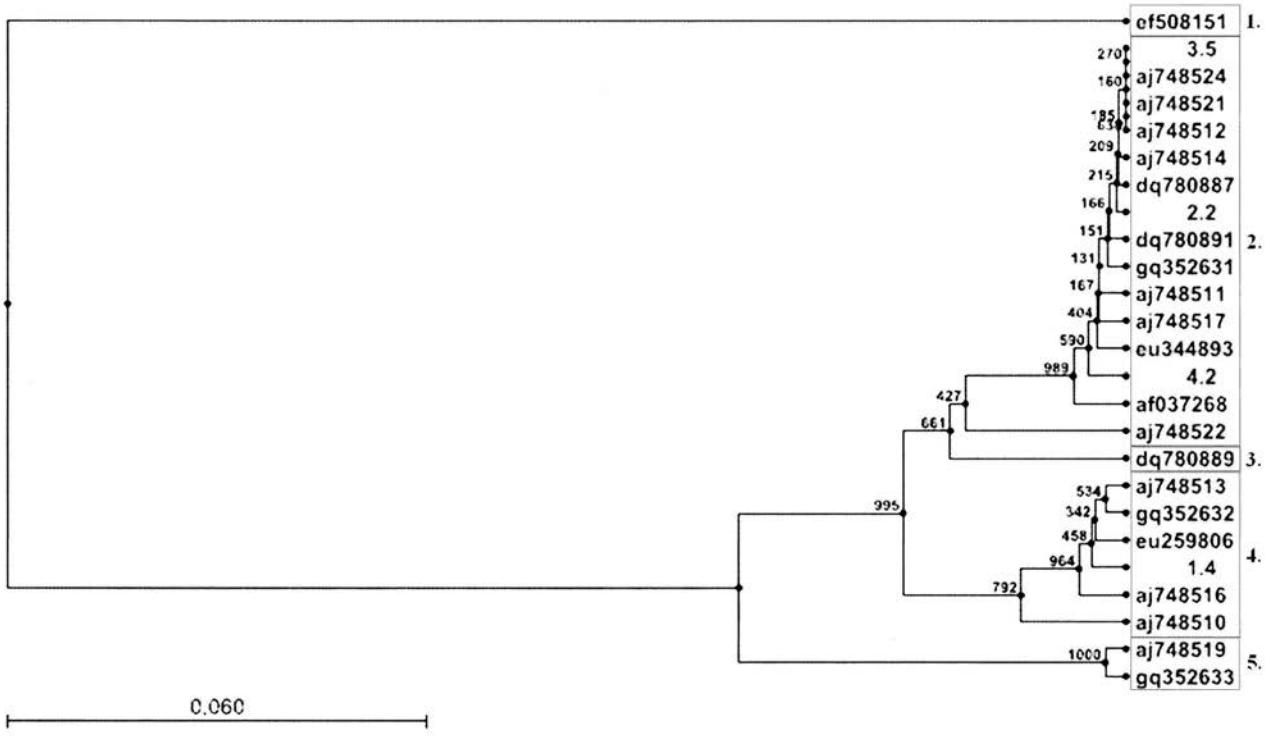

Fig. 2. Phylogenetic tree of the 500 bp part of the HSP70h gene of GLRaV-3 constructed using the UPGMA method. Numbers at nodes represent bootstrap values. The five phylogenetic groups are shown on the left. Isolates from Hungary belong to group 2 and 4. Abbreviations: ef508151 (New-Zealand), aj748524 (Israel), aj748521 (Italy), aj748512 (Austria), aj748514 (China), dq780887 (China), dq780891 (USA), gq352631 (South Africa), aj748511 (Austria), aj748517 (Syria), eu344893 (Chile), af037268 (USA), aj748522 (Tunisia), dq 780889 (China), aj748513 (Austria), gq352632 (South Africa), eu259806 (South Africa), aj748516 (Syria), aj748510 (Austria), aj748519 (Italy), gq352633 (South Africa), Hungarian isolates: 3.5, 2.2, 4.2 and 1.4

Currently very few molecular data are available on the comparison of GLRaV-3 isolates from Middle-European countries. These are the first data reported from Hungary.

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FUNGICIDE SENSITIVITY PROFILES OF THE *PLASMOPARA VITICOLA* POPULATIONS IN THE LUXEMBOURGIAN GRAPE-GROWING REGION

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SUMMARY

Resistance of grape downy mildew caused by *Plasmopara viticola* against different fungicide classes has been reported in Europe. The main objective of the present study was to measure the sensitivity status of *P. viticola* populations to four fungicide active ingredients used in the Luxembourgian vine-growing region and to investigate the presence of resistant populations. Thus, leaf samples with downy mildew symptoms were collected in 19 vineyards along the river Moselle in 2010 and 2011. Samples were analysed in both years for carboxylic acid amides (CAA) and the quinone outside inhibitors (QoI), using a microplate conidia growth inhibition assay. In 2011, a leaf disc assay was used in addition to determine the sensitivity profile of the sampled populations to phenylamides (PA) and to cymoxanil. Results showed that in average, 64% (2010) or 81% (2011) of the zoospores were resistant to QoI fungicides, and 91% (2010) or 85% (2011) to the CAA fungicide dimethomorph. Furthermore, in 2011, all populations were classified as extremely resistant to the PA fungicide kiralaxyl, indicating that the sampled Luxembourgian populations contained at least 50% of resistant strains. No population was controlled by cymoxanil at the rate of 100 mg/l. In average, 84% of the populations showed a minimum inhibitory concentration (MIC) between 300-800 mg/l, and 16% a MIC reaching 800 mg/l. The presented results indicate that fungicide resistance against CAAs, QoIs, PAs and cymoxanil is currently widespread in the Luxembourgian wine-growing area.

Key words: Grape downy mildew, quinone outside inhibitors, carboxylic acid amides, phenylamides, cymoxanil.

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INTRODUCTION

Downy mildew (DM), caused by *Plasmopara viticola* (Berk. et M.A.Curtis) Berl. et De Toni is one of the major constraints to grape production worldwide and in particular in the Luxembourgian vine-growing area.

As in many vine-growing regions, disease management of downy mildew requires several applications of fungicides during the growing season, eventually starting as early as five leaves are unfolded (BBCH 15) in the typical grape (*Vitis vinifera*) cultivars grown in Luxembourg, i.e. Rivaner, Elbling, Riesling, Auxerrois, Pinot gris and Pinot blanc.

One major problem in the management of downy mildew epidemics is the appearance of fungicide resistance in pathogen populations (Gisi *et al*., 2008). Indeed, the introduction of selectively active site-specific fungicides into agricultural production systems has been systemically followed by the development of resistant strains, decreasing the efficacy of the chemicals. The situation becomes worrying in specific locations such as in northern Italy, Switzerland, and France, where more than one half of the natural populations are resistant to one or more classes of fungicides (Gisi *et al*., 2008).

The active ingredients most widely used to protect grapevine against *P. viticola* belong to several fungicide classes. Whereas multi-site inhibitors such as the dithiocarbamates and relatives are of moderate susceptibility of resistance, active ingredients belonging to the phenylamides (PA), the quinone outside inhibitors (QoI) and the carboxylic acid amide (CAA) are classified as highly susceptible due to their specific mode of action (Fungicide Resistance Action Committee, FRAC, 2011). As a consequence of the frequent application of such fungicides, the appearance of fungicide-resistant pathogens has been reported around the world and especially in Europe.

One of the first reports of *P. viticola* resistance dates from 1982 for active ingredients belonging to the PA group (Clerjeaux *et al*., 1985). PA are highly systemic fungicides specifically used to control diseases caused by water molds and foliar diseases such as late blight, downy mildew, and white rust. PA inhibit fungal growth by disrupting RNA synthesis. Resistance problems with

PA, specifically metalaxyl, were observed shortly after their introduction. Problems with the efficiency occured particularly when PAs were used exclusively and under high disease pressure. Resistance is governed by one or two genes and a low frequency of resistant individuals may exist in wild populations prior to the use of these fungicides. Resistance can increase rapidly through selection of the naturally occurring strains. Cross resistance occurs with other phenylamide fungicides, but not with fungicides from other mode of action groups (Clerjeau *et al*., 1985; FRAC, 2011; Fourie, 2004).

In 1993, a decrease in disease control was reported in northern Italy which was attributed to the development of cymoxanil resistance. Since then, much data were published on the level of cymoxanil sensitivity of European *P. viticola* populations, with contrasting results (Genet *et al*., 1999). Cymoxanil is the only representative of the cyanoacetamide-oximes and is used for the control of several fungi belonging to the peronosporales (Genet *et al*., 1999; Gullino *et al*., 1997). This fungicide exhibits a strong curative activity against *Phytophtora infestans* on potato and tomato and against *P. viticola* on grapevine. Due to its short lifetime and its quick metabolization by the plants, cymoxanil is mainly used in mixture with various other fungicides with contact and/or systemic properties. Its mode of action is still unknown; reported studies have suggested that several biochemical processes are disrupted, including the synthesis of nucleic acids and amino acids. Mycelial growth and germ tube formation are also inhibited (Genet *et al.,* 1999; Gullino *et al*., 1997).

Resistance to QoIs in grape downy mildew was first observed in Europe in 2000 and has become common in many grape-growing areas (Baudoin *et al*., 2006; Gullino *et al*., 2004). This resistance is commonly conferred by a point mutation of the mitochondrial cytochrome b gene that gives rise to a substitution from glycine to alanine at position 143 of the amino acid sequence (G143A) (Gisi *et al*., 2002, 2008; Grasso *et al*., 2006; FRAC, 2006). Strobilurin fungicides (QoI) are synthetic analogues of a naturally occurring compound produced by a wood rotting fungus (*Strobilurus tenacellus*) that inhibit respiration in fungal cells by targeting the protein cytochrome bc-1. These fungicides penetrate plant leaves and move in a translaminar way from one side of the leaf to the other. They act on a broad range of fungal processes including spore germination, fungal growth, and reproduction (e.g. sporulation). Strobilurins have been registered on numerous crops because of their broad-spectrum activity and excellent human and environmental safety profiles. However, fungicide resistance phenomena have been described shortly after their introduction in the late 1990s (Gisi *et al*., 2008; Gessler *et al*., 2011).

Resistance to CAA fungicides has been reported in nature for several oomycetes including *P. viticola* (Albert *et al*., 1991; Cohen *et al*., 1995; Gisi *et al*., 2008). CAA fungicides exhibit translaminar movement in the plant and although their mode of action is still not certain, it was suggested that they inhibit phospholipid biosynthesis and interfere with cell wall deposition (Gisi *et al*., 2007). The target gene(s) have not yet been identified and no mutations conferring resistance to CAAs are known, despite the detection of CAA-resistant field isolates (FRAC, 2006).

Because of the costs of frequent applications of fungicides, the desire to reduce pesticide levels in the environment, to preserve the fungicide utility and to prolong their efficacy, considerable efforts have to be made to manage fungicide resistance (Freier *et al*., 2008). An essential part of such management strategies is the monitoring of fungal pathogen populations for their sensitivity against crop protection compounds. These monitoring efforts should be based upon comparisons with baseline sensitivity data and the techniques must be suitably precise and reliable to detect relevant shifts in sensitivity within populations (Brent, 1995).

In the laboratory of Biorizon (France), the sensitivity of *P. viticola* has been monitored for over a decade, allowing comparison of sensitivities to some of the most commonly used fungicides. However, to our knowledge, the current sensitivity status of *P. viticola* population in the Luxembourgian grape-growing region has not yet been investigated.

Hence, leaf samples showing downy mildew symptoms were taken in 19 vineyards along the river Moselle in the seasons 2010 and 2011. Samples were analysed using a microplate assay for conidia growth inhibition and a leaf disc assay to determine the sensivity profiles of *P. viticola* to the major classes of single-site-fungicides used in the Luxembourgian vine-growing region, and to investigate the presence of resistant populations.

MATERIALS AND METHODS

Sampling. Sampling took place on 23-08-2010 and 05-09-2011; leaf samples were collected in 19 vineyards located along the river Moselle close to the following villages: Ahn, Bech-Kleinmacher, Bous, Contz-les-Bains, Dreiborn, Ehnen, Erpeldange, Greiveldange, Grevenmacher-Wecker, Lenningen, Mertert, Perl, Remerschen, Remich, Scheierberg, Schengen, Stadtbredimus, Wellenstein, Wintrange (Fig. 1). Vineyards were located in Luxembourg except the ones in Contz-les-Bains (France) and Perl (Germany). However, even those two vineyards were managed by Luxembourgian grapegrowers.

In each vineyard, a sample of at least 50 infected leaves with fresh sporulating lesions was harvested randomly and bulked. The fungal material thus consisted of sporangia samples of *P. viticola* representing the diversity of the population at the date of sampling.

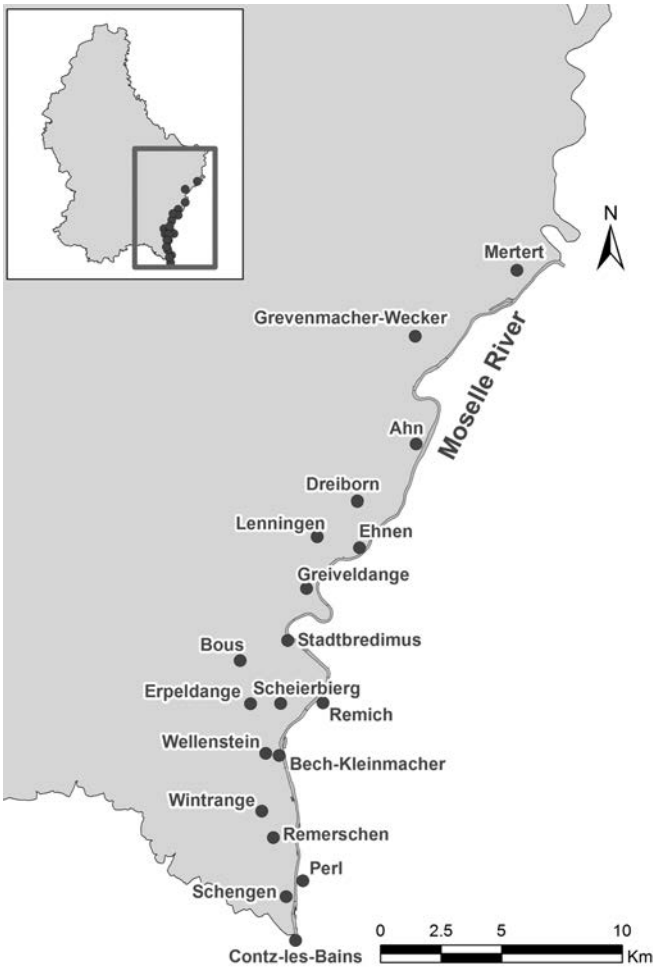

Fig. 1. Map showing the locations of the vineyards where leaf samples were collected in 2010 and 2011 in the Luxembourgish winegrowing region.

The material was dispatched to the Biorizon laboratory, where leaves were washed in demineralized water and put in a moist chamber for 24 h at 21°C to induce fresh sporulation. The sporangia were collected and inoculated by deposition of droplets (10 µl) onto untreated leaves. After 7 days, fresh sporangia were harvested from these leaves and used to contaminate the test units.

Fungicides. Dimethomorph (CAA, 15% active, Forum, BASF, Germany), pyraclostrobin (QoI, 25% active, Comet, BASF), azoxytrobin (QoI, 25% active, Quadris, Syngenta, France) and kiralaxyl (PA, benalaxyl-M, 20% active, Isagro, Italy) were used as formulated products in the different assays. The technical-grade cymoxanil (99% active, Syngenta, France) was dissolved in water during 1 h before use.

Microplate test to determine sensitivity to CAA and QoI (2010 and 2011). In the case of *P. viticola*, a positive cross resistance among all CAA fungicides is reported. The same conclusion was also observed for all members of the QoI group, allowing to use a single member of each group to determine the resistant profile of the studied populations (FRAC, 2011).

A microplate test developed by Steva (2002a, 2002b) was used in which 50 µl of fungicide and 50 µl of sporangia suspension (25 x 10^3 non-granulated sporangia per ml) were deposited in each well of a microplate. For each fungicide concentration, 5 wells were used representing 5 replicates of the test unit. For the microplate test, the following fungicide classes and active ingredients were tested at the following concentrations as discriminatory rates (Steva,2002a, 2002b): (i) CAAs (carboxylic acid amides): dimethomorph 0.3 mg/l (2010 and 2011); (ii) QoIs (quinone outside inhibitors): pyraclostrobin 3 mg/l (2010); azoxytrobin 10 mg/l (2011).

The microplates were incubated in a climatic chamber at 21±2°C. After 6 h, a droplet of "cotton blue" [500 g methylene blue (Sigma-Aldrich Chemie, France) and 417 ml lactic acid (Prolabo-Merck, USA)] was incorporated in each well of the microplate in order to block the development of infectious units.

Characterization of the percentages of resistant phenotypes to CAA and QoI (2010 and 2011). QoIs are strong inhibitors of zoospore germination and able (at low rates) to destroy the zoospores before the germination process. CAA fungicides are also strong inhibitors of zoospore germination. Consequently, for both fungicide classes, the number of germinated zoospores per surface unit is a suitable criterion to characterize the proportion of resistant phenotypes within a natural population. The characterization technique consisted in the observation of each oomycete unit under a light microscope (magnification 400X), counting the percentage of germinated zoospores.

