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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5th International Workshop on Grapevine Downy and Powdery Mildew - Proceedings

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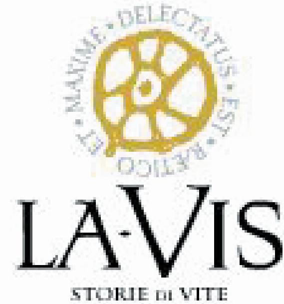

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Preface

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.

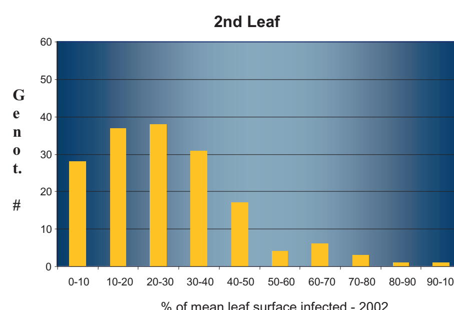

Fig. 2. Example of downy mildew infection response distribution in the MxR offspring. The character is clearly quantitative. Classes were made only for graphical purpose.

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).

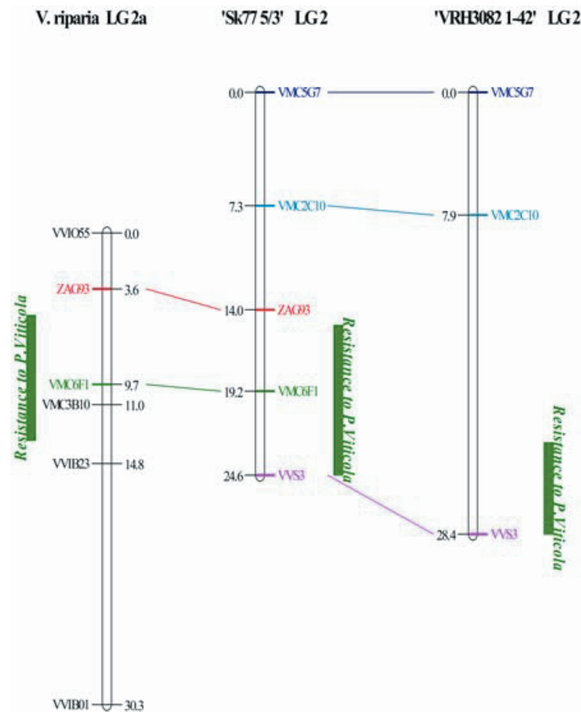

Fig. 3. Comparative QTL analysis in the linkage group 2 between *V. riparia*, ‘Sk77 5/3’ and ‘VRH3082 1-42’

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).

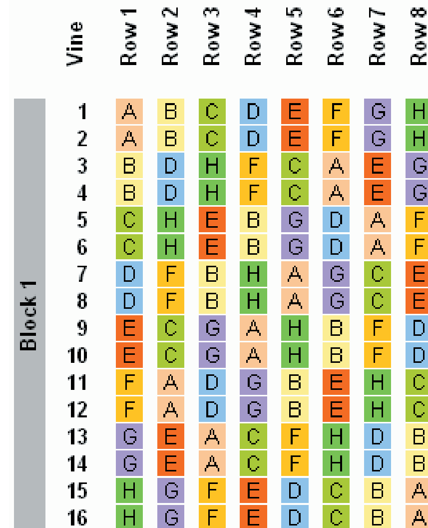

Fig. 1. Vine positioning in one of the four cultivar mixture blocks (Block 1). Each letter indicates one vine of a cultivar (A: Regent, B: Merlot, C: Isabella, D: RxS, E: Solaris, F: Bianca, G: Gamaret, H: Chambourcin).

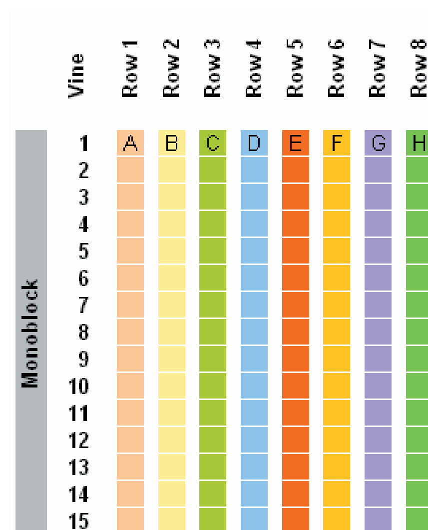

Fig. 2. Vine positioning in the monoblock (Block 5). Each letter indicates one vine of a cultivar (A: Regent, B: Merlot, C: Isabella, D: RxS, E: Solaris, F: Bianca, G: Gamaret, H: Chambourcin).

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.

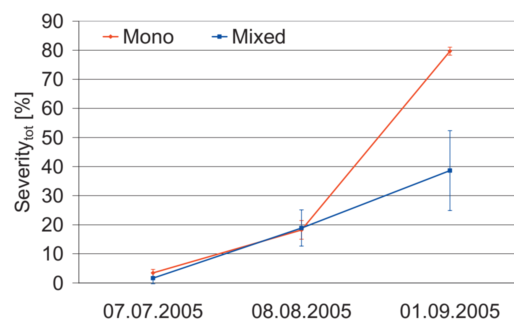

Fig. 4. Downy mildew severity assessed for the cultivar RxS throughout the period 7th July – 1st September 2005 in Cugnasco (scale:0-90%).

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

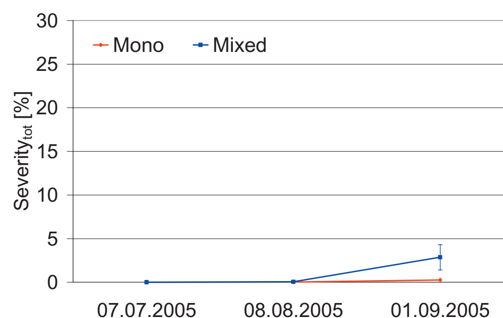

Fig. 6. Downy mildew severity assessed for the cultivar Chambourcin throughout the period 7th July – 1st September 2005 in Cugnasco (scale:0-30%).

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

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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).

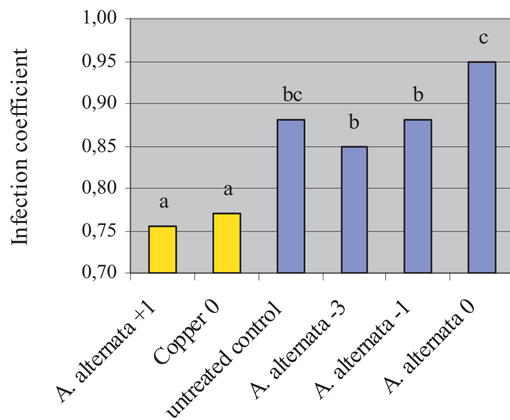

Fig. 1. Infection coefficient (CT *V. vinifera* / CT *P. viticola*) in the grape tissues treated with *A. alternata* at different times from *P. viticola* inoculation. Means followed by different letters are significantly different (least significant difference [LSD]; P≤ 0.05).
Yellow columns=no sporulation.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

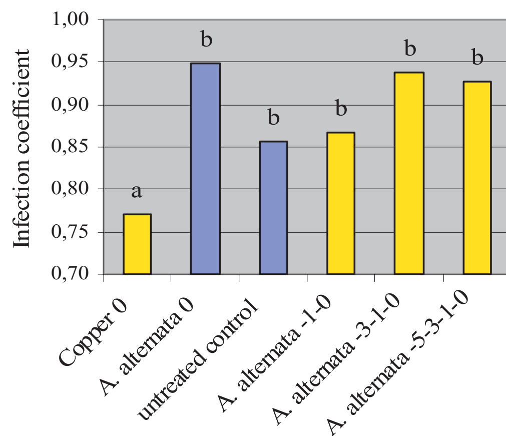

Fig. 2. Infection coefficient (CT *V. vinifera* / CT *P. viticola*) in the grape tissues treated once, twice, three and four times with *A. alternata* at different times from *P. viticola* inoculation. Means followed by different letters are significantly different (least significant difference [LSD]; 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.

