Istituto Agrario di San Michele all'Adige
SafeCrop Centre

PROCEEDINGS

5th International Workshop on Grapevine Downy and Powdery Mildew

Edited by
I. Pertot, C. Gessler, D. Gadoury, W. Gubler, H.-H. Kassemeyer, P. Magarey

San Michele all'Adige, Italy, 18-23 June 2006

PROCEEDINGS

Istituto Agrario di San Michele all'Adige

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5th International Workshop on Grapevine Downy and Powdery Mildew
San Michele all'Adige, Italy, 18-23 June 2006

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Preface

The origin of this story is in the wise recognition of some researchers that the knowledge on *Plasmopara viticola* is incomplete and presents many contradictions. So, as a logical consequence for scientists, a meeting of colleagues was called (Geneva, USA, 1991). After this first meeting some of us recognized the trueness that the subject is far to be fully understood and we may have to change our questions and approach. Moreover we realized that we must join our efforts and stop working alone in our labs and fields to just show amazing presentations in congresses (Freiburg, Germany, 1994). Some of the purposes remained empty words, but progresses were slowly made. In the third meeting (Loxton, Australia, 1998) we have seen some new ideas, approaches and data emerging. The fourth meeting (Davis, USA, 2002) had to reaffirm the necessity of collaboration and, in some particular topics, finally more cooperation was started. Being tempted to do our research alone, come to a workshop, present results and go back home without confront is a risk always present. We should always remember that doubt and discussion is the driving force for research. Truly comparing progresses and results, exchanging our information, doubting that our approach is the only correct one will help us in getting a continuous improvement in our knowledge. The strict contact with agriculture and markets is an opportunity that will help us focusing real problems. We are now at the beginning of the fifth meeting and, as scientists working on powdery and downy mildew of grape, we have few questions to answer at the end of this week. What are the most important discovers on these two important diseases? What are the positive benefits to growers and what it is still needed to be solved? Which approach should we choose: a small group with a clear focused topic and a common research program discussed prior or a large open group, as wide as possible, so to exchange information, and pick up new ideas? What do we need to foster common research and/or development, funding?

This fifth International Downy and Powdery Mildew Workshop will enjoy the hospitality of Trentino. The reception of the proposal to hold this meeting in San Michele was absolutely great and, as you will experience, most the major wineries participate with enthusiasms. However this is also a clear message to us: “bring us better solutions to control downy and powdery mildew than we currently have”.

We also thank the Istituto Agrario di San Michele all’Adige (IASMA), which hosts us and offers its infrastructure and experience in organising international scientific meeting. It is important to mention here that IASMA played and plays, in relation to research on grape and wine, a prestigious role in Italy. Not at last, we would like to thank all the collaborators of Safecrop Centre, whose help made this meeting possible.

We thank the “Provincia Autonoma di Trento”, which funded SafeCrop, a centre for research and development of crop protection with low environment and consumer health impact.

Cesare Gessler and Ilaria Pertot

San Michele all’Adige, 28 May 2006

Session 1:

Host resistance, induced resistance, mechanisms, breeding

Host-Pathogen-Interactions and resistance responses of different *Vitis* genotypes after infection by *Plasmopara viticola*

Hanns-Heinz Kassemeyer, Tobias Seibicke, Sabine Unger

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Grapevine is capable of defending itself against attacks of potential pathogens by a broad spectrum of preformed barriers and inducible defence reactions. All cultivars of *Vitis vinifera* are susceptible for *Plasmopara viticola*, whereas wild *Vitis* species from North America typically express significant resistance to this pathogen. The colonization of susceptible and resistant *Vitis* genotypes has been studied to get an insight in the time course of resistance response. In addition the resistance response has been characterized on a molecular level.

The course of colonization of the mesophyll by the causal agent of grapevine downy mildew, *Plasmopara viticola*, in susceptible and in resistant grapevines was examined in order to characterize the development of the pathogen in compatible and incompatible host-pathogen interactions. Within a few hours after inoculation, the pathogen had established in the susceptible *Vitis vinifera* cv. Müller-Thurgau and formed primary hyphae with a first haustorium. No further development occurred in the following 10 to 18 h. The next developmental step, in which the hyphae grew and branched for colonizing the intercellular space of the host tissue, was observed 36 h after inoculation. After 3 days the intercostal fields were entirely filled with mycelium. The first infection steps were essentially identical in the resistant hybrid *V. x vinifera* cv. Merzling and *V. rupestris*. However, further development of the pathogen was incomplete, and the intercostal fields were filled with dense mycelium only in low frequency. In contrast to cv. Müller-Thurgau, *P. viticola* sporulated rarely on resistant grapevines.

One of the prominent responses after fungal attack is the expression of pathogen related proteins (PRP´s). In order to investigate the response of grapevine to the infection by *Plasmopara viticola*, the expression of PRP´s has been characterized. For the analysis of a glucanase from grapevine and studies on its induction by *P. viticola* the structure of the entire gene was elucidated by sequencing of a cDNA clone. Starting from a 3´-fragment of a putative extracellular *Vitis*-Glucanase a target region was cloned. For this purpose a 3´-primer of the coding sequence was designed and a PCR was performed using a 5´-primer specific for the vector SK-1. As template for the amplification a cDNA library from a cell suspension culture of *Vitis vinifera* cv. Pinot noir was available which had been induced by a yeast elicitor. The gene encodes a protein with 345 aminoacids and a molecular mass of 37.5 kDa. The analysis of the sequence, performed with the SMART program, revealed the particular glucanase (*VGluc*) showing a sequence motif typical for glycosyl hydroxylases. Homology to glucanases from other plants was found; the highest level was 77% identity to an extracellular β-1,3 glucanase from *Prunus persica*. A signal peptide motif at the N-terminus with 36 aminoacids responsible for the transport of proteins in the apoplast indicates this *Vitis*-glucanase to be extracellular. To confirm the localisation of the expression, parsley protoplasts were transformed with a *VGluc-GFP*-construct. The expression of the 5´ *VGluc-GFP* 3` fusion protein constitutively took place under the control of a 35S-*CaMV* promoter. Microscopical analysis of the protoplasts showed a green fluorescence in the cytoplasma but no fluorescence in the vacuoles. This indicates expression of *Vgluc* in the cytoplasma and a lacking transport into the vacuoles.

Because glucanases have been described as pathogenesis related proteins the transcription of *VGluc* after challenge infection should confirm its role in the defence response of grapevine. For this purpose leaves of the same insertion and size from the susceptible *Vitis vinifera* cv. Mueller-Thurgau and the resistant *Vitis rupestris* were inoculated with *Plasmopara viticola*. Within nine days on the susceptible cultivar the manifestation of the pathogen was evident by clearly expressed symptoms and sporulation. In contrast, four to six days after inoculation necrotic spots without any sporulation appeared on the resistant grapevines. In distinct time intervals inoculated and uninfected leaves were collected to extract RNA. Northern blot analysis using a *VGluc*-cDNA probe revealed an induction of the transcript two days after the challenge infection both in the susceptible and the resistant grapevines. The transcript abundance increased and reached a transient maximum six days after infection.

By means of quantitative PCR (Real-Time-PCR) the course and level of transcript expression of *Vgluc* was studied in resistant and susceptible *Vitis* genotypes. In addition further pathogen induced genes of grapevine such as stilbensynthase and callosesynthase were analyzed by this method. The results indicate differences between resistant and susceptible genotypes confirming that resistance is quantitative and depends on the induction rate after infection.

Candidate genes mapping and comparative QTL analysis for powdery and downy mildew resistance in grape

Alberto Madini, Flavia M. Moreira, Rosanna Marino, Luca Zulini, Hanns-Heinz Kassemeyer, Pal Kozma, Riccardo Velasco, Marco Stefanini, M. Stella Grando

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Grapevine (*V. vinifera* L.) is highly susceptible to fungal pests as downy mildew (*Plasmopara viticola*) and powdery mildew (*Erysiphe necator*, syn. *Uncinula necator*).

Unfortunately, the most effective way to fight these dangerous diseases still consists in applying a chemical protection, with all the obvious drawbacks to the environment. Thus, understanding the genetic resistance determinism, which is reported in *non-vinifera* species and acts when grape is challenged by a fungal attack, is undoubtedly of great relevance.

Combining the "*vinifera*" fruit quality together with resistances that other *Vitis* species bear in their genome is a difficult task, because the resistance is quantitatively determined and therefore the governing genes are possibly located in several genomic regions. In this frame, resistance trait evaluation and molecular linkage maps can be coupled to identify quantitative trait loci (QTLs) responsible for phenotypes in selected progenies.

Our goal is to generate tools for assisting the traditional breeding programs by means of molecular markers tightly linked with the resistance trait.

For this purpose we are managing two hybrid grape progenies: 174 F1 plants resulted by crossing the susceptible *V. vinifera* cv. 'Moscato bianco' and a downy mildew resistant individual belonging to the *V. riparia* species, and 96 F1 plants derived from the cross between 'VRH3082 1-42' (a BC4 descending from *V. rotundifolia* and *V. vinifera*, extremely resistant to powdery- and resistant to downy mildew) and 'Sk77 5/3' (a BC2 from *V. amurensis* and *V. vinifera*, resistant to downy mildew).

Fig. 1. Cuttings of the 'VRH3082 1-42' x 'Sk77 5/3' offspring growing in the San Michele all'Adige's greenhouse. The original population is grown and maintained in the field at the FVM Research Institute for Viticulture & Oenology - Pécs (Hungary).

For each cross, we built the framework molecular maps of the parents, based on a backbone consisting of SSRs mainly from the VMC initiative and added of more informative markers as CG and RGA derived SSCP markers, EST-derived microsatellites, and SNPs derived from BACend sequences. We used for the mapping analysis the software JoinMap® 3.0, setting LOD 5 and rf 0.45 for 'Moscato bianco' x *V. riparia* and LOD 3 and rf 0.45 for 'VRH3082 1-42' x 'Sk77 5/3'. In both cases, the Kosambi's mapping algorithm was applied. Consensus maps were generated for each experiment and consisted of 123 and 121 markers aligned into 19 linkage groups covering a combined map lengths of 973 and 691 cM respectively.

The response of the MxR progeny to downy mildew infection was scored for three years in the field (under artificial and natural infections) and for one year in the greenhouse (artificial inoculation). Mean diameter of the infection spots and percentage of leaf surface infected were evaluated.

Concerning the 'VRH3082 1-42' x 'Sk77 5/3' offspring, response was scored, after artificial inoculations during years 2003-2004-2005, according to the OIV descriptors.

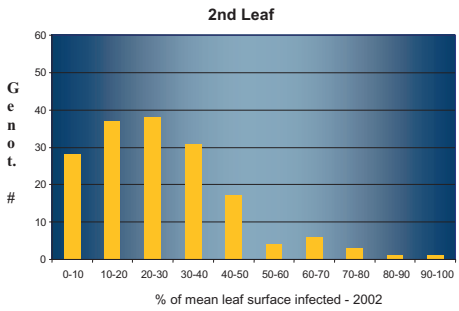

Fig. 2. Example of downy mildew infection response distribution in the MxR offspring. The character is clearly quantitative. Classes were made only for graphical purpose.

Afterwards, by means of the MapQTL® 4.0 software, we performed a QTL analysis by integrating the segregation data of each cross together with the phenotypic data of the respective progeny previously scored. For this analysis we used the interval mapping approach coupled with a permutation test that enabled us to determine, for each linkage group, the appropriate LOD threshold for the presence of the QTLs.

Significant QTLs controlling downy- and powdery mildew resistance were found on different linkage groups. Furthermore, two SSCP markers were located inside the confidence interval of QTLs for downy mildew resistance identified in *V. riparia*, and one in 'SK77 5/3'; deeper investigations could confirm them as candidate genes for these important traits. The comparative QTL analysis for downy mildew revealed interesting conserved QTLs among the different species under study (see an example in Fig. 3).

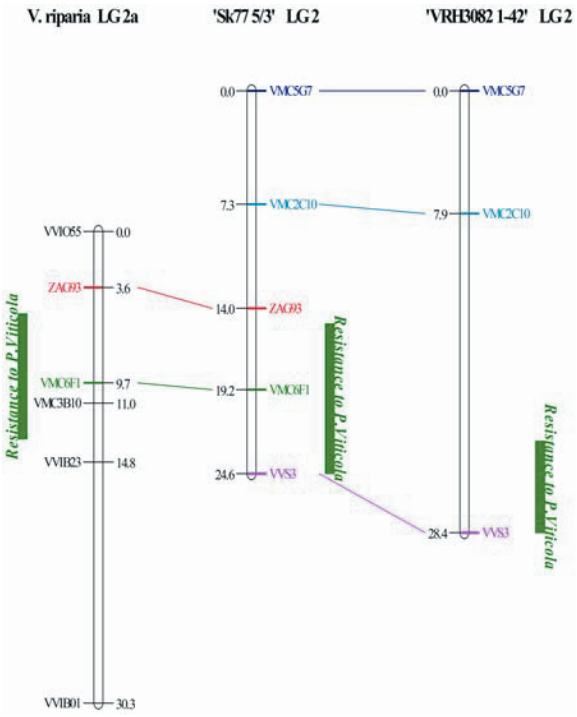

Fig. 3. Comparative QTL analysis in the linkage group 2 between *V. riparia*, 'Sk77 5/3' and 'VRH3082 1-42'

In this work we describe the comparative QTL analysis results concerning the traits "resistance to downy mildew" in both crosses as well as "resistance to powdery mildew" in the 'VRH3082 1-42' x 'SK77 5/3' one. The generation of novel molecular markers genetically linked to fungi-resistance will be presented.

Technical abbreviation used:

CG Candidate Gene
EST Expressed Sequence Tag
LOD Logarithm Of Odds
QTL Quantitative Trait Locus
RGA Resistance Gene Analog
SSR Simple Sequence Repeat
SSCP Single Strand Conformational Polymorphism

Influence of cultivar mixtures on grapevine Downy mildew epidemic

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Variety mixtures have been shown to be an effective disease control method in various crops ranging from cereals to tree crops. Disease reduction operates in three principal ways: (i) dilution of inoculum, (ii) physical barrier effect and (iii) induction of defense reactions in the host. Within the European project REPCO (Replacement of Copper Fungicides in Organic Production of Grapevine and Apple in Europe) we investigated if grapevine cultivar mixtures are effective in reducing the severity of *Plasmopara viticola* compared to a plot whose rows are composed by a single cultivar (monoblock).

For this purpose a vineyard (632 vines, distance between vines: 0.8 x 2.0 m, distance between blocks: 1.6 m) with a pattern consisting of mixed cultivar blocks (Fig. 1) and one monoblock (Fig. 2) was established in May 2004 in Cugnasco (Southern Switzerland). Plants of cultivars Gamaret, RieslingxSylvaner (syn Müller-Thurgau), Merlot, Regent, Isabella, Solaris, Bianca and Chambourcin, differing in susceptibility toward *P. viticola* infections and allowing different sporulation intensity were chosen. They were planted in five blocks: four cultivar mixture blocks (consisting of 8 rows with 2 plants of each cultivar planted nearby and in one monoblock (consisting of 8 rows with 15 plants of each cultivar).

Severity was assessed visually using the Horsfall-Barratt rating scale after three important infective events (7th July, 8th August, and 1st September). For each block one branch per cultivar and row was chosen. The midpoint severity value was assigned for each class number and the obtained value was divided by the total number of leaves of the selected branch.

In the end of May 2005 the first disease symptoms were detected but the disease severity remained <1% for approx. five weeks on all cultivars. On 8th August the most diseased cultivar was RxS, showing about 18% severity both in the mixed (18.2%) as in the monoblocks (18.9%; Fig. 4). The most resistant variety Chambourcin showed less than 0.1% severity in both block types (Fig. 6).

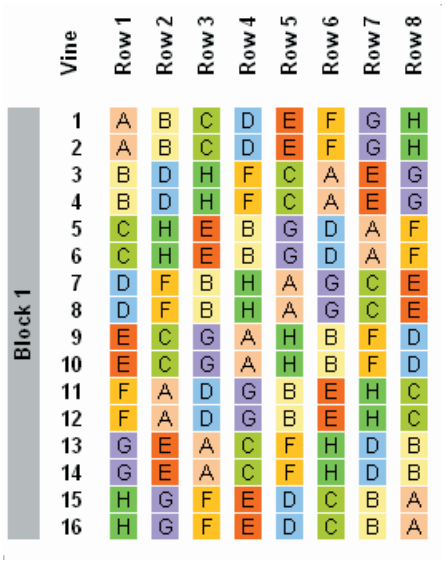

Fig. 1. Vine positioning in one of the four cultivar mixture blocks (Block 1). Each letter indicates one vine of a cultivar (A: Regent, B: Merlot, C: Isabella, D: RxS, E: Solaris, F: Bianca, G: Gamaret, H: Chambourcin).

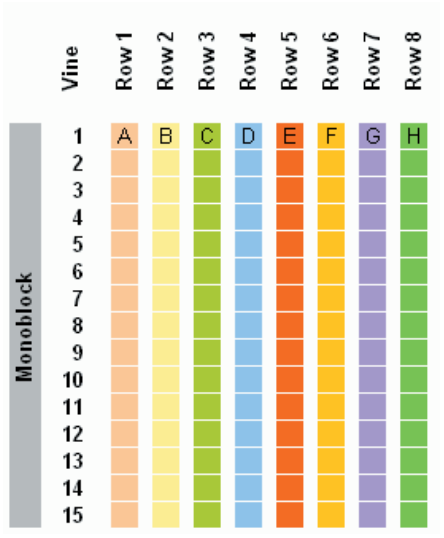

Fig. 2. Vine positioning in the monoblock (Block 5). Each letter indicates one vine of a cultivar (A: Regent, B: Merlot, C: Isabella, D: RxS, E: Solaris, F: Bianca, G: Gamaret, H: Chambourcin).