The average score (5 observations) for each fungicide concentration was converted to a percentage of resistant units by comparison with the untreated samples according to the formula:

$$\%R = 100 \times [(\%D)/\%T].$$

where %D is the percentage of germinated infectious units in the fungicide wells and %T is the percentage of germinated infectious units in the control wells.

Four classes of resistance were determined: group 1, resistance-free populations; group 2, less than 5% of resistant phenotypes; group 3, between 5.1 and 30% resistant phenotypes; group 4, more than 30% of resistant phenotypes according (Steva, 2002a, 2002b).

Leaf disc assay to determine PA sensitivity (2011). This method was firstly described by Clerjeau *et al.* (1985). Leaf discs were cut from healthy leaves of the *V. vinifera* cv. Cinsault and placed into plastic Petri dishes. The concentrations used were 0 (untreated control) and 10 mg/l kiralaxyl. The leaf disc lower surfaces (10 discs per fungicide concentration) were inoculated with 3

droplets (50,000 spores/ml, 5,000 spores/ml and 500 spores/ml) per disc from a sporangiospore suspension. After 7 days of incubation at 22ºC, the growth was evaluated on a scale of 0 to 4: 0, no growth; 1, very small lesion without sporulation; 2, sporulating lesions smaller than a droplet; 3, sporulating lesion of the same size as the original droplet; 4, sporulating lesion exceeding the size of the droplet. The total score (10 leaf discs) for each sporangia concentration was calculated for the different concentrations of the fungicide. The sensitivity of the population was determined according to Clerjeaux *et al.* (1985). The populations of *P. viticola* were classified as: S: no resistant strains to phenylamides; R+: *ca.* 1% of resistant strains to PA; R++: *ca.* 10% of resistant strains to PA; R+++: 50% or more resistant strains to PA.

Leaf disc assay to determine cymoxanil sensitivity (2011). This sensitivity test was performed on grape leaf discs treated with cymoxanil and inoculated 24 h later with three droplets of a suspension of *P. viticola* sporangia. The doses tested of cymoxanil were 100, 300 and 800 mg/l. After 7 days of incubation at 22ºC, the fungal growth was evaluated on a scale of 0 to 4 as above. Minimum inhibitory concentrations [MIC values; lowest concentrations where sporulation (rating of 2 to 4) was not observed (Genet and Vincent, 1999)] were determined from the raw data for each population. Details of the method were described by Genet *et al.* (1997, 1999).

RESULTS AND DISCUSSION

QoI phenotype resistance Situation (2010-2011). The percentages of resistant phenotypes to QoIs (pyraclostrobin for the season 2010) detected on each sample of grape downy mildew, collected in several fields are given in Table 1. None of the samples was free of resistant infectious units. In average, zoospores resistant to 3 mg/l of pyraclostrobin represented 64% of the infectious units during the 2010 season. As previously described (Steva, 2002b), the studied populations were splitted into 4 classes. Only the population of one vineyard contained less than 5% of resistant strains and the population of another vineyard between 5.1 and 30% of resistant phenotypes. A majority (88%) of *P. viticola* populations collected in the Moselle grape-producing area in 2010 consisted of more than 30% of strains resistant to QoI fungicides.

The percentages of QoI (azoxystrobin) resistant phenotypes detected on each sample of grape downy

Table 1. Percentages of resistant phenotypes of grape downy mildew to QoI and to CAA in the samples collected in the Luxembourgian grapegrowing region (n=19) during the 2010 and 2011 seasons.

Season	2010		2011	
Location	QoI Pyraclostrobin 3 mg/l	CAA Dimethomorphe 0,3 mg/l	QoI Azoxystrobin 10 mg/l	CAA Dimethomorphe 0,3 mg/l
Ahn	88,8	96,9	78,6	87,6
Bech-Kleinmacher	64,2	100,0	85,3	78,6
Bous	47,6	94,3	3,7	88,3
Contz-Les-Bains	62,9	100,0	7	93,5
Dreiborn	1,8	40,5	95,3	85,6
Ehnen	78,0	96,4	59,3	89,1
Erpeldange	78,0	98,6	100	100
Greiveldange	31,6	98,2	97,8	42,5
Grevenmacher-Wecker	85,0	47,8	89,5	87,4
Lenningen	59,5	100,0	94,5	85,4
Mertert	40,8	99,6	92,3	85,4
Perl	79,1	85,4	100	94,5
Remerschen	73,9	90,3	97,5	87,4
Remich	75,7	97,8	78,9	74,1
Scheierbierg	-	-	100	100
Schengen	100,0	99,8	98	89,5
Stadtbredimus	88,1	100,0	87,6	78,4
Wellenstein	85,0	64,3	98,3	89,4
Wintrange	24,6	100,0	91,3	84,1
Mean value	64,7	89,4	81,8	85,3
Median value	74,8	98,0	92,3	87,4

mildew collected in several fields in 2011 are presented in Table 1. None of the samples was free of resistant infectious units. In average, zoospores resistant to 10 mg/l of azoxystrobin represented 81% of the infectious units during the 2011 season.

Only the population of one vineyard contained less than 5% of resistant strains and the population of another vineyard contained between 5.1 and 30% of resistant phenotypes. A majority (90%) of the *P. viticola* populations collected in the Moselle grape-growing area in 2011 was constituted by more than 30% of strains resistant to QoI fungicides.

The appearance of QoI resistance was described for the first time in 2000 in European *P. viticola* populations. The evolution was fast with a rapid increase of resistant isolates, reaching a frequency of 70% to 80% in France in 2003. A further increase to a frequency of 90% was detected in 2006. In the north of Italy and in Switzerland, frequencies reached high levels in certain areas, whereas they are still low in other countries like Germany (Sierotzki *et al*., 2008). In specific trials in Brazil, a decline of the resistance was observed when the QoI applications were stopped, followed by a rapid increase when the use of QoI was resumed (Sierotzki *et al*., 2008). In this context, the resistance evolution appeared to be the result of the strategy of QoI applications. This might lead to the possibility to manage QoI resistance based on a reduced fitness of the QoI-resistant isolates. The web site of the FRAC indicates that, in 2010, a high level of resistance was detected in France, Switzerland, Italy and Germany. For the 2011 season, a high level of resistance was detected in Germany, Czech Republic and Slovakia and a moderate level of resistance was observed in France, Italy and Switzerland.

CAA phenotype resistance situation (2010-2011). The percentage of phenotypes resistant to CAA detected on each sample of grape downy mildew collected in several fields in 2010 are presented in Table 1. None of the samples was free from resistant infectious units. The percentages of strains resistant to 0.3 mg/l of dimethomorph varied from 40% to 100% of the zoospores. Zoospores resistant to CAA represented 89% of the population (average of all samples) while 100% of the *P. viticola* populations collected in the Luxembourgian vine-growing area in 2010 were constituted by more than 30% of resistant strains to CAA fungicides.

Also in 2011, none of the samples were free of resistant infectious units. The percentages of strains resistant to 0.3 mg/l of dimethomorph varied from 42% to 100% of the zoospores. Zoospores resistant to CAA represented 85% of the population (average of all samples) while 100% of the *P. viticola* populations collected in the Luxembourgian wine-growing area in 2011 were constituted by more than 30% of strains resistant to CAA fungicides.

For both seasons (2010 and 2011), all samples were composed by a high proportion of strains able to grow at a concentration of 0.3 mg/l dimethomorph. Dimethomorph-resistant isolates of *P. viticola* were detected in French vineyards as early as in 1994 and in 2000 (Gisi *et al*., 2007). Since then, the FRAC website has reported that sensitivity-monitoring studies performed by agrochemical companies over several years revealed that in the populations of grape downy mildew pathogen, resistant isolates are consistently detected in France and in Germany with high frequencies.

Phenylamide sensitivity situation (2011). The sensitivity profile of the PA active ingredient kiralaxyl is presented in Table 2. Using the methodology adopted by Clerjeaux *et al.* (1985), 100% of *P. viticola* populations of were classified as extremely resistant to the PA (R+++) (Table 2), indicating that they contained at least 50% of strains resistant to PA. In France, the resistance in *P. viticola* populations varied between 15 and 75% for the seasons 1987 and 1988, respectively (Gisi, 2002).

Fourie (2004) classified the grape downy mildew populations according to their MIC values: populations exhibiting a MIC up to 200 mg/l were considered as highly resistant. For the 3 years of the survey (1999 to 2002), the majority of the isolates (94%), collected from various vineyards in the Western Cape province (South Africa), were designated resistant to highly resistant. In China, Sun *et al.* (2010) found a high proportion of local isolates resistant to PA, i.e. 61% of the samples were designated as resistant.

Additional data showed high frequencies of PA resistance in vineyards that had no recent history of PA usage, indicating the stability of PA resistance in *P. viticola* (Fourie, 2004). This last observation holds serious implications for the management of PA resistance in the Luxembourgian grape-growing region.

Cymoxanil sensitivity situation (2011). The sensitivity profile of cymoxanil and the MIC values are presented in Table 2. No population was controlled by treatments at the rate of 100 mg/l of a.i. (active ingredient). The efficacy varied from 0 to 70% with a mean value equal to 7%. Growth and sporulation of *P. viticola* was also observed on leaf discs treated with 300 mg/l cymoxanil. None of the populations was controlled and the efficacy ranged from 0 to 95% with a mean value equal to 20%. Three populations were not controlled by the highest rate of cymoxanil (800 mg/l of a.i.); the mean efficacy for this rate was 81%. In average, 84% of the populations had a MIC between 300-800 mg/l, 16% up to 800 mg/l.

Gullino *et al.* (1997) found many populations in Italy showing a reduced sensitivity to cymoxanil in combination with decreased performance in disease control: in 1994, 62% of the populations sampled in the Trentino

Table 2. Sensitivity profile of grape downy mildew populations to cymoxanil* and kiralaxyl** collected in the Luxembourgish winegrowing region (n=19) during the 2011 season.

Location	Acetamides efficacy of the cymoxanil (mg/l)				Phenylamides kiralaxyl (mg/l)
	100	300	800	MIC	10
Ahn	0	0	100	300-800	R+++
Bech-Kleinmacher	0	12,5	100	300-800	R+++
Bous	66,7	95,8	100	300-800	R+++
Contz-Les-Bains	0	35	100	300-800	R+++
Dreiborn	0	0	96,7	>800	R+++
Ehnen	0	0	93,3	>800	R+++
Erpeldange	70	93,1	100	300-800	R+++
Greiveldange	0	28,3	100	300-800	R+++
Grevenmacher-Wecker	0	25	100	300-800	R+++
Lenningen	0	0	100	300-800	R+++
Mertert	0	36,7	100	300-800	R+++
Perl	0	0	95	>800	R+++
Remerschen	0	0	100	300-800	R+++
Remich	0	0	100	300-800	R+++
Scheierberg	0	0	100	300-800	R+++
Schengen	0	0	100	300-800	R+++
Stadtbredimus	0	0	100	300-800	R+++
Wellenstein	0	55,8	100	300-800	R+++
Wintrange	0	1,7	100	300-800	R+++

For the the cymoxanil*, the leaf disc assay provides for each population an efficacy for the studied concentration. MIC: Minimum Inhibitory Concentrations: lowest concentration where sporulation was not observed. For the PA (phenylamides)**, the population of *P. viticola* were classified as: S : no resistant strains to Phenylamides - R+ : about 1% of resistant strains to PA - R++ : about 10% of resistant strains to PA - R+++ : 50% and more of resistant strains to PA.

region showed a reduced sensitivity to cymoxanil with a MIC value of 100 mg/l or more. In 1995, 41% of the studied populations had MIC values of up to 250 mg/l. Moreover, populations with a MIC of >200 mg/l were able to grow on potted vined treated with cymoxanil at concentrations up to 1000 mg/l. Hence, Gullino *et al.* (1997) collected evidence of an increased resistance to cymoxanil.

Genet *et al.* (1999) carried out a European monitoring on 278 fungal populations; their sensitivity to cymoxanil was also determined using a leaf disc assay. The results revealed a wide distribution with a MIC ranging from 10 to more than 800 mg/l: 13% of the tested population were inhibited by 100 mg/l, 66% had a MIC ranging from 300 to 800 mg/l and 23% were still growing at 800 mg/l.

CONCLUSIONS

Fungicide resistance has been reported in several European countries. Our data indicate that fungicide resistance against CAAs, QoI, PA and cymoxanil is currrently widespread in the Luxembourgian grape-egrowing region. The high frequency of QoI resistance in *P. viticola* detected in Luxembourg is similar to that recorded in several European countries. Studies show that QoI-resistant isolates of *P. viticola* are less fit in the absence of selection pressure than sensitive isolates, resulting in a decline of this population when QoI treatments are stopped. This observation can be the basis for a new strategy in the Moselle area. Concerning CAAs, inheritance studies performed by Gisi *et al.* (2007) delivered important informations for the management of CAA resistance: first, sexual crosses between sensitive

and CAA-resistant isolates of *P. viticola* lead to a co-segregation of the resistance to CAA; secondly the resistance to CAA fungicides is inheritated in a recessive manner. Based on these results, the FRAC had classified the resistance risk as moderate for the CAA and has issued recommendations for their use. Nevertheless, high frequencies of CAA-resistant isolates were detected in the Moselle area for two seasons, indicating the need of complementary studies on field performances and new appropriate chemical strategies. The high frequency of PA resistance in *P. viticola* detected in the Moselle area was similar to that reported in South Africa and China. Due to the cross-resistance between the PA compounds and the presumed stability of PA resistance, the use of PA formulations should be reconsidered in the Luxembourgian vineyards. The MIC to cymoxanil of the Luxembourgian populations is high. Based on the study of Gullino *et al.* (1997), where potted plants were treated with cymoxanil at concentration up to 1000 mg/l, additional investigations are necessary to characterize the Luxembourgian *P. viticola* isolates with a reduced sensitivity for their potential of danger for the protection strategies against grape downy mildew.

Based on these present results from the Luxembourgian Moselle area, future studies are needed to:

(i) Analyse the nature and number of chemical treatments applied in the vineyard in the present and the previous seasons in order to find correlations with the different levels of sensitivity detected:

(ii) Determine the impact of resistant phenotypes on the fungicide efficacy and the field performance;

(iii) Follow the progress of the resistance levels in the Luxembourgian vine-growing region in the years to come.

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CHARACTERISATION OF LACCASE ACTIVITY OF TWO STRAINS OF *BOTRYTIS CINEREA*

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SUMMARY

The purpose of the present study was the characterization of the polyphenol oxidase activity, which can mainly be ascribed to a fungal laccase, produced and released in grape juice by two different strains of *Botrytis cinerea.* This fungal laccase belongs to the group of polyphenol oxidases (PPO) and is mainly responsible for the oxidation of polyphenols contained in grape must. The analysis of the SDS-PAGE band profile of the proteins released in the grape must showed huge differences between the investigated strains. These differences regard both the proteins with a high molecular weight, among which presumably the laccase is present, and the proteins with a molecular weight below 40 kDa. The "laccase activity" of the enzymes, expressed and secreted by the two fungal strains in the growth medium, was tested at pH 5.0 by the syringaldazine test and at pH 3.5 by an oxygraphic method using three substrates present in the must: caffeic acid, caftaric acid and malvidin-3-glycoside. Also in this case significant differences among the apparent Michaelis-Menten constant values of the two strains for the various polyphenols were found. Although the activity behaviour cannot be unquestionably attributed to laccase, it could be of practical importance to wine producers since the kinetic data were obtained in experiments carried out at pH 3.5, i.e. under a condition typical of must.

INTRODUCTION

The grey mould fungus *Botrytis cinerea* has a very broad range of hosts (Agrios, 2005) among which the grapevine. *B. cinerea* can be seen either as a blessing or as a curse for wine production. In the first case, under suitable climatic conditions, *B. cinerea* infections can lead to the so-called "noble rot" when bunches are infected in a very late ripening stadium with high sugar and low acid concentrations. These conditions are essential for the production of sweet wines e.g. "Trockenbeerenauslese" or "Eiswein". On the other hand, when the infection occurs in an early ripening stage with low sugar and high acid concentrations, the infection can cause the opposite scenario. In fact, in combination with unfavourable weather conditions and acetic acid bacteria, an acid rot can develop, leading to a definitive spoilage of the crop.

B. cinerea possesses several ways to infect its host. The aim of this study was to emphasize the enzymes secreted during the infection process. These enzymes first of all facilitate penetration of the berry skin, then promote hyphal growth inside the host (Touzani *et al.*, 1994). A very important enzyme released by *B. cinerea* is laccase, a polyphenol oxidase (PPO) much more active than other PPOs, such as tyrosinase, an enzyme naturally occurring in grapes (Ribéreau-Gayon *et al.*, 2004). Laccase is responsible for the oxidative browning of the must and the apperance of polyphenols in the bunches. This fact is very important for wine makers.