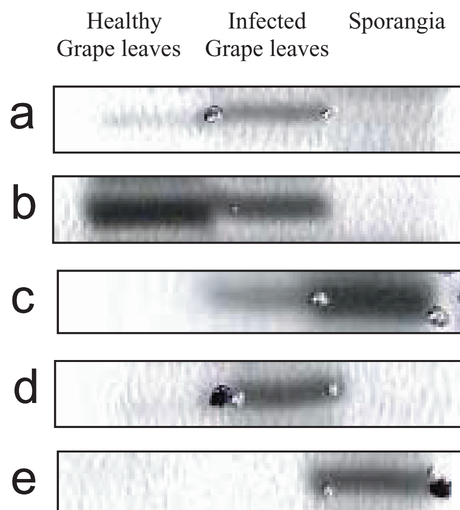

Fig. 1. Examples of cDNA-AFLP bands corresponding to grape or *P. viticola* transcripts, expressed during the infection process at the oil spot stage (a to d), and *P. viticola* transcripts identified from sporangia samples (e).

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.

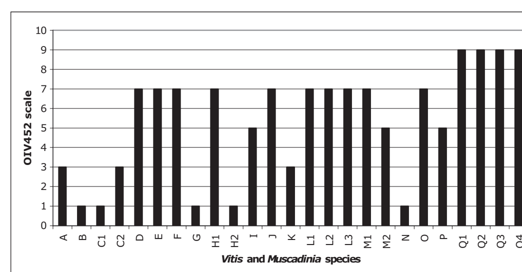

Fig. 1. Resistance level evaluation of *Vitis* species and *Muscadinia rotundifolia* varieties using the OIV 452 descriptor (1= susceptible, 9= totally resistant). A: *V. aestivalis*, B: *V. arizonica*, C1: *V. berlandieri* Planchon, C2: *V. berlandieri* Thyers, D: *V. candicans*, E: *V. cinerea*, F: *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

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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 $\pm$ 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).

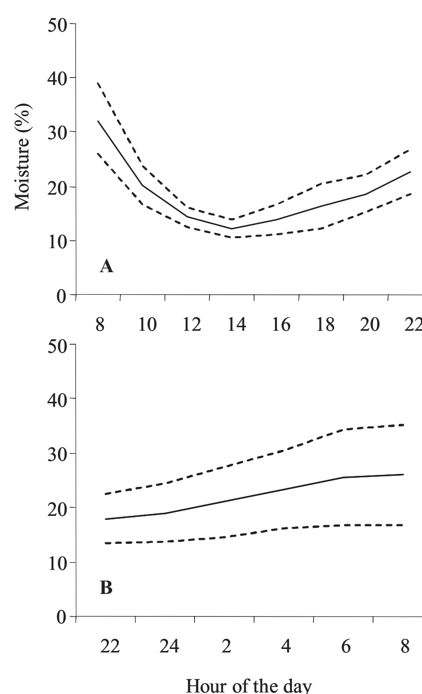

Fig. 1. Diurnal periodicity of the percent moisture of grape leaves in the leaf litter above the vineyard ground: average data of several replicate leaves exposed between March and June, in the experiments made in daytime (A) and nighttime (B). Dotted lines represent 99% confidence intervals.

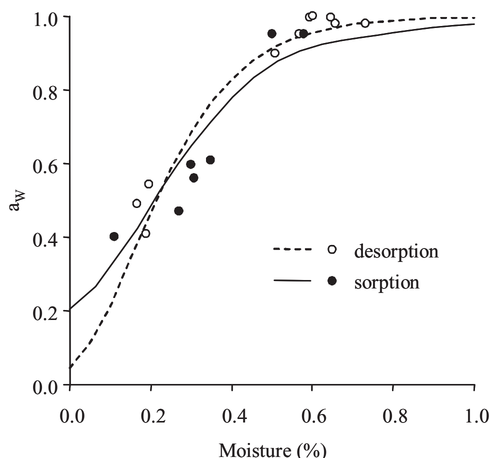

Fig. 2. Percent moisture *versus* water activity in grape leaves which have been dried to mimic leaves of the leaf litter covering the vineyard ground. Dots represent absorption and desorption data at 10°C, while lines represents the moisture sorption isotherm according to the modified Chen-Clayton equation.

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).

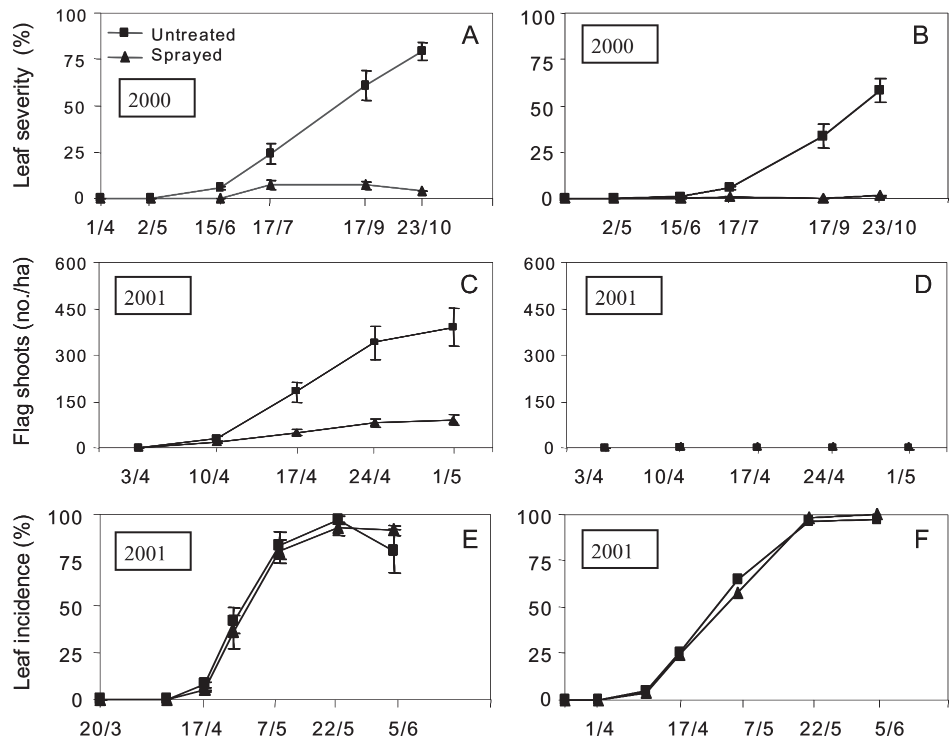

Fig. 1. The effect of powdery mildew development on the leaves of *cv.* Carignane (A, C and E) and Cabernet (B, D and F) vines in 2000 and the subsequent intensity of flag shoots and leaf disease in 2001. Experimental plots were either sprayed with fungicides or left untreated in 2000 (A and B) while 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.

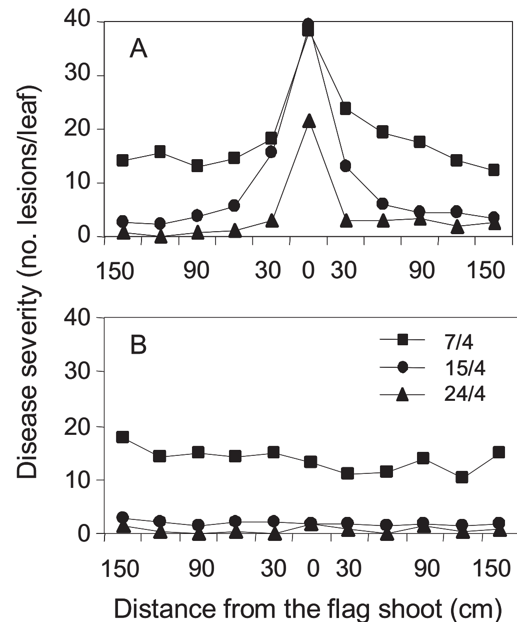

Fig. 2. The spatial and temporal development of powdery mildew around vines with flag shoots (A) and in areas without flag shoots (B). Disease was recorded in vines of Carignane in the year 2001.

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.