On September 1st, the highest severity was assessed on RxS (79.7%) and on Merlot (71.2%) in the monoblock (Table 1). The lowest severities were

assessed on vines of the cultivar Chambourcin (0.3%) and Bianca (1.4%) in the monoblocks.

Tab. 1. Severity of grapevine downy mildew in the monoblock and in the four mixed blocks on September 1st, 2005 in Cugnasco (Switzerland).

Cultivar	Severity in monoblock	Severity in mixed blocks
RxS	79.7%	38.6%
Merlot	71.2%	45.8%
Gamaret	58.7%	35.3%
Isabella	37.7%	21.4%
Solaris	5.3%	14.3%
Regent	2.9%	10.1%
Bianca	1.4%	7.8%
Chambourcin	0.3%	2.9%

Severity was lower in the mixed blocks then in the monoblock for cultivars where high severity was assessed (RxS, Merlot, Gamaret, and Isabella). This indicates an effect of cultivar mixtures on the severity of *P. viticola* which could depend from a dilution of inoculum, a barrier effect or an induction of resistance in the host. The opposite situation was observed for the more resistant cultivars (Chambourcin, Bianca, Regent, and Solaris) where the severity was lower in the monoblock than in the mixed blocks. This could be related to a lower inoculum due to the presence of a larger number of resistant plants in the monoblock than in the mixed blocks. On the contrary resistant cultivars interposed among susceptible varieties are subjected to a higher disease pressure (neighboring effect) than in the monoblocks.

Fig. 3. Vines of cultivar RxS in the monoblock on September 23, 2005.

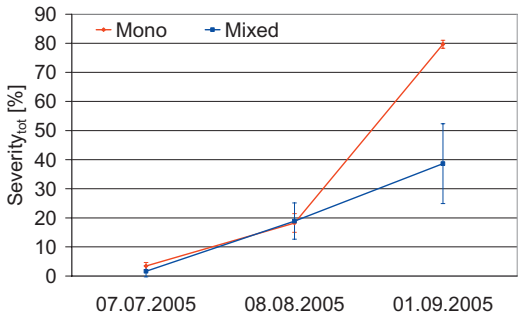

Fig. 4. Downy mildew severity assessed for the cultivar RxS throughout the period 7th July – 1st September 2005 in Cugnasco (scale:0-90%).

Fig. 5. Vines of cultivar Chambourcin in the monoblock on September 23, 2005.

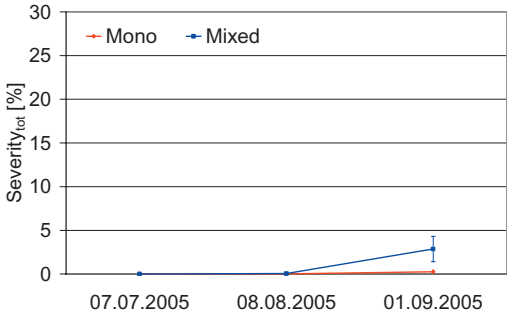

Fig. 6. Downy mildew severity assessed for the cultivar Chambourcin throughout the period 7th July – 1st September 2005 in Cugnasco (scale:0-30%).

For the season 2006 severity on bunches and quantitative (yield) and qualitative (pH, soluble solids (Brix), acidity and yield) aspects will be considered in the analysis.

Activity of the grapevine endophyte Alternaria alternata on infection, colonization and sporulation of Plasmopara viticola in relation to application time

Ilaria Pertot, Silvia Dagostin, Rita Musetti, Davide Gobbin

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The grapevine endophyte *Alternaria alternata* (Fr.) Keissl completely inhibits the sporulation of *Plasmopara viticola* on grapevine leaves. Ultrastructural analyses carried out to observe cellular interactions between *P. viticola* and *A. alternata* in the grapevine leaf tissue show that, even without close contact with *A. alternata*, the *P. viticola* mycelium has severe cytological alterations, such as the presence of enlarged vacuoles or vacuoles containing electron-dense precipitates. Haustoria appear necrotic and irregularly shaped or enclose in deposits of callose-like substances. Three diketopiperazines: cyclo(L-phenylalanine-trans-4-hydroxy-L-proline), cyclo(L-leucine-trans-4-hydroxy-L-proline) and cyclo(L-alanine-trans-4-hydroxy-L-proline) produced by *A. alternata* in culture broth were isolated and characterised. A mixture of the three diketopiperazines was very efficacious in limiting *P. viticola* sporulation both on artificially inoculated grapevine leaf disks and greenhouse plants. A toxic action of *A. alternata* against *P. viticola* was therefore hypothesized.

The aim of this research is to compare the activity of *A. alternata* on the pathogen when applied at different times from *P. viticola* inoculation.

Agar plugs with *A. alternata* mycelium, maintained on potato dextrose agar (Sigma) at 4°C, were transferred to Nutrient Broth (Oxoid) and incubated with shaking for 5 days at 25° C. Grapevine plants of the susceptible cultivar Pinot gris, having two shoots with four fully expanded leaves each, were used in the bioassays. Five plants (replicates) were used for each treatment. The plants were sprayed with the *A. alternata* culture broth and cells (table 1).

Tab. 1. Application times (days prior and after *P. viticola* inoculation) of *A. alternata* culture broth on grapevine leaves. "Zero" refers to *P. viticola* inoculation time

Days from *P. viticola* inoculation				
-5	-3	-1	0	+1
x	x	x	x	
	x	x	x	
		x	x	
	x			
		x		
			x	
				x

The treatment on the same day (0) was done one hour before of inoculation. Copper hydroxide (Kocide 2000, DuPont) and water sprayed plants were used as treated and untreated controls. Water suspension of *P. viticola* sporangia ($4.5x10^5$ sporangia/ml) were prepared by washing fresh sporulating lesions. The inoculation was done on 10 leaf disks randomly cut from the each treated plant, by floating them on the *P. viticola* sporangia suspension, overnight at 20° C.

Five disks per treatment were kept in moist chambers until sporulation was seen on the untreated control. Sporulation was visually assessed on the leaf disks using a stereomicroscope. The forty five leaf disks were transferred in multiwell plates for the DNA extraction and analysis. They were freeze-dried and the DNA was extracted with NucleoSpin Multi-96 Plant Kit (Macherey-Nagel, Duren, Gremany). Instead of the lysis buffer supplied, CTAB was used and instead of one final elution of 180 µl, three elution of 60 µl were obtained. The DNAs collected in the second elution were afterwards analysed with a quantitative real time polymerase chain reaction. The protocol for DNA extraction and PCR as described by Valsesia et al. (2005) was followed. DNA of samples, pure *P. viticola* DNA, pure *V. vinifera* DNA and a no-DNA control template were amplified. Multiplex reaction containing 1x TaqMan Universl Master Mix, 250 nM *P. viticola* VIC-labelled probe (Giop P), 250 nM *V. vinifera* FAM-labelled probe (Res P), 900 nM *P. viticola* forward and reverse primers, 120 nM *V. vinifera* forward and reverse primers and 5 µl template DNA. Amplification were performed with the standard short cycling parameters (50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min). Results are shown as ratio of CT *V. vinifera* over CT *P. viticola* (infection coefficient) A high ratio (IC) between *P. viticola* DNA / *V. vinifiera* DNA indicated a successful infection and tissue colonization by *P. viticola* and a low control activity. Conversely, a low IC indicated a low P. viticola colonisation and therefore a good disease control.

A. alternata cells and culture broth, applied once before inoculation with *P. viticola* did not inhibit *P. viticola* development. DNA is present in a similar amount to what is found in the untreated control and sporulation is also visible. Copper treatment one hour before inoculation significantly reduced *P. viticola* DNA and inhibited sporulation (fig. 1). 24 Application of *A. alternata* (cells with culture broth) 24 hours after inoculations, led to a low amount of *P. viticola* DNA in leaf disks treated and complete inhibition of sporulation

These results show that *A. alternata* when applied any time before inoculation is not able to prevent *P. viticola* infections, colonization and sporulation. *A. alternata* is active if leaves are treated one day after

inoculation. Similar results were obtained using the mixture of three diketopiperazines (data not shown).

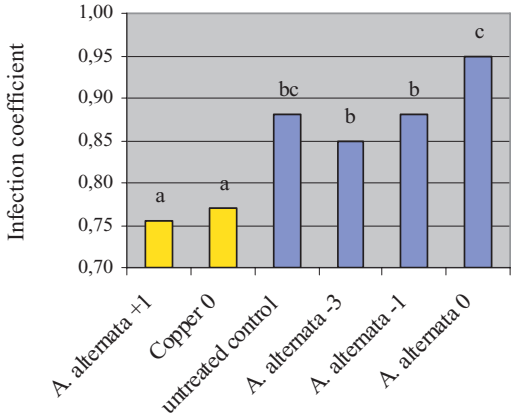

Fig. 1. Infection coefficient (CT *V. vinifera* / CT *P. viticola*) in the grape tissues treated with *A. alternata* at different times from *P. viticola* inoculation. Means followed by different letters are significantly different (least significant difference [LSD]; P≤ 0.05).
Yellow columns=no sporulation. Blue columns=sporulation.

Repeated applications of *A. alternata* before *P. viticola* inoculation did not reduce the presence of *P. viticola* DNA in tissues, but significantly inhibit sporulation (fig. 2).

A. alternata does not act as resistance inducer in the plant since the high content of *P. viticola* DNA demonstrate that leaf colonization by the pathogen is present, however it is able to interact with the sporulation process, when several treatments are applied.

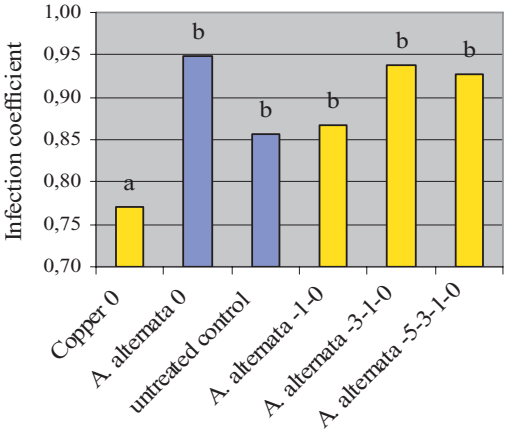

Fig. 2. Infection coefficient (CT *V. vinifera* / CT *P. viticola*) in the grape tissues treated once, twice, three and four times with *A. alternata* at different times from *P. viticola* inoculation. Means followed by different letters are significantly different (least significant difference [LSD]; P≤ 0.05).
Yellow columns=no sporulation. Blue columns=sporulation.

A. alternata is an endophyte that colonises grape, but when the organism with its culture broth is applied it shows antagonistic effects against *P. viticola*. It is effective only if applied after *P. viticola* inoculation preventing tissue colonization, but repeated applications before infection have an inhibiting effect on pathogen sporulation. It produces pathogen-inhibiting metabolites belonging to the groups of diketopiperazines, which showed similar post-infection efficacy compared to cells with culture broth but it seems not able to promote plant disease resistance.

If the antisporulating efficacy against *P. viticola* and the activity after infections will be confirmed also in field experiments, *A. alternata* or its metabolites could represent a new biological tool to be further developed for grapevine protection against downy mildew.

Acknowledgements

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Identification of differentially expressed genes in grape following infection with *Plasmopara viticola*

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Plasmopara viticola is an obligate biotrophic pathogen of grape which uses one of the most specialized infection strategies of plant pathogens. This oomycete obtains its nutritional resources from the living cells of its host and is completely dependent on living plant tissue for its growth and propagation. Hyphae grow in the intercellular spaces of the leaves and form a close association with host plant cells through haustoria. The haustorium-host cell interface is thought to be a dynamic interaction involving extensive molecular trafficking and signaling processes. However, the nature of these exchanges remains unknown. A comprehensive analysis of transcriptional changes associated to the infection process of *Plasmopara viticola* in susceptible grapevine (cv. Riesling) has been undertaken, with the aim to identify differentially expressed genes from the plant and from the pathogen side, in infected leaves at the oil spot stage.

The approach chosen for the analysis of transcriptional changes is the cDNA-AFLP technique which allows the identification of the largest possible inventory of differentially expressed genes, without previous knowledge about their sequences. The protocol applied is a recent modification of the original technique (Bachem et al., 1996; Breyne et al., 2003), improved in the fact that it permits the visualization of one single cDNA fragment for each messenger originally present in the sample and thus completely abolishes the problem of redundancy of the results obtained. cDNA-AFLP has been shown to be robust, reliable and reproducible (Breyne et al., 2003; Reijans et al., 2003) and has already been used to investigate other plant-pathogen interactions, including *Peronospora parasitica* infection on Arabidopsis (van der Biezen et al., 2000). As we were interested in the identification of both grape and *Plasmopara* modulated transcripts, a preliminary bio-informatic survey has been carried out on grape sequence database (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=grape) and on sequence database of *Phytophthora infestans* (http://www.pfgd.org/pfgd), which is the oomycete with the widest genomic information available, with the aim to identify the best restriction enzyme combination to use to produce cDNA fragments from the highest possible number of grape and *Plasmopara* genes. While plant sequences have an average GC content of about 42%, oomycetes are estimated to have an average GC content of about 58% (Kamoun, 2003). The standard enzyme couple used on plant material in our laboratory is *BstYI* (RGATCY) / *MseI*, (TTAA) which is expected to generate few polymorphisms in *Plasmopara* cDNAs. The best enzyme couple identified for *Phytophthora* was *BstYI* / *HhaI* (GCGC) - which was predicted to provide fragments in 40% of *Phytophthora* transcripts, in comparison to an estimated 30% of the *BstYI* / *MseI* couple, and thus was supposed to be able to identify a higher number of differentially expressed cDNA fragments also from *Plasmopara*.

RNA was extracted from leaves of *in vitro* grape plants either infected with *P. viticola* or healthy, as a control, as well as from sporangia. Preliminary cDNA-AFLP experiments were carried out in parallel with both enzyme couples; the observed number of polymorphisms were compared to the expected. Surprisingly, there were no significant differences in the number of cDNA fragments that could be visualized with the two restriction enzyme combinations, at least in the selectivity conditions adopted (two selective bases on one primer and one selective base on the other). Thus we decided to start the analysis with the *BstYI* / *MseI* restriction enzymes. Amplifications with all 128 primer combinations were performed. We estimate that about 7000 transcripts have been visualized on the whole (about 50-60 bands per lane on each gel) and a huge modulation of transcriptional activity was observed, with 1653 differentially expressed fragments characterizing the interaction. Most of them are predicted to be of plant origin, also in consideration of the very low concentration of *P. viticola* RNA in the infected leaves sample. However, a small percentage of *Plasmopara* genes are also expected to be identified. The origin of the transcripts cannot be assessed from the profiles, as both partners can produce *de-novo* transcripts during the interaction, but a tentative prediction was made anyway and the cDNA fragments were first classified in 4 categories, according to their expression profiles: a) 213 transcripts (12.9 %) positively modulated during grape-*P. viticola* interaction and possibly deriving from the plant, considering that a band of the same length is also present in the sample from healthy leaves (Fig. 1a); b) 885 transcripts (53.5%) negatively modulated during the interaction and also possibly deriving from the plant, as above (Fig. 1b); c) 293 transcripts (17.7%) detected in the interaction and possibly deriving from the fungus, predicted as above from the presence of a band of identical size in the sample from sporangia, although this is difficult to predict for the reasons mentioned above; it is possible that many of these fragments are of plant origin, but are just of the same size of a fungal cDNA fragment from sporangia (Fig. 1c); d) 199 *de-novo* synthesized cDNAs, which cannot be attributed to any of the two organisms because the corresponding band is absent from either the healthy plant and the sporangia samples (Fig. 1d). Only 63 bands are migrating at the

same position in the gel in all three samples and have not be considered for sequencing (not shown). All the other fragments are being sequenced.

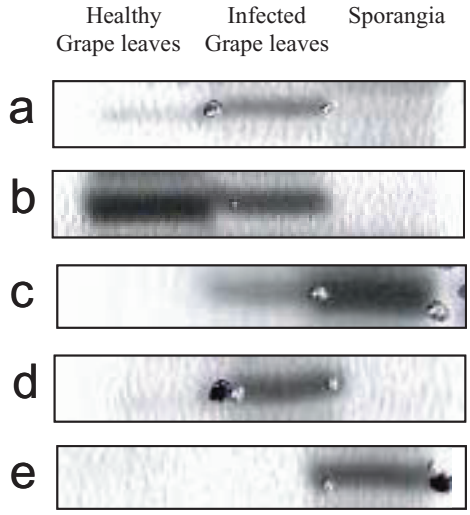

Fig. 1. Examples of cDNA-AFLP bands corresponding to grape or *P. viticola* transcripts, expressed during the infection process at the oil spot stage (a to d), and *P. viticola* transcripts identified from sporangia samples (e).

Up-to-date, eighty-one fragments have been successfully sequenced, chosen among the putative grape transcripts, both induced and repressed during infection. Homology search in databases revealed that all of them find a significant homology in the TIGR grape database, except for 8 with no homology in any published database. Complete results will be presented at the meeting.