In 2010, during harvest and sampling procedure, a severe *Botrytis* infection was observed, which was not correlated with a high laccase activity. Different vineyards planted with cv. Riesling were inspected and, although the visual impression showed high infection rates, a comparatively low laccase activity was detected. This finding disagrees with those of studies conducted in France in which a direct relation between a strong *Botrytis* infection level and an elevated laccase activity was observed (Grassin and Dubordieu, 1989). Furthermore, Dubernet and Ribéreau-Gayon (1973) and Mayer (1978) pointed out that laccase represents a suitable indicator for grey mould infections. Macheix *et al.* (1991) and Perino *et al.* (1994), however, ascertained that an increase of laccase activity cannot necessarily be correlated with a rising fungal infection. This multi-faced behaviour, among other reasons, could be attributed to different strains of *B. cinerea* (Kovac, 1982).

Within the framework of the EU-Project SAFE-

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GRAPE (www.safegrape.eu), having as a goal the development of a biosensor able to measure laccase activity, the polyphenol oxidase activity and some general enzymatic features of two genetically dissimilar strains of *B. cinerea* selected in different vineyards were investigated.

MATERIAL AND METHODS

Organism and growth conditions. The *B. cinerea* strains BcK and BcV, selected in 2008, were kindly provided by the Institute of Phytomedicine, Hochschule Geisenheim University (former Geisenheim Research Center, Germany). Strains were grown on PDA (39 g/l) at 24°C, then transferred to Erlenmeyer flasks with a growth medium consisting of sterile grape juice, $CuSO_4$ (3.9 g/l) and gallic acid (1 g/l) as inducers, and incubated at 24°C. Sampling was conducted every third day from three replicate Erlenmeyer flasks.

Sample preparation. To test the laccase activity of proteins secreted by the fungus, aliquots (1 ml) of growth medium were centrifuged 13,000 g for 4 min at room temperature. Pellets were discharged while supernatants, containing the secreted proteins, were promtly analysed or stored at -80°C.

Syringaldazine test according to Grassin and Dubordieu (1989). This test, which according to the authors is specific for *B. cinerea* laccase, was carried out using a photometer UV/VIS - spectrometer Lambda 2 (Perkin Elmer, Germany) and using syringaldazine (Sigma Aldrich, Germany) as substrate according to Grassin and Dubourdieu (1989) with few modifications. The syringaldazine stock solution was prepared solubilizing 0.06 g/l syringaldazine in ethanol, whereas the fresh test solution was prepared with 200 µl of syringaldazine and 600 µl of Na-succinate 0.1 M, NaCl 30 mM, at pH 5.0. After the addition of 200 µl infected grape juice sample to the syringaldazine/Na-succinate buffer solution, syringaldazine oxidation to violet chinon was measured at 530 nm at 35°C ($\propto$= 65000 $M^{-1}cm^{-1}$). Measurement lasted 10 min and laccase activity was expressed as ULac, i.e. the amount of enzyme oxidizing 1 nanomol of syringaldazine per minute. Analyses were made in triplicates for each sample.

Protein content. The total protein content in the sample was calculated by the Bradford (1976) dye binding method, using bovine serum albumin (BSA) as standard (Bradford, 1976). Analyses were made in triplicates for each sample.

SDS-PAGE analysis. Supernatants from *B. cinerea* strain cultures were analyzed using gradient gels [Mini-Protean TGX™ 4-20 % Precast Cells; 30 µl/well (Bio-RAD, USA)]. Samples were denatured by heating for 5 min at 95°C after the addition 30 µl of buffer containing 10% sodium dodecyl sulphate (SDS) and 0.2 M dithiothreitol (DTT) (final concentration). Gels were fixed, stained and destained according to Hesse and Jahn (2008), modifying the staining procedure elaborated by Neuhoff *et al.* (1988) to the effect that ethanol was used in the staining solution instead of methanol.

K_m and v_{max} measurement. K_m and v_{max} constants were determined using three selected polyphenols naturally occurring in white- and red-berried grape musts: caffeic acid, caftaric acid and malvidin-3-glycoside. Polyphenols were solubilized in ethanol at a final concentration of 10 mM. Each polyphenol was added at different concentrations in a solution containing an aliquot of supernatant from fungal cultures corresponding to 12 ULac/ml in tartrate buffer at pH 3.5, previously equilibrated with atmospheric oxygen at 35°C. The rate of oxygen consumption, due to laccase activity, was detected with a Clark electrode (Zennaro *et al.*, 2007). K_m and V_{max} values were calculated by non-linear regression analysis, fitting the Michaelis-Menten equation to the experimental data, using the SigmaPlot 9.0 Systat Software (USA).

RESULTS

Fig. 1a and 1b report the laccase activity measured according to the syringaldazine test in various samples of growth medium of *B. cinerea* BcK and BcV strains. From these figures it appears that activity significantly increases starting from day 10 and reaches a plateau around day 21 in the case of strain BcK and day 17 in the case of strain BcV. Furthermore, it appears that the activity is about four times higher in the growth medium of strain BcK in comparison with that released by strain BcV notwithstanding the lower final protein content of BcK respect to BcV (BcK 25 mg/l and BcV 45 mg/l).

As to protein profiles, it can be noticed that strain BcK protein bands of *ca.* 122 and 95-99 kDa in size increased after the 12^{th} day (Fig. 2a, 2b). A similar behaviour was observed for strain BcV, but the proteins bands of *ca.* 110-120 and 89-98 kDa were much less intense with respect to those yielded by strain BcK (Fig. 3a, 3b). This result could be correlated with the low syringaldazine activity of strain BcV, suggesting that the high molecular weight proteins (110-120 kDa and 89-98 kDa) might be responsible for the laccase activity measured in the growth medium. Additionally, in the BcV gels, two strong bands of *ca.* 34 and 25-26 kDa were present, while they were negligible in the BcK gels.

It should be noted that, notwithstanding the difference both in the laccase activity and in the intensity of protein bands in the three parallel samples of growth

medium, a quite good correlation appears between the protein bands of high molecular weight (120-95 kDa) and laccase activity measured according to the syringaldazine test.

Fig. 4a and 4b show the plots of the initial rate of molecular oxygen consumption of samples of grape musts supporting the growth of the two *B. cinerea* strains, in the presence of various concentrations of three selected polyphenols. Each kinetic measurement was carried out using an amount of culture broth having an activity of 12 U Lac/ml according to the syringaldazine test, taken as reference.

In Table 1, the "apparent" V_{max} and the K_m values calculated from the best fit of the Michaelis-Menten equation to the experimental data of Fig 4a and 4b are reported. These data indicate that the apparent V_{max} values are quite similar independently of strains and substrates (range of the values 13.5-17.9 µM/min).

From the comparison of the apparent affinity (K_m values) of the enzymes with laccase activity produced by the two *B. cinerea* strains, it appears that the affinity for caftaric acid is 2-3 times lower (K_m values *ca*. 2-3 times higher) than that found for caffeic acid and malvidin-3-glycoside, regardless of the strain used.

DISCUSSION

Syringaldazine is a substrate showing a high specificity towards laccase (Harkin and Obst, 1973), which re-

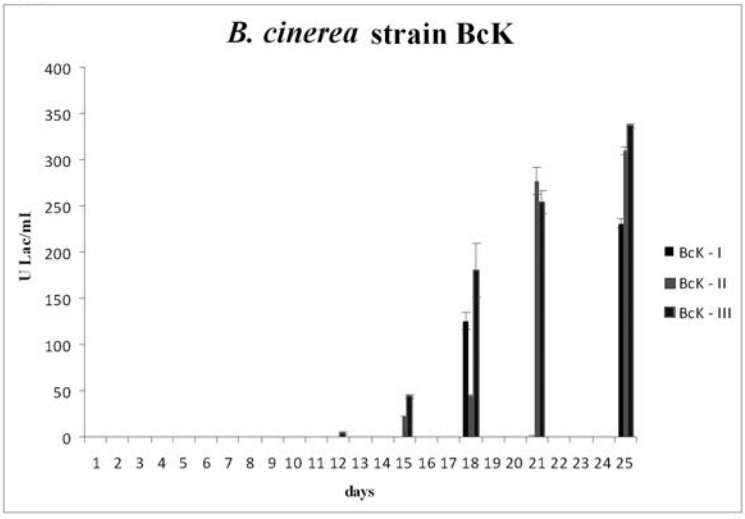


Fig. 1a,b. Laccase activity of the growth medium of *B. cinerea* strains BcK and BcV in U Lac/ml determined by the syringaldazine test (Grassin and Dubourdieu,1989). Samples were taken in triplicate.

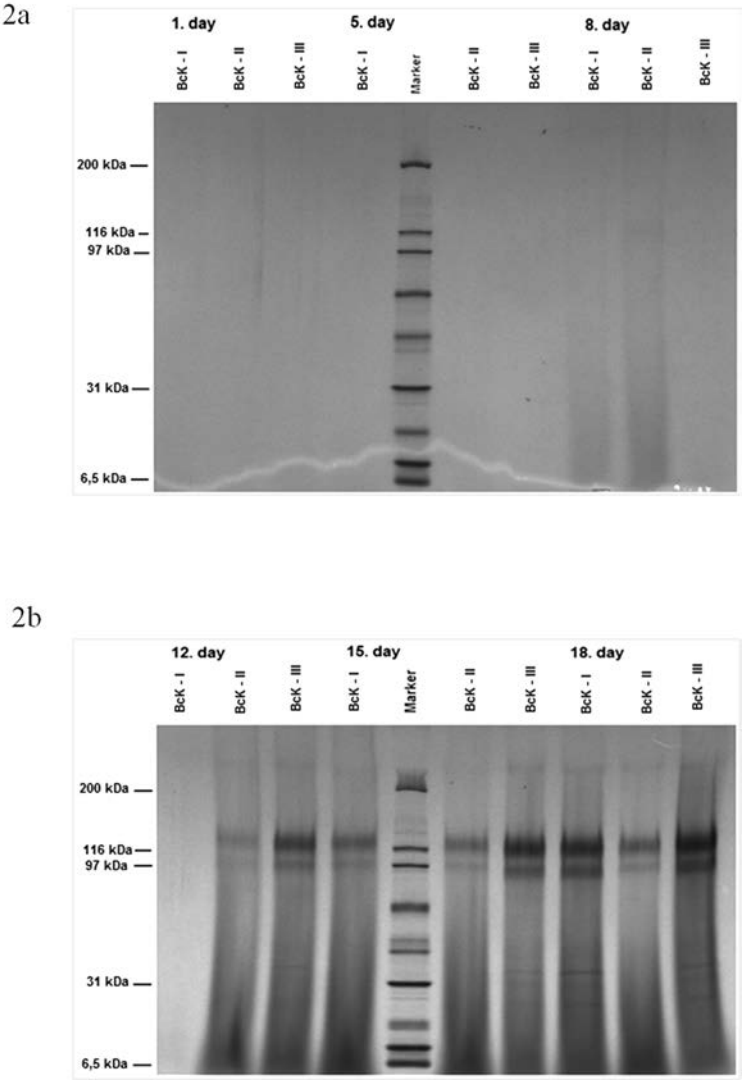


Fig. 2a,b. Electrophoretic gels of *B. cinerea* strain BcK of sampling days 1, 5, 8, 12, 15 and 18. Protein standards are shown in lane "Marker". Samples were taken in triplicate. Each lane was loaded with the same amount (20 µl) of denatured sample.

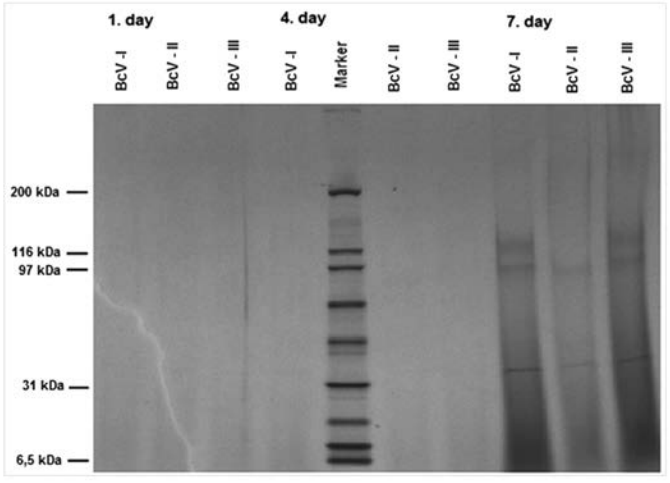
duces the importance of measuring the activity of other possibily present PPOs.

Grape juices infected by *B. cinerea* strain BcV showed a protein concentration higher than that in the samples infected by strain BcK, which had shown extreme high laccase activities in ULac/ml [ULac/mg = 12000 and 1440 for BcK and BcV] respectively. Interestingly, the visual observation of grape juice from infected samples showed no differences concerning mycelial growth. All strains employed showed a good and uniform mycelium development.

Regarding the protein secretion profiles analysed by SDS-PAGE, no protein band was detected during the first week, although the mycelium grew significantly in this period. This indicates that all the bands found in later stages of infection were of fungal origin. Also the presence of enzymes naturally occurring in healthy and infected grapes such as tyrosinases (Ribéreau-Gayon *et al.*, 2004) can be excluded. Interestingly, based on the Bradford assay and syringaldazine test, both BcK and BcV strains showed the appearance of proteins concomitantly with the development of laccase activity.

Both strains yielded protein bands around 90-99 kDA which, according to Baldrian (2006), can be attributed to laccase, differently from Slomczynski *et al.* (1995), who reported that laccase from *B. cinerea* has a mol. wt of about 74 kDA. This diverse attribution can be justified by the different grades of glycosylation of

3a



3b

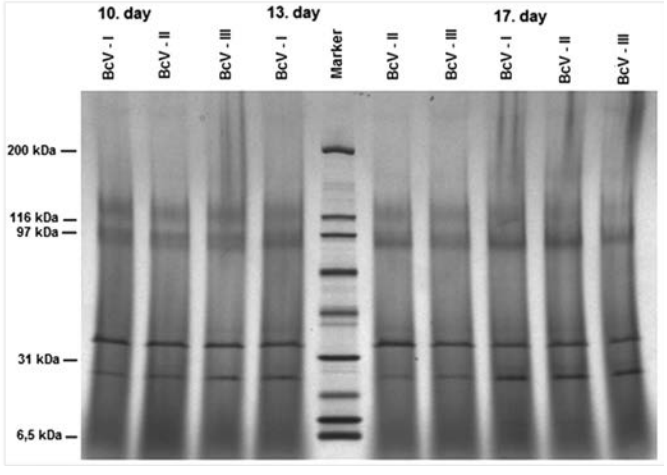
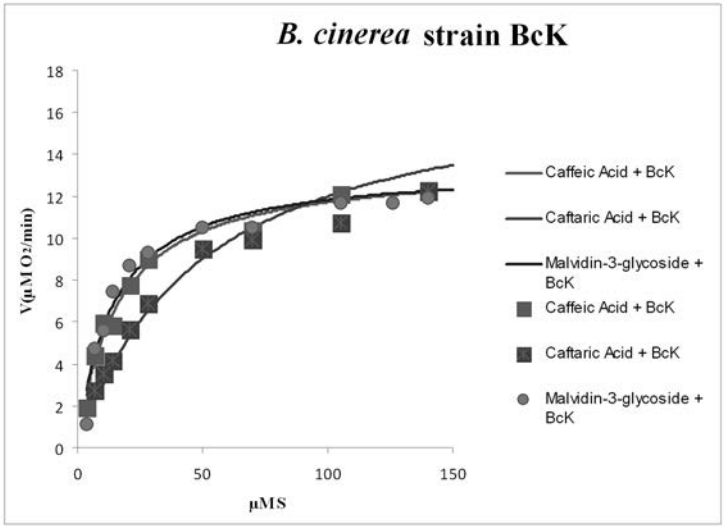


Fig. 3a,b. Electrophoresic gels of *B. cinerea* strain BcV of sampling days 1, 4, 7, 10, 13 and 17. Protein standards are shown in lane "Marker". Samples were taken in triplicate.

4a



4b

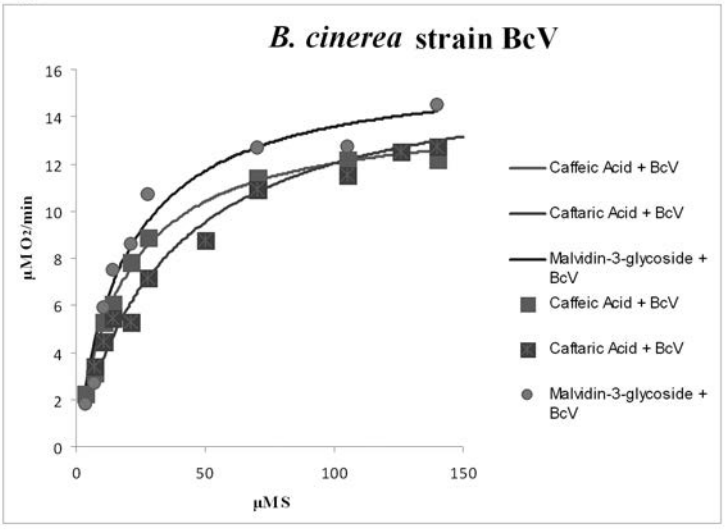


Fig. 4a,b. Oxidation rate (µM O_2/min) *vs* polyphenol concentrations (caffeic acid, caftaric acid and malvidin-3-glycoside; µM S) catalyzed by *B. cinerea* strains BcK and BcV. Continuous lines are the best fit of the Michaelis Menten equation to the experimental data.