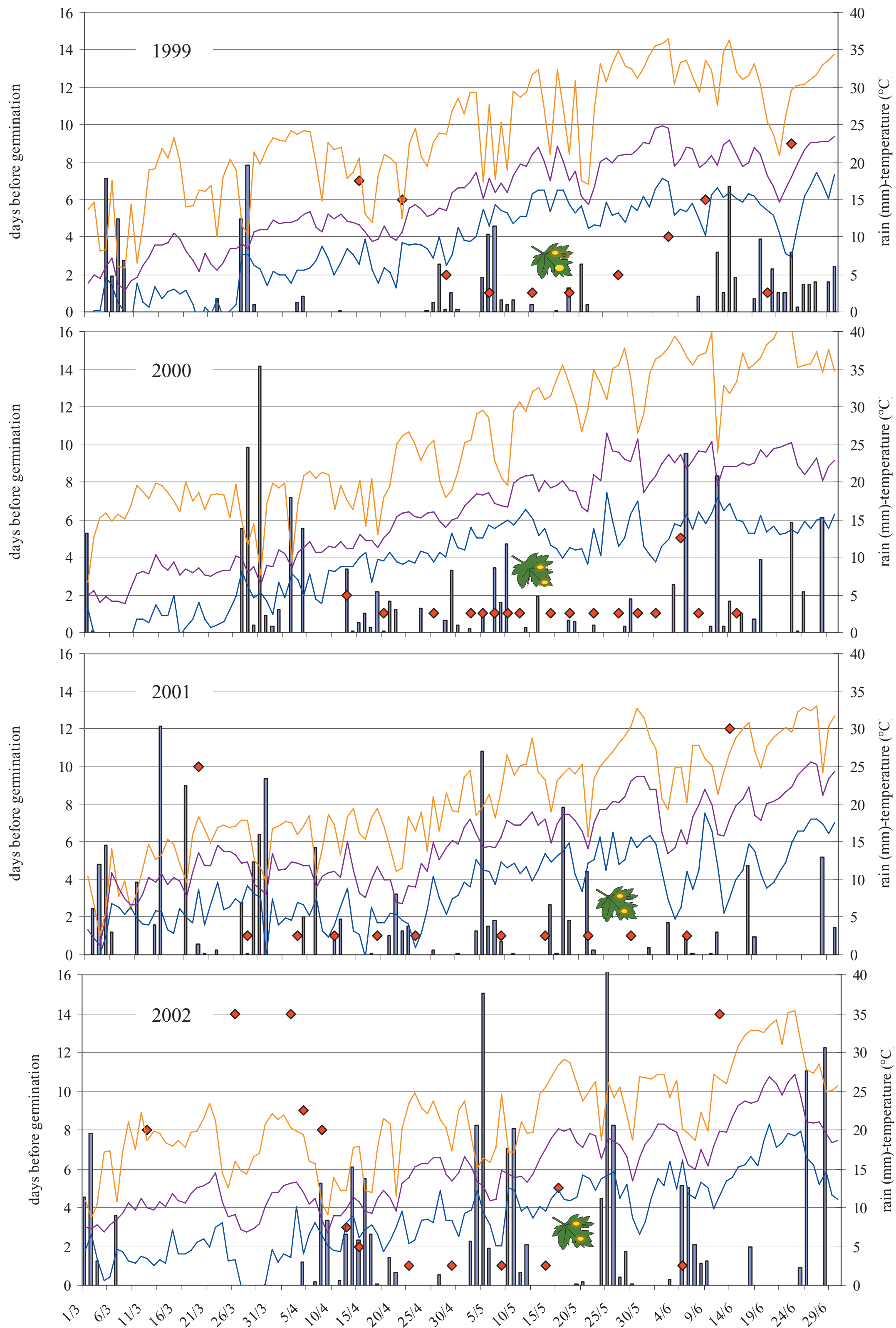

Fig. 1. Germination delay of overwintered oospores (Rovereto, Northern Italy) under controlled conditions (red square marker), minimum, average and maximum temperatures (blue, violet, and yellow line) and rain (blue histogram). Leaf indicates when the first oil spot symptoms were found in the vineyard.

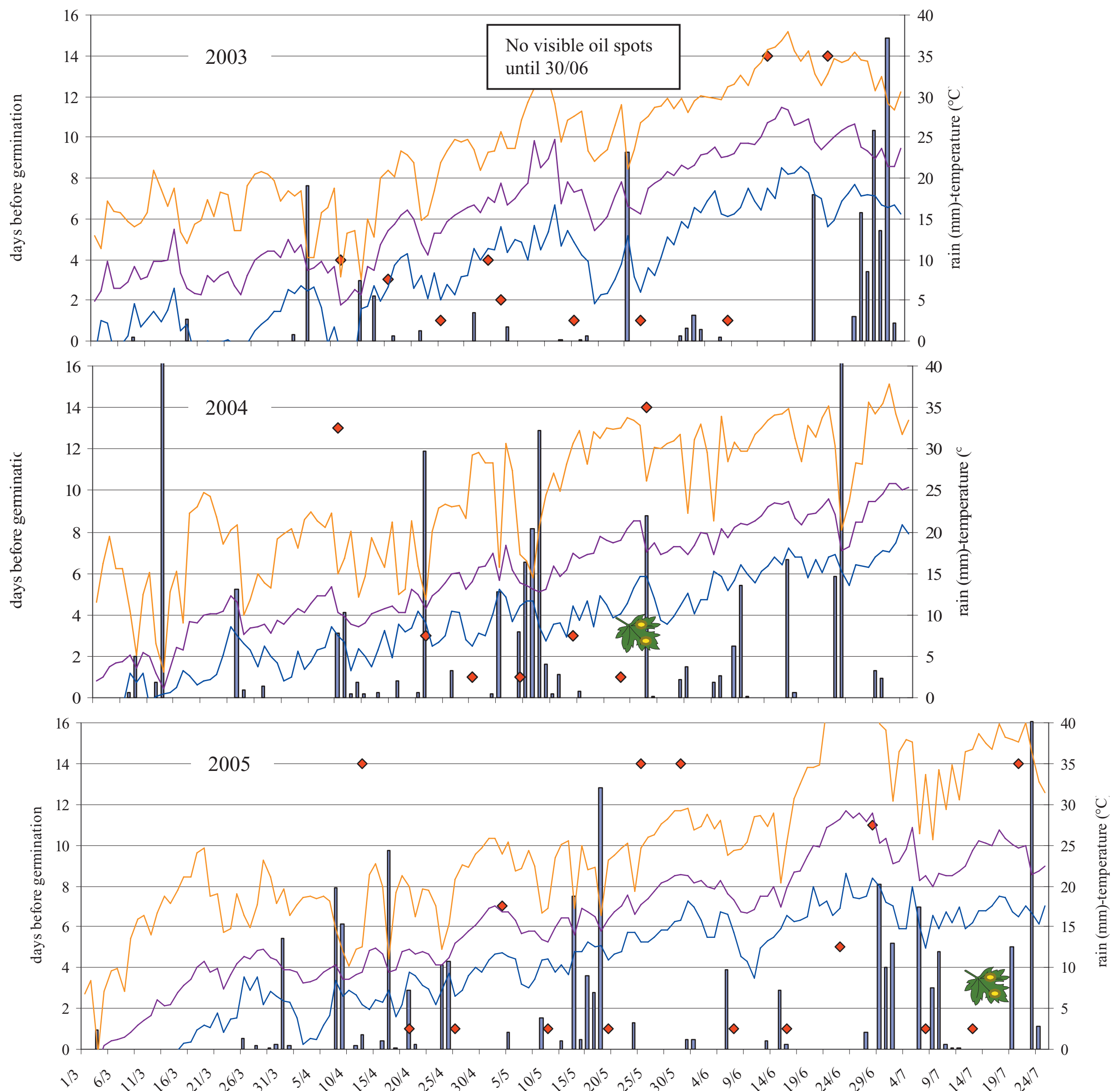

Fig. 2. Germination delay of overwintered oospores (Rovereto, Northern Italy) under controlled conditions (red square marker), minimum, average and maximum temperatures (blue, violet, and yellow line) and rain (blue histogram). Leaf indicates when the first oil spot symptoms were found in the vineyard. 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.

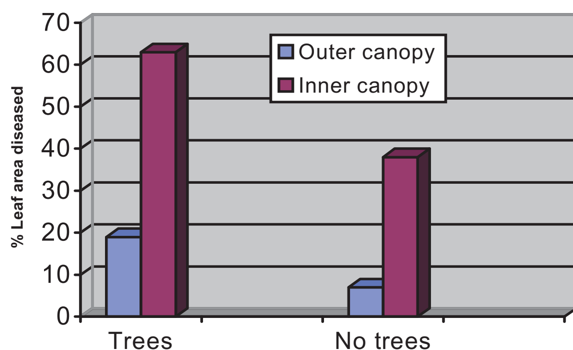

Fig. 1. Effect of two sources of shading on the development of powdery mildew. Evaluated leaves were from shoots on the outer (sun-exposed) or inner (shaded) portions of the vines, which were (a) adjacent to a group of tall trees (morning shade) or (b) away from the trees and exposed to available sunlight 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°C higher than for leaves in the shade-cloth treatments. Similarly, Chardonnay leaves in the direct sun were 6-11°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°C, whereas those in full sunlight measured 38-43°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.