After sequencing, cDNA fragments have been classified in functional categories. The results presently available for 81 sequences show that modulated transcripts correspond to genes putatively involved in photosynthesis and basic metabolism, but also in stress response and signal transduction, while a significant proportion (about 36%) correspond to "tentative consensus" sequences, without any further annotation. A special attention will be devoted to grape genes that are functionally related to signal transduction cascades. Their expression will be confirmed by means of other techniques, such as Real-time RT-PCR and Northern analysis.

cDNA-AFLP has the potential to identify "new" and rare transcripts, specifically associated to downy mildew infection. All cDNA fragments will represent the basis for future production of a specific microarray resource. Additionally, about 2000 cDNA fragments from sporangia have been identified (one example is given in Fig. 1e). They are going to represent a possible wide integration of the presently scarce knowledge on expressed *P. viticola* sequences.

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Resistance to downy mildew derived from *Muscadinia rotundifolia*: genetic analysis and use of molecular markers for breeding

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Downy mildew is one of the major diseases of grapevine. All the traditional grapevine varieties are susceptible to downy mildew. Disease control is mainly achieved by application of various fungicides. Breeding for resistant varieties is an alternative to chemical control of the disease. Marker assisted selection (MAS) is useful to combine in a same variety several resistant genes or QTLs. Various sources of resistance were described in the American *Vitis* species, in the Asian *Vitis* species and also in *Muscadinia rotundifolia* which is tightly related to the true*Vitis vinifera*.

In order to study these resistances, we developed a bio-assay where, seven days after inoculation, resistance level is assessed with various qualitative and quantitative parameters.

We firstly evaluated various accessions from American and Asian *Vitis* and from *Muscadinia rotundifolia.* According to the OIV 452 resistance descriptor, *Muscadinia rotundifolia* and *Vitis* species showed various resistance level. *Muscadinia rotundifolia* was totally resistant and all tested accessions of this species presented the same resistance level. *V. aestivalis, V. arizonica, V. berlandieri, V. doaniana, V. palmata* and *V. rupestris* were susceptible. In contrary, *V. candicans, V. cinerea, V. cordifolia, V. Monticola, V. riparia* and *V. titania* were highly resistant. *V. linsecumii* and *V. vulpina* were partially resistant. Inside a species, the resistance level can change according to the accessions, as it was the case for *V. labrusca* and *V. rubra* (Figure 1).

We have chosen to analyse the resistance derived from *Muscadinia rotundifolia* because this species is totally resistant to downy mildew. We used a BC2 mapping population obtained from a cross between the susceptible *Vitis vinifera* parent "Syrah" and the resistant parent "22-8-78" derived from *Muscadinia rotundifolia* var. Dearing. Bulked segregant analysis revealed 8 SSR markers correlated with the downy mildew resistance, all of them being located on the same linkage group. We mapped them on a 15 cM long region. The interval mapping analysis revealed the presence of a QTL with a LOD score of 19. This QTL accounted for 76 % of the total phenotypic variation. We considered this QTL as a major gene and we called it *Rpv1*.

In order to test the application of markers in a marker-assisted selection (MAS) program, two SSRs polymorphic markers flanking *Rpv1* and separated by 8 cM were chosen.

Six breeding populations were studied. The male parent was a BC6 individual derived from *Muscadinia rotundifolia* and carrying *Rpv1* while the female was derived from various resistant origins. The result of this MAS will be presented.

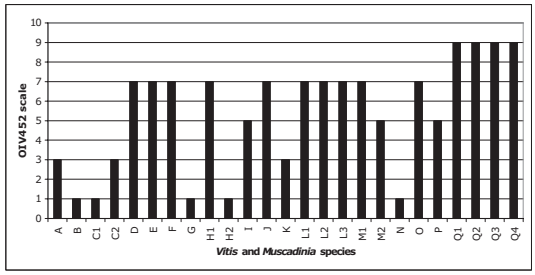

Fig. 1. Resistance level evaluation of *Vitis* species and *Muscadinia rotundifolia* varieties using the OIV 452 descriptor (1= susceptible, 9= totally resistant). A: *V. aestivalis*, B: *V. arizonica*, C1: *V. berlandieri* Planchon, C2: *V. berlandieri* Thyers, D: *V. candicans*, E: *V. cinerea*, F: *V. cordifolia*, G: *V. doaniana*, H1: *V. labrusca* Concord, H2: *V. labrusca* Isabelle, I: *V. linsecumii*, J: *V. monticola*, K: *V. palmata*, L1: *V. riparia* Gloire, L2: *V. riparia* Millardet, L3: *V. riparia* Muller, M1: *V. rubra* 1, M2: *V. rubra* 2, N: *V. rupestris,* O*: V. titiana,* P*: V.vulpina,* Q*1: Muscadinia rotundifolia* Regale*,* Q2*: Muscadinia rotundifolia* Carlos*,* Q3*: Muscadinia rotundifolia* Dulce*t,* Q4*: Muscadinia rotundifolia* YxC

Efficay evaluation of the resistance inducer Benzothiadiazole against grapevine downy mildew

Silvia Dagostin, Antonella Vecchione, Luca Zulini, Alessandro Ferrari, Ilaria Pertot

First, fourth and fifth authors: SafeCrop Centre c/o Istituto Agrario di S. Michele all'Adige, Second and third authors: Agricultural Resources Department, IASMA Research Center, via Mach 1, S. Michele all'Adige (TN), Italy.

Downy mildew, caused by the obligate biotrophic oomycete *Plasmopara viticola* (Berk. & Curt.) Berl. & de Toni, is one of the most important grapevine disease worldwide. Control of downy mildew is currently based on application of several chemical treatments. Future prospect in chemical pesticide development is limited and the alternatives currently used in organic farming are less effective and inconsistent in controlling downy mildew. The use of plant defense elicitors has arisen in the last years as a new possible disease control measure (Walters et al., 2005) and a range of chemical or natural inducers have been identified in various plant/pathogen systems.

A benzothiazole derivate benzo(1,2,3)thiadiazole-7-carbothic acid S-methyl ester, also known as acibenzolar-S-methyl or BTH, was shown to be a strong systemic acquired resistance (SAR) activator and is registered and used on several crops with the commercial name of Bion® (Syngenta), containing 50% of active ingredient. It does not exhibit noticeable direct-activity against pathogens, but increase crop resistance to diseases, caused by viruses, bacteria and fungi, by activating SAR signal transduction pathway in several crops. Just as an example, reduction of bacterial and fungal disease of tobacco, of cucumber and Japanese pear disease and of disease caused by *Colletotrichun gloesporioides*, *Erwinia amylovora* and *Peronospora parasitica* were demonstrated (Brisset et al., 2000; Cole, 1999; Godard et al. 1999; Ishii et al. 1999; Lopez and Lucas, 2002).

The aims of this study were to test the ability of BTH to induce resistance in grape (*Vitis vinifera*) against downy mildew caused by *Plasmopara viticola*. The efficacy was evaluated under greenhouse and field condition, and the time frame in which resistance induction can be triggered was correlated.

The effect of the applied product and plant reaction to the disease development was studied under controlled greenhouse conditions (20° C, 70 ± 10 % RH), using potted plants of the susceptible cultivar (Pinot gris) having two shoots with four fully expanded leaves. Five plants (replicates) were used in each treatment. The plants were sprayed with 0.1% Bion (Syngenta Crop Protection, Italy) aqueous solution as listed in table 2. As standard references water and copper hydroxide (Kocide 2000, Du Pont De Nemorous) or mancozeb (Dithane DG, Dow AgroSciences Canada) were sprayed 6 hours before inoculation. On each plant 40 ml of treatment solution was sprayed. Fresh sporangia of *P. viticola* were used as inoculum, using a concentration of $1x10^5$-10^6 sporangia/ml. Sporangia suspension was sprayed on the underside of wet leaves and plants were incubated overnight in darkness at 80% RH and 20 °C. Between seven to ten days after artificial inoculation, severity (percentage of infected leaf area) and incidence (percentage of infected leaves) were assessed on all leaves. Experiments were repeated twice.

Tab. 1. Application of BTH in greenhouse tests against grapevine downy mildew.

Treatment	15 days *	7 days*	4 days*	6 hours *
BTH 3X		x	x	x
BTH 2X			x	x
BTH 1X				x
BTH 15	x			
BTH 7		x		
BTH 1				x

* before inoculation with *P. viticola* ($2.5x10^5$ sporangia/ml)

In 2005 a field experiment was carried out according to EPPO guidelines in an experimental organic vineyear of IASMA, in Rovereto, Italy, on cultivar Cabernet Sauvignon grafted on Kober 5 BB. Completely randomized blocks design with four replicates was used. Plot size was 16 m^2 containing 7 plants. BTH was weekly sprayed, at concentration 100 g/hl (12 hl/ha), giving a total of 14 applications. BTH was integrated with three copper treatments during bloom. Copper hydroxide treated plots were used as a reference. The unchecked epidemic was followed on an untreated larger size plot (untreated control). Disease severity and incidence were weekly evaluated scoring 50 leaves and 50 bunches in each replicate.

Greenhouse and field data were statistically analyzed by ANOVA and Tukey's test was used to separate means, using Statistica Software.

The results obtained in the first greenhouse experiment, where BTH was applied 15, 7 and 1 day before inoculation, show that the plants exhibited application time-dependent resistance to downy mildew (table 2). These results, as already reported by Godard at al. (1999) for crucifers downy mildew, show that when BTH is applied to grapevine with an interval between treatments and inoculation of 24 hours, no control against *P. viticola* was achieved. Optimal induction of resistance is achieved with a lag of one week. A lag of 15 day is conversely too long and it does not guarantee adequate disease control.

In the second experiment the efficacy of one (6 hours before inoculation), two (4 days and 6 hours before inoculation) or three (7, 4 days and 6 hours before inoculation) applications of BTH were compared (table 3). The best results were achieved on the plant treated three and two times with BTH. A low effect, statistically not different from untreated, was observed when BTH was applied only once 6 h before the inoculation. This confirms to the short application-inoculation interval does not allow the buildup of host resistance and probably that several application can reinforce the effect.

Tab. 2. Effect of a single preventive BTH treatment on leaf downy mildew in controlled greenhouse conditions. Application on Pinot gris plants were done 15, 7 and 1 days before inoculation with *P. viticola* sporangia. Numbers with a letter in common are not significantly different according to Tukey's test ($P \leq 0.05$).

Treatments	Severity (%)	Incidence (%)
BTH 15	15.5 a	73.8 b
BTH 7	9.8 a	42. 6 a
BTH 1	30.0 b	86.7 b
Mancozeb	4.3 a	29.4 a
Untreated	65.3 c	95.5 b

Tab. 3. Effect of repeated BTH treatments on leaf downy mildew in controlled greenhouse conditions. Pinot gris plants were treated one, two and three times (6 hours, 6h+4days, and 6h+4d+7d) before artificial inoculation Numbers with a letter in common are not significantly different according to Tukey's test ($P \leq 0.05$).

Treatments	Severity (%)	Incidence (%)
BTH 3X	5.9a	36.79 ab
BTH 2X	6.88 a	37.83 ab
BTH 1X	16.54 ab	73.15 ab
Copper Hydroxide	7.42 a	27.33 a
Untreated	44.66b	76.88 b

In the field trial, plant treated with BTH had a significantly lower disease severity and incidence compared to untreated plants and did not differ from copper treated standard reference (tab. 4). However disease pressure was low during grape growing season in 2005. The data on bunches was not informative: because of the low incidence of infections on the untreated control (5.5%) efficacy can not be evaluated. As in greenhouse experiments, no phytotoxicty was detected on BTH treated plants under field condition.

Tab. 4. Field trial. Downy mildew severity and incidence on leaves of grape. Assessment at harvest. Numbers with a letter in common are not significantly different according to Tukey's test ($P \leq 0.05$).

Treatments	Severity (%)	Incidence (%)
BTH	7.0 a	41.5 a
Copper Hydroxide	12.35 a	52.0 a
Untreated	59.3 b	99.0 b

The current results suggest a good activity of BTH as resistance inducer against grapevine downy mildew, but it is important to attain a certain the number of application and a suitable interval between application and infection. Moreover in this study leaf treatment was considered the sole mode of application. However, other studies (Anfoka et al, 2000) suggest that other methods could be used for induction of SAR. These factors should be optimized in order to develop a reliable and effective control measures against grapevine downy mildew under field condition.

Acknowledgments

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Session 2:

Biology of the two pathogens, climate interactions and disease expression

Water availability in the leaf litter and germination of *Plasmopara viticola* oospores

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Sexual spores of the Oomycete *Plasmopara viticola* (Berk *et* Curt.) Berlese *et* de Toni are the overwintering structures of the fungus. Oospores are formed in autumn in the infected leaf tissue and they remain in the leaf litter above the vineyard ground. During this period they complete their morphological maturation and overcome dormancy, a physiological stage that does not allow oospores to germinate in winter even if climatic conditions are favorable. Oospores then germinate in a macrosporangium that releases zoospores which are responsible for grape downy mildew primary infections in spring.

It is well known that temperature is a main factor regulating dormancy and germination of *P. viticola* oospores (Laviola *et al*., 1986). It was also observed that rainfall distribution in spring influences the time when oospores germinate: frequent rainfall accelerates the process while long dry periods delay it (Rossi *et al.*, 2002). The role of rainfall has been demonstrated empirically, but there are no experimental findings to explain it from a biological point of view. It is likely that rainfall moistens the leaf litter and provides water for the physiological processes involved in overcoming dormancy and promoting germination. Water activity (a_w) is a measure of the relative availability of water in a substrate and it is a measure of water that an organism can use to support its growth. Minimum a_w varies from species to species; however, most fungi grow better at 0.75-0.95.

To find out more about the role of water in oospore biology, a study was carried out to investigate: i) dynamics of the leaf litter moisture during winter and early spring; ii) the relationship between moisture and a_w; iii) relationship between a_w and oospore germination.

In a first study, full-grown grape leaves were dried and exposed on the vineyard ground for 96 hours; this was replicated many times between March and June, on days without rainfall. Leaves were weighed every 2 hours during both daytime and nighttime, and their moisture content was calculated as a percentage of the dry weight; the dry weight was determined at the end of each exposure by keeping leaves at 120°C for 2 days.
The percent moisture of the grape leaves in the leaf litter showed a daily periodicity (Fig. 1). In daytime the moisture is frequently lower than 20% with a minimum level around 14.00 h. Between 22.00 and 8.00 h moisture is usually higher than 20% and increases to reach the maximum at 8.00 h. Nighttime variability between replicate leaves and experiments was higher than in daytime.

A second experiment was aimed at studying the relationship between percent moisture, which is simply the percent of water within leaves of leaf litter, and a_w, which measures the relative availability of water in these leaves. Water availability is equal to the equilibrium relative humidity divided by 100, at constant temperature and pressure: a_w then depends on water exchange between the substrate and the atmosphere, in the form of absorption and desorption. This relationship is usually depicted graphically, the resulting construct being called a moisture sorption isotherm (Fig. 2).

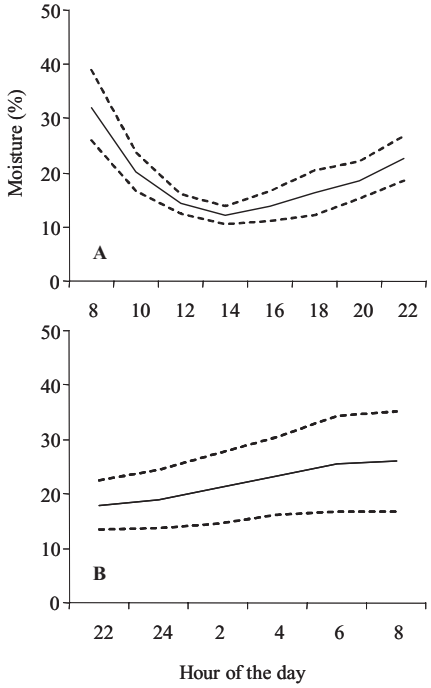

Fig. 1. Diurnal periodicity of the percent moisture of grape leaves in the leaf litter above the vineyard ground: average data of several replicate leaves exposed between March and June, in the experiments made in daytime (A) and nighttime (B). Dotted lines represent 99% confidence intervals.

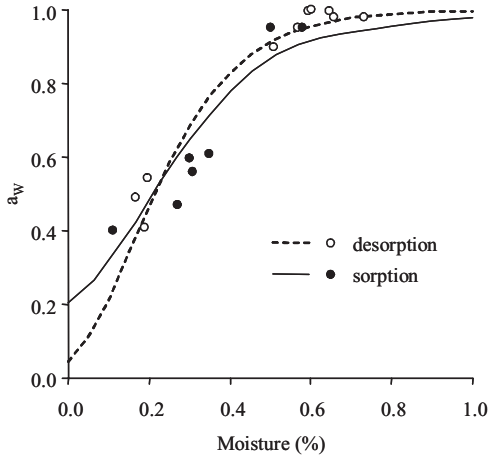

Fig. 2. Percent moisture *versus* water activity in grape leaves which have been dried to mimic leaves of the leaf litter covering the vineyard ground. Dots represent absorption and desorption data at 10°C, while lines represents the moisture sorption isotherm according to the modified Chen-Clayton equation.