Table 1. "Apparent" K_m and V_{max} values calculated by applying the Michaelis-Menten equation to the data of Fig. 4.

	BcK		BcV	
	V_{max} [μM/min]	K_m [μM]	V_{max} [μM/min]	K_m [μM]
Caffeic acid	13.5 ± 0.4	15 ± 2	14.2 ± 0.3	19 ± 2
Caftaric acid	17.9 ± 0.9	49 ± 8	16.2 ± 0.8	35 ± 5
Malvidin-3-glycoside	13.4 ± 0.5	14 ± 2	16.2 ± 0.9	19± 3

laccase and the fact that, according to Leach *et al.* (1980), glycopeptides in polyacrylamide gels show abnormally high mol. wts.

Furthermore, the presence needs to be pointed out of a band with a very low mol. wt in the protein profile of strain BcV but not in that of strain BcK. According to Pezet (1998), this component can be attributed to a "stilbene-like-oxidase", a PPO with a mol. wt of 32 kDA, thus indicating that not all *B. cinerea* strains produce this type of PPO during the infection process.

As to the kinetic characterization of laccase activity of *B. cinerea* strains, it is important to mention that only the apparent K_m values can be compared (they do not depend on enzyme concentration but on the type of enzyme) for the selected phenols used as substrates, because the enzymes are not pure and their concentration is not exactly known, as shown by the SDS-PAGE analysis. In addition, it appears that the enzyme(s) with "laccase activity" expressed by the two fungal strains have an apparent lower affinity for caftaric acid than for the "more" simple caffeic acid and malvidin-3-glycoside, a molecule with a large steric hindrance.

In conclusion, our results indicate that diverse strains of *B. cinerea* are characterized by a different expression of proteins (with high and low mol. wt), and a different polyphenol oxidase activity both in terms of syringaldazine test ($ULac/mg_{protein}$ = 12000 and 1440 for BcK and BcV, respectively) and towards the substrates present in the must. Although the activity behaviour cannot unquestionably be attributed to laccase, it could be of practical importance to wine producers since the kinetic data were obtained in experiments carried out at pH 3.5, which is typical of must.

ACKNOWLEDGEMENTS

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EFFECTS OF CLIMATE CHANGE ON GRAPEVINE PROTECTION. I. Pertot [1], A. Caffarra[1], M. Rinaldi[1], E. Eccel[1], B. Roatti[1], M. Storari[3], C. Gessler[3] and V. Rossi[2]. *[1]Research and Innovation Centre, Fondazione Edmund Mach, S. Michele all'Adige, Italy. [2]Università Cattolica del Sacro Cuore,Isitituto di Entomologia e Patologia Vegetale, Piacenza, Italy. [3]Institute of Integrative Biology, ETH Zurich, 8092 Zürich, Switzerland. E-mail: ilaria.pertot@fmach.it*

The result of climate change is not commonly perceived as a threat to grapevine productivity, especially where water is not a limiting factor. However even small changes in the environmental condition may strongly impact biological systems. A plant disease is the result of interaction between a susceptible plant, a virulent pathogen and the environment. The environment significantly (directly or indirectly) influences plants, pathogens and their antagonists, therefore changes in environmental conditions are commonly associated with differences in the level of losses caused by a disease. For these reasons, the changes associated with global warming (i.e., increased temperatures, changes in the quantity and pattern of precipitation, increased carbon dioxide and ozone levels, drought, etc.) may affect the incidence and severity of plant diseases (Chakraborty, 2005). As a consequence, climate change could substantially impact plant protection strategies. In a specific case study we refined current assessments of climate change impacts on pest and disease pressure on grapevines by considering pest/pathogen-host interactions. Our simulations suggest that under the environmental conditions of northern Italy increasing temperature will result in a marked increase in the mean number of generations completed by the European grapevine moth. On the other hand, the increase in pest pressure due to the increased number of generations might not be as severe as expected on the basis of the pest model only, due to the advance in harvest dates limiting damages from late-season generations. Simulations for powdery mildew highlighted a decrease in simulated disease severity, especially in years with a later onset of disease symptoms and in a climate scenario with higher temperature increases (Caffara *et al.*, 2012). Microbial antagonists to the pathogen may also play a role in the development of a disease, but they are in turn influenced by environmental conditions. In addition, abiotic stresses (such as drought, extreme temperatures and oxidative stress) leads to physiological and molecular changes that may interfere with the ability of the plant to assemble the resistance against pathogens. For example, drought stress combined with heat stress may interfere with the beneficial microorganism-induced resistance and lead to a reduction of the level of protection against downy mildew. Not only grapevine pathogens can be influenced by climate change; the members of the *Aspergillus* section *Nigri* (black aspergilli) responsible for the ochratoxin A (OTA) and fumonisins contamination of wine may also be affected. Although at present the contamination is almost absent in northern Italy (Trentino region) projections of mean daily temperatures and monthly rainfall indicate that the presence of black aspergilli on grapes grown in these vineyards will probably increase in the future (Storari *et al.*, 2012). The consequences of climate-driven changes are not easily predictable in complex agro-ecosystems as vineyards. In fact the biology of pests/pathogens is interdependent from the host plant; therefore any change in each side can be reflected on the entire system. However climate change will add an additional layer of complexity to grapevine protection.

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GRAPEVINE LEAF STRIPE DISEASE, ESCA AND OTHER TRUNK DISEASES OF GRAPEVINE: A MULTIPLE CHALLENGE FOR VINE GROWERS. L. Mugnai. *Dipartimento di Biotecnologie Agrarie, Sezione Protezione delle Piante, Università degli Studi di Firenze, Italy. E-mail: laura.mugnai@unifi.it*

Fungal trunk diseases of grapevine are gaining the increasing attention of growers and plant pathologists, being one of the major factors causing losses in terms of number of vines killed, and amount and quality of the yield. As soon as a new research flow started, it was clear that grapevine wood represents a considerably complex environment and that the interactions among pathogens were going to be one of the relevant and striking aspects of these diseases. The sudden increase of "esca disease" in several European countries and the expansion of a decline condition of young vineyards fostered investigations that were focussed on single diseases until it was soon realize that multiple pathogens were involved. The most damaging fungal trunk disorder of grapevine in Europe is "grapevine leaf stripe disease" (in the past misinterpreted as one of the symptoms caused by decay agents), a tracheomycosis causing typical foliar symptoms, which is induced by vascular fungi, i.e. primarily *Phaeomoniella chlamydospora* and, very often, *Phaeoacremonium aleophilum*. Another quite common disease is the wood decay caused by various basidiomycetes (in Europe mainly *Fomitiporia mediterranea*), and different canker agents (*Eutypa lata* and species of Botryosphaeriaceae are the most common, but not the only ones). Many records of a general decline of young vineyards ended up to be ascribed to wood fungal infections. Typical collar and root rots, characterizing the so-called "black foot disease", were determined to be caused by species of the "*Cylindrocarpon* complex" [e.g. *Cyl. Liriodendri* (the main species causing black foot on grapevine), *Cyl. Destructans* and *Cyl. macrodidynum*)] and by *Campylocarpon* (*Campyl. fasciculare* and *Campyl. pseudofasciculare*). Tracheomycotic fungi causing leaf stripe disease can invade the canes of affected vines so that the cuttings used for propagation are infected and disseminate the disease. The presence of infections in nursery propagating material and soils, highlights an emergent and most important aspect of grapevine trunk diseases, that represents an additional challenge for the industry. Disease symptoms are linked to particular virulence factors that cause specific effects on wood texture, vessels function, and leaf activity. Up to now, even the identifcation of the activity of single pathogens has not been an easy task, so despite the big steps forward done in the last 10 years, there is still much to do to elucidate the single virulence factors and their effects. Nevertheless, new metabolites were described and some knowledge on their activity was secured. Studying the single agents and their "weapons arsenal" remains an essential step to be taken. In addition, all of the grapevine fungal trunk diseases cited above have to be now treated also, though not only, as a multiple agents problem as a whole. Grapevine represents a really unique environment for all microorganisms. Studies on endophytic bacterial and fungal microflora are showing an incredible variety of microorganisms present in healthy as well as in diseased vines. Colonization is highly favoured by cultural practices, and the vine anatomy and wound repair activity encourage and facilitate microorganisms entrance in its large vessels. Not only: beside the usual, relevant role of the environment on diseases, grapevine is more subject than all other woody plants to the influence of cultural factors.

Not only infection courts are commonly provided by pruning practices, but also the way the plant is cultivated and forced in an un-natural shape affects its physiology and therefore the effect od diseases, expecially that of vascular disorders. Anyone can easily hypothesise that these microbes and/or their metabolites, can interact and affect the physiology of the host plant and its defence reactions. It is a hard task to understand all this without a large view approach. Proteomic and metabolomic studies are developing searching tools towards a more holistic approach, able to give us a global view on the changes taking place in a symptomatic vine. A holistic approach is surely the one to be followed to set a control on these diseases. Nursery practices need to establish specific risk stages and sanitation practices to be adopted, cultural practices need to be accompanied by the consciousness of the vine life-long impact of several field operations applied in each single year. Winter wood sanitation, wood protection, treatments to the canopy that help inhibiting symptom development will have to become normal practices to bring to a sustainable level the presence of these unavoidable diseases.

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STATE-OF-THE-ART OF ESCA SYNDROME AND PETRI DISEASE IN THE PORTUGUESE WINE REGION OF DÃO.

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Esca syndrome and Petri disease, two major grapevine trunk diseases (GTDs) are commonly considered responsible for substantial losses while inducing premature decline and dieback of grapevines all over the world, and in the Portuguese wine region of Dão. Although recognizing the existence of GTDs-related problems in their vineyards, local growers are often incapable of correctly identifying which one is affecting their vines and this frequently causes misleads on management decisions of the affected vineyards. Simultaneously, there is an uncomfortable lack of perception of the real dimension of these problems (extension, incidence, etc.) and their economic consequences for the local wine industry. Assessing the real exent of each of the four GTDs, i.e. Esca, Young grapevine decline, Black dead arm and Phomopsis cane and leaf spot in this important Portuguese wine region, would allow an economic perception of their consequences and, if damages were considered significant, to put pressure on the public powers in order to obtain more support for research and control of the GTDs. To promote the growers knowledge on GTDs, a simple brochure with color pictures and succinct symptoms description has been produced and widely distributed to local viticulturists. To assess the incidence and importance of the different GTDs on the Dão wine region, while receiving the brochure, local growers were invited to carry out a simple survey. The results of this initiative show that the divulgation of the publication improved the knowledge of local growers on GTDs and the results of the survey gave a first perception of the real situation of the main GTDs in the region. During the period of the survey, several samples of wood, collected from Esca symptomatic grapevines, reported during the field work, throughout the entire region, were analyzed and the fungi recovered from the different observed lesions were registered.

PHYTOTOXIC SECONDARY METABOLITES FROM THE GRAPE BLACK ROT FUNGUS *GUIGNARDIA BIDWELLII*.

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One of the most devastating diseases of grapevine is black rot caused by the ascomycete *Guignardia bidwellii* (Molitor, 2009). In some German grapegrowing regions disease outbreaks can result in significant crop losses ranging from 5 to 80% (Ramsdell and Milholland, 1988). Reasons for the establishment of the pathogen are the increased number of abandoned vineyards serving as reservoir for fungal spores (Ullrich *et al*., 2009), and the increasing temperature due to global warming (BMELV, 2004). In integrated plant protection programs the disease can easily be controlled by the application of modern synthetic fungicides. However, such control agents are not registered in organic viticulture and can therefore not be applied in these vineyards (Molitor, 2009). As a consequence, there is a strong demand for alternative control methods. In order to develop new vine protection strategies it appears mandatory to understand the molecular basis of the *Vitis vinifera/G. bidwellii* interaction. Phytopathogenic fungi often produce phytotoxins for successful colonisation of the plant. Such low-molecular compounds are often found in necrotrophic fungi in which they are believed to contribute to disease symptom formation. Bioactivity-guided isolation from submerged cultures of the grape black rot fungus led to the identification of new phytotoxic secondary metabolites. These compounds are structurally related to guignardic acid, a dioxolanone moiety containing metabolites isolated previously from *Guignardia* species (Rodrigues-Heerklotz *et al*., 2001). However, in contrast to guignardic acid, which is presumably synthesised via deamination products of valine and phenylalanine, the biochemical precursors for the biosynthesis for the other phytotoxins appear to be alanine, phenylalanine or tyrosine. Potentially, the secreted phytotoxins serve as important virulence factors within pathogenesis.

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IMPACT OF ACTINOMYCETES ON PLANT PHOTOSYNTHESIS DURING PATHOGEN ATTACK: A COMPLETE NEW STORY.

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Botrytis cinerea is highly pathogenic to grapevine (*Vitis vinifera*), producing the characteristic grey mould symptom and causing great losses to the yield and also to the final product, the wine. Control of the disease caused by *B. cinerea* is mainly achieved by pre and/or post-harvest pesticide applications. However, the chemical control of this pathogen is a challenge because of its adaptability to various environmental conditions and development of fungicide resistant strains. This is why biological control methods have gained quite a lot of attention since a few years. In this work we report on the use of *Streptomyces anulatus* strain S37, a biocontrol agent, in order to reduce the use of pesticides in viticulture. Strain S37 is an actinomycete able to colonize *in vitro*-grown grapevine plants and inhibit the growth of *B. cinerea*. This strain was isolated from the rhizosphere soil of *V. vinifera* in Morocco and has a great potential for production of antifungal metabolites. The objective of this study was to understand the modification of photosynthesis during infection by S37 and S37 plants inoculated with *B. cinerea*. Measurement of chlorophyll a fluorescence, which reflects the functionality of the photosynthetic apparatus, showed that in the S37 pre-treated plants inoculated with *Botrytis* the maximum efficiency of PSII photochemistry is comparable to that of the control, contrary to *Botrytis*-infected plants at 48 h post inoculation. The effective quantum yield and the photochemical quenching were higher in the S37 pre-treated plants than in *Botrytis*-infected plants. These differences were significantly different and might indicate that an alteration of carbohydrate metabolism is implied in the mechanisms by which this *S. anulatus* strain operates to reduce disease incidence. Further studies are necessary to evaluate the protective effect of this potential biocontrol agent under greenhouse and field conditions, and also to purify and characterize the secondary metabolites produced by this actinomycete.

ORGANOLEPTIC MODIFICATIONS OF WINE QUALITY IN RELATION WITH PARASITIC FUNGI DEVELOPMENT ON GRAPES.

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Parasitic fungi as downy mildew (*Plasmopara viticola*), powdery mildew (*Uncinula necator* syn. *Erysiphe necator*) or *Botrytis cinerea* are of much concern for viticulture. Even when these fungi do not produce yield losses (example of diseased vines by downy mildew, powdery mildew), they are responsible for modifications of grape composition and usually of an altered enological quality, consequent to the degradation of numerous grape components (Cilindre *et al.*, 2008; Ky *et al.*, 2012). The pathogens alone, or accompanied by saprophytic fungi in the context of secondary bunch rots, can also lead to the presence of undesired metabolites (off odors and mycotoxins) lowering the enological and the hygienic quality of the crop. In recent years, the characterization of key compounds implicated in the wine typicality or its depreciation has given the opportunity to study more precisely the organoleptic impact of the main pathogens on wine quality. With grapes diseased by powdery mildew (*Uncinula necator*), the sugar content did not decrease usually. However, phenolic and varietal aroma compounds were affected when the proportion of diseased berries increased (Calonnec *et al.*, 2004; Stummer *et al.*, 2005). Surprisingly, the characteristic mushroom odor of grapes diseased by powdery mildew disappeared or, at least, was attenuated after alcoholic fermentation. This is consequent to the fact that some compounds responsible for mushroom aroma, particularly 1-octen-3-one and (Z)-1,5-octadien-3-one, are reduced by an enone reductase of *S. cerevisiae* during alcoholic fermentation (Wanner *et al.*, 1998; Darriet *et al.*, 2002). However, *B. cinerea*, alone or associated with saprophytic fungi in the context of bunch rot complexes, is usually one of the major causes of deterioration of wine quality. The problem of earthy off odors, related to the presence of (-)-geosmin has been deeply studied. Now it is clear that this alteration is due to bunch rot complexes associating *B. cinerea* and various *Penicillium* sp, particularly *P. expansum* (La Guerche *et al.* 2006, 2007; Morales-Valle *et al.*, 2011). Other wine defects with fresh mushroom nuances have been studied, 1-octen-3-one and 1-nonen-3-one, being the main contributors (Pons *et al.*, 2011). These compounds originate from grapes affected by bunch rot complexes in which a variety of saprophytic fungal species belonging to *Penicillium* sp., *Clonostachys* sp., *Trichothecium roseum*, *Verticillium* sp., and *Trichoderma* sp. can be involved, through their metabolism with the pathogen *B. cinerea*. But *B. cinerea* is not always the cause of deterioration of grapes and wines quality, for it is the key factor for the development of noble rot that makes the searched dessert wines. It was shown that wines obtained from botrytised grapes had a higher concentrations of some varietal odoriferous thiols as 3-sulfanylhexan-1-ol, since the infection of healthy grapes by *B. cinerea* impacts on the plant biosynthesis metabolism (Sarrazin *et al.*, 2007; Thibon *et al.*, 2009). The interpretation of this phenomenon was given while working with plant cells incubated with the fungus (Thibon *et al.*, 2011).