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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.

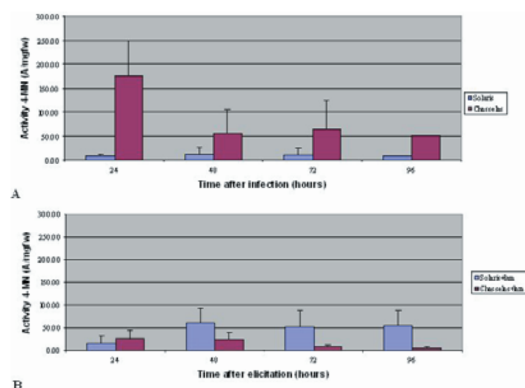

Fig. 1. Peroxidase oxidation of 4 methoxy-1-naphtol (4-MN) in leaves of Solaris and Chasselas at different time after infection (A) and elicitation with laminarin (1g/ml) (B).

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).

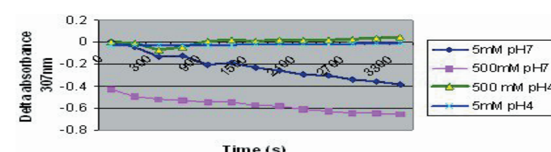

Fig. 2. Resveratrol stability as a function of time, pH and hydroxide peroxide concentration

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).

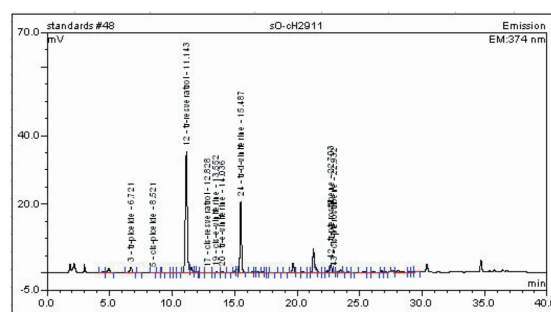

Fig. 3. HPLC-analysis of resveratrol oxidation product: resveratrol (10µg) was oxidized by the peroxidase fraction isolated from Chasselas in 100mM Tris-acetate, pH4 supplemented with 5µM of H2O2. Spectral emission (374nm) reveal specific pick of resveratrol and viniferin at 11.04 and 15.38 minutes respectively, 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).

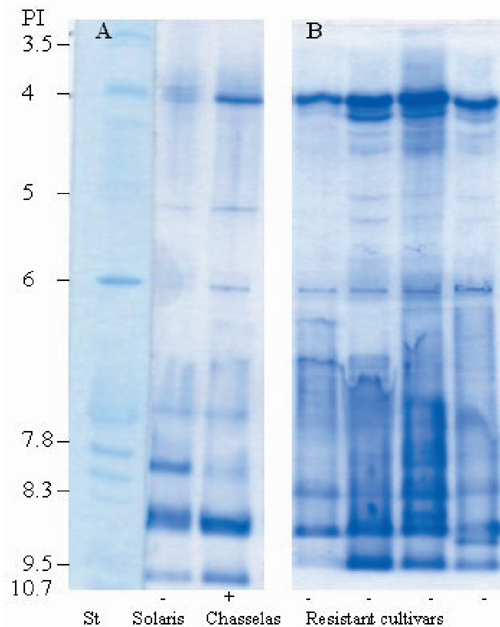

Fig. 4. Isoenzyme pattern of peroxydases separated by IEF and stained with 4-MN in presence of hydroxide peroxide

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).

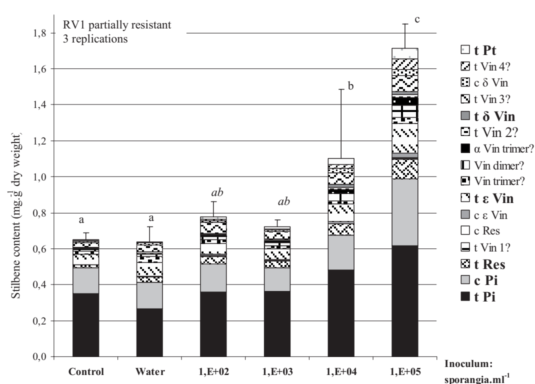

Fig. 1a. Relation between inoculum concentration of *P. viticola* and stilbene content in leaf disc of RV1 (partially resistant) 6 dpi. t for the isomer *trans*, c for the *cis*

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.

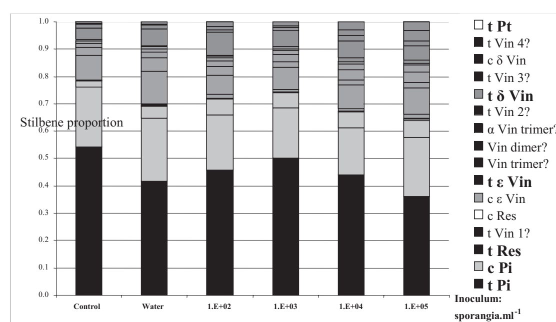

Fig. 1b. Relation between inoculum concentration of *P. viticola* and stilbene proportion in leaf disc of RV1 6 dpi.

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.

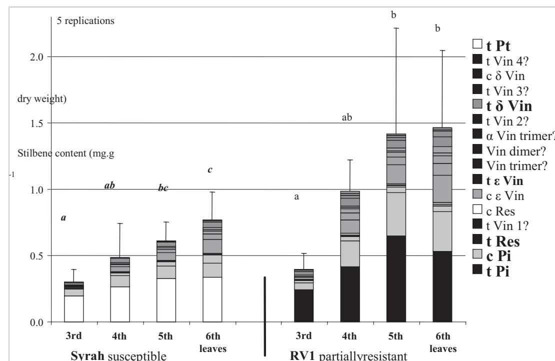

Fig. 2a. Relation between plant leaf age (first at the top) and stilbene content in leaf discs of Syrah (susceptible) and RV1 (partially resistant) after inoculation with *P. viticola* (10^5 sporangia.ml^{-1}) at 5 dpi. t for the isomer *trans*, c for the *cis*

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 6th leaf: 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.

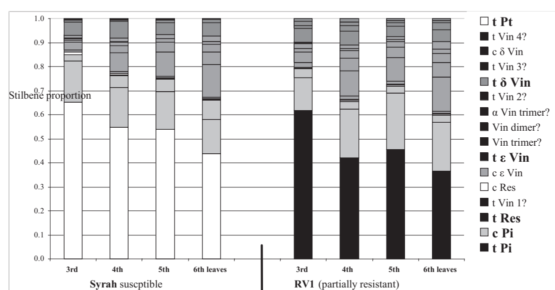

Fig. 2b. Relation between leaf level on the plant (first at the top) and stilbene proportion in leaf disc of Syrah and RV1 after inoculation of *P. viticola* (10^5 sporangia.ml^{-1}) at 5 dpi.

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.

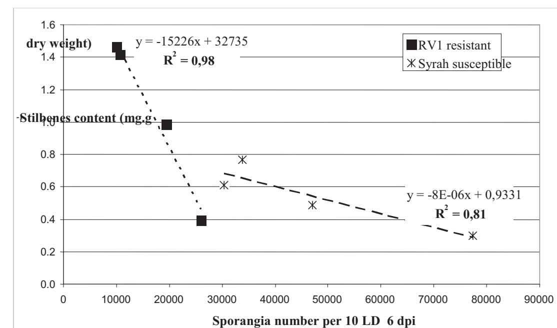

Fig. 3. Relation between stilbene content in leaf discs after inoculation with *P. viticola* (10^5 sporangia.ml^{-1}), and the number of sporangia per 10 leaf discs at 5 dpi.

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.

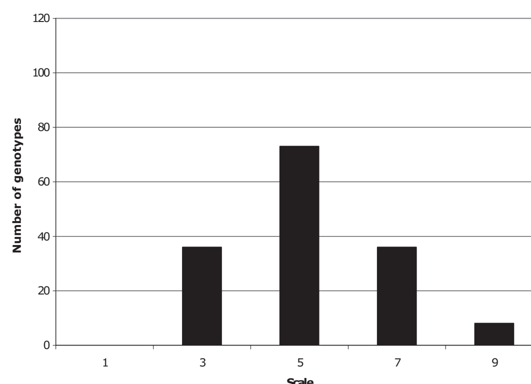

Fig. 1. Vineyard evaluation. Distribution of genotypes according to OIV 452 descriptor where infection degree is rated. 1= no

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.