To obtain the moisture sorption isotherms for the grape leaves of the leaf litter, leaf discs were dried and put in both absorption and desorption conditions under 4 different temperature regimes between 5 and 20°C. Different sorption conditions were obtained using glass jars as humidity chambers that were prepared with distilled water to obtain water absorption, or sodium chloride, to obtain desorption; inside the glass-jars, samples were not in contact with either water or salt. Both a_W and percent moisture were measured with a time step of 30 minutes. The experimental data were then fitted using five different equations widely used in literature for this purpose for various substances. The modified Chen-Clayton equation was the best for both absorption and desorption at the different temperature regimes.

It was observed that in grape leaf discs, similar to the leaf residues forming the leaf litter containing *P. viticola* oospores, moisture percentage lower than 30-40% correspond to water availability that makes any fungal growth theoretically impossible.

In order to assess the role of water availability during oospore germination, a laboratory experiment was carried out using *P. viticola* oospores. Grape leaves infected by downy mildew and containing a high density of oospores were collected from the leaf litter of a vineyard which had been severely affected by downy mildew at the end of the previous season. In early spring, when the first oospores were able to germinate, leaves were dried at room temperature and milled. Distilled water was then added to milled leaves to obtain different water contents: a_W equal to 0.123, 0.292, 0.487, 0.785, 0.954, and 0.991. Leaf samples were incubated at 20°C for two to fifteen days in Petri dishes where the a_W was maintained constant. Afterwards dishes were flooded with water and oospore germination was measured by means of the floating leaf disc assay (Hill, 1998). The numbers and dimensions of the infection sites which appeared on the leaf discs were determined and expressed as disease severity (percentage of the total leaf surface becoming infected). The average number of days required to reach 50% and 90% of disease severity was then calculated for each level of a_W (Tab. 1).

A significant difference was found between the oospores maintained at 0.785 or higher a_W and those incubated at 0.487 or lower water availability. In the former leaves first infections always appeared 1 day after the leaf discs remained in water, while in the latter it occurred between 2 to 10 days in water. Similarly times required to reach 50% and 90% of disease severity were about two times longer in the leaves maintained at the lowest a_W (Tab. 1). Therefore, development of *P. viticola* oospores was significantly influenced by the water available within the grape leaf residues where they were held: $a_W \leq 0.487$ (about 20% moisture) delayed germination while $a_W \geq 0.785$ (about 40% moisture) promoted it.

This study is the first published investigation on the relationships between the water content of the leaf litter holding *P. viticola* oospores and oospore development. It was ascertained that the leaf residues must have a moisture greater than 20% to support optimal development of the oospores. In the measurements made in this work during the season when oospore usually overcome latency and germinate, that is between March and June, the moisture content of the leaf residues in days with no rainfall is lower than the above mentioned level for many hours of the day. Therefore, rainfall has a key role in wetting and moistening the leaf litter and consequently promoting oospore germination.

The dynamics of the available water in the leaf litter covering the vineyard ground can not be ignored in the simulation models aimed at producing warnings for primary downy mildew infections on grapevine.

Tab. 1. Comparison between the number of days of incubation in water at 20°C required to reach 50% and 90% of downy mildew severity in grape leaf discs holding *P. viticola* oospores that have been previously maintained at different levels of available water (a_W).

Disease severity	a_W					
	0.123	0.292	0.487	0.785	0.945	0.991
50%	4.0	5.5	4.5	2.8	2.9	1.9
90%	6.5	8.9	7.2	4.5	4.7	3.2

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Do flag shoots serve as the main source of primary inoculum in grape powdery mildew epidemics in Israel?

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Powdery mildew in grapes, caused by *Erisyphe necator*, is the most destructive vine disease in Israel. The pathogen infects all the green parts of the grapevine, but the most important damage is to the bunches - the disease may cause total loss of the yield. Disease management in Israel is primarily based on fungicidal sprays which are applied at regular intervals from shortly after bud break until the veraison stage. Flag shoots are considered as the only source of primary inoculum in Israel but several lines of evidence suggest that other sources of inoculum are involved. In this study we examined this possibility under natural epidemics of the disease.

The appearance of flag shoots was surveyed on six varieties in commercial vineyards in the years 1998-2003. In each vineyard, 0.1 to 0.3 ha were examined from bud break to blossoming. Flag shoots were observed only in plots of *cv.* Carignane y, with single instances in two other varieties. Flag shoots appeared when healthy shoots were already present and continued to appear for about a month, at numbers ranging from 70 to 760 per ha.

The coincidence between flag shoot incidence and subsequent leaf infections was studied in the year 2001. Variation in flag leaf infections between experimental plots was created by spraying, or not spraying the vines with fungicides in the previous season (year 2000). All experimental plots remained untreated in 2001 and the number of flag shoots and the subsequent development of powdery mildew on the leaves was determined. The experiments were carried out in vineyards of Carignane, which produces flag shoots and Cabernet, which does not produce flag shoots. In the plots sprayed with fungicides in 2000, powdery mildew severity on the leaves remained low throughout the entire growing season (Figs. 1 A and B). In the following season, significantly less flag shoots were observed in plots that were protected in 2000 than in the untreated plots of Carignane (Fig. 1C) but they were not observed at all in both treatments of the Cabernet experiment (Fig. 1 D). Despite these differences, powdery mildew developed at the same level on the leaves of both varieties in 2001 (Figs. 1 E and F).

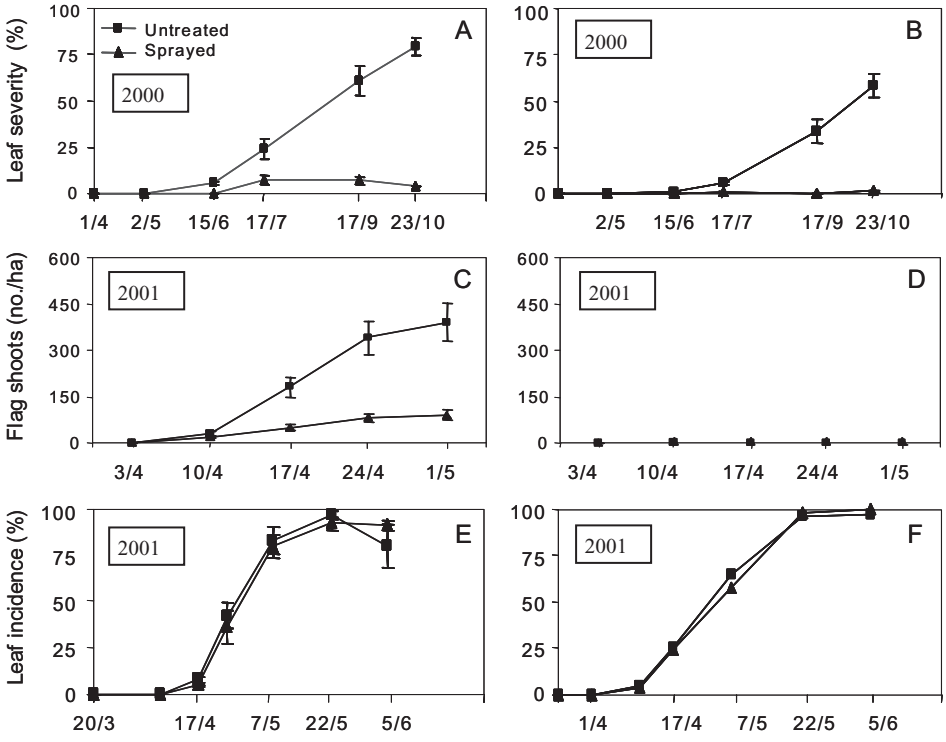

Fig. 1. The effect of powdery mildew development on the leaves of *cv.* Carignane (A, C and E) and Cabernet (B, D and F) vines in 2000 and the subsequent intensity of flag shoots and leaf disease in 2001. Experimental plots were either sprayed with fungicides or left untreated in 2000 (A and B) while the response was assessed in 2001 in which all plots remained untreated in 2001. Flag shoot intensity (C and D) and powdery mildew development (E and F) were recorded in the same plots in 2001. Vertical bars represent the SE.

The significance of flag shoots as a source for powdery mildew inoculum for the adjacent leaves was determined in experiments conducted in Carignane vineyards in 1999 to 2002. In each year, shoots exhibiting the typical flag shoot symptoms were marked with colored plastic tags. For comparison, healthy shoots in areas without flag shoots were marked as well. The spatial development of the disease around the marked shoots was recorded over time. In this report, data from 2001 are presented. Powdery mildew severity was high in the vicinity of the flag shoots and disease severity decreased gradually with increasing distance from the infected shoot. At distances of >60 cm from the flag shoots disease severity (Fig. 2A) was at the same magnitude of the severity recorded at areas without flag shoots (Fig. 2B).

Results recorded in the present study raise questions regarding the significance of flag shoots as the main source of primary inoculum in grape powdery mildew epidemics in Israel. Powdery mildew development was not related to the incidence of flag shoots in a variety that produces flag shoots (Carignane, Fig. 1E) and equivalently high disease levels was recorded in the same year in plots of Cabernet, a variety that does not produce flag shoots (Fig. 1F). Furthermore, flag shoots seem to induce high disease levels only in their immediate vicinity – up to 60 cm away (Fig. 2).

In summary, we conclude that the powdery mildew infections may originate from a different source of primary inoculum. In a follow-up study it was found that ascospores, produced in overwintering cleistothecia, are the main source of primary inoculum in grape powdery mildew epidemics in Israel. Results of this study will be presented elsewhere.

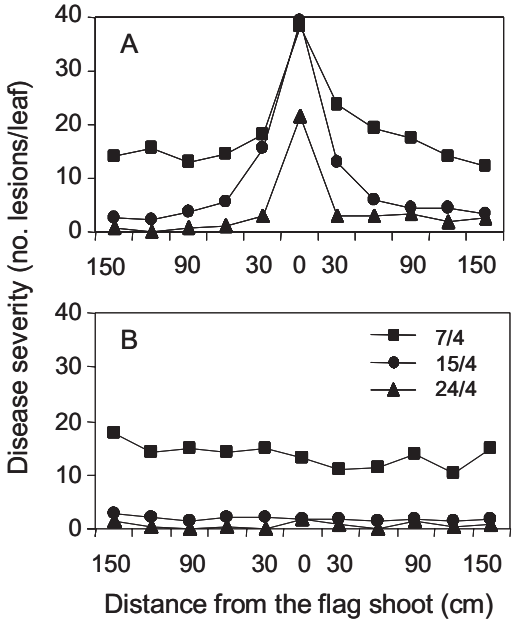

Fig. 2. The spatial and temporal development of powdery mildew around vines with flag shoots (A) and in areas without flag shoots (B). Disease was recorded in vines of Carignane in the year 2001.

Germination delay under controlled conditions of overwintered oospores of *Plasmopara viticola* in Trentino Region (Northern Italy)

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Recent studies on downy mildew populations showed that primary infections have an important role in *Plasmopara viticola* epidemics. European populations were shown to be highly genetically variable and composed by a large number of genotypes. Genetic heterogeneity indicates a high occurrence of oospore driven infections as a consequence of massive sexual reproduction (Gobbin et al., 2003). In contrast to the existing beliefs, it was shown that subsequent periods of primary infection outbreaks occur, with different extents, from May to August, depending on the epidemic. However, oospore quantitative contribution to the epidemic decreases with the progress of time (Gobbin et al., 2005). Overwintered oospores, collected in different time of the season even if put in optimal condition (water and 20°C) require a certain time to germinate (Hill, 1998). In some periods they are very fast in germinating (one day or less) in others they do not germinate at all.

Materials and methods

From 1999 to 2005 oospores germination delay was evaluated using the leaf disk germination test as described by Hill (1998). From 1998 to 2004 *P. viticola* infected leaves were collected in an untreated vineyard (Rovereto, Northern Italy) in late summer (September) and air dried. Leaves were crushed in a blender adding distilled water. Leaf material was put on the surface of small holed boxes (used in cheese industry) filled for 2/3 with sand. Boxes were buried in the same vineyard in such a way that leaf material was at ground level. Starting from the end of March, a fixed quantity of leaf material was weekly collected and placed into boxes, in water, letting 15 leaf disks of healthy grapevine to float on water surface, under controlled temperature (20° C). Leaf disks were daily changed and incubated in humidity chambers for 7 days at 20° C to evaluate if an infection succeeded Time for germination and percentage of infected leaf discs were evaluated. When time for germination was more than 14 days, it was assumed that oospore do not germinate at all. In 1999 germination delay of oospores originating from leaf material coming from a second location (S. Michele all'Adige), but overwintered in Rovereto was also analysed.

Daily minimum, average and maximum temperature and total daily rain were collected by a weather station located inside the untreated vineyard. Presence of first oil spots was weekly checked in the untreated vineyard.

Results and discussion

Oospores germination delay (days at optimal conditions before germination), temperatures and rain are presented in figs. 1 and 2, starting from 1 March until 30 of June. In 2005 the germination delay was evaluated until 20 July. Spring of 1999 was relatively mild and dry (with several days in April with average daily temperature higher than 10°C). The oospore germination delay dropped to one day after a period of rains (25-30 April). After two weeks without rain (21 May-8 June) the time required for germination increased and decrease again after another period of rain. Oospores from leaf material collected in the two locations, but overwintered in the same place showed exactly the same germination delay. The temperatures during winter in S. Michele are lower than in Rovereto (Σ T_{aver} S.Mich. -T_{aver} Rov. form 1/12/1998 to 31/03/1999= -33) and it rains more (208 mm more in S. Michele than in Rovereto during the above mentioned period). 2000 and 2001 were warm and wet with frequent rains and the oospores germinated very quickly for a very long period. In 2001 after a warm and dry period they increased to 12 days their germination delay, starting from 12 June. In 2002 oospore germination delay was fluctuating: one day required for germination after periods of rain and no germination at all after dried periods. 2002 was relatively dry; germination delay was higher than one day except for the sample of 22 April and in the period 12 April-4 June. Oospore stopped to germinate from 10 June. 2003 was relatively cold in March with average daily temperature lower than 10° C until beginning of April. After 30 mm of rain on 19 April germination delay dropped to one day. After 9 May there was no rain (except for 0.8 mm on 12 May) and oospore stopped to germinate. In 2005 a situation similar to 2002 was seen with a fluctuation in germination delay and alternation of dry and rainy periods. It seems that oospores, at least in some years, are able to reduce their promptness of germination, but they start to germinate quickly if weather conditions change. First oil spots were always seen after periods of fast oospore germination delay.

Even if it was not possible until now to find a mathematical correlation between germination delay and weather conditions, it is clear that they need a certain number of days with average daily temperature of more than 10° C and a period of rain. Warm and dry period in summer prevent oospores ability to fast germinate.

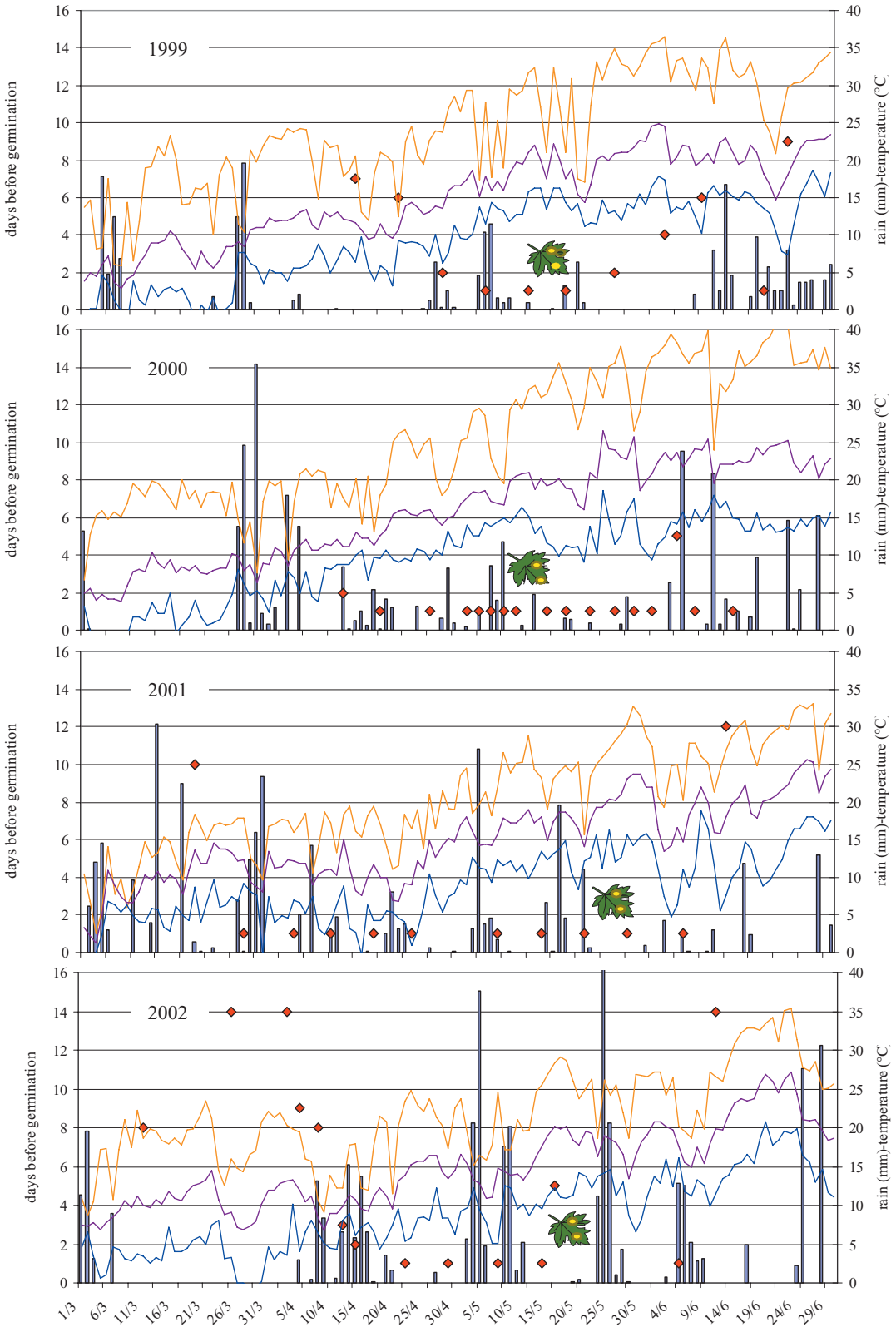

Fig. 1. Germination delay of overwintered oospores (Rovereto, Northern Italy) under controlled conditions (red square marker), minimum, average and maximum temperatures (blue, violet, and yellow line) and rain (blue histogram). Leaf indicates when the first oil spot symptoms were found in the vineyard.