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CROPPING CONDITIONS AND MYCOTOXIN CONCERN IN GRAPES AND WINE.

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Mycotoxins are toxic secondary metabolites produced by fungi, known since 1960. Grapes and derived products were first reported as sources of ochratoxin A (OTA) for humans (Zimmerli and Dick, 1996). Many surveys followed which confirmed the relevance of OTA, mainly in red wine produced in southern Europe. OTA is a mycotoxin produced in the vineyard by fungi belonging to *Aspergillus* section *Nigri* (black aspergilli), mainly by *A. carbonarius* but also by *A. niger* and *A. tubingensis* which are prevalent in bunches, but with a low percentage of toxigenic strains. In 2007, the production of fumonisin B2 and B4 by *A. niger* was demonstrated and with following studies FB2 contamination was detected in musts, but at very low levels. Therefore, OTA is confirmed as the main mycotoxin of concern in grapes and derived products. Black aspergilli overwinter in the vineyard soil, and can be isolated from bunches with increasing frequency from setting to ripening. OTA is detected in bunches around ripening; higher contamination is found in damaged berries showing black mould. The presence of OTA in the berries only during ripening seems not to be strictly related to the inadequacy of the substrate in previous stages. The production of OTA by *A. carbonarius* on homogenised grapes of different varieties at different growth stages was confirmed, with potentially higher synthesis in earlier stages. Environmental conditions and berry skin thickness and soundness are supposed to be the crucial factors contributing to OTA contamination of grapes at about ripening. The ecology of black aspergilli has been deeply studied and quantitative data on the role played by temperature, water activity, pH on fungal growth and OTA production are available and the development of a predictive model able to describe fungal behaviour during the grape growing season is ongoing. The crucial role of the grape-growing area and meteorological conditions was pointed out by several authors and following a geostatistical approach, significant correlation between *A. carbonarius* incidence and geographic coordinates of fields sampled in Europe has been shown. A positive correlation with longitude and a negative correlation with latitude was reported; therefore, *A. carbonarius* incidence increased from west to east and the positive gradient was confirmed also towards the south of Europe. Good agricultural practices, and good management of pests and diseases in particular, are very efficient preventive actions. A correlation was found between OTA content in berries and *Lobesia botrana* attacks, probably related to both wounds caused and spore dissemination operated by the larvae with a significant reduction of OTA contamination after effective pest control. Among pathogens, grey mould proved to be unrelated to OTA contamination while powdery mildew seemed the most conducive for black aspergilli; severely infected berries often split open, mainly during ripening when the inoculum of black aspergilli is more relevant. All data published confirm that good vineyard management, in term of pest and disease control, guarantees a relevant decrease of OTA content in the berries. Even if the control of mycotoxin-producing fungi is not commonly done, many efforts have been devoted to the identification of biological control agents active against black aspergilli, which were supported by good results. Grapes varietal differences are reported to play a role in fungal abundance as well as OTA incidence either due to berry morphology as skin thickness, cuticle characteristics or chemical composition. Nevertheless, data useful in practice are not yet available regarding the role of grape varieties; similar conclusions can be reported for the trellising system. It was recently stated that frequent soil tillage increases the overall fungal population in the vineyard, especially with dry soil and/or during windy periods. Therefore, it should be avoided to limit the spread of toxigenic fungal population. Finally, the long stay of grapes on the vines before harvest influences grape susceptibility to fungal attack resulting in higher OTA contamination. Additional OTA is not synthesised during wine making, but it increases during post-harvest with delayed processing and during drying, both for raisin or dessert wine production, especially with sun drying. Based on available information, it can be concluded that cropping conditions are crucial for OTA contamination in grapes and an accurate management of the production-processing system is mandatory to optimise the safety of grapes and derived products.

FUNGAL AND BACTERIAL BIOTA ON THE CARPOSPHERE OF RIPENING GRAPE CLUSTERS

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The ascomycete *Botrytis cinerea*, the causal agent of grey mould of grapes, induces also rotting of the bunches, thus representing one of the most important fungal diseases for central European viticulture. Besides *B. cinerea*, several other fungal and bacterial species are involved in the development of bunch rot. In addition, the putative fungal or bacterial antagonists present on the grape carposphere, might be relevant for the progression of bunch rot epidemics. However, little is known on the composition of microbial communities on grape berries and how these are influenced by different crop management systems or plant protection strategies in viticulture. In 2010 and 2011 grape clusters (*Vitis vinifera* cv. Riesling) were collected during the ripening period at three sampling dates (BBCH 81, 85 and 89) from a

vineyard located in the German grape-growing region of Rheingau (49°59'N, 7°57'E). This experimental vineyard contained plots which were cultivated according to three different management systems (integrated, bio-organic and bio-dynamic) for the last six years. Total microorganisms from the berry skin surface were washed off and DNA was isolated. High-throughput tag-encoded FLX amplicon pyrosequencing of an ITS fragment and bacterial 16S rDNA fragment was used to characterize fungal and bacterial communities on grape berry skins. By sequencing about 18,000 bacterial and 42,000 fungal amplicons, more than twelve different fungal and 26 bacterial taxa could be identified. Data showed, that the abundance of members of the Sclerotiniaceae (amongst others *B. cinerea*) increased during ripening while the extent of fungal biodiversity (calculated on the basis of the Shannon-Index) decreased. The extent of biodiversity and composition of fungal and bacterial communities was different depending on the management system. On grape berries from integrated viticulture, a higher diversity of fungal communities was observed compared to berries from bio-organic or bio-dynamic plots. In addition, putative fungal and bacterial antagonists were found. These species were particularly present on grape berries from integrated plots in 2011. A detailed knowledge about the functional and structural diversity of the microbial community of berry skin surfaces is relevant to promote indigenous antagonists in a stable microbial community with the aim to suppress bunch rot of grapes. Considering the recent effects of climate change on the extent of bunch rot epidemics, such an approach might be an important tool of future pest control programs in viticulture.

LOOKING AT THE MICROBIAL COMMUNITY OF GRAPEVINE. C. Pinto, V. Custódio, S. Sousa and A.C. Gomes
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Vitis vinifera is a remarkable crop with a relevant economic importance in the world. As a plant, it is naturally colonised by a wide variety of microorganisms, both beneficial and phytopathogenic, which interact with it and play a major role in its growth, vigour and clearly influence the wine quality. The natural microecosystem from grapevine is very dynamic and is mainly affected by spatial and temporal factors as well as by the application of plant protection products that are mostly based on chemical compounds. In this study we have extensively characterized the natural microbiome present on grapevine during the vegetative cycle using a metagenomic approach. The analysis revealed a surprising and complex microbiome associated with *V. vinifera* and a balance between the phytopathogenic and beneficial microorganisms. This is of utmost importance for the grapevine phytosanitary status, vine performance and quality of the wines. Furthermore, among prokaryotic population the most frequent microorganisms were represented by proteobacteria, actinobacteria and firmicutes whereas at the level of the eukaryotic population, the ascomycota phylum was the most abundant. Our samples were mainly characterized by the dominance of the *Aureobasidium pullulans* and members of the family Enterobacteriaceae, which are considered as beneficial microorganisms. However, phytopathogens as *Botrytis, Phomopsis* or *Guignardia* were also detected. Overall, the study of the global population of a vineyard allowed unveiling a great and complete microbial biodiversity during the vegetative cycle, inferring about the interactions between plant-microbe communities and reflecting about the impact of the co-habitation of both beneficial and phytopathogenic microorganisms on vine performance and wine quality.

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RED WINE QUALITY AFFECTED BY GRAPEVINE VIRUSES. F.J. Legorburu[1], E. Recio[1], E. López[2], J. Baigorri[2], M. Larreina[2], A. Remesal[2], J.F. Cibriaín[3], L. Caminero[3], J. Suberviola[3] and F. Aguirrezábal[3].
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Some grapevine-infecting viruses have thoroughly been studied from the point of view of yield reduction. The impact on fruit composition has also been studied for the leafroll complex, which is known to decrease sugar content and titratable acidity (Cabaleiro and Segura, 1996; Borgo and Angelini, 2002). However, only some wine parameters can be extrapolated from fruit analysis. Sugar is stochiometrically converted to alcohol during fermentation, but red wine usually undergoes a malo-lactic fermentation that decreases the malic acid content. Phenolic compounds are extracted from grape skins and seeds by the alcohol produced during fermentation. Anthocyanins and tannins, responsible for red colour and long-term stability, respectively, are among them. Moreover, while sugar and acidity measurements in fruit and/or must are standarized (Anonymous, 2006), this is not the case for phenolics. Thus, colour intensity, a critical parameter of red wine quality, cannot be reliabily predicted from fruit analyses. In comparison with the infuence of viruses on fruit quality, reports on their effect on the elaborated wine are scarce (Legorburu *et al.*, 2009; Malossini *et al.*, 2009). Separate microvinifications were done from infected and uninfected plants from the same vineyards over three years. One experiment studied the effect of the severe viruses *Grapevine fanleaf virus* (GFLV) and *Grapevine leafroll-associated virus 3* (GLRaV-3), whereas another experiment studied the effect of the mild viruses *Grapevine fleck virus* (GFkV) and *Grapevine leafroll-associated virus 2* (GLRaV-2). The effect of the severe viruses on the time course of grape ripening was studied in a third experiment. All the work was done with the red cv. Tempranillo in the Rioja appellation (Basque Country and Navarre, northern Spain). The effect of virus infection on wine quality was smaller than that of the vineyard or the vintage, but still statistically significant. The two severe viruses had opposite effects. GFLV-infected plants yielded a more concentrated wine, with a higher alcoholic degree, titratable acidity and colour intensity. This is probably explained by the severe fruit yield loss induced by this virus. GLRaV-3 was found to decrease the final alcohol content by half a degree and to seriously diminish colour intensity. The colour intensity differences were strong enough to be detected in tasting, not only by spectrophotometry. Contrary to expectation, no increase in wine acidity was detected. The follow up of grape maturation detected a higher concentration of malic acid in leafrolled vines, but this would disappear upon malo-lactic fermentation.

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BIOCONTROL: A PROMISING WAY TO MANAGE GRAPEVINE FUNGAL PATHOGENS. E. Ait Barka, S. Dhondt-Cordelier, A. Aziz, P. Trotel Aziz, F. Fontaine, A. Spagnolo, M. Magnin-Robert, F. Mazeyrat-Gourbeyre, S. Dorey and C. Clément. *Université de Reims Champagne Ardenne, URVVC EA 4707, Laboratoire de Stress Défenses et Reproduction des Plantes, Reims Cedex 02, France. E-mail : christophe.clement@univ-reims.fr*

For several years, biocontrol appeared as a promising way to manage pathogens in many crops. This question is of special interest in Europe because of the pesticide reduction trend, revealed by the constant deletion of authorized active molecules usable for plant treatments. Also, GMOs cultivation for alimentary purposes is not allowed in Europe, requiring developing new strategies to face pathogen attacks. Viticulture is one of the greater pesticide consumers in Europe although it represents less than 4% of the cultivated area. Therefore, developing environment-friendly procedures to protect grapevine against fungal diseases is of considerable importance.In viticulture, biocontrol using micro-organisms was shown to be possible focusing on *Botrytis cinerea* and wood decay diseases. Various experiments will be presented to demonstrate the efficacy of some biocontrol agents (BCA), focusing on the bacterium *Burkholderia phytofirmans*. The colonization of grapevine by this BCA leads to protection against *B. cinerea* and cold temperature under lab conditions. Additionally, the mechanisms of positive interactions between grapevine and *B. phytofirmans* will be detailed in some ways. It appears that *B. phytofirmans* is perceived by plant cells but that defense reactions are much less important than those generated by pathogenic bacteria, strongly suggesting that *B. phytofirmans* may avoid plant defenses. Also, *B. phytofirmans* helps grapevine neutralizing the toxic effects of defense reactions such as the biosynthesis of reactive oxygen species. Altogether, this bacterium acts by priming grapevine defense reactions to stress conditions. For practical application purposes, it filed experiments must be carried out, either spraying the bacterium or growing in nurseries plantlets that have previously been colonized by *B. phytofirmans*.

STATUS OF BOTRYTICIDES RESISTANCE IN FRANCE AND TOOLS FOR RESISTANCE MANAGEMENT. A.-S. Walker. *INRA-UR BIOGER-CPP, 78850 Thiverval-Grignon, France. E-mail: walker@versailles.inra.fr*

Grey mould, caused by *Botrytis cinerea*, is responsible for qualitative and quantitative damages to the grapevine. Therefore, it is controlled mainly by chemical fungicides, which have led to the selection of resistance towards most botryticides, especially in vineyards suffering high disease pressure, as in the Champagne area (France). This presentation will review the resistance status (evolution and frequency) of most fungicidal modes of action used to control grey mould in France (Leroux et al, 2002). It will also provide information about the mechanisms at work to determine resistance, with new insights about hydroxyanilides, SDHIs and QoIs resistance genetics (Bilalrd *et al.*, 2012; Fillinger *et al.*, 2008; Leroux *et al.,* 2010). A particular look will be also given to the emerging multidrug resistance (MDR) mechanism, inducing cross resistance between unrelated modes of action (Kretschmer *et al.*, 2009). In a second part, risk management will be dicussed, considering the population genetics diversity, fitness penalties occuring in resistant mutants and local selection pressure and available strategies.

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INNOVATIVE APPROCHES FOR REDUCING FUNGICIDE APPLICATIONS: A WEB-BASED DECISION SUPPORT SYSTEM FOR INTEGRATED VINEYARD MANAGEMENT. F. Salinari[1], T. Caffi[2], S.E. Legler[2], N. Ciliberti[2], V. Rossi[2]. *[1]Horta Srl., Piacenza, Italy. [2]Università Cattolica del Sacro Cuore, Istituto di Entomologia e Patologia Vegetale, Piacenza, Italy***.** *E-mail: vittorio.rossi@unicatt.it*

MoDeM_IVM (A web-based system for real-time Monitoring and Decision Making for Integrated Vineyard Management) is a project funded under the EU-FP7 Capacities programme and aims to develop an interactive, web-based decision support system (DSS) for integrated vineyard management. The prototype DSS, which will be delivered as the final product of the project within 2012, integrates: (i) a wireless sensor network (WSN) for the measurement of micrometeorologicial data and soil conditions; (ii) hand-held devices, such as a digital camera (for assessing the canopy status and berry maturation) and a tablet (for guiding the user in scouting activities on the plant status and the presence of diseases and insect pests), and (iii) a web-based portal that hosts the technological infrastructure of the DSS. The DSS provides decision supports and alerts on the basis of mathematical models and of the best options for managing the vineyard according to integrated pest management (IPM) principles. The following functionalities are embedded in the DSS: decision supports for canopy management and for disease and pest control, alert systems on potential abiotic stresses (such as low temperature injury and water stress), and estimate of the yield. Concerning disease control, the

DSS includes mechanistic models that simulate downy and powdery mildew epidemics (both primary and secondary infections) which have been previously developed and validated (Rossi *et al.*, 2008; Caffi *et al.*, 2010, 2011, 2012). Models for grey mould and black rot were also developed within the project. All these models use weather data, received in real-time from the WSN installed in the vineyard, for calculating at hourly intervals disease risk indexes, such as the available inoclum dose, the different stages of infection, and incubation and latency periods. In respect to insect pests, managemant suggestions are given for grape berry mouth, Mediterranean vine mealybug, and American grapevine leafhopper. The vineyard manager, to whom the DSS is addressed, can consult the DSS and access information via the Internet with a user-friendly interface at two levels of detail: (i) a synthetic overview of the diseases and pests risks, provided in the form of a dashboard; and (ii) detailed outputs on all key infection processes, provided in the form of graphs. To prove the viability of the DSS, demonstration trials were set in 2012 in Italy and Spain. The trials were designed in such a way to compare the farmer's usual vineyard management to the DSS-based management. A small plot was always maintained untreated against fungal diseases and pests, so as to monitor the natural dynamic of epidemics and infestations. Results of this first year of comparison are very promising since they showed that the DSS allowed to reduce fungicide applications by correctly scheduling the treatments and targeting them to the actual need of control. The same level of protection was obtained in the plots managed following the grower's practice or according to the DSS, but a lower number of treatments was performed in the latter.