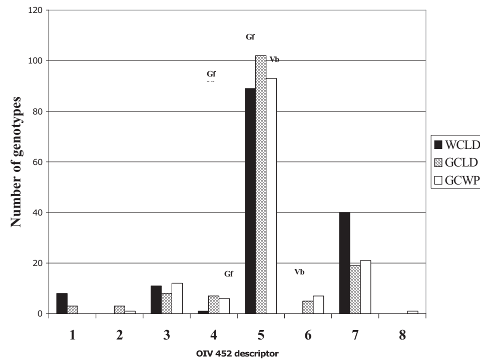

Fig. 2. Laboratory evaluation. Comparison among methods. WCLD = wood cutting leaf disc, GCLD = green cutting leaf disc, GCWP= green cutting whole plant. Gf= Gf.Ga 47-42, resistant parent 1, Vb = Villard blanc, resistant parent 2.
OIV 452 resistant scale = 1, 2, 3, 4, for susceptible scores. OIV452 =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

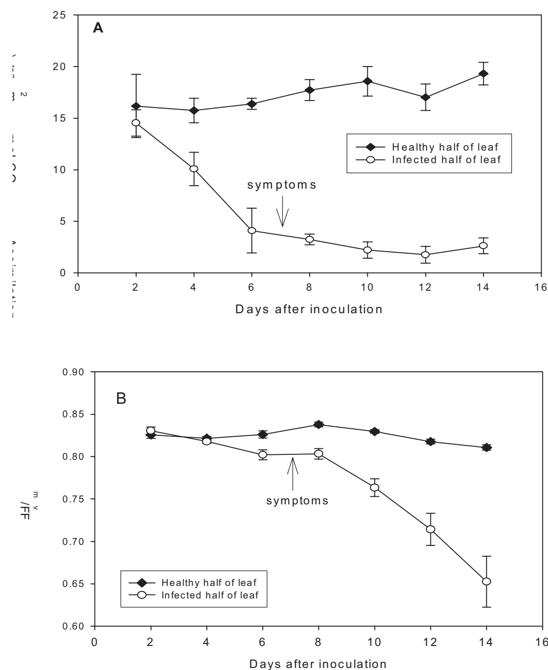

Fig. 1. The effect of downy mildew development on: (A) the rate of photosynthesis at ambient CO_2 levels, and (B) chlorophyll fluorescence expressed as the ratio of F_v/F_m in leaves of two-year-old potted 'Niagara' grapevines. Means are averages of five replications. Error bars indicate the standard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

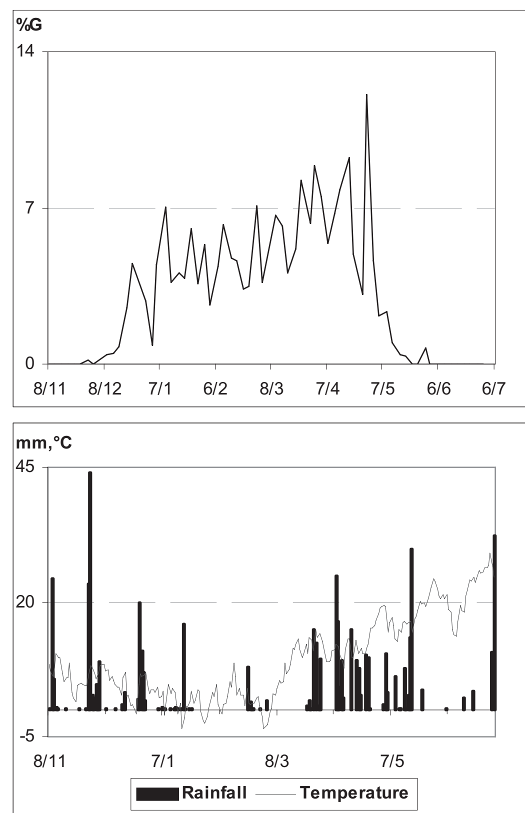

Fig. 1. Climatic conditions and germination percentages (%G), Montorio

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.

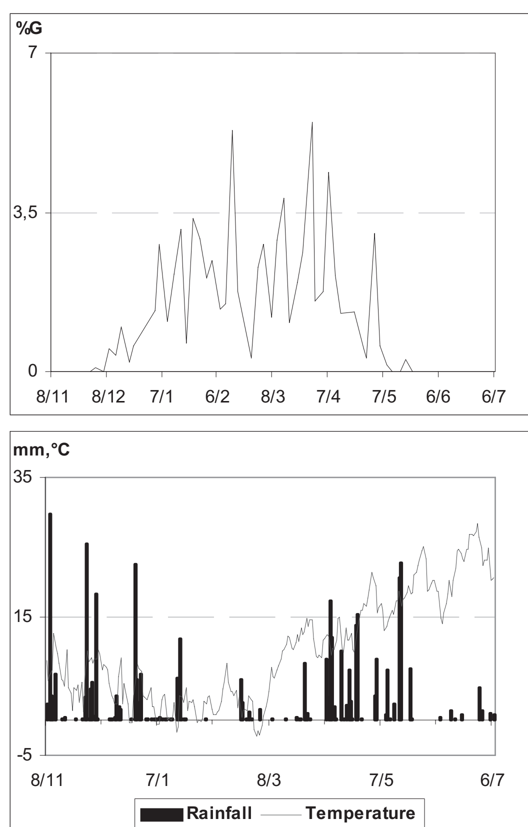

Fig. 2. Climatic conditions and germination percentages (%G), Monselice

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".

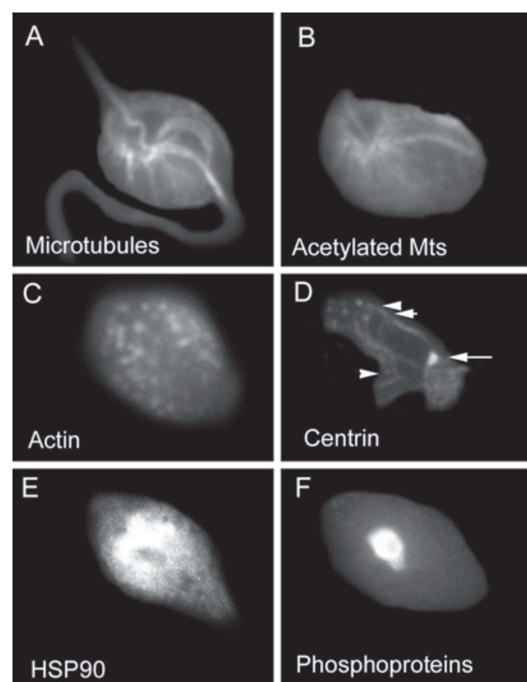

Fig. 1. Zoospores of *Plasmopara viticola* are presented with the ventral surface facing the reader and anterior to the left. (A) Typical oomycete microtubule arrangement. Microtubules (Mts) are acetylated (B) which is usually an indicator of stabilization. Actin plaques are present (C) probably just 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 microorganisms 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).

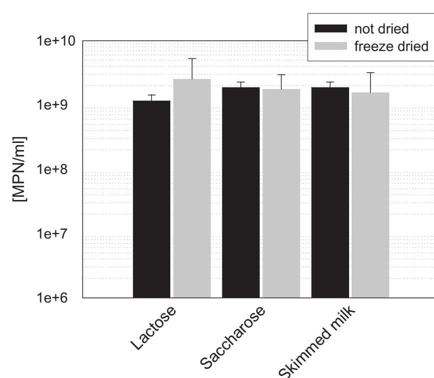

Fig. 1. Comparison of the viability of Pf 153 before and after freeze drying formulated with three different protectants.

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.

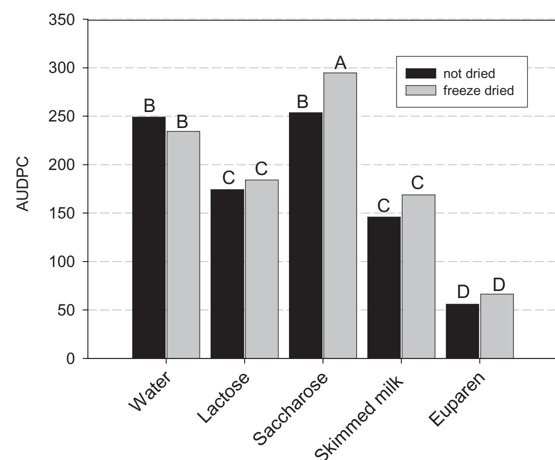

Fig. 2. Effectiveness of freshly produced and freeze dried cells of Pf 153 formulated with different protectants on the disease symptoms caused by *Botrytis cinerea* on detached leaves of *Vicia faba* (AUDPC=Area under disease progress curve).