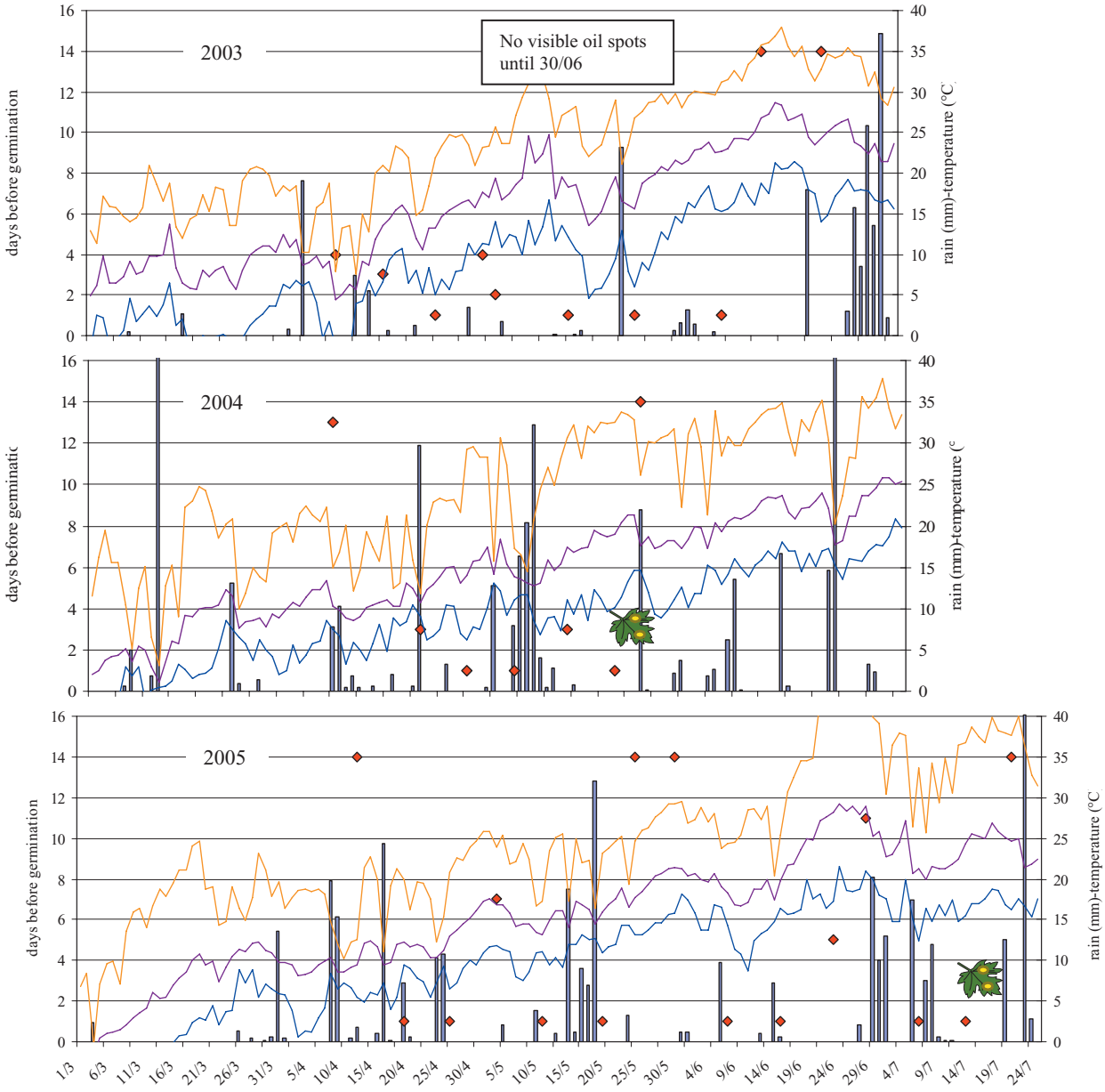

Fig. 2. Germination delay of overwintered oospores (Rovereto, Northern Italy) under controlled conditions (red square marker), minimum, average and maximum temperatures (blue, violet, and yellow line) and rain (blue histogram). Leaf indicates when the first oil spot symptoms were found in the vineyard. In 2005 the germination delay was assessed until it reached 14 days (until 20 July).

Acknowledgements

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Influence of Sun Exposure on Powdery Mildew Development

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Powdery mildew is often most severe in portions of the vineyard and/or individual canopies that are subject to shading. Although this phenomenon is widely recognized, it has received relatively little formal study. Willocquet et al. (1996) showed that UV-B radiation negatively impacted both conidial germination and mycelial growth of *Uncinula necator*. They suggested that solar radiation is an important factor in the development of grape powdery mildew epidemics, and that training systems that increase exposure of the vines to sunlight could help to reduce disease development. Zahavi et al. (2001) monitored powdery mildew development in vineyard blocks with different row spacings and training systems, and associated increased disease development with decreased light intensity in the fruit zone. Keller et al. (2003) related the increased development of powdery mildew in low UV environments with various physiological characteristics of vines growing under such conditions, including low concentrations of constitutive phenolic compounds that might hinder fungal pathogenesis and reduced cuticular wax deposition.

Our field experience suggests that powdery mildew may be a particular problem not only in specific vineyard and canopy locations subjected to shading, but also regionally in seasons with reduced solar radiation resulting from prolonged periods of cloudy weather. However, there are few data to which we can refer for guidance in quantifying these effects. Therefore, in 2005, we initiated a multi-year study whose objective is to better document and explain the relationship between sunlight exposure and powdery mildew development on fruit and foliage. Our results are as yet preliminary, but they are offered here for the purposes of further consideration and discussion.

In one set of experiments in a vineyard of the inter-specific hybrid cv. Chancellor, vines were maintained (i) in full sunlight; (ii) beneath a single layer of shade cloth, which admitted a daily average of 45% of available solar radiation (as measured in the 400 to 1100 nm range using a LI-COR pyranometer); and (iii) two layers of shade cloth, which admitted 20% of available solar radiation. On three separate occasions, eight different replicate shoots per treatment were inoculated with *U. necator* conidia, and disease severity (% leaf area infected) was assessed on each leaf 2 weeks later. Across all runs of the experiment, mean disease severities were 21, 51, and 61%, respectively, in these three treatments.

Air temperature, relative humidity, and solar radiation in the range of 400-1100nm were measured every 15 min with a data logger. Additionally, ultraviolet radiation was measured with an Ultraviolet Multi-Filter Rotating Shadowband Radiometer (UV-MFRSR). Shading had no effect on ambient air temperature or relative humidity. The proportion of UV radiation that was reduced by the shade cloths was similar to that within 400-1100 nm range measured with the pyranometer.

In another, factorial experiment in a vineyard of cv. Chardonnay (Umbrella-kniffen training system), we examined the effects of natural shading. One group of vines was located at the edge of the vineyard, immediately west of a line of tall trees that provided morning shade; the second was in the same row, but in a cleared area not shaded by the trees. Within these two groups, we inoculated *U. necator* conidia onto shoots that were either (a) on the outer edge of the canopy, fully exposed to the sun, or (b) trained into the center of the canopy, which was dense and provided substantial shading at the time that the experiment was conducted in August. Disease severities were determined 2 weeks after inoculation.

The results are summarized in Figure 1. Note that both sources of shading increased disease development. Shade from the trees roughly doubled disease severity for both the outer and inner portions of the canopy. Similarly, severity on leaves within the canopy was three to five times greater than on leaves comprising the outer edge, for both sets of vines. And these effects were cumulative, with fully 63% of the leaf area diseased on the inner shoots of vines shaded by the trees, versus only 9% on shoots that were provided full sunlight exposure. Once again, there were no differences in ambient air temperature or relative humidity among these treatments. However, UV radiation measured within the center of the most heavily shaded canopy was reduced by 92 and 99% relative to a reference UV sensor in full sunlight, on two different occasions.

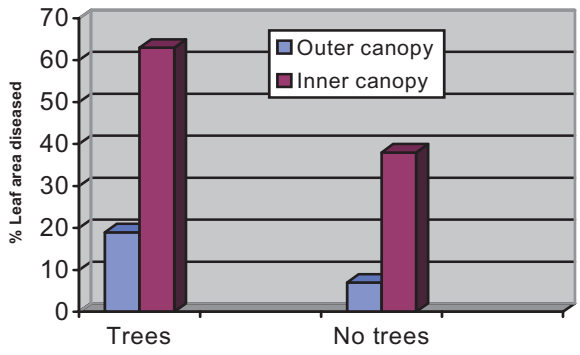

Fig. 1. Effect of two sources of shading on the development of powdery mildew. Evaluated leaves were from shoots on the outer (sun-exposed) or inner (shaded) portions of the vines, which were (a) adjacent to a group of tall trees (morning shade) or (b) away from the trees and exposed to available sunlight all day.

Although there was no effect of sunlight on ambient air temperatures in the different shading treatments, we hypothesized that leaf temperatures in full sunlight might be higher than those in the shade. Therefore, leaf temperatures were measured with a hand-held infra-red thermometer. On a calm sunny day in August, temperatures of leaves in direct sunlight were 8-14^{o}C higher than for leaves in the shade-cloth treatments. Similarly, Chardonnay leaves in the direct sun were 6-11^{o}C warmer than the naturally-shaded leaves within those canopies; such leaves were near ambient temperature to a few degrees cooler. These vines were not irrigated. In separate sets of measurements of irrigated and water-stressed vines in different locations, the temperature elevation of sun-exposed leaves was greatest for vines that appeared to be drought stressed. Nevertheless, these data suggest that under some conditions, the temperature of shaded leaves might be in the optimum range for disease development, whereas the temperature of those in full sun might be suboptimal or even lethal for the causal fungus. For example, in one set of measurements in our experiment, leaves in the shade at midday measured 30^{o}C, whereas those in full sunlight measured 38-43^{o}C.

We examined the interactive effects of drought stress and leaf temperature in another set of experiments with potted Chardonnay vines maintained outdoors during the summer. One group of vines was watered regularly, whereas another was watered to induce a level of stress that would cause stomates to close. In two of the three repeats of the experiment, drought-stressed vines developed no signs of the disease, and in the third, only 8% of the leaf area was infected (i.e., <3% on average across all three runs of the experiment). In contrast, the well-watered plants averaged 25% leaf area infected across the three runs, with disease severity on some individual leaves as high as 85% in one experiment. Leaf surface temperatures were 7-10°C higher on the drought-stressed versus well-watered vines, reaching values in the low 40's during midday. All vines were in full sunlight. Other vine responses to the imposed water deficit were not quantified.

Although this project is still young, our initial data confirm previous observations and experimental results indicating that full sun exposure can dramatically limit powdery mildew development relative to what might otherwise occur in its absence. In addition to the previously-suggested effects of UV radiation on disease development, our data also suggest that sun exposure might have a limiting effect by elevating the temperature of irradiated leaves, and that this effect might be further exacerbated under water-stressed conditions. Current data, and practical experience, suggest that solar radiation and perhaps some measure of plant water status might be useful environmental variables in models designed to forecast the development of grapevine powdery mildew.

Refereces

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Session 3:

Poster presentation

Host resistance, induced resistance, mechanisms, breeding

Biology of the two pathogens, climate interactions and disease expression

Peroxidases activity in susceptible and resistant grapevine after inoculation with *Plasmopara viticola*

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Stilbenes analysis after *Plasmopara viticola* infection were shown to be an effective discriminating tool to identify susceptible and resistant grapevine. Resistant cultivars can transform resveratrol to viniferins, a more toxic product for the pathogen. Susceptible cultivar accumulates preferentially a glycosylated form of resveratrol known as piceid, a non-toxic product for the pathogen even at high concentration (Pezet et al., 2004). The objective of this research was to investigate the role of enzymes that could convert resveratrol in viniferins in resistant cultivar, and serve as a marker for resistance. Peroxidases (POX) are a class of enzymes that could play this role. They are involved in many parts of plant defence processes like oxidative burst, lignification of cell wall at the papillae or phytoalexin production.

Time course experiment. Two cultivars of *Vitis vinifera* were chosen as model for resistance and susceptibility to downy mildew, respectively Solaris and Chasselas.

Peroxidase activity was measured on crude extract of protein for both cultivars, on abiotic and biotic stress, after elicitation by laminarin (fig. 1B) or at the site of infection of *Plasmopara viticola* (fig. 1A). Stilbenes were analysed by HPLC on the same set of samples. Higher peroxidase activity was observed in resistant variety after elicitation but that was not the case after infection. Elicitation with laminarin does not produce hypersensible response (HR) and no stilbenes accumulation could be measured. In contrary the downy mildew inoculation produce the classical HR and viniferins accumulation in the resistant cultivar.

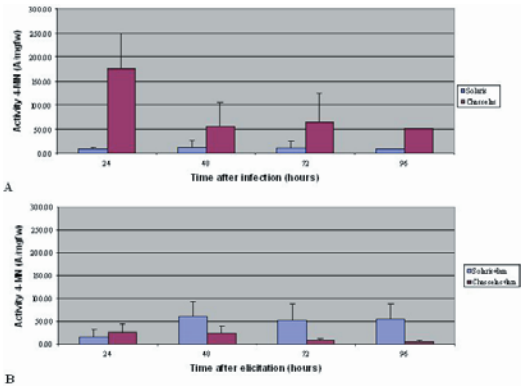

Fig. 1. Peroxidase oxidation of 4 methoxy-1-naphtol (4-MN) in leaves of Solaris and Chasselas at different time after infection (A) and elicitation with laminarin (1g/ml) (B).

Abiotic and biotic stress could produce burst of POX activity that could be linked or not to phytoalexin synthesis. So we decide to develop a test to directly measure peroxidase activity on resveratrol.

Resveratrol oxidation Stability of resveratrol in aqueous solution was measured during one hour at different pH and hydroxide peroxide concentrations. At acidic pH and low concentration of hydroxide peroxide resveratrol absorbance is stable at 307nm and enzymatic oxidation could be monitored (fig. 2).

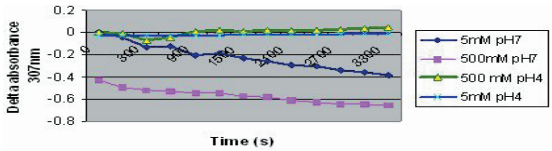

Fig. 2. Resveratrol stability as a function of time, pH and hydroxide peroxide concentration

Proteins were purified from the resistant and the susceptible cultivar before and 5 days after inoculation using FPLC and a UNO-Q1 ion exchange column. POX activity was recorded and the fraction with highest POX activity was tested on resveratrol. In an *in vitro* test, resveratrol was added to the enzyme extract and decrease of absorbance at 307nm was recorded as resveratrol oxidation, according to Morales et al, 1997. The decrease of resveratrol and the *de novo* synthesis of viniferins were confirmed by HPLC (fig. 3).

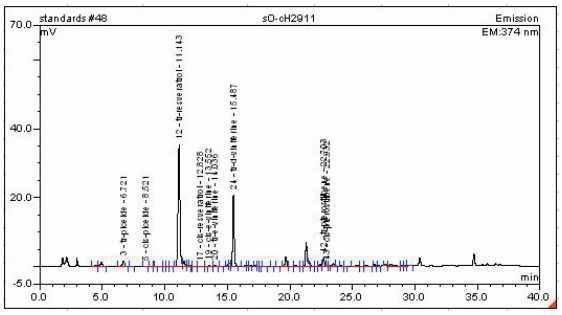

Fig. 3. HPLC-analysis of resveratrol oxidation product: resveratrol (10µg) was oxidized by the peroxidase fraction isolated from Chasselas in 100mM Tris-acetate, pH4 supplemented with 5µM of H2O2. Spectral emission (374nm) reveal specific pick of resveratrol and viniferin at 11.04 and 15.38 minutes respectively, and with specific spectrometric profile (not show).

Isoelectric focusing (I EF) Profiles Apoplastic fluid was collected from non-infected grapevine leaves by infiltration and peroxidase activity was measured on 4-MN and resveratrol. IEF of the apoplastic proteins showed differential POX profiles between Solaris and Chasselas (figure 4A). POX profile was also investigated

in other cultivars obtained from our breeding program, known to be resistant (-) or susceptible (+) according to their stilbenes accumulation during infection (Figure 4B). Decrease of activity during infection by *Plasmopara viticola* in apoplastic compartment of Chasselas has been measured (preliminary results).