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ENHANCED PHYTOSANITARY PERFORMANCE IN THE VINEYARD AND PHYSIOLOGICAL HOST RESPONSES INDUCED BY UV-C RADIATION. B. Berkelmann-Loehnertz[1], S. Klaerner[1], O. Baus[1], G. Herrmann[2], B. Flemming[2], R. Keicher[3], H.-P. Schwarz[3], M. Pfliehinger[4] and O. Loehnertz[4]. *[1]Geisenheim Research Center, Department of Phytomedicine. [2]Uv-Technik Meyer Gmbh, 63683 Ortenberg, Germany. [3]Department of Viticultural Engineering. [4]Department of Soil Science and Plant Nutrition, 65366 Geisenheim, Germany. E-mail: berkelmann@fa-gm.de*

UV-C radiation is a non-chemical method suitable for crop protection purposes. Normally, this approach is applied in the food industry and medicine technology for surface disinfection of wrapping compounds and packaging (e.g. plastic beaker, aluminium cover, bandaging material). Disinfection is achieved by short time radiation of the target area with tubular UV-C lamps at a range of 254 nm. Here, the biological efficacy reaches at most 99.9%, depending on quantity and quality of previous microbial spoilage and other technical features. Up to now, this method was applied particularly to greenhouse-grown horticultural crops. In viticulture, protection schedules are dominated by fungicides, especially against *Plasmopara viticola*, *Erysiphe necator*, *Botrytis cinerea* and *Guignardia bidwellii*. This is true for both integrated and organic viticulture. With the aim of reducing fungicide treatments in viticulture, we started a project for adopting UV-C technology. First, the dose-response relationship *in vitro* was elaborated for different propagation units of the above mentioned fungi. This was achieved using a small scale UV-C radiation unit for the use on Petri dishes and detached vine leaves. These data were confirmed *in situ* for pathogen development on potted vines after UV-C radiation at different stages of pathogenesis. These studies were carried out in the greenhouse with a double branch UV-C radiation unit. Phytotoxicity for the host was also checked. Finally, a large UV-C unit for vineyard application was developed. In 2012, a field trial was conducted in our experimental vineyard (cv. Riesling). Treatments were as follows: (i) control plots for each pathogen (apart from *G. bidwellii*); (ii) integrated crop protection standard programme; (iii) 50% reduction of the integrated standard programme alone and (iv) in combination with UV-C radiation of the canopy and the cluster zone. In addition, several host physiology parameters were investigated (e.g. amino acid concentration, antioxidative potential, stress indicator). Side effects of UV-C radiation on must quality and fermentation process were tested by microvinification using bunches of vines treated in 2011. A sensory panel tasted the young wines organoleptically. Different propagation units of the target microorganisms differed with respect to UV-C sensitivity *in vitro*. Dose-response relationships indicated that the biological efficacy *in vitro* was always less than 100%. In order to ensure a satisfactory biological efficacy *in situ* and to avoid phytotoxicity, the applied UV-C dose should range between 80 and 160 mWs/cm. The results of the field trial clearly revealed a potential for fungicide reduction. This was most obvious for *E. necator* and *B. cinerea*. In order to reach *P. viticola* on the lower leaf surface, it is essential to turn the leaves during the UV-C treatment. No negative side effects on host physiology and must fermentation were observed. However, an interesting increase of the amino acid concentration in berries occurred when leaves were treated with potentially phytotoxic doses of >160 mWs/cm.

DEVELOPMENT OF AN UNMANNED HELICOPTER AS AN ENVIRONMENTALLY FRIENDLY AND DRIFT-REDUCING SPRAYING DEVICE FOR GRAPEVINES IN STEEP-SLOPE VITICULTURE. S. Freimut. *DLR Mosel, Department Weinbau und Oenologie, 54470 Bernkastel Kues, Germany. E-mail. freimut.stephan@dlr.rlp.de*

The steep-slope vineyards are a characterising feature of the cultural landscape of the river valleys in the German viticultural areas. Spraying them by helicopter is one of the most important preconditions for the preservation of steep-slope viticulture. It can be assumed that by using a small unmanned helicopter the environmental impact (noise and spray drift) might be reduced. This as the aim of the project to achieve a high drift reduction by using this technology. It is also to be expected that this technology can considerably improve the quality of the pesticide application in comparison to the conventional helicopter and that it might be possible to preserve especially vineyards that are parceled out into very small plots (cost and effectiveness). The development of an autonomous control for such a small helicopter is absolutely necessary for meeting the aim of the project. The

main focus is put on the field tests which are used to assess the application quality. Primarily, these tests focus on the analysis of the coating and drift studies, as well as on determining the biological efficacy. Scoring according to the EPPO guidelines of the most important grapevine disease agents, *Plasmopara viticola*, *Uncinula necator*. The analyses of the coating concentrate for determining the amount of coating on the leaves and the bunchstem. The scheduled drift studies are going to be performed according to the BBA (German Federal Biological Research Centre) guideline VII.

ONTOGENETICAL RESISTANCE OF GRAPES - A CHANCE TO REDUCE FUNGICIDE RESIDUES IN WINES? W.K. Kast and K. Bleyer. *State Institute for Viticulture, Oenology and Fruit Technology, Weinsberg, Germany. E-mail: Walter.Kast@lvwo.bwl.de*

Residues of pesticides in wines are extremely low in comparison to fruits (Cabanis and Cooper, 1991). However, even extremely low residues cause serious problems in wine marketing in Germany. Daily newspapers and professional gourmet journals refer to negative quality criteria in wines although values are much lower than the maximum of tolerable residual values. Investigations by Stark-Urnau and Kast (1999) and Gadoury *et al.* (2003) indicated that developing grapevine bunches are highly susceptible to infections by *Erysiphe necator* exclusively between one week before anthesis and the point of time when berries reach a diameter of 2 mm, the so called "open window period" (OWP). *Plasmopara viticola* penetrates its host exclusively through stomata (Pearson and Goheen, 1988). No functional stomata are present on the surface of grape berries, when they reach the size of peas. Only few functional stomata are present on the rachis, which are mainly damaged by a high number of infectious spores, which are normally produced on the upper leaves and reach the bunches during rainfall (Hill 2012). Ontogenetical resistance should offer the chance to reduce fungicide residual without any risk of loss. This paper deals with the disease scores of field trials, if we leave out the grape zone for the last sprays and the effect on fungicide residuals on grapes and wines.

Materials and methods: Four field experiments against downy mildew were carried out in a cv. Riesling vineyard and three against powdery mildew in a vineyard planted with cv. Cabernet Dorsa. The experimental design and disease evaluation criteria are described in detail by Kast and Bleyer (2011), i.e. single row plots with untreated and artificially infected rows on both sides, application by tunnel sprayer, four replications. Vitimeteo-Plasmopara and Vitimeteo-OiDiag were used to plan the start and the intervals of either seven or eight sprays.

Results: Leaving out fungicides against downy and powdery mildew in the grape zone in the last spray did not cause any effect on the amount of downy mildew attack of grapes and leaves, even under a high disease pressure. No differences on the leaves in three powdery mildew experiments were detected in 2009-2012. Differences on grapes did not exceed the significance level of 5%. No residues of any of the applied fungicides (Copper, Dithianon, Dimethmorph, Folpet, Myclobutanil) were found in wines, if the last two sprays were left out. For Boscalid residues were within the detection limit. On the grapes of normally treated vines (until mid of August), Boscalid, Dithianon, Cyprodinil and Myclobuthanil residues were detected, though at low level. In wines, only Boscalid and Myclobuthanil could be detected, in extremely low values, which were about 1/100 of the tolerable level. Thus, we recommend not to spray fungicides on grapes but on leaves, because bunches are ontogenetically resistant at this stage in contrast to leaves.

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AN INHIBITOR-FREE MULTIPLEX POLYMERASE CHAIN REACTION ASSAY FOR THE EARLY DETECTION AND IDENTIFICATION OF *PLASMOPARA VITICOLA* AND *BOTRYTIS CINEREA* SPORES IN AIR-BORNE ENVIRONMENTAL SAMPLES TO REDUCE PESTICIDE IMPACT IN THE VINEYARD. V. Huerga, B. Salas and A.M. Díez-Navajas. *NEIKER, Plant Production and Protection Department, Box 46, 01080 Vitoria-Gasteiz, Spain. E-mail: adiez@neiker.net*

Plasmopara viticola, a specific obligate biotrophic agent causing downy mildew and *Botrytis cinerea*, a non-specific pathogen that causes grey mould, are two of the most important pathogens affecting grapes worldwide. Both of them decrease yield and fruit quality and lead to important economic losses. Air is probably the most important agent for the transport of spores of these pathogenic fungi. Tools for a quick monitoring of the presence and severity of infection in the vineyard, before disease symptoms appear on the vines, would help growers to control the diseases earlier and apply chemicals more sparingly along the season, so as to reduce phytochemical inputs as much as possible and without crop loss risk. Passive traps, consisting of microscopic slides coated with an inert sticky substance, are the simplest method to collect air-borne biological particles (Iturritxa and Ganley, 2007). However, not only biological particles adhere to the traps. Copper-based fungicides, composed of copper hydroxide or copper sulphate, are sprayed to control *P. viticola* and *B. cinerea* in the vineyard. Drops of these compounds fix to spore traps. In the laboratory, traps are processed to be analyzed by multiplex PCR, that allows the simultaneous identification of two or more target DNA sequences directly from traps. But PCR is inhibited by copper particles, yielding unsatisfying results. To obtain a good quality and inhibitor-free DNA, traps with different combinations of pathogen spores and copper solution ($CuSO_4$) aliquots, were washed in two steps previously to DNA extraction: first using a 100 mM EDTA solution, a copper chelating agent, and a second wash with water to remove the remaining particles. Then the trap content was placed into a tube, to which glass beads were added for spore breaking and efficient DNA extraction. Pathogen-specific primers were used: (i) for *P. viticola*, GiopR: TCCTGCAATTCGCATTACGT and GiopF: GGTTGCAGCTAATGGATTCCTA (Valsesia *et al.*, 2005), generating a 208 bp PCR product; (ii) for *B. cinerea*, BOTYF4: CAGCTGCAGTATACTGGGGGA, and BOTYR4: GGTGCTCAAAGTGTTACGGGA, generating a 533 bp fragment (Ma and Michailides, 2005). These primers, together with BSA, were

added to the reaction mix to increase and assure the specificity of the method. Results showed a correct amplification of *P. viticola* spores from $CuSO_4$-treated traps washed as above. Samples of *P. viticola* spores from $CuSO_4$-treated traps and no washing showed PCR inhibition. In the same samples, *B. cinerea* was amplified simultaneously with *P. viticola* and correctly in a second amplification round, adding to the first reaction amplicon a pair of internal primers of the sequence generated by the BOTY pair, designed with the program Primer3 (http://www.frodo.wi.mit.edu/primer3) in order to perform a nested PCR. These internal primers were BOTYF′: GATCCATCACT CCCACCACT and BOTYR′: CCTCTAGGGTCACGTGGAAG, generating a 235 bp fragment. This proved an effective, rapid and inhibitor-free method for the simultaneous and early detection of *P. viticola* and *B. cinerea* in copper-contaminated airborne environmental samples, before downy mildew and grey mould disease symptoms appear in the vineyard and helping to make culture management decisions.

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TIMING OF FIRST SHOOT TOPPING AND ITS IMPACT ON GRAPEVINE CANOPY AND CLUSTER MORPHOLOGY AS WELL AS ON SUSCEPTIBILITY TO BUNCH ROT.

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Shoot topping, hedging or summer pruning is an essential practice in the vertical shoot positioning trellis system, which respresents the standard management system in many cool climate viticultural regions. It is targeting a light canopy and a maximal sunlight exposure. Furthermore, shoot topping prevents extensive self-shading of the canopy, leads to a better ventilation and a faster drying process in the cluster zone. In practical viticulture, the timing of the first shoot topping strongly varies depending on the canopy architecture, the region and the practices of the grapegrowers. Investigations under cool climatic conditions according to Hügelschäffer (1990) using *Vitis vinifera* cvs Riesling and Müller-Thurgau indicated that the timing as well as the intensity of the first shoot topping has an impact on grape physiology. Results showed that yield in particular is influenced by the time but hardly by the intensity of first shoot topping. Indeed, early shoot topping (pre-bloom) led to an increase in yield (Hügelschäffer, 1990). However, little is known about the impact of the timing of first shoot topping on canopy and cluster morphology as well as on bunch rot. Hence, the aim of the present work was to determine the optimal timing of this management practice in relation to canopy structure, cluster morphology and the establishment of bunch rot or grey mould caused by *Botrytis cinerea*, representing one of the economically most important grape diseases worldwide. Field trials were conducted in 2012 in Remich/Luxembourg using the white *Vitis vinifera* cvs Riesling and Pinot gris, which are commonly grown in Luxembourg and highly susceptible to grey mould attack because of their frequently compact cluster structure. First shoot topping was done at seven different time points between pre-bloom (BBCH 57) and four weeks after the end of bloom (BBCH 75) at weekly intervals (treatments 1 to 7) [for BBCH scale see Eichhom and Lorenz (1977)]. Moreover, shoot tips remained uncut and were wrapped around the upper wire of the trellis (treatment 8). Each treatment was replicated four times. The lateral shoot growth (biweekly measurements), the canopy structure [Point Quadrat according to Smart and Robinson (1991)], the cluster morphology using bunch density index according to Ipach *et al.* (2005), the bunch rot disease progress, the yield as well as quality parameters of the must such as total soluble solids, total acidity and nitrogen content were assessed. The development of the canopy was followed using digital color photographs, taken under standardized conditions. An image segmentation routine was developed to distinguish between green canopy elements and other image segments. This approach allowed to quantitatively assess the increase of the canopy surface in the different treatments over time. First results showed that, the earlier the shoot tips were removed, the faster the lateral shoots began to sprout in the upper part of the canopy. However, in the cluster zone, the assessments of lateral shoot lengths, Point Quadrat (Smart and Robinson, 1991) as well as digital image analyses pointed out that the lateral shoot growth and, consequently, the cluster exposure was only slightly modulated by the timing of shoot topping. However, the density index (Ipach *et al.*, 2005) at bunch closure (BBCH 79) indicated a clear influence of the timing of the first shoot topping on cluster morphology. Indeed, the clusters were most compact in the early treatments (shoot topping around flowering) and cluster density declined with temporal distance to bloom (Fig. 1). According to the literature, higher cluster compactness is associated with a higher predisposition of grape clusters to bunch rot (Molitor *et al.*, 2012), which besides yield losses can result in decreased wine quality by the generation of off-flavours, unstable color, oxidative damage, difficulties in clarification and premature aging. In conclusion, the presented results indicate that postponing the date of first shoot topping to the latest possible date may have potential to improve the grape health status and, as a consequence, the wine quality.

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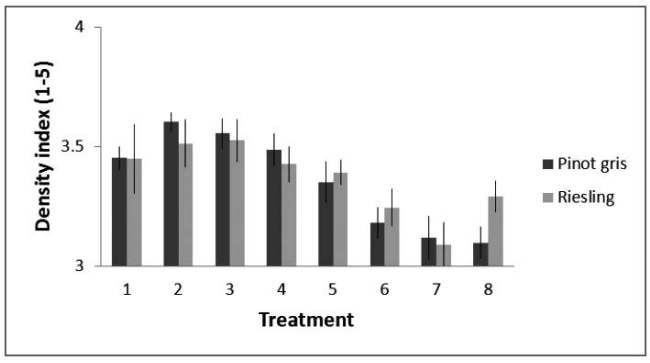

Fig. 1. Average values of the density index in the cvs Pinot gris (assessment date: 07.08.12) and Riesling (14.08.12); n = 100 clusters per plot; error bars = standard error. Treatment 1: shoot topping approximately one week before bloom (BBCH 57); treatment 2: beginning of bloom (BBCH 61); treatment 3: end of bloom (BBCH 68); treatment 4: one week after the end of bloom; treatment 5: two weeks after the end of bloom; treatment 6: tree weeks after the end of bloom; treatment 7: four weeks after the end of bloom; treatment 8: shoots remained uncut and were wrapped around the upper wire of the trellis.
Bunch density index: 1: very loose bunch, flexibility >90°; 2: loose bunch, flexibility 45 to 90°; 3: compact bunch, flexibility 10 to 45°; 4: very compact bunch, flexibility <10°; 5: tight bunch without any flexibility.

WHAT ABOUT CARBOHYDRATE METABOLISM OF GRAPEVINE FLOWERS DURING *BOTRYTIS* ATTACK?

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Botrytis cinerea is highly pathogenic to grapevine, and causes grey mould disease, thus affecting the yield and wine quality. Control of grey mould is mainly achieved by pre- and/or post-harvest chemical applications. However, controlling *B. cinerea* is a challenge because of its adaptability to various environmental conditions and development of fungicide resistant strains. A better understanding of plant/pathogen interaction during the early stages of infection is the key factor to improve control. The aim of our study was to understand the modification in carbohydrate metabolism in the flowers of grapevine during infection by *B. cinerea* VD 22 strain which is resistant to the benzimidazole class of fungicide. During infection, an alteration in net photosynthesis, accompanied by changes in stomatal conductance was observed. Measurement of chlorophyll a fluorescence, which reflects the functionality of the photosynthetic apparatus, showed that relative quantum yield of photosynthetic system II might be involved in the alteration in the net photosynthesis. In addition to this, our results demonstrate significant changes in the starch content in infected flowers as compared to control as soon as 12 hours post infection. The level of soluble sugars like glucose and saccharose also varied during infection. Thus, taken as a whole, our results demonstrate that during infection by *B. cinerea* in grapevine inflorescence, carbon uptake is altered the plant ability to assimilate carbon through photosynthesis is disturbed. Further investigations into defence signalling pathway in grapevine inflorescence during infection would help to decode several mechanisms during plant pathogen infection and would allow improving control of the pathogen in the fields.