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 fertilition 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*.

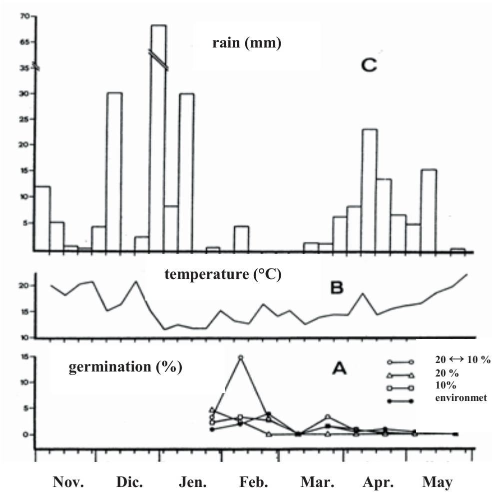

Fig. 1. 1989-'90: germination of *P. viticola* oospores kept at different soil moisture level (20% or field capacity; 10% or wilting point), (A), mean weekly temperature (B) and rain fall during assay (C).

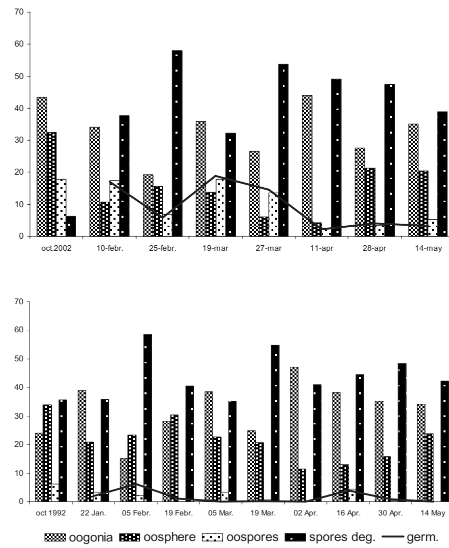

Fig. 2. Two year trial of observations on percentage of the different *P. viticola* gamic structures and of the oospore germination

Session 4:

Genetic of the pathogens: population genetics, virulence-avirulence, variability and fungicide resistance

Spatial genetic structure of grapevine downy mildew epidemic

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To evaluate the magnitude of the primary and secondary cycles of downy mildew of grape (*Plasmopara viticola*), it is important to determine the respective contribution of sexual and asexual reproduction to the epidemic. Recently, the population dynamics of downy mildew populations has been investigated using genetic markers (Gobbin et al. 2003, 2005). These studies have provided the first quantitative analysis of the proportion of *P. viticola* genotypes that undergoes secondary multiplication and of the genotype-specific distribution during an epidemic. These authors observed a continuous input of new genotypes into the epidemic and evaluated that migration during secondary cycles was low. These results consistently contradict current assumptions in viticulture that postulated the great importance and the long-range migration of secondary sporangia.

In our study, we used eight recently isolated microsatellite markers (Delmotte et al. 2006) to characterize 908 oil-spots collected at two dates in four different sites (Champagne and Bordeaux vineyards). The combination of a large number of highly polymorphic loci ensured an excellent resolution, which is necessary when studying genotypic structure of populations undergoing clonal reproduction. In addition, such markers were shown very convenient to assess genetic relationships between multilocus-genotypes and spatial genetic structure of mapped strains. This allowed estimating for the first time the dispersion level of both primary and secondary inoculum at a very fine spatial scale.

According to Gobbin et al. (2005), we found a high genotypic richness with a majority of genotypes sampled once (55%-70%). Most repeated multilocus genotypes were found in low number of copy while, at particular dates, a few proportion of genotypes was highly amplified. The second date always presented more repeated genotypes than the first one and we found an important number of new genotypes that were not sampled before. This can be explained by three non exclusive hypotheses: sampling bias, arrival of secondary inoculum through long distance dispersion of sporangia, oospores germination late in the season.

Autocorrelation analyses revealed a high and significant pattern of spatial genetic structure for both secondary and primary inoculum (Fig. 1): we first confirm the low dispersion of secondary sporangia (several stocks). More surprisingly, we also found that genotypes constituting the primary inoculum were highly genetically related at the wine stock scale suggesting a low dispersal of oospores in the early season of the epidemic.

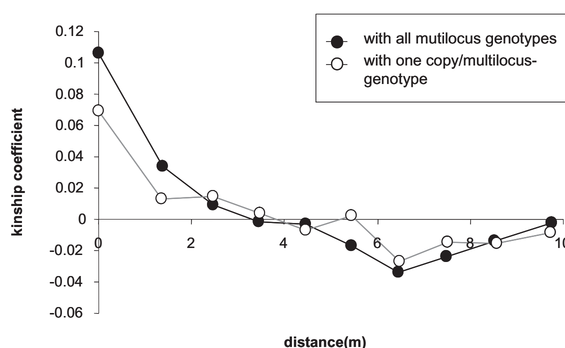

Fig. 1. Spatial autocorrelation of kinship for 241 oil spots over all microsatellite loci. Two data sets were analysed: in black including all sampled multilocus genotypes, in white including only one copy per multilocus genotypes.

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Genetic structure and epidemiology of *Plasmopara viticola* populations from Australian grape growing regions

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Plasmopara viticola, the causal agent of grapevine downy mildew, originating from North America, was introduced at different times into the Australian states on the east and west coast (Victoria: 1917; New South Wales: 1918-1920; Western Australia: 1998) whereas it is not known if there was an early single introduction with a subsequent spread inside Australia or if there were several separate introduction events. The estimated annual crop loss in an average year in Australia is 22.5 million AUD, with an additional 10 AUD spent on control measures. In wet years, direct crop losses in Australia may be as high as 64 million AUD (Magarey and Butler, 1998). Downy mildew population structure as well as epidemiological data were reported for European populations but no information was available for populations growing in hot and dry regions as Australia.

In this study the quantitative contribution of primary vs. secondary inoculum to epidemic of a particular Australian grapevine downy mildew population was investigated. Six-hundred and thirty-six lesions were collected from an unsprayed vineyard of the table grape cultivar "Ribier" in Caversham (Swan Valley, Western Australia) from 12 November 2004 until 19 January 2005 (six sampling dates, 68 days survey time) and genetically analyzed by the four microsatellite markers ISA, CES, BER and GOB (Gobbin *et al.*, 2003). Genotypes presenting the same allele pattern were considered as clones (derived from the same oospore through asexual reproduction), while the ones presenting a different allele pattern were considered to have derived from different oospores. Following this principle, oosporic infections could be differentiated from the clonal ones.

Thirty-three mm rain fell on the vineyard during the 68 days survey, distributed in eight events. Average daily temperatures were about 17°C at the beginning of the survey, increased to 30°C toward Christmas and then decreased to 19°C in January. Average daily relative humidity ranged from 40% to 80% with a maximum of 98%. As the sampling began the disease was already present. Disease severity increased until the fourth sampling (8 December 2004) and then decreased abruptly as a consequence of a dry and hot period. Genetic analysis showed 31 distinct genotypes among the 636 successfully analyzed samples. Sixteen different genotypes produced one lesion each, nine genotypes produced from two to 22 lesions each, and four genotypes produced from 63 to 268 lesions. The most frequent genotype amounted to 42% of the lesions sampled, suggesting that about the half of the disease symptoms in Caversham was originated by a single oosporic infection. The population structure on the most diseased vine on 19 January 2005 was featured by the presence of nine different genotypes; the most frequent genotype occurred in 2/3 of the 73 lesions sampled. The most salient conclusions drawn from this study comprehend both the dominance of secondary inoculum (88%) since the very beginning of the epidemic and the continuous but low occurrence of putatively primary lesions (Fig 1a,b). The dominance of the secondary cycle can be explained by the fact that secondary infections are triggered by dew or rain events while primary infections require intensive rains to allow macrosorangia/zoospores to be splash-dispersed throughout the vines.