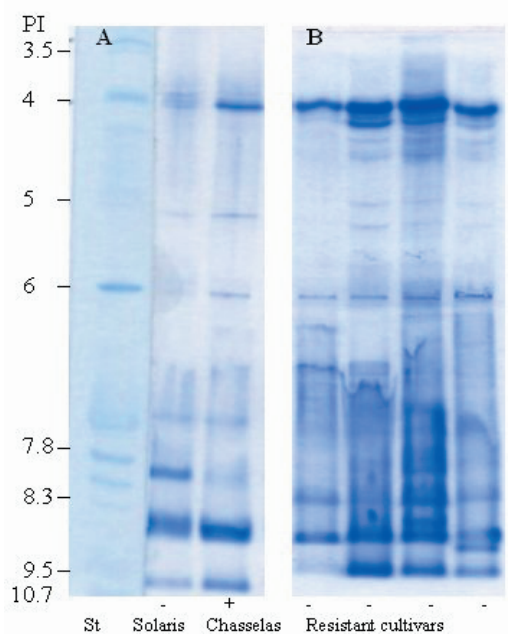

Fig. 4. Isoenzyme pattern of peroxydases separated by IEF and stained with 4-MN in presence of hydroxide peroxide

Conclusion

Peroxidase activity can be induced by biotic or abiotic stress on grapevine leaves. Specific oxidative process and specially the conversion of resveratrol into viniferins require more specific methods for proteins collection and stilbenes oxidation test.

Resistant and susceptible cultivars have the enzymatic package to synthesize viniferins. However, this does not happen to the same extent to susceptible species after *P.viticola* inoculation.

Different hypothesis should be formulated to understand this phenomenon:

1. Different isoforms of POX exist that could have more or less affinity to resveratrol. Affinity of different peroxidase isoforms to stilbenes should be accessed comparing resistant to susceptible grapes.
2. The cellular compartment of specific peroxidase is not accessible to viniferins formation. Cellular compartment of resveratrol and POX should be identified.
3. Most of the peroxidase are mainly involved in another process of plant defence, as cell wall lignification. Localisation of peroxidase activity during infection by electronic microscopy should be accessed.
4. The substrate accumulation itself, resveratrol, induce peroxidase activity.
5. Pathogen progression could induce glycosylase activity that would balance the synthesis of piceid.

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Analysis of the expression of defense-associated genes in grapevine leaves upon infection by *Plasmopara viticola*

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Downy mildew of grapevine caused by the Oomycete *Plasmopara viticola* is one of the most important diseases affecting *Vitis spp*. The pathogen attacks grapevines worldwide causing important economical losses. The current strategy to fight the disease relies totally on the use of fungicides. This practice not only is expensive (in France alone its cost is estimated to be around 150 million euros per year) but also causes a slow and progressive damage to the environment. On top of this, the arising of pathogen strains resistant to fungicides diminishes the efficiency of the practice. Thus, alternative strategies are necessary to achieve sustainable management of grapevine downy mildew.

Accordingly, at INRA Colmar we are developing a breeding programme for resistance to grapevine downy mildew that exploits mainly the resistance to *P. viticola* found in *Muscadinia rotundifolia*, whereas other sources of resistance are being characterised. In that context, we are studying the molecular mechanisms underlying the interaction between grapevine and *P. viticola*, because we believe that a sound knowledge of the pathosystem is necessary to achieve an efficient management of the breeding programme.

Part of our research is devoted to the study of the compatible interaction between grapevine and *P. viticola*. Previous research in our laboratory has characterised the different stages of the infection process by means of fluorescence microscopy. To complement this work, we aim to analyse the changes in plant defense-related gene expression associated to the different stages of pathogen development in a susceptible host using semi-quantitative RT-PCR (Reverse Transcription – Polymerase Chain Reaction). Before we could address such question it was necessary to perform some preliminary work devoted to find the best experimental conditions:

First, because we would be performing a considerable amount of RNA extractions, we tested different RNA extraction methods that will allow a considerable throughput from small amounts of starting material. We tested a column-based RNA extraction kit (Qiagen), a method using a plant-specific RNA-extraction reagent (Invitrogen) and a "classic" method using organic solvents an Lithium Chloride precipitation, scaled down to be used with 100 mg of plant tissues. In our hands, only the "classic" method consistently produced RNAs of good quality to be used in RT-PCR.

Another important point was the choice of plant material. Our genotype of choice is *Vitis vinifera* cv. Muscat Ottonel (MO), because it is a highly susceptible variety from which we know the time course of the infection by *P. viticola*. Preliminary experiments using plants grown in the greenhouse revealed differences in the expression levels of some candidate genes between non-inoculated plants. This observation leads us to the use of in vitro plants for further experiments.

Finally, to be sure of the significance of the association between stage of pathogen infection and the expression of a given plant gene, we were interested in observing pathogen infection in the same leaves that were used for RT-PCR experiments. Because fluorescence microscopy is time consuming and renders the samples useless for RNA extraction, we chose to monitor *P. viticola* development by means of RT-PCR. Thus, we designed primers to amplify the actin from *P. viticola* based on sequences from other Oomycetes present in the databases. PCRs performed on DNAs from grapevine and *P.viticola* confirmed the specificity of the primers, and preliminary experiments demonstrated their efficacy to monitor the process of infection.

Based on previous knowledge in other pant-pathogen systems, we selected the following defense-related genes for the expression analysis: pathogenesis-related protein 1 (PR1), β-glucanase (PR-2), chitinase I (PR3), chitinase III (PR8), defensin-like protein (PR12), thaumatin-like protein (PR5), glutation peroxidase and phenylalanine ammonia lyase (PAL). Primers specific for each gene were designed based on the grapevine sequences available in databases. All primer pairs amplified fragments of the expected size, and the identity of the amplified product was confirmed by sequencing. Results concerning the changes of expression of those genes associated to the different stages of infection by *P. viticola* will be presented.

Analysis of stilbene content in leaf discs of grapevine upon inoculation with *Plasmopara viticola*

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Stilbenes are considered as the main phytoalexins in grapevine. They are known to contribute to the protection against various pathogens among which downy mildew (*Plasmopara viticola*). The major molecules involved in this phenomenon are the 3,5,4'-trihydroxystilbene resveratrol (Res) and its derivatives: pterostilbene (Pt), a 3,5-dimethylated resveratrol, ε and δ viniferin (Vin), dehydrodimers of resveratrol, piceid (Pi), a glucoside of resveratrol. Their fungitoxicity differs from one to another and numerous biotic and abiotic factors can induce their synthesis.

The final aim of our research program is to test the hypothesis of a relation between grapevine resistance to downy mildew and stilbene content in leafs after inoculation. An HPLC-DAD method was adapted to assay the stilbene present in different sources of resistance. Then different factors supposed to influence stilbene content in leaf like the concentration of sporangia in the inoculum and the leaf level on the plant have been investigated.

Two genotypes were studied: *Vitis vinifera* Syrah (susceptible to *P. viticola*) and a hybrid called RV1 (partially resistant, BC1 between *Muscadinia rotundifolia* and *V. vinifera*). Leaves were harvested on green cutting grapevine of eight to twelve leaf stage cultivated in greenhouse. Sixteen leaf discs were placed in a Petri dish of 75 mm of diameter and sprayed with 1 ml of a suspension of *P. viticola* with a concentration ranging from 0 to 10^5 sporangia.ml^{-1}. HPLC-DAD was performed at 5 or 6 days post inoculation (dpi).

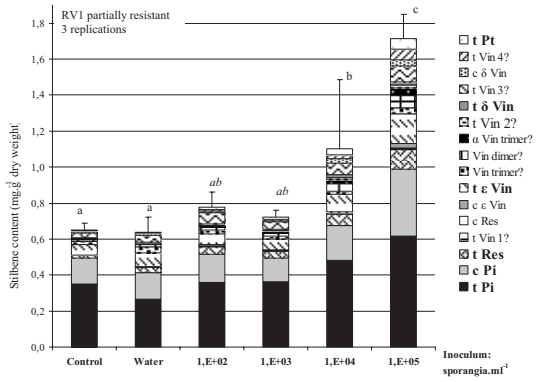

Fig. 1a. Relation between inoculum concentration of *P. viticola* and stilbene content in leaf disc of RV1 (partially resistant) 6 dpi. t for the isomer *trans*, c for the *cis*

Our results show that numerous stilbenes were induced after RV1 inoculation (Fig. 1a). Sixteen stilbenes were detected with our HPLC-DAD method. Stilbenes noted in bold (Fig. 1) where identified from controls based on their retention times and their UV-spectrum. The remaining ones were identified by there UV spectrum and extrapolation of existing data. There determination is in progress. To calculate the concentration of these last molecules, we applied the absorption coefficient of the molecule known with the closer spectrum.

Other experiments have shown that stilbene content in entire leaf without inoculation was very low (generally <0.1 mg.g^{-1} dry weight). Thus the stilbene content of 0.65 mg.g^{-1} in control and water modalities resulted of the wound reaction induced by making the leaf disc.

The relation between the stilbene content and the inoculum concentration is linear between 10^3 sporangia.ml^{-1} to 10^5 (R^2=0.98). The undetermined molecules seemed to be in low concentration and didn't modify drastically the tendency of the results.

For the inoculum of 10^5 sporangia.ml^{-1} we sprayed about 23 sporangia per mm^2 while the number of stomata was approximately 70 per mm^2. As there are about 8 zoospores per sporangia we could consider that at a concentration of 10^5 sporangia.ml^{-1} all the stomata are potentially infected. At a concentration of 10^4 sporangia.ml^{-1} 10 times less zoospores infect potentially the stomata.

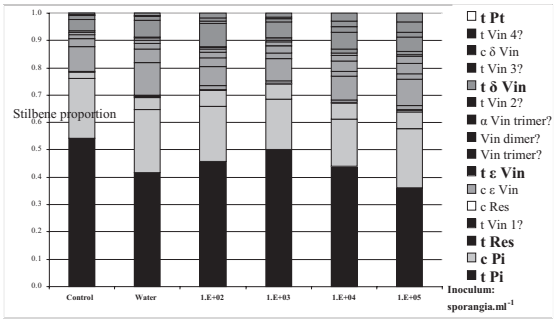

Fig. 1b. Relation between inoculum concentration of *P. viticola* and stilbene proportion in leaf disc of RV1 6 dpi.

The proportion of each molecule was different according the presence or the absence of inoculum (Fig. 1b). The proportion of high fungitoxic stilbenes like Pt and Vin increased with the concentration of inoculum, and Pi decreased.

Working on leaf disc provoked a wound reaction which could interfere with reaction induced by inoculation. So, its looks more appropriate to inoculate

entire leafs to measure only the reaction linked to the inoculation.

As stilbene content and proportion depend on the concentration of the inoculum, good standardisation of the inoculum appears important to study resistance.

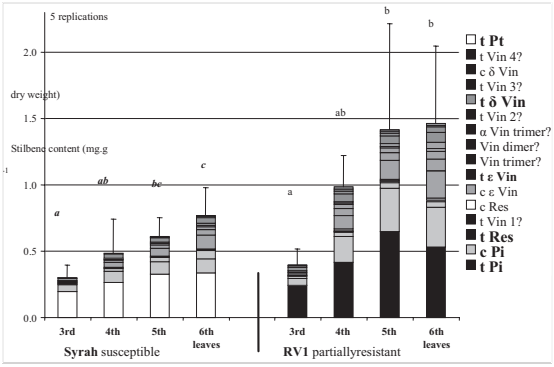

Fig. 2a. Relation between plant leaf age (first at the top) and stilbene content in leaf discs of Syrah (susceptible) and RV1 (partially resistant) after inoculation with *P. viticola* (10^5 sporangia.ml^{-1}) at 5 dpi. t for the isomer *trans*, c for the *cis*

Next, we studied the effect of the age of the plant leaf on stilbene content (Fig. 2a). In leaf discs inoculated with *P. viticola*, the stilbene content increased with the level of the leaf on the plant (age) in the susceptible grapevine Syrah (linearity from the 3rd to the 6thleaf: R^2=0.99) and in the resistant RV1 (linearity from the 3rd to the 5th leaf: R^2=0.87). Ontogenic resistance could be linked to this observation.

We present Syrah *trans* piceid differently because its UV spectrum was slightly different from the control but its retention time was similar. Its molecular structure has to be validated.

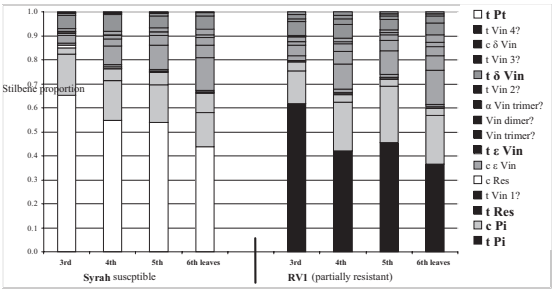

Fig. 2b. Relation between leaf level on the plant (first at the top) and stilbene proportion in leaf disc of Syrah and RV1 after inoculation of *P. viticola* (10^5 sporangia.ml^{-1}) at 5 dpi.

The proportion between stilbenes varied depending on the level of leaf on the plant (Fig. 2b). The lower on the plant the higher proportion of fungitoxic stilbene.

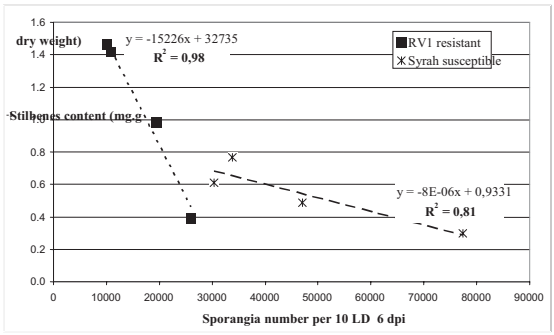

Fig. 3. Relation between stilbene content in leaf discs after inoculation with *P. viticola* (10^5 sporangia.ml^{-1}), and the number of sporangia per 10 leaf discs at 5 dpi.

We found a strong relation between the stilbene content in leaf discs and the level of sporulation at 5 dpi for the two genotypes studied: the stronger the stilbene content the lower the level of sporulation (Fig. 3).

The relation between stilbene content and sporulation varied depending on the genotype. For RV1 (partially resistant) a stilbene concentration of 0.4 mg.g^{-1} dry weight allowed the development of 26000 sporangia per 10 3rd leaf discs at 5 dpi while the same concentration of stilbenes allowed the development of 65000 in Syrah (susceptible).

Different hypothesis could be put forward to explain this difference:
1) The number of stomata was higher in susceptible plants allowing a better development of the pathogen. However, we can rule out this possibility because the number of stomata per surface area is nearly the same for these two genotypes.
2) Another mechanism present in the partially resistant RV1 reduces the infection by the fungus. Other genotypes and level of leafs have to be studied.
3) Spatial distribution of the stilbene in the leaf is not homogenous. Stilbene content was performed on a large area compared to the level of organisation of the mechanism of infection (length of stomata about 20 µm). High and fungitoxic stilbene content could be located around the stomata blocking or limiting the development of the pathogen.

Comparison of laboratory and vineyard evaluation of grapevine downy mildew resistance in a population derived from a cross between two partially resistant parents.

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Plasmopara viticola is an oomycete pathogen causing downy mildew of grapevine, a severe disease in temperate winegrowing regions. Disease control is mainly achieved by application of various fungicides. The repeated use of fungicides has led to long-term accumulation of molecules in soils, creating a negative biological and ecological impact, as well as a potential danger for human health. Moreover, systematic use of fungicides has led to the appearance of resistant pathogen strains.

Breeding for resistant varieties is an alternative to chemical control of the disease. Marker assisted selection for resistance to downy mildew is an important challenge for breeders. To find markers associated to resistance genes or QTLs, large populations issued from crosses must be evaluated for resistance with a powerful screening method combining high throughput and reliability.

Work presented here correspond to the analysis of a 150-individual population derived from a cross between two partially resistant parents « Villard blanc » and « Gf.Ga 47-42 ». The population has been produced at the Institute for Grapevine Breeding Geilweilerhof in Germany, where it has been assessed during four years in the vineyard.

We have developed laboratory bioassays using artificially inoculated leaf discs or whole plant. Plants used for evaluation are produced by wood or green cuttings. To rate partial resistance, twelve resistance parameters linked to sporulation and necrosis have been defined. Data were compared with vineyard infection evaluation.

Vineyard evaluation

Natural occurrence of downy mildew infections on the leaves were rated in August/September. Fungicide applications were totally omitted during the whole period. The scoring was done according to the appropriate OIV 452 descriptor whereas not the degree of resistance but the degree of infection was rated.

Laboratory methods

For the leaf disc protocol (LD), leaves (4 and 5) were harvested on twelve leaves stage wood (WC) or green cutting (GC), grown in greenhouse. Sixteen leaf discs (1 cm diameter) were placed in a 75mm Petri dish and sprayed with a suspension of a local *Plasmopara viticola* isolate (100'000 sporangia/ml). Petri dishes were incubated at 21°C for 7 days.

For the whole plant protocol (WP), two leaves (4 et 5) of twelve leaf stage green cuttings were inoculated by a sporangia suspension at the same concentration as for LD. The inoculated plants were incubated under plastic containers for 7 days.

Resistance is assessed using twelve parameters. OIV n°452 descriptor is a semi-quantitative parameter method based on a visual global evaluation. The method was completed by adding newly developed qualitative, semi quantitative and quantitative parameters. In this way, five parameters linked to sporulation and six parameters related to necrosis were defined.