ECO-FRIENDLY METHODS TO CONTROL *BOTRYTIS CINEREA* INFECTION DURING THE PRODUCTION OF PROPAGATING MATERIALS OF GRAPEVINE.

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Botrytis cinerea has many host plants (235 listed) and induces gray mould (Coley-Smith *et al.*, 1980), a disease harmful also to the grapevine nursery industry as it damages stored canes or grafted plants during the callusing period. Conditions for fungal infections are ideal during the pre-forcing stage and storage, because of the high temperature and humidity, thus *B. cinerea* can easily attack the outbursting buds. Many plants may be concentrated in storage rooms in small paces or in callusing boxes. Although the fungicide-based management of fungal attacks is still commonly implemented, the number of chemicals authorised by the European Union regulation is becoming progressivley narrower. Therefore it is necessary to develop eco-friendly control methods. Our aim was to develop a technology, which combines the use of natural materials with techniques used in organic farming. Based on the findings of Nagy (2003) and Nigro *et al.* (1998) we studied the effect of ultraviolet light (UV-C, 190-280 nm wavelength) on *B. cinerea* grown *in vitro* (Petri dishes) and in infected grape canes. A UV lamp was placed 250 mm above Petri dishes and various doses (0, 1, 3, 10, 30, 60 minutes) of UV-C light were applied. We also studied the evolution of the pathogen in daylight and dark conditions and its reaction after treatment with cowmilk. Experiments were also performed *in vitro* with acetic acid assessing its impact on the growth of the fungus. Results showed that the 253.7 nm UV-C light can be used effectively to destroy the already developed conidia. However, since the radiation stimulated the development of immature propagules the UV-C radiation dose and the treatment distance need to be adjusted. More conidiophores and conidia were formed in daylight than in the dark, while the different conditions did not significantly change the mycelial growth characteristics. The tests with cowmilk showed that "bacteria drops" in Petri dishes prevented the growth of the fungal mycelium, although the smooth development of propagules that occurred did not change the vitality of the colony. Furthermore, the growth of *B. cinerea* was strongly inhibited by acetic acid. A field experiment is in progress on cv. Muller Thurgau, where the impact of cowmilk and acetic acid will be evaluated, either to prevent or control the infection.

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THE INFLUENCE OF FLAVOR-RELEASING β-GLUCOSIDASES IN WINERY TECHNOLOGY.

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The sensory perception of wine is very complex and is formed by a variety of taste and odor-active compounds, such as terpenes, esters, aldehydes and methoxypyrazines (Styger *et al.*, 2011). Terpenoid compounds, that play a major role in wine aroma determination, can be present either as bound or as free glycosides in

grapes or wine. The glycosidically-bound monoterpenes occur more frequently than the free ones (Günata *et al.*, 1985). Even though glycosidic monoterpenes have no direct influence on the flavor of grapes and wines, they can act as precursors for a variety of typical wine aroma compounds by selective enzymatic release, thus enhancing the varietal aroma of the wine (Mateo and Jiminez, 2000; Rapp, 1992). Enzymes with hydrolytic activity such as pectinylases, polygalacturonases, pectin esterases, cellulases, cinnamylesterases or β-glucosidases are used in oenology for must clarification, colour and juice extraction from grape skin, stabilization of polyphenols and release of flavors. In particular, mixtures of these enzymes are often used to improve the flavor quality during fermentation by cooling. To avoid that too much flavor is released by the β-glucosidase activity, the enzyme mixture is precipitated by a bentonite fining and is thus lost. Besides the loss of active enzyme, it is disadvantageous that new enzyme preparation must be used for each new fermentation batch, which constitutes a considerable cost for winemakers. Another disadvantage is that not all enzymes are precipitated, thus some activity is retained in the wine. So treated wines strongly reduce the sensory perception after six months up to a year. Pectinolytic enzyme preparations with a β-glucosidase side activity are especially interesting as they can help forming the typical varietal wine aroma (Rapp, 1992), due to the release of aromatic terpenes. In the framework of the cooperation project MAGNENZ, β-glucosidase and other effective glycoside hydrolytic enzymes were immobilized on iron oxide magnetic particles and their efficiency determined during alcoholic fermentation. In a first step, the enzyme preparations were characterized for assessing their potential use in wine making. For determining the activity of the enzymes utilized, the use of p-nitrophenyl-β-D-glucopyranoside (pNPG) as substrate emerged as an efficient and appropriate method. The following enzyme preparations were used: two oenological preparations (Lallzyme Beta and AR 2000), a cellulase and a βglucosidase from almonds. Furthermore, it was found that the origin of β-glucosidase is critical for the enzyme activity in the wine (Ketudat Cairns and Esen, 2005). Results demonstrated that Lallzyme Beta and AR 2000, both of which are obtained from the fungus *Aspergillus niger* had a satisfactory activity even at low pH. In contrast, the cellulase obtained from *Trichoderma longibrachiatum* showed a lower activity compared with the commercial preparations Lallzyme Beta and AR 2000. Since β-glucosidase from almonds did not perform well, AR 2000 and Lallzyme Beta proved to be the more suitable for use in wine-making. The selected cellulase was found to be only of limited applicability and the β-glucosidase from almonds was ineffective. Of interest in the characterization was that AR 2000, cellulase and Lallzyme Beta gained activity by increasing ethanol levels, similarly with increasing fructose concentrations up to 100 g/l. Glucose, however, is an inhibitor of all enzyme preparations. Even a concentration as small as 5 g/l it inhibits the enzyme preparations up to 50%. In addition, inhibitory effects by the typical wine acidic pH values and low temperatures were also detected, in agreement with literature reports (Palmeri and Spagna, 2007; Caldini *et al.*, 1994; Murray *et al.*, 2004). The results indicate that it is useful to add the enzyme preparations at the end of fermentation.

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INFLUENCE OF LEAF REMOVAL ON MICROCRACK FREQUENCY OF GRAPE BERRIES.

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Leaf removal in the cluster-zone between early bloom and bunch closure is an efficient option to reduce grey mould (causing agent: *Botrytis cinerea*) infection in grapevine (*Vitis vinifera*) clusters under various climatic conditions. These effects are mainly assumed to be caused by: (i) an improved microclimate due to better sun and wind exposure in the cluster-zone and (ii) an open cluster structure (Molitor *et al.*, 2011). Surface moisture induces microscopic cracks (microcracks) in the cuticular membrane (CM) of the grape berries, thereby reducing the CM barrier function against pathogens like *B. cinerea* and against uncontrolled water transport which promotes cracking of the fruit (Becker and Knoche, 2012). Improved sun and wind exposure of grape clusters should reduce surface moisture and therefore possibly the frequency of microcracks on the berries. However, to our knowledge, it is not known whether leaf removal in the cluster-zone indeed reduces the frequency of microcracks on grape berries. To answer this question a field trial (8 plants per plot, 4 times replicated, randomized block design) was conducted in Remich/Luxembourg in 2010 in the white-berried cvs Pinot gris and Riesling comparing leaf removal at BBCH 73 (berries groatsized) to the untreated control. Around harvest, 25 berries per treatment and replication were randomly sampled from each cultivar and the frequency of microcracks around the stylar scar analyzed. Fruits were incubated for 15 min in the fluorescence dye acridine orange (0.1% w/v), rinsed for 10 sec with deionized water and blotted with tissue paper. Thereafter, images of the stylar scar region of the berries were taken with a fluorescence microscope (MZ10F, Leica Microsystems, Germany; 440-480 nm excitation wavelength, ≥ 510 nm emission wavelength; camera DP71, Olympus Europa Holding, Germany). To index the number of microcracks, the area infiltrated by the dye was quantified using a rating scheme and image analysis. For rating, five people assessed the fluorescing area on a 0 (no infiltration) to 3 (heavy infiltration) scale. For image analyses the Cell^P software was used (Olympus Europa Holding, Germany). Data were subjected to analysis of variance (SAS version 9.1.3; SAS Institute, USA) and presented as means ± SE. The number of microcracks varied between fruits. In cv. Riesling, no significant difference in the number of microcracks were observed between leaf removal (visual examination: score 1.43 (±0.08); image analysis: 8.24 (±1.32)% dye infiltrated area) and the untreated control (visual examination: score 1.36 (± 0.08); image analysis: 6.98 (±1.23)% dye infil-

trated area). In cv. Pinot gris the frequency of microcracks was lower in the defoliated vines (visual examination: score 1.30 (± 0.07); image analysis: 2.89 (± 0.43)% dye infiltrated area vs. visual examination: 1.56 (±0.08); image analysis: 3.65 (±0.47)% dye infiltrated area for the control, respectively). This effect, however, was significant only for the rating scores, but not for image analysis. The preliminary data suggest that the effect of cluster-zone leaf removal on the frequency of microcracks may depend on the cultivar. While in cv. Riesling there was no observed effect, in cv. Pinot gris leaf removal -besides other treatments- may be effective in decreasing the frequency of microcracks.

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"VITIMETEO BLACK ROT": DEVELOPMENT AND VALIDATION OF A NOVEL GRAPE BLACK ROT MODEL.

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Grape black rot, caused by *Guignardia bidwellii* (Ellis) Viala et Ravaz [anamorph: *Phyllosticta ampelicida* (Engelman) Van der Aa] is a fungal disease native to North America. In Europe, it was already reported at the end of the 19th century but limitedly to regions with particularly humid conditions in spring and early summer. Since the beginning of the 21st century, the disease appeared in several European grapegrowing regions such as the Moselle and Rhine valley (Germany + Luxembourg), Tuscany and Sardinia (Italy), Dão, Bairrada, Minho, Alentejo (Portugal), Austria, Hungary and Romania, where it was previously almost unknown. Severe epidemics caused yield losses of up to 100%, making black rot one of the economically most important fungal diseases of grapevine in these regions. Consequently, the control of grape black rot is one of the major challenges for the grapegrowers in the affected regions. So far, black rot management strategies are focused mainly on routine applications of efficient fungicides containing strobilurins, triazoles or dithiocarbamates. However, a precise decision support system aiming at a more targeted timing of fungicide treatments is still missing. To fill this gap, the grape black rot model "VitiMeteo Black Rot" taking into account meteorological data and the biology of the pathogen was developed based on the existing decision support platform "VitiMeteo", an internationally established forecasting system for grape disease models and management of weather data (see http://www.vitimeteo.info). "VitiMeteo Black Rot" uses the following input parameters:

- local meteorological data and a five-day-weather-forecast (hourly temperatures, precipitation, wetness periods),
- a simulation of the success and the intensity of infection events on grape organs depending on temperature conditions and wetness duration according to Molitor (2009),
- the susceptibility of grape organs for infections depending on their phenological development according to Molitor and Berkelmann-Loehnertz (2011) and Kuo and Hoch (1996),
- the length of the incubation period depending on temperature conditions and phenological development according to Molitor *et al.* (2012).

After processing, the model delivers the following output parameters (past and five day forecast):

- occurrence and intensity of infection events
- present susceptibility of grape organs
- present status of incubation period progress

To validate the efficiency of the model under different climatic conditions and for different grape cultivars, a European black rot monitoring network was created in 2012 including seven locations in five European countries (Remich/Luxembourg, Florence/Italy, Nelas/Portugal, Freiburg and Oppenheim/Germany, Changins and Cugnasco/Switzerland) and seven cvs (Rivaner, Sangiovese, Trebbiano Toscano, Jaen, Gutedel/Chasselas, Portugieser, and the hybrid IRAC 2091). The monitored plants remained either untreated or were treated only with fungicides without known black rot activity. In each location, the appearance and severity of black rot symptoms were assessed two to three times a week on main leaves as well as on clusters throughout the whole season. The first results of the validation show that infection events as well as the lengths of the incubation periods were accurately simulated by the model. Indeed, the observation of new symptoms could directly be connected with predicted infection events. Adjustments respecting differences in the susceptibility and ontogenetic resistance of different cultivars should further improve the precision of the model.

The final version of the model is supposed to constitute a precise decision support system for a targeted timing of fungicide applications against black rot, which allows for a combination of: (i) an optimum disease control and (ii) a reduction of pesticide input. "VitiMeteo Black Rot" is foreseen to be available via the internet platform "VitiMeteo" starting in 2013.

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RECOVERY OF LOST WINE FLAVOR BY FRACTIONATED CONDENSATION.

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Flavors are an important quality parameter of wines although they occur only in small amounts. They are the basis for the character and uniqueness of different wine types and growing areas (Drawert and Rapp, 1966; Rapp, 1990). The sensory impression is

caused by the high volatility of flavor active substances, whose volatility is a problem during production and processing. Although repeated recirculations and other wine treatments provoke a great loss of flavor, it is the fermentation that causes the main leakage. Carbon dioxide, produced by yeasts during fermentation, is an efficient flavor carrier that transports a lot of flavors out of the tank. This fact was stimulated our research that was aimed at developing a cooling system to recover the lost flavor compounds. We used the technique of Peltier elements to reach -20°C with continuously adjustable temperature setting. Hence, it was possible to condense the lost flavors and analyse their composition using gas-chromatography coupled with mass spectrometry. In addition, it was investigated if these volatile compounds have an antioxidative potential and sanitary effects. Therefore, internationally applied tests like the TEAC, DPPH or FRAP were used. Our studies showed that the flavor condensate consists of water, ethanol and a mixture of different flavor-active compounds especially fruit esters, i.e. secondary flavors built during fermentation. These flavors have exclusively positive sensory characteristics (Li *et al.*, 2008; Perestrelo *et al.*, 2006; Takeoka *et al.*, 1992) so that their recovery is beneficial. Adding this condensate to an alcohol-free or a low alcohol wine results in a more tangy, more fruity note and a reduction of the perception of acid. Furthermore, an antioxidative potential of the flavor condensates was found. It was negligible compared to the potential of polyphenols due to the low amount of flavors in wine (Kähkönen *et al.*, 1999). Currently, a number of experiments are underway to prove the influence of grape cultivar, fermentation temperature, yeast type and addition of enzymes on the composition of the recovered flavor condensate. However, further improvements of the condensator are also needed. Through the analysis of the flavor condensate it is conceivable that diseases or microbial disorders of the grapes which may cause anomalous fermentation can be detected timely. This will make the early identification of a possible damage feasible, thus saving time and money. Further researches are, however, necessary to determine typical off flavor compounds that are built and get lost as a consequence of microbial affections of grapes.

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PLASMOPARA VITICOLA: COMPARISON OF CONTROL STRATEGIES UNDER HIGH DISEASE PRESSURE.

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Grape downy mildew (causal agent: *Plasmopara viticola*) is one of the most devastating diseases of grapevines. In cool climate regions with high humidity more than five fungicide applications are necessary to restrain disease development. The number of treatments depends primarily on disease-favouring weather conditions in May and June (bud burst and bloom). In organic viticulture, pesticide use is severely restricted, but not copper-containing fungicides which are employed to control *P. viticola*. Due to their generally lower efficacy, farmers are forced to apply more treatments compared to integrated viticulture (maximum level of copper input: 3 kg pure copper per ha and year). Because of this our research was aimed at a general reduction of both synthetic fungicides and those containing copper as active ingredient. In order to highlight potential differences between the reduction strategies under trial, intense disease pressure conditions were established by artificial inoculation with *P. viticola*. In 2012, a field trial to control *P. viticola* was performed in an experimental vineyard of the Geisenheim Research Center, Rheingau region, Germany (49°59'N, 7°57'E). Treatments were as follows:

- untreated control (water)
- copper – 3 kg/ha * year (Cuprozin Progress)
- copper – 2 kg/ha * year (Cuprozin Progress)
- protective standard (Folpan 80 WDG)
- systemic standard (Profiler)
- protective standard+phosphonate 1 (Folpan 80 WDG + Veriphos)
- copper – 3 kg/ha * year+phosphonate 2 (Cuprozin Progress + Frutogard)

The trials were conducted in our experimental vineyard (cv. Müller-Thurgau) according to the EPPO-Guideline PP 1/31. At the developmental stage BBCH 55 young shoots were inoculated with a suspension of *P. viticola* sporangia to facilitate natural infection. Treatments were carried out with a pneumatic application equipment (Schachtner, Germany). Applications started at the end of June and the spray interval between treatments was 10 days. At two time points (BBCH 77 and BBCH 81), the incidence of *P. viticola* infection on leaves and clusters was assessed. The infection level increased dramatically until the first assessment date (BBCH 77; mid of July). Disease incidence on the leaves was about 91% in the untreated control plots, while in the copper plots it was 60% (3 kg/ha * year) and 69% (2 kg/ha * year), respectively. The "protective standard" came up to 55% and both "systemic standard" and "protective standard+phosphonate 1" reached about 10% disease severity. Infection level on the bunches in the untreated control plots and in all treatments with copper increased very much (more than 90% disease incidence). The treatment "protective standard" showed a degree of infestation of about 52%. In contrast to these results, "systemic standard" amounted to 9% and "protective standard+phosphonate 1" showed 11% disease severity. At the time of the second assessment (BBCH 81; beginning of September) only the treatments "systemic standard" and "protective standard+phosphonate 1" could control disease development sufficiently under the given conditions with extremely high disease pressure.

EVIDENCE OF MYCOVIRUSES AND TRANSPOSONS IN *BOTRYTIS CINEREA* STRAINS COLLECTED FROM GRAPE BERRIES AND THEIR INFLUENCE ON MYCELIAL GROWTH.