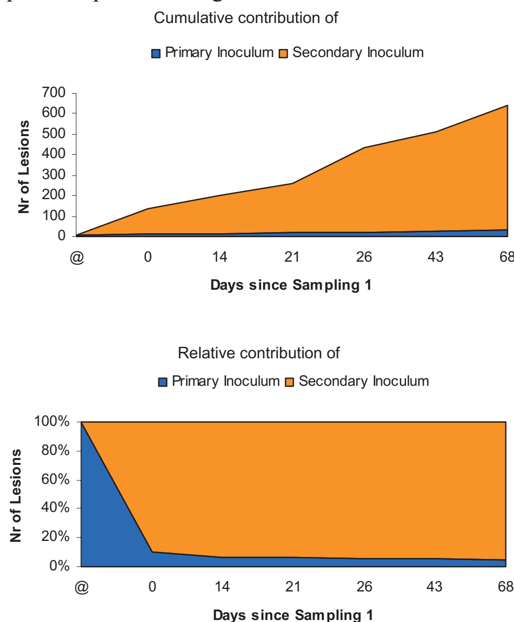

Fig. 1. Number of lesions (upper graph) and relative contribution of primary and secondary inoculum to the Caversham epidemic 2004-5 (lower graph). "@" indicates a date before the first sampling.

A second objective of this study was to assess genetic diversity of five oosporic (clone-corrected) Australian populations: Caversham (cav, WA), Henley Brook (heb, WA), Pemberton (pem, WA), Lovedale/Pokolbin (hun, NSW), Yarra Glen (yar, Vic). From those populations 483 lesions were collected. Normalized Shannon indices (E_H) showed low within-population diversities, especially in cav (0.28), heb (0.33) and pem (0.34), while in hun (0.62) and yar (0.84) higher diversities were found.

Fixation indices were significantly low when comparing the Western Australian populations cav, heb and pem among themselves ($0.02 < F_{ST} < 0.06$). Higher

significant F_{ST} were found comparing western populations with hun and yar ($0.20 < F_{ST} < 0.22$). Among *P. viticola* populations, low to large genetic distances (Shriver's D_{sw}) were observed, especially between east and west coast populations. This genetic differentiation was evident in the Neighbor Joining phylogenetic tree showing clear substructure and distinguishing mainly three clusters based on geographic origin (Fig. 2). Including one American and ten European populations (data from Gobbin *et al.* 2003, 2005, Rumbou and Gessler 2004, unpublished D. Gadoury) into the phylogenetical analysis, Australian populations seem to be directly related to one French population and to the Geneva population (USA). Greek and German populations are displayed at the opposite of the three, indicating a minor genetic relationship.

To explain this population substructuring three possible explanations must be taken into account: first, the populations seem to have experienced low genetic exchange between each other, thanks to natural barriers and interstate quarantine measures. Second, *P. viticola* was introduced from outside of Australia at different times into the states on the east and west. Third, the Western Australian climate is adverse to the development of the pathogen. The fungus is forced to go through regular cycles of extinction and re-colonization or at least extreme bottleneck situations.

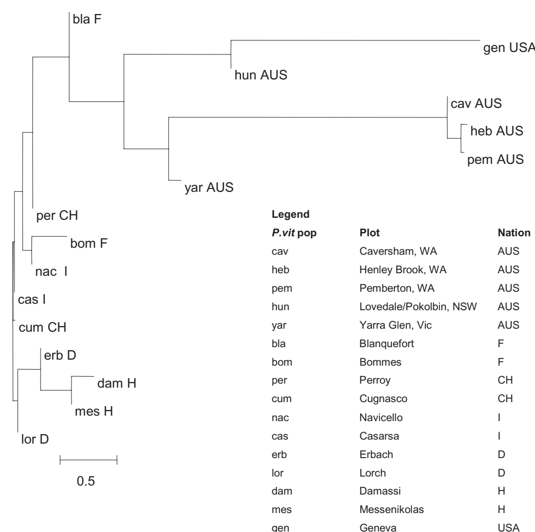

Fig. 2. Unrooted Neighbour-joining tree based on D_{sw} (Shriver *et al*, 1995), representing phylogenetic relations amongst five Australian populations (AUS), one American population (USA), two Swiss (CH), two French (F), two Italian (I), two Greek (H) and two German populations (D) for *Plasmopara viticola*. Population data sets were clone corrected.

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Genotype fitness and fungicide resistance of *Plasmopara viticola*

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Plasmopara viticola is known as a high risk fungus for development of resistance. Resistance was recently found to be well established and widespread for the fungicide Metalaxyl (Magnien, 2001) but resistance is also reported for QOL-fungicides, strobilurins, famoxadone, fenarimol (Panon and Panigai, 2002) and for Cymoxanil (Genet and Vincent, 1999).

Plasmopara viticola is genetically very variable (Stark-Urnau et al., 2000; Gobbin et al., 2002). Great differences in aggressiveness were found by KAST et al. (2000). The aim of the present work was to test the effect of genotypic aggressiveness on the resistance against some fungicides with curative properties.

Materials und Methods

Test procedure

The leaf disc test method, described in detail by KAST et al. (2000), was used for this test series. The number of leaf discs was modified to four instead of 15. Fungus isolates were propagated on leaves grown in a glasshouse. Spores were produced by 16h darkness and 20° C and saturated moisture. A suspension incorporating $3x10^4$ sporangia per ml was used for infection (0,007ml per cm^2, uniformly distributed) which was produced by diluting a sample after counting the sporangia. Use a haemacytometer??

Fungicides were applied 24 hours after infection (curative treatment). 0,007ml fungicide solution per cm^2 were used. Six fungicide concentrations were tested: 100 %, 33.3 %, 11.1 %, 3.7 %, 1.2 %, 0 % of the recommended dose.

Sample collection

The pathogen was sampled in August 2002. Isolates 121, 122, 131 - 133, 142 - 145 and 151 - 153 were selected from different vine varieties grown in the experimental station "Burg Wildeck" (KAST, 2004). (This station was run organically since 1993 without using fungicides except copper and sulphur preparations). Isolates 161 - 164 were found on untreated vines of the interspecific hybrid variety Regent grown at Weinsberg in a small vineyard surrounded by normally sprayed vines. Isolates 171 - 172 originated from an experimental plot in a field trial at Weinsberg which was treated 7 times with Aktuan (Dithianon + Cymoxanil), 181 - 183 from a plot treated with copper. This experimental vineyard, bearing isolates 171 - 183 was curatively treated several times in 2001 using Dimethomorph and Metalaxyl-M with poor results. Isolates 191 - 192 were collected from a vineyard at Heilbronn, which was treated with Forum, Ridomil Gold Combi and Melody Multi, but was severely attacked by downy mildew.

Fungicides

The fungicides user in the experiments and their active substances and recommended dose are shown in table 1.

Each fungicide was tested in a separate experiment.

Tab. 1. Tested fungicides ([1]protective (contact) agent; [2]curative agent)

Product	Active substances	Recommended dose = 100 %	
Ridomil Gold C.	5 % Metalaxyl-M[2], 40 % Folpet[1]	0.15 %	75 mg/l 600 mg/l
Forum	15 % Dimethomorph[2]	0.12 %	180 mg/l
Equiation Pro	22,5 % Famoxadone[1] 30 % Cymoxanil[2]	0.04 %	90 mg /l 120 mg/l
Melody Multi	0,06 % Iprovalicarb[2], 0,375 % Tolylfluanid[1]	0.20 %	120 mg/l 750 mg/l

Evaluation

Eight days after infection the disease severity on each leaf disc was evaluated visually. An index was used by estimating % sporulating leaf area in 4 classes: 0 = no, 1 = < 25 %, 2 = 25 - 50 %, 3 = 50 - 75 %, 4 = 75 - 100 %.

The Box and Cox procedure according FEURER and KAST (1989) was used to check for optimum transformation. Regression analysis using the 24 values of eachcombination of isolate and fungicide was used to calculate:

1. mean lethal dose MLD100 (disease index = 0)
2. mean disease index at fungicide-dose = 0.

Results and Discussion

The Box and Cox transformation did not result in lower values for skewness and kurtosis- Original values were used for further calculations. The hypothesis of a linear relationship between dose and disease index values could not be rejected. Linear regression was used to calculate disease severity (mean index) at fungicide concentrations 0 = water control (FC0), and MLD100 (in % of recommended dose), the mean concentration necessitated to kill 100% of the fungus mycelium.

Extreme differences were found for the mean disease severity at a dose of 0 (water check). It has to be mentioned that some isolates could not be included in the test because there was no relevant reproduction. These results support the findings of Stark-Urnau et al. (2000) and Gobbin et al. (2003), who reported extreme differences of fitness from different genotypes. Only a

few genotypes contribute overwhelmingly to the epidemic.