Data analysis

Data were analysed using SAS with a general linear model. ANOVA was applied to test for each parameter the presence of significant differences among methods used. Correlation analysis was used to find any relationship between resistance parameter. Similarly correlation analysis was used to find any relationship of each of the parameters assessed with laboratory methods and the resistance level found in the vineyard..

Results

Concerning vineyard evaluation, the maximum value score obtained for the degree of infection over four years of observations is presented in Figure 1. With natural infection in the vineyard, the population distribution has shown a range in downy mildew resistance among genotypes. Two thirds of the population presented a partial resistance rated 5 or 3 and one third was susceptible, rated 7 or 9. No totally resistant genotype has been observed. This data shows also that a cross between partially resistant parents may produce susceptible progenies.

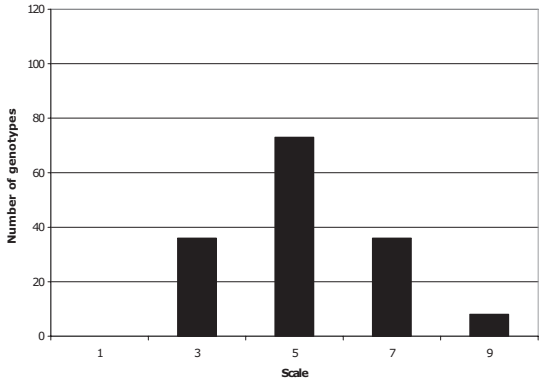

Fig. 1. Vineyard evaluation. Distribution of genotypes according to OIV 452 descriptor where infection degree is rated. 1= no

infection (resistant genotype), 9 = high infection (susceptible genotype).

Data produced by laboratory methods has shown a quite different distribution among genotypes (Figure 2). With the OIV 452 descriptor, a high proportion of individuals from the population was evaluated as resistant (scored 5, 6, or 8), while 20% of the population was scored as susceptible (scored 1,2,3,4). Analysis of variance for OIV 452 scores was applied to test the presence of significant differences between methods. A leaf disc test established with green cuttings (GCLD) was significantly different from the two other methods. Besides, for each resistant parameter related to sporulation or necrosis, comparison between methods revealed a highly significant effect of the method.

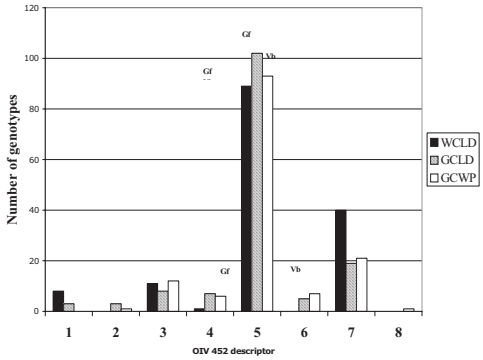

Fig. 2. Laboratory evaluation. Comparison among methods. WCLD = wood cutting leaf disc, GCLD = green cutting leaf disc, GCWP= green cutting whole plant. Gf= Gf.Ga 47-42, resistant parent 1, Vb = Villard blanc, resistant parent 2.
OIV 452 resistant scale = 1, 2, 3, 4, for susceptible scores. OIV452 = 5, 6, 7, 8, used for moderately to highly resistant scores.

Correlation between vineyard and laboratory methods was studied by correlation coefficient (*r*). *r* was calculated for each resistance parameter compared to the vineyard maximum infection value. Concerning OIV 452 descriptor, correlation coefficient *r* value obtained for WCLD *vs* vineyard maximum infection value ranged from *r* = 0,36 to *r* = 0,56 (Table 1). WCLD presented the higher correlation coefficient.

Tab. 1. Correlation coefficient (*r*) between vineyard maximum infection value and laboratory methods using OIV452 descriptor as resistance parameter.

OIV 452	WCLD	GCLD	GCWP
Vineyard	-0,56	-0,40	-0,36

Effects of Downy Mildew on Photosynthetic Parameters in 'Niagara' Grape Leaves

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Downy mildew (*Plasmopara viticola*) is an important disease of grapes in the Eastern United States. The leaves of *Vitis labrusca* 'Niagara', a native grape variety used for the production of white grape juice and wine, are highly susceptible to downy mildew. Severe infections can result in premature leaf drop. Despite apparent susceptibility, cluster infections are rare. In this study, the effects of foliar downy mildew on photosynthetic parameters were assessed. Analysis of gas exchange in leaves of plants infected with pathogens provides a means of understanding the mechanisms leading to reductions in photosynthesis. Furthermore, since it is non-destructive, it allows for sequential measurements to be taken as the disease progresses, enabling the separation of individual mechanisms that cause reductions in photosynthesis.

Two-year-old, potted *Vitis labrusca* 'Niagara' grapevines were inoculated with an aqueous suspension of *P. viticola* sporangia ($5x10^4$ per ml) collected from naturally infected vines in the field. On the first or second fully expanded leaf, an area approximately 2.5 cm in diameter on the abaxial surface was inoculated using an atomizer. An equivalent area on the opposite side of the leaf was left uninoculated. The entire leaf was enclosed in a plastic bag for 6 hours to retain leaf wetness. Gas exchange measurements were taken every 48 hours after the initial measurement for 14 days after inoculation. Each measurement was taken on the inoculated area of the leaf, and the non-inoculated area was used as a control.

Gas exchange measurements were taken with a CIRAS I infrared gas analyzer and a Parkinson leaf cuvette. Light values of 0, 50, 100, 200, 300, 400, 500, 700, 900, 1100, 1300, and 1500 µmol m^{-2} s^{-1} PAR (photosynthetically active radiation) and ambient CO_2 concentrations were applied to the leaves to develop light response curves. Concentrations of 0, 100, 200, 300, 400, 500, 700, 900, 1100, 1300, and 1500 µmol mol^{-1} of CO_2 and 1400 µmol m^{-2} s^{-1} PAR light were applied to leaves to develop CO_2 response curves. Measurements were taken during the day between 10:00 and 16:00 hours. Temperatures ranged from 21-27ºC. Data obtained from the light experiments include the maximum assimilation rate (A_{max}), light compensation point, and photosynthetic efficiency (Φ). Data obtained from the CO_2 response curves included the maximum assimilation rate (A_{max}), assimilation rate at ambient CO_2 (A_{amb}), carboxylation efficiency (k), CO_2 compensation point (Γ), and intercellular CO_2 concentration (C_i). Stomatal conductance (g_s) was obtained directly from the measurements. The stomatal limitation ($l_{g(ii)}$) was calculated.

Fluorescence measurements were taken with a Hansatech plant efficiency analyzer. Leaves were dark-acclimated for 30 minutes before measurements were taken. Infected and control areas were measured twice (close to the same time) every 48 hours and the average was used in the analysis. Fluorescence was expressed as the optimal yield of Photosystem II, which is the ratio between variable fluorescence and maximum fluorescence (F_v/F_m). Statistical analyses were done with Sigmastat statistical software using a repeated measures analysis of variance.

Symptoms first appeared on leaves 7 days after inoculation as translucent, pale yellow lesions, which became darker and more distinct as infection progressed. Infected leaves showed differences from healthy leaves in photosynthetic efficiency, carboxylation efficiency, intercellular CO_2, the CO_2 compensation point, stomatal limitation, and A_{max} and A_{amb} before symptoms appeared. However, chlorophyll fluorescence, the light compensation point, and stomatal conductance were not significantly affected until after symptoms appeared (Table 1).

Tab. 1. Temporal effects of downy mildew infection on photosynthetic parameters. A "+" indicates a significant difference from healthy areas on leaves (Tukey's HSD at $P<0.05$).

	Days after inoculation						
Parameter	2	4	6	8	10	12	14
Symptoms				+	+	+	+
Fluorescence				+	+	+	+
Light compensation point				+	+	+	+
Photosynthetic efficiency			+	+	+	+	+
Carboxylation efficiency		+	+	+	+	+	+
Intercellular CO_2			+	+	+	+	+
CO_2 compensation point			+	+	+	+	+
Assimilation rate at ambient CO_2		+	+	+	+	+	+
Maximum assimilation rate		+	+	+	+	+	+
Stomatal conductance				+	+	+	+
Stomatal limitation			+	+		+	

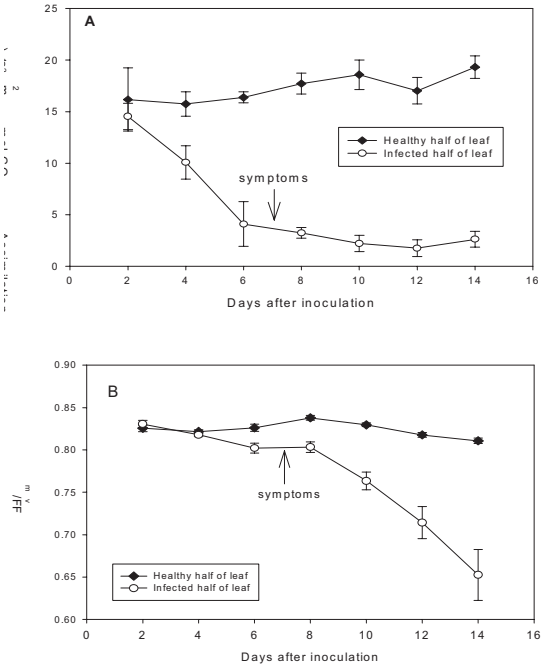

Fig. 1. The effect of downy mildew development on: (A) the rate of photosynthesis at ambient CO_2 levels, and (B) chlorophyll fluorescence expressed as the ratio of F_v/F_m in leaves of two-year-old potted 'Niagara' grapevines. Means are averages of five replications. Error bars indicate the standard error of the mean.

Similar effects were seen for maximum photosynthesis values. Carboxylation efficiency (*k*) was significantly lower in infected leaves at 4 DAI and decreased sharply thereafter. Photosynthesis at ambient CO_2 levels decreased sharply at 4 days after inoculation (DAI) and was reduced by more than two-thirds compared to control values at 6 DAI (Figure 1). Photosynthetic efficiency (θ) in infected leaf areas decreased significantly at 6 DAI compared to healthy leaves, and remained significantly lower than healthy leaf areas throughout the remainder of the experiment. The intercellular CO_2 concentration (Ci) in infected leaves also increased significantly at 6 DAI, as did the CO_2 compensation point (Γ). The light compensation point also increased and was significantly different from control values at 8 DAI, but fluctuated some thereafter. Chlorophyll fluorescence and stomatal conductance in infected leaf areas did not decrease significantly until 8 DAI.

The data show that negative effects from downy mildew infection already occur prior to the appearance of visible symptoms. The CO_2 and light response curves suggest that the reduction in photosynthesis in downy-mildew infected grape leaves is due to a mesophyll limitation rather than reduced photosystem II efficiency or a stomatal limitation. Reduced rates of photosynthesis are likely bi B ical in nature and could be the result of damage to photosynthetic enzymes such as ribulose-1,5-biphosphate carboxylase (Rubisco) and/or decreased rates of ribulose-1,5-biphosphate (RuBP) regeneration, possibly due to damage to electron transport components.

Germination dynamics of *Plasmopara viticola* oospores and occurrence of primary infections in Veneto

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Plasmopara viticola (Berk. *et* Curt.) Berl. & De Toni causes the grapevine downy mildew, a very serious disease in areas characterized by abundant rainfall and moderate temperature during late spring and summer. While detailed information on the effect of environmental conditions on the occurrence of secondary infections is currently available, factors affecting the occurrence of primary infections are not yet investigated. The inoculum for primary infections is provided by germination of oospores, the sexual and only overwintering structures of the pathogen, differentiated at the end of grapevine growing season. Germination in *P. viticola* oospores involves numerous ultrastructural changes, such as wall solubilization, dissolution of the ooplast and nuclear division, leading to the differentiation of a germination tube with a pyriform macrosporangium containing zoospores. Occurrence of primary infections requires water availability and temperature above 10 °C, as summarized in the three ten rule, when the shoots are 10 cm long. Rain is very effective in sporangial dispersal and provides the wetness required for the germination of both sporangia and zoospores, while temperatures ranging from 15 and 20 °C allow the highest sporulation and infection efficiency. Previous studies carried out in different grapevine growing areas suggest that the climatic conditions during the overwintering period may affect the germination dynamics of the oospores.

In order to evaluate the effect of water availability and temperatures, the germination of oospores differentiated and overwintered in two untreated and naturally infected vineyards, was investigated during 2004-2005. The first vineyard of cv Corvina was located near Verona (Montorio) and the second of cv Merlot near Padova (Monselice). Leaf zones with a high number of oospores were collected in October in order to prepare 60 samples with three repetitions each. Oospores isolated from leaves were incubated on water agar (1 %) at 20 °C. Macrosporangium formation was checked daily at the microscope. Percentage of germinated oospores was estimated on 1200 oospores per sample. The assays were repeated twice a week from November since June. Occurrence of primary infections was recorded in untreated vines nearby the overwintering site. The number of infected leaves and the disease incidence were assessed every week until August. The infection index, I%I, was assessed in 4 untreated plots, taking into account 100 leaves and clusters per plot.

Numerous rainy events, more abundant in Montorio, occurred in both vineyards in November and December, while the following months, January and February, were almost completely dry. Frequent rainfall was recorded in Montorio and Monselice since the middle of March until the end of May. Average temperature reached the lowest values between January and the beginning of March and steadily remained over 10 °C since the end of April. The oospores overwintered in both vineyards produced the first macrosporangia at the end of November. The germination capability increased until the end of December and reached the highest values at the end of March in Monselice and one month later in Montorio. The germination percentages decreased abruptly at the end of April and no macrosporangia were differentiated from the beginning of June onwards.

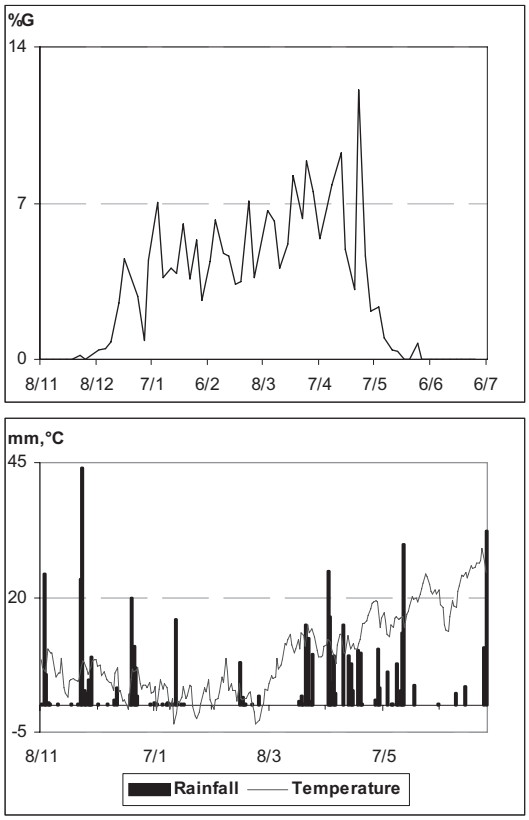

Fig. 1. Climatic conditions and germination percentages (%G), Montorio

Primary infections, due to the inoculum dispersed at the middle of May, were detected in vineyard between the end of May in Montorio and the beginning of June in Monselice. In Montorio the number of infected leaves suddenly increased at the beginning of June and very slowly until the end of the month. On the contrary, rainfall recorded at the end of June and July caused a constant increase of infection on both leaves and clusters: the final I%I was 38 % on leaves and 63 % on clusters.

In Monselice a lower number of infected leaves was observed: moreover its increase was limited in comparison with Montorio. The I%I recorded in Monselice reached 10 % on leaves and 7 % on clusters.

Germination capability was acquired by the oospores at the end of November, demonstrating that the maturation period was concluded. Lack of rainfall during January and February caused the fluctuation of the germination percentages around the value reached at the beginning of January, except from the first maximum of 5 % recorded in Monselice in the middle of February, probably due to the snow coverage. Rainfall recorded from the end of March until the end of April was associated with subsequent increases in the germination percentage. Even in presence of abundant water availability, the germination percentage widely fluctuated, indicating that endogenous factors are involved in the regulation of the process. Oospores did not germinate from the beginning of June onwards. Since the period of time required by the oospores for macrosporangium formation was about a week, it is likely that effective inoculum was available for primary infections until the 7th of June in Montorio and the 27th of May in Monselice.