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Mycovirus infections of fungal strains and the presence of genetic elements such as transposons can have significant effects on distinctive phenotypic traits of phytopathogenic fungi. The ubiquitous fungus *Botrytis cinerea* is known to host two filamentous single-stranded RNA viruses, *Botrytis virus X* (BVX, genus *Botrexvirus*) and *Botrytis virus F* (BVF, genus *Mycoflexivirus*), as well as two transposable elements (TEs), Boty and Flipper. In the present study we assessed the genetic diversity of 100 *B. cinerea* isolates, sampled from cv. Riesling within a small-sized geographical area (Rheingau region, Germany; 49°59'N, 7°57'E) by examining and classifying them according to the presence of mycoviruses and TEs. The sampling locations differed in management system (integrated, bio-organic and bio-dynamic), level of nitrogen fertilization (0, 60 and 150 kg of N/ha per year) and soil properties. In addition, the influence of mycoviruses and TEs on mycelial growth of the respective strains was examined *in vitro* at different temperatures (4, 7, 10, 15, 20, 25 and 30°C). In 37% of the analysed isolates the presence of one or both *B. cinerea* mycoviruses was confirmed, representing the first record of *B. cinerea* mycoviruses in German isolates. Mycelial growth of *B. cinerea* strains containing BVF was significantly reduced at lower temperatures. Furthermore, nearly all isolates were screened positive for the presence of a transposon. Such knowledge may contribute to a better understanding of the evolutionary processes and the genetic structure of *B. cinerea* populations associated with grapevine plants. If negative impacts of virulent *B. cinerea* isolates would be reduced by the presence of other *B. cinerea* strains harbouring a mycovirus, a selective support of certain strains in the vineyard might have implications for innovative future control programs.

CONTROLLING BLACK ROT (*GUIGNARDIA BIDWELLII*) AND DETERMINATION OF ITS MYCOTOXINS. B. Schildberger, M. Mehofer and A. Grießbacher. *Federal College and Institute for Viticulture and Pomology, Klosterneuburg, Austria, Institute of Chemistry, Biology and Plant Protection. E-mail: barbara.schildberger@weinobst.at*

After the first infrequent observation of black rot (*Guignardia bidwellii*), an increased incidence of infections has been observed in different grape-growing regions of Austria since 2010. Therefore the aim of this work was to investigate the influence of different active agents on the growth of black rot. A further aim was to detect if black rot produces mycotoxins. To test the efficacy of the particular active agents, the growth of black rot was investigated with protective as well as with curative treatment in the laboratory. With the plate diffusion test, particular active components were tested at different concentrations with respect to the formation of zones of inhibition. These investigations confirmed the good effect of different plant protecting agents such as strobilurins and triazoles. However, the plant protecting agents containing copper octanoate (Cueva) and copper hydroxide (Cuprozin flüssig) did not show sufficient effects against black rot. Because some fungal species that reside on grapes produce the mycotoxins ochratoxin A and patulin, an attempt was made to find out if these mycotoxins are also produced by black rot. Analyses carried out at different times points showed that ochratoxin A and patulin, which are two of the most frequent mycotoxins on grapes, could not be detected.

ESCA (BLACK MEASLES) OF GRAPEVINES: NEW INSIGHTS IN PLANT PATHOGEN INTERACTIONS. J. Fischer[1], S. Schwarz[2], L. Licht[3], T. Opatz[4] and E. Thines[1]. *[1]Institute of Biotechnology and Drug Research, 67633 Kaiserslautern, Germany. [2]Max Delbrück Center For Molecular Medicine, Research Team for Cell Differentiation and Tumorgenesis, 13092 Berlin, Germany. [3]Environmental Campus Birkenfeld, Institute of Process Engineering, Hoppstädten-Weierbach, Germany. [4]Johannes Gutenberg-University Mainz, Institute of Organic Chemistry, 55128 Mainz, Germany. E-mail: fischer@ibwf.de*

Esca is a destructive disease of the grapevine caused by fungal pathogens that can behave as endophytes, like *Phaeomoniella chlamydospora, Phaeoacremonium aleophilum* and the wood decaying fungus *Fomitiporia mediterranea*. It has been suggested that phytotoxins, which are responsible for disease development, are secreted by these fungi. Several toxins produced by Esca-associated fungi have been reported previously. However, in order to characterize phytotoxic metabolites produced by the pathogenic fungi, these were grown individually and in co-culture as batch or fed-batch fermentations. Several new metabolites were identified by bioactivity-guided isolation, HPLC-MS as well as by NMR-analysis. Amongst the novel bioactive metabolites identified there were siderophores, e.g. triacethylfusigen, as well as linoleic acid, phaeofuran and methoxycoumarin. Also substances produced by *Vitis vinifera* as defence were found to be modified by the pathogenic fungi. It was found that the production of the compounds was altered under stress conditions. Some efforts were also made for analysing plant-fungus interactions. To this aim, fluorescent-transformants of the associated fungi were generated and characterized. A further attempt was to generate transformants of *F. mediterranea*, the only basidiomycete involved. First results indicate that only species-specific promoter sequences may work as support for the transcription. These investigations lead to new insights in plant pathogen interactions of Esca disease.

MICROSCOPICAL INVESTIGATION OF EARLY INFECTION EVENTS OF *GUIGNARDIA BIDWELLII* ON GRAPE CULTIVARS WITH DIFFERENT LEVELS OF RESISTANCE AND EUROPEAN WILD GRAPES. C. Tisch[1], R. Eibach[2], P. Nick[3] and A. Kortekamp[1]. *[1]Dienstleistungszentrum Ländlicher Raum (DLR) Rheinpfalz, 67435 Neustadt an der Weinstrasse, Germany. [2]JKI, Institut für Rebenzüchtung Geilweilerhof, 76833 Siebeldingen, Germany. [3]Karlsruher Institut für Technologie, Botanik I, 76128 Karlsruhe,Germany. E-mail: christine.tisch@dlr.rlp.de*

Guignardia bidwellii, the causal agent of black rot (BR) of grapevine, is an important fungal pathogen from North America that can lead to severe crop losses. In the last decade, it had a significant impact on European grape production especially in organic farming, where effective protection strategies are currently not available but urgently needed. It is known that some *Vitis vinifera* cultivars differ in their tolerance towards BR. However, there is still a lack of knowledge on the interaction between *G. bidwellii* and different *Vitis* species, the mechanisms of resistance, and the initial infection process in general. Fluorescence microscopy was used to characterise and analyse the early infection structures of the asexual cycle of *G. bidwellii*. In addition, differences in the infection process on leaf discs of European wild grapes, cv. Müller-Thurgau (susceptible) and cv. Börner (tolerant) were investigated in terms of pycnidiospore attachment, spore germination and development of appressoria. In a first infection step, pycnidiospores need to attach firmly to the leaf sur-

face. Subsequently, a germ-tube emerges and an appressoria develops and matures, giving rise to a penetration peg. Branched hyphae start to colonize the host tissue subcuticularly above the anticlinal cell walls of the epidermal cell layer. To compare germination and appressoria formation, leaf discs of different genotypes were inoculated, incubated for 24 h at 21°C, stained, and conidia were distinguished according to their developmental stages. Preliminary results showed that less pycnidiospores germinated and formed appressoria on European wild grapes compared to cvs Müller-Thurgau and Börner. This phenomenon could be caused by a deficient attachment of pycnidiospores to the leaf surface. To prove this hypothesis, leaf discs were inoculated, incubated and washed in ethanol and the remaining pycnidiospores were counted. More pycnidiospores were able to attach to the leaf surface of cvs Müller-Thurgau and Börner than on leaves of the European wild grapes. To secure more information on the characteristics of the leaf surfaces of the different genotypes, an analysis of the adhesion power was started, since low temperature scanning electron microscopy results revealed that the genotypes investigated differ in regard to wax layers.

GENOMIC AND PROTEOMIC STUDIES ON THE PATHOGENIC GRAPE FLORA IN LUXEMBOURG. M. Behr, T. Serchi, E. Cocco, S. Legay, D. Molitor, C. Guignard and D. Evers. *Centre de Recherche Public-Gabriel Lippmann, Département Environnement et Agro-biotechnologies (EVA), 4422 Belvaux, Luxembourg. E-mail: evers@lippmann.lu*

During the years 2000, several wines were spoiled by various off-flavours of fungal origin. These fungi, mainly *Botrytis cinerea* and, to a lower extent, *Penicillium expansum*, are able to produce a large number of organoleptic modificatins, among which earthy-muddy and fresh mushroom flavours. In order to study the fungal population prevailing in the Luxembourgian part of the Moselle, a survey was carried out between 2007 and 2011, and the isolated fungi were identified by molecular techniques. i.e. sequencing of the ITS of rDNA and β-tubulin regions. Of the identified species *Botrytis cinerea*, *Mucor fragilis*, *Chaetomium globosum* were associated with grey mould, whereas *Penicillium expansum*, *Penicillium minioluteum* and *Cladosporium* spp. were recovered from berries affected by green mould. *P. expansum* is able to produce geosmin, an earthy-muddy smelling sesquiterpenoid. Fourteen strains of this fungus were screened for their geosmin production. Intra-strain and inter-strain variations (with factors up to 20 and 50, respectively), were observed and the various impacts that an infection might have on grape and wine quality were highlighted. A proteomic study using 2D-DIGE and Maldi-Tof-Tof MS analysis was conducted to understand the different geosmin producing abilities of four selected *P. expansum* strains. Eightysix differentially expressed proteins were detected and used to create a PCA (principal component analysis) and build a hierarchical clustering. The two high-producing strains clustered together, while the low producer showed a different position. One strain displayed a heterogeneous geosmin production clearly visible in its proteomic profile. The differentially regulated proteins were those involved in redox status (catalase, peroxiredoxin, HSP-70, HSP-60 and HSP-p23), amino acid and protein metabolism (serine carboxypeptidase, fungal proteinase A, elongation factor, ribosomal proteins), cellular cycle (CDC/septin GTPase) and glycolysis-TCA cycle (phosphoglycerate kinase, enolase, acetyl CoA C-acetyltransferase, malate dehydrogenase).

FURTHER STEPS TOWARDS THE MOLECULAR IDENTIFICATION OF *PENICILLIUM* SPECIES ON GRAPES. R. Walter and A. Kortekamp. *State Service Center Rheinpfalz (DLR Rheinpfalz) for Research, Teaching and Consulting in Viticulture, Horticulture and Rural Development, Department Phytomedicine, 67435 Neustadt/Weinstrasse, Germany. E-mail: ruth.walter@dlr.rlp.de*

Penicillium species causing green mould on grapes are capable to produce metabolites that can interfere with must and wine quality. Some of these metabolites represent important mycotoxins such as patulin, citrinin, and ochratoxin that may also have an impact on human health. Since 2004, berries affected with green mould were collected, and a total of 724 *Penicillium* strains were cultivated on suitable media and identified based on morphological and molecular features. *P. expansum* was the most prevalent species (673 strains), followed by *P. miniluteum* (25), *P. crustosum/P. commune* (13), *P. purpurogenum* (6), and *P. spinulosum* (3). Several other species, such as *P. aurantiogriseum*, *P. janthinellum/P. griseovulvum*, *P. solitum/P. echinulatum*, and *P. thomii/P. purpurescens* were found unfrequently. *P. expansum* was identified by a species-specific PCR assay using the primer pair PEF and PER (Marek *et al.*, 2003) that generates a 404 bp product. Other *Penicillium* species were successfully identified based on differences within the ITS (internal transcribed spacer) regions. For this purpose, PCR was carried using the primer pair ITS4 and ITS5 (White *et al.*, 1990), that amplify products of *ca.* 600 bp, that were sequenced and aligned. However, this method was unable to discriminate closely related *Penicillium* species such as *P. crustosum* and *P. commune*, whose reliable identification is of particular importance since *P. crustosum* is also capable to produce the nephrotoxic and cancerogenic mycotoxin ochratoxin A (OTA), at least *in vitro.* In order to develop a specific method for the identification of this putative mycotoxin producer, first a PCR utilizing primer pair PenF1 and AspR1 (Seifert *et al.*, 2007) which leads to an amplification of a part of the cytochrome oxidase-gene (cox1) was performed. The PCR product was digested subsequently with the restriction enzyme HpyF3I. Cleavage of the cox1 PCR-product yielded one fragment in the case of *P. commune* and two fragments in the case of *P. crustosum*, which was verified by using reference strains from the CBS (Centraalbureau voor Schimmelcultures, Utrecht/Netherlands). Thus, this method allows the identification of the *Penicillium* species most frequently found on grape berries, including putative mycotoxin producers, but needs to be adapted for the identification of other *Penicillium* species which are rarely observed on grapevine.

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***GUIGNARDIA BIDWELLII*: EPIDEMIOLOGY AND SYMPTOMS DEVELOPMENT IN MEDITERRANEAN ENVIRONMENT. P. Rinaldi[1], M. Skaventzou[1], M. Rossi[1], C. Comparini[1], J. Sofia[2], D. Molitor[3] and L. Mugnai[1].** *[1]Dipartimento di Biotecnologie Agrarie, Sezione Patologia Vegetale, Università degli*

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Guignardia bidwellii is an ascomycetous fungus and the causal agent of black rot disease of grapes. It appeared in Europe, coming from North America at the end of 19th century and spread through the main areas in which the grapevine is cultivated. In North American and the central European countries, where the life cycle and epidemiology of the pathogen had already been studied in the past, the fungus affects all young tissues (leaves, peduncles, tendrils, shoots, petioles), but the main symptoms appear on leaves and berries. Damages lead to quality losses and yield reduction. In those areas, spraying strategies against black rot are focused on the period of highest susceptibility: between flowering and 6-8 weeks afterwards. In the Mediterranean viticultural areas, typically characterized by warmer and dryer springs and summers than in central Europe, the pathogen creates new problems to the viticultural industry. First devastating damages on grapes were reported from Bairrada, Portugal in 2006 (Rego and Oliveira, 2007) and Tuscany, Italy in 2011 where yield losses reached 100% (Rinaldi and Mugnai, 2012). Beside the attack to berries, in some vineyards heavy damages to vegetative organs were recorded prior to or shortly after flowering. In such cases, the cankers on the green shoots, on the clusters rachis and on the peduncles of the small bunches, can further reduce the yield significantly. Under field conditions, symptoms appear almost simultaneously and pycnidia become visible on the lesions after one day. Also young berries (1 to 2 mm diameter) can be attacked by the pathogen, and are mummified in the earliest stages of their development. Consequently, new spraying strategies for the Mediterranean areas were tested and adapted to the local plant/pathogen match. Current investigations focus on the relevance of leaf infections as inoculum source for secondary infections.

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TRANSCRIPTIONAL CHANGES IN RESPONSE TO ELICITATION OF GRAPEVINE AGAINST *PLASMOPARA VITICOLA*, THE CAUSAL AGENT OF DOWNY MILDEW.

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Grapevine (*Vitis vinifera*), the most widely cultivated and economically important fruit crop worldwide, is susceptible to several pathogens including *Plasmopara viticola*, the causal agent of downy mildew. Therefore, grapevine requires intensive use of fungicides. Induced resistance represents an important element of an integrated pest management (IPM) to control the pathogen. This work aimed at activating the grapevine's defence mechanisms using some resistance inducers known as elicitors applied before inoculation. Microarray analyses (NimbleGen), based on the 12X *Vitis vinifera* genome sequence, of leaf tissues (cv. Riesling) was carried out to obtain an overview on the differentially expressed genes and, possibly, to identify genes that play a role in grapevine resistance against *P. viticola*. Differentially expressed genes were functionally characterized using MapMan analysis tool. Microarray analyses revealed several thousands of genes with significantly altered expression (FDR adjusted $p<0.05$). In the leaves of potted vines, 3,466 genes were differentially expressed after inoculation with *P. viticola*, most of them being down-regulated (1,328 up, 2,138 down). Most of the genes involved in stress pathways such as the secondary metabolite pathway (e.g. stilbene synthase and terpene synthase), cell wall generation (e.g. cellulose synthase) and hormone metabolism (e.g. lipoxygenase and salicylic acid carboxyl methyltransferase) were down-regulated. Plants treated with the elicitors "phosphonate" and "phosphate" 24 h before inoculation showed less differentially expressed genes (2,850 and 3,394, respectively). Upon elicitation, most of the differentially expressed genes with phosphonate and phosphate were up-regulated (phosphonate: 1,832 up, 1,018 down; phosphate: 1,867 up, 1,527 down). Genes involved in stress pathways were mainly up-regulated, such as the aforementioned genes and genes coding for pathogenesis-related proteins (PR-proteins), e.g. PR-3 and PR-10. Moreover, genes involved in signalling pathways (e.g. receptor kinases and heat shock proteins) were also up-regulated. Plants treated with the elicitor Frutogard and subsequently inoculated with *P. viticola* showed only 47 differentially expressed genes, seven of which were involved in stress pathways.

Interestingly, leaves of potted vines treated with Frutogard, phosphonate and phosphate but not inoculated showed fewer differentially expressed genes (462, 1422 and 1,529, respectively). This indicates that elicitors alone have less impact on the plant, which preserves the plant´s energy resources under disease-free conditions.