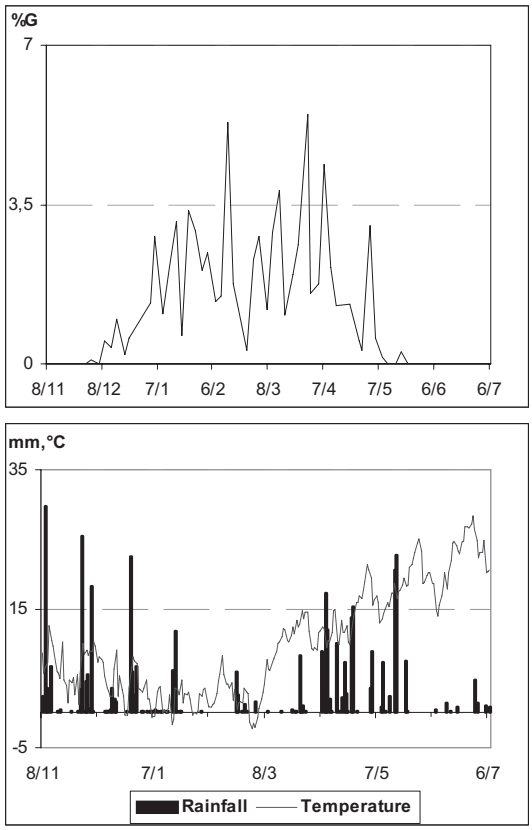

Fig. 2. Climatic conditions and germination percentages (%G), Monselice

Primary infections were probably caused by the inoculum dispersed by the rain recorded from middle till the end of May. Subsequent waves of primary infections were likely to take place, since both effective inoculum and rainy events occurred in vineyard. The more abundant and frequent rainfall recorded in Montorio determined a more efficient dispersion of the macrosporangia formed by the oospores, more suitable conditions for macrosporangia and zoospore germination and consequently more primary infections. Disease progress observed in July was affected by either the number of infection foci already present in vineyard and the frequency and amount of rainfall. In Montorio numerous infected organs allowed the differentiation of very high amount of asexual spores, efficiently dispersed by rain.

Oospore germination seems to be influenced mainly by water availability and endogenous factors, among which maturation affects the acquisition of germinating capability. Macrosporangia are available only for limited period of time, until the end of May-beginning of June, during which dispersion by rainfall is essential in order to allow the occurrence of primary infections. Subsequent epidemic progress is influenced by either the frequency of primary foci and climatic conditions.

Date	MS		MT	
	IL	I%I	IL	I%I
4/5	0	0	0	0
12/5	0	0	0	0
19/5	0	0	0	0
26/5	0	0	125	0.01
2/6	78	0.01	2563	0.5
9/6	807	0.05	3375	0.8
16/6	807	0.05	3500	0.8
23/6	846	0.06	3813	0.9
30/6	846	0.06	3922	0.9
9/7	879	0.06	-	4.1
19/7	-	0.3	-	6.1
27/7	-	3.4	-	21.3
1/8	-	10.5	-	38.6

Tab. 1. Number of infected leaves/ha (IL) and infection index (I%I) on leaves, Monselice (MS) and Montorio (MT)

Immunofluorescence Microscopy of the Cytoskeleton and Associated Components in Zoospores of *Plasmopara viticola*

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Downy mildew is an economically important disease of grapevines. The disease is caused by the oomycete *Plasmopara viticola,* which is an obligate parasite related to other disease causing species such as *Phytophthora cinnamomi* (dieback 'fungus') and *Phytophthora infestans* (cause of potato blight). Current control of downy mildew in grapevines relies on the strategic application of biocides, however resistance to these is increasing and new methods of control are being sought. The aim of our research is to identify likely targets for biocides by directing antibodies to the cytoskeleton and other cellular components of the motile biflagellate zoospores, of *P. viticola*. Immunofluorescence detection of cell components was performed on zoospores using established techniques (Harper et al. 1995. *Protoplasma* 188: 225-235).

In *P. viticola*, microtubules (Mts; A) form the structural basis for the anterior and posterior flagella and the precise arrangement of rootlets within the zoospores. This configuration is found in other zoospores such as those of *P. cinnamomi*. As shown here (B) the zoospore microtubules are also post-translationally modified by acetylation which is an indication, not a cause, of microtubule stabilisation.

Actin labelling of zoospores (C) shows distinct plaques which are also typical of other oomycete zoospores (S. Jackson Uni. Canterbury, NZ, Pers. comm.).

Centrin antibody, in oomycetes, such as *P. viticola,* labels the basal body connector (D; arrow) along with the R1 Mt rootlet ($R1^{cen}$; right hand arrowheads) and the anterior flagella (left arrowhead). In this case the cell has been lysed with 0.1% Triton-X 100 revealing the close nucleus-connector association which may be centrin-based.

Heat shock protein 90 (HSP90) is member of the chaperone family and is abundant in several oomycete zoospores we have tested, including *P. viticola* (E).

The anti-phosphoprotein antibody MPM-2 detects phosphoproteins in the nuclei of zoospores, which may indicate that this post-translational modification is necessary for zoospore activity.

These components have not been described in *P. viticola* zoospores before. Our studies indicate that zoospores in oomycete species have these proteins and supramolecular structures in common and an increase in our understanding of them may lead to the discovery of an 'Achilles' heel' in these "weapons of plant destruction".

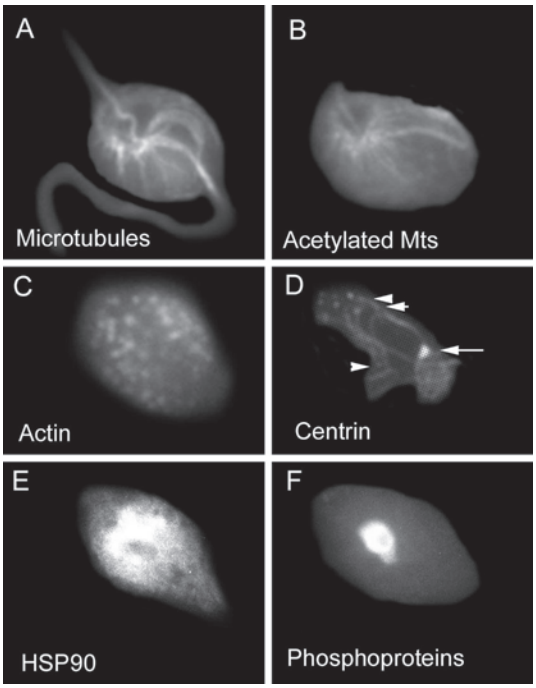

Fig. 1. Zoospores of *Plasmopara viticola* are presented with the ventral surface facing the reader and anterior to the left. (A) Typical oomycete microtubule arrangement. Microtubules (Mts) are acetylated (B) which is usually an indicator of stabilization. Actin plaques are present (C) probably just under the surface of the zoospore membrane. Lysing zoospores in mild detergent reveals that the configuration of the centrin-basal body connector (D arrowhead) and rootlet system are as in other oomycetes such as *P. cinnamomi* with the R1 Mt rootlet containing centrin and looping across to the opposite side of the cell (two arrowheads). One of the flagella also labels with anti-centrin antibodies (left arrowhead). Moreover lysing cells reveals the nucleus (below the connector) remains attached to the flagella apparatus. HSP90 chaperone proteins (E) are abundant in zoospores with a higher concentration anterior to the pear-shaped nucleus. MPM-2 reactive phosphoproteins (F) appear to be abundant in the nuclei. Zoospores are approximately 20 microns long.

Fermentation and formulation of *Pseudomonas fluorescens* strain CHA0 and Pf 153 and their influence on the control of *Botrytis cinerea*

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The production, formulation and application of micro-organisms are important steps within the development of biocontrol agents. Although it has been demonstrated that *Pseudomonas fluorescens* is an effective antagonist to control several plant diseases, including *Botrytis cinerea*, limited information on the production and formulation of living cells of *P. fluorescens* is available.

Therefore, we investigated the influence of media composition and fermentation regimes on the quantity and quality of *P. fluorescens* strains Pf 153 and CHA0. Additionally, research was carried out on the development of freeze drying protocols for *P. fluorescens.* Furthermore, the influence of fermentation on the freeze drying process was investigated.

In the first experiments *P. fluorescens* was grown in 35 different liquid media in microtiterplates. Within these experiments clear differences in the consumption of various nitrogen and carbon sources were visible. Additional results on the influence of the media composition on the efficacy of living cells to control *B. cinerea* will be discussed.

In a following step the freezing and drying process was optimised for both *P. fluorescens* strains and different protectants were compared. The results indicate that the two isolates require different freezing rate. When 22 different protectants were compared, cells were mainly protected by sugars.

After optimising the freeze drying process the viability before and after freeze drying of Pf 153 was not significantly different (Figure 1).

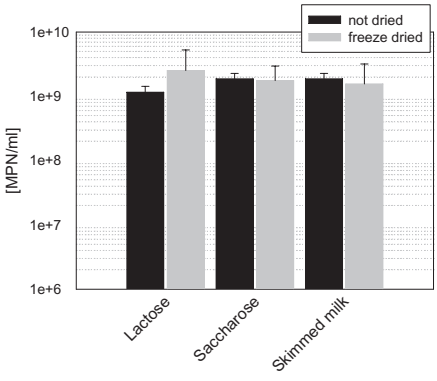

Fig. 1. Comparison of the viability of Pf 153 before and after freeze drying formulated with three different protectants.

These results demonstrate that after the optimisation of the drying process the desiccation sensitive *P. fluorescens* can survive a drying process without loss of viability.

The efficacy of freshly produced and freeze dried pseudomonads were compared *ad planta* on detached *Vicia faba* leaves inoculated with *Botrytis cinerea*. With exception of the protectant saccharose the efficacy of freshly produced and freeze dried cells did not differ (Figure. 2). Additionally, the efficacy of *P. fluorescens* was influenced by the protectants. *P. fluorescens* was effective when formulated in lactose and skimmed milk but not when formulated in saccharose.

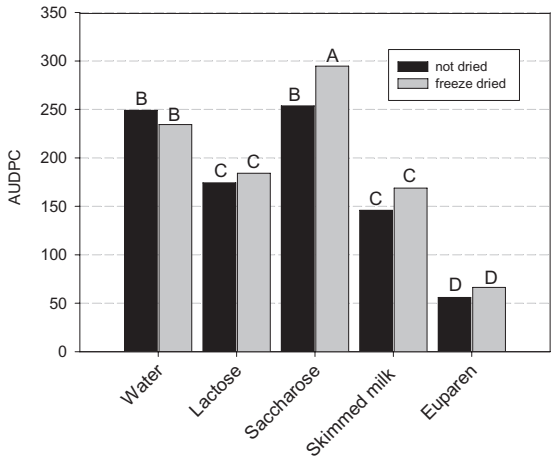

Fig. 2. Effectiveness of freshly produced and freeze dried cells of Pf 153 formulated with different protectants on the disease symptoms caused by *Botrytis cinerea* on detached leaves of *Vicia faba* (AUDPC=Area under disease progress curve).

In further experiments these protectants were added to agar media to proof whether *B. cinerea* can utilize the additives. Despite of the *ad planta* results the best growth was obtained when skimmed milk was added and high sporulation took place especially on this medium.

The results on the formulation of pseudomonads demonstrate that freeze drying is an interesting technique for the conservation of *P. fluorescens*. The results also indicate that the selection of the right protectant influences the viability and efficacy. All these parameters have to be taken in consideration for further investigations on the development of practicable formulations.

Plasmopara viticola: three decades of observation in Sicily

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Primary infections of *Plasmopara viticola*, in western Sicilian vineyards, occur during May-June, causing two or three infective cycles; afterwards their severity decreases until the middle of July, when the infection stops. In some very hot seasons, oomycete do not produce agamic structures, and the first typical oil-spots, necrotize in a few days. Usually, the pathogen remains latent (2-3 months) in the host, until the end of summer and the beginning of autumn when rain occurs, the sporulation, appearing as white mould on the leaf surface below the oil spots, is stimulated. Moreover, the oomycete can infect leaves in autumn, inducing typical mosaic leaf spots, inside which it forms oospores, the only means of its overwintering. Oospores, originate from fertilization of oogonia by antheridia, are already formed in the infected leaves at the end of July, reaching the highest density during the autumnal months. By use of fluorochrome stains (DAPI, Aniline) and observations either with fluorescent or electronic microscope, we followed the nuclear and cytological development of the gamic structures in natural infected foliar tissues. As matter of fact, young gametangia contain many small, probably haploid gametic nuclei, the products of gametangial meiosis in this diploid *Straminipilous.* The oogonium fertilitation starts when a nucleus of the antheridium, through the fertilization tube, migrate and approach another oogonial nucleus while the others nuclei reaching periplasm, until they completely degenerate. The binuclate stage defines the oosphere, which wall is constituted by one thick external layer and a thin inner one. Periplasmic nuclei are also observed in unfertilized oogonia but their degeneration is a consequence of the microrganisms degradation. In the oosphere, single antheridial and oogonial nuclei fuse while periplasmic nuclei degenerate. The nuclear fusion characterizes the oospore and seems to be the necessary condition for its physiological maturation; then follows an "after-ripening period", during which the oospore is dormant and will not germinate even if exposed to optimal conditions. Several years (1980/1990) of study demonstrate that oospores, even under optimal conditions of temperature (20-24°C) and humidity (> 95%) do not germinate before January; under the Sicilian conditions oospore germination reaches an optimum within the end of February and the middle of March. Moderate amount of oospores germinate in April and, to a lesser extent, during the remaining spring. Researches on the effect of uniform and/or environmental fluctuating temperatures on oospore germination show that the process occurs from a minimum of 10°C to a maximum of 28 °C, with an optimum at 20 and 22°C. Germination is rather slow at low temperatures and reaches the shortest duration at 22-24°C; germination time can last from 17 to 46 days, at 10°C, but only 2-3 days at 22-24°C.

The duration of oospore "after-ripening" period depends upon the interaction of environmental factors (as temperature, frequency of the distribution of rain and correlate moisture of the soil in which the structures overwinters). As matter of fact, the prolong of low temperature associated to an adequate rainfall during summer can increase oospore maturation, extending the germination period (until December); moreover, these processes are improved by a variable condition of soil moisture, included within the field capacity and the wilting point (Fig. 1).

Nuclear staining and germination assays show correlation between the percentage of mononucleate spores in autumn and in spring, and their germination efficiency, after natural overwintering conditions (Figure 2). Thus the nuclear stage of gamic structures of *P. viticola* before the fall of the leaves would affect their ability to germinate. Such germinating oospore can contain over 40 mitotic nuclei and one or more germ tubes (until 3). During the next 24 hours, at the apex of the germination tube a sporangium is formed, the nuclei migrate in it leaving the oospora and germ tube empty. Then mitotic divisions occur within the sporangium, as partially confirmed by the presence of groups of two nuclei and by the total number of nuclei which is double of that found in the oospore just prior the emergence of the germ tube. The mature sporangium releases mostly mononucleate zoospores, the only source of primary inoculum, that rain and wind spread on the abaxial surface of a grape leaf. Mobile zoospores, possibly by autotactism, reach the open stomata (by light) and germinate. One or more germ tubes can penetrate the same stoma, developing a substomatal vesicle and giving origin to the intercellular mycelium with many haustoria in mesophyll. Four-five days after the infection, the typical oil-spot lesions appear on the adaxial surface. Then, if high humidity occurs (over 90%) with temperatures between 14-28°C, hyphal coils grow in sub-stomatal cavity giving origin to sporangiophores bearing sporangia. Thus, in 24 hours, *P. viticola* emerges from host infected tissues, and the mature sporangia (with papilla and 4-5 nuclei) germinate in 20-30', releasing zoospores, which are the source of secondary infections. As matter of fact, from May to October, incubation period of disease is nearly constant (4 days) even if moderate variations (± 1 day) could be correlated to extremes of temperature. On the other hand, the length of latent period, strictly depending upon the level of environmental humidity, is usually very variable (from 8 to 16 days in spring and to up 52 days in Sicilian summer). Forecasting latent period length is very difficult and of a doubt value in protection program. While considering the development of gamic and agamic structures of the pathogen in our region, we made the first attempt to forecast the date of primary infection. Recently, an hyphomycete of genus *Acremonium* was either isolated or visualized in symptom-less tissues of several wine cultivars; this endophytic fungus is able to prevent *P. viticola* "*in vitro* and *in vivo*". Current studies focus on the interaction between several strains of *Acremonium* –

Vitis vinifera - P. viticola.

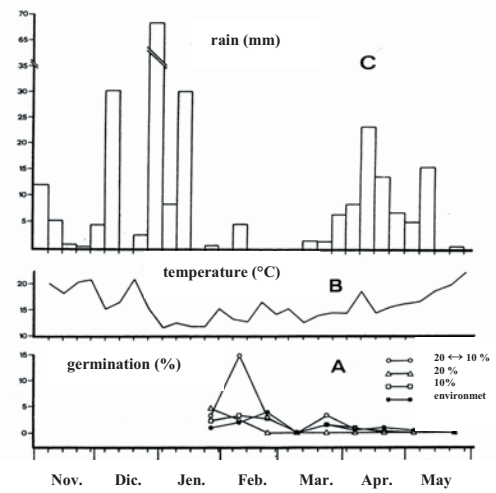

Fig. 1. 1989-'90: germination of *P. viticola* oospores kept at different soil moisture level (20% or field capacity; 10% or wilting point), (A), mean weekly temperature (B) and rain fall during assay (C).

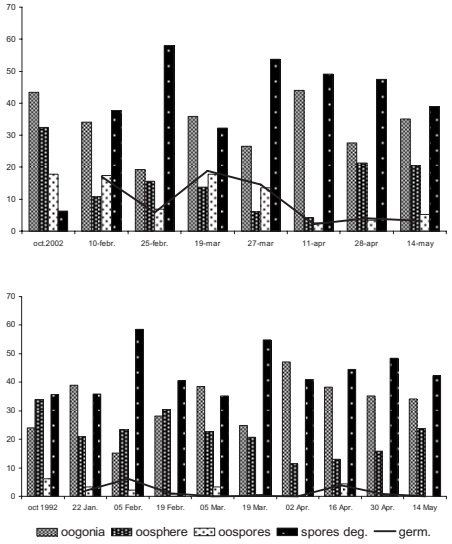

Fig. 2. Two year trial of observations on percentage of the different *P. viticola* gamic structures and of the oospore germination