The MLD100 was highly correlated to FC0:

Ridomil Gold Combi	r=0.88
Equiation Pro	r=0.91
Forum	r=0.81
Melody Multi	r=0.77

A test for homogeneity of these correlations indicated differences. For Cymoxanil (in Equation Pro) 82 % of the differences in MLD100 between the fungus isolates is explainable by differences in fitness (Coefficient of determination in %), for Iprovalicarb this value is only 59%. Adjustment of the MLD100 using this covariance diminished some extreme outliers in the calculated values for MLD100. (KAST 2004).

Theoretical considerations and model simulations for host-parasite interaction of obligatory biotroph fungal diseases indicated a balanced system, where the mean aggressiveness of the fungus was limited by some negative effects (Leonard 1977; Leonard and Czochor 1980; Geiger et al. 1980). This equilibrium is dependent on the strength of selection towards fitness. If overall fitness is a main component for the resistance of the fungus against fungicides, a strong selection towards higher fitness (=aggressiveness) would take place, when these fungicides are used in the vineyards.

Mean disease index (=fitness) of isolates which originated from the organic vineyard (0.77) was lower than that of isolates from intensively sprayed vineyards (1.05) but the difference was not significant.

Problems caused by *Plasmopara viticola* in Germany have not decreased over the last 50 years in spite of the introduction of much better fungicides, much better application techniques, an increasing knowledge about the biology of the fungus, better education and information of winegrowers and the use of models. Maybe this is a result of selection pressure towards higher aggressiveness?

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Something new about *Plasmopara* viticola? A microscopy and molecular biology-based contribution to its general biology and its effect on grapevine.

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Many downy mildew pathogens, such as *Plasmopara viticola*, invade the leaf via the stoma and carry out the major part of their life cycles inside the leaf. They are therefore not detectable at the beginning of an infection and many aspects of the infection process at the cellular and molecular level are still unknown. In such a case, an investigation of the infection process may help to understand the initiation, establishment, and progression of infection and to find the Achilles´ heel of the pathogen. Therefore, well-established and new staining techniques were used to identify infection structures and their chemical composition in semi-thin sections and whole-leaf samples. After application of appropriate dyes, it was possible to distinguish plant from fungal cell walls, haustoria from fungal cell walls, and haustorial necks from haustorial heads, indicating a different underlying chemical composition. Furthermore, the occurrence and development of septa in *P. viticola* and other *Peronosporaceae* was investigated. Enzymatic digestions and suitable staining techniques were applied to verify chemical constitution of septa, which are mainly composed of 1,3-glucans. In *P. viticola*, septa were found in the stem and branches of the sporangiophores but not in the mycelium, which is in contrast to other downy mildews (Tab. 1).

Tab. 1. Occurrence of septa in downy mildews.

Species	Host	Septa in Mycelium	Septa in Sporophore
Bremia lactucae	lettuce	-	+
Peronospora destructor	onion	-	-
Peronospora tabacina	tobacco	+	-
Peronospora valerianellae	Lamb´s lettuce	-	-
Plasmopara crustosa	parsley	-	-
Plasmopara viticola	grapevine	-	+
Pseudoperonospora cubensis	cucumber	+	+
Pseudoperonospora humuli	hop	+	-

As β-1,3-glucans are the major component of septa, the potential of 2-deoxy-D-glucose (2-DOG) to inhibit fungal growth and septal-development was assessed. Development of the intercellular mycelium and the occurrence of septa were unaffected after treatment with 2-DOG at low concentrations (1-5 mM). However, sporangiophores showed untypical branching and a failure of sporangiospore production. At a concentration of 10 mM 2-DOG, spreading of the intercellular mycelium was reduced and completely blocked at a concentration of 50 mM 2-DOG. Interestingly, septal development seemed not to be influenced except for the highest concentration used. In this case, leaf tissue also became necrotic.

In addition to microscopic studies, first steps in gene expression and gene analysis were performed, since analysis of genes and gene function in *Plasmopara viticola* has lagged significantly behind other oomycetes. In fact, there is nearly no history of genetic research except in the case for genome size determination and analysis of phylogenic relationships. This is mainly due to difficulties in obtaining enough fungal material to extract stage-specific mRNA. Gene disruptions and replacements are also not established due to the biotrophic growth of *P. viticola*. In search for specifically expressed genes, we first used a non-radioactive differential display strategy to identify genes involved in the life cycle of *P. viticola*. This included the isolation of pure zoospores, the isolation of RNA from the respective fungal structures (zoospores, sporangia, mycelium in oil spots), the detection of putative differentially expressed genes or gene fragments using agarose-gel electrophoresis, and the confirmation of origin and differential expression profile by Southern hybridization and RT-PCR analysis, respectively. After electrophoresis, differentially displayed cDNA bands were directly excised from the gel, PCR amplified, cloned into pGEM-T Easy vector, and used for sequence determination. Alignment and comparison of the DNA sequences was conducted through GenBank (BLAST), Phytophthora Functional Genome Database (PFGD), and the Joint Genome Initiative (JGI).

Several genes isolated are ones that show homologies to genes well characterized in other fungal systems or necrotrophic/hemibiotrophic oomycetes such as *Phytophthora*. Nevertheless, many cDNA sequences derived from *P. viticola* did not show significant similarity to known sequences in database searches, and as such, they may represent novel oomycete proteins. As oomycetes are fundamentally different from true fungi in terms of cell-wall composition, reproductive biology, and genetics, which may explain why most fungicides against major fungal pathogens are ineffective in controlling diseases caused by oomycetes, these features are certainly reflected in oomycete specific genes.

Molecular aspects of the interaction between grape and *P. viticola* are also being investigated by cDNA-amplified fragment length polymorphism (AFLP) transcriptional analysis. cDNA-AFLP is a powerful technique, able to identify the almost complete picture of the transcriptional changes occurring in grape following *P. viticola* infection, without any previous sequence information. This technique has been already used in

several model systems, including plant pathogen interactions and is particularly suited for non-sequenced organisms, in which gene discovery is still an important issue. The analysis is being carried out on cDNA from infected leaves (cv. Riesling) at the oil spot stage, compared to healthy plant samples and samples from *P. viticola* sporangia. Amplifications with all the 128 primer combinations have been performed. An example of the profiles observed is given in Fig. 1.

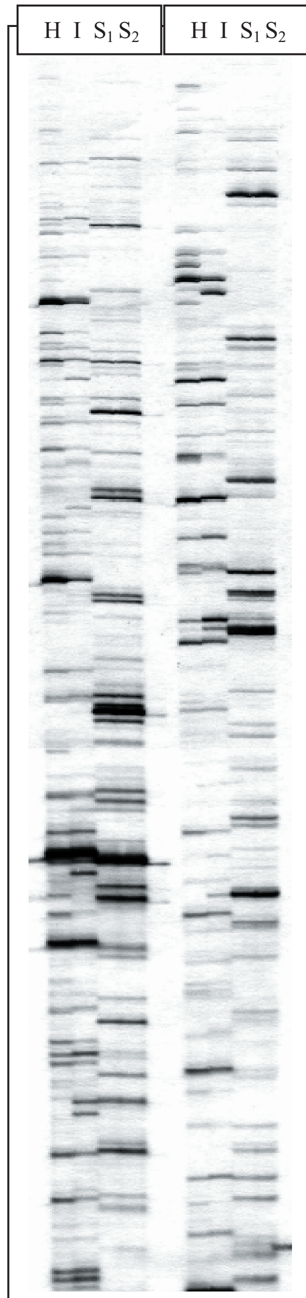

Fig. 1. The two groups of four lanes represent cDNA-AFLP amplifications with two different primer combinations on cDNA samples from healthy (H) and infected (I) grape leaves and on two replicas of cDNA from *P. viticola* sporangia (S_1 and S_2) as a control.

About 2000 cDNA fragments have been visualized from the sporangia samples, which could contribute to the creation of a wider database on the pathogen's expressed sequences. Unfortunately, cDNAs made from sporangiospore mRNA is unlikely to be representative of all *P. viticola* genes, and in particular would exclude genes solely expressed *in planta*. Some genes of *P. viticola* seem only to be induced during its interaction with the grapevine, and it is likely that the pathogen also induces and/or represses the expression of host genes.

Indeed, the analysis revealed a huge modulation of the grape transcriptome in response to infection: about 1500 differentially expressed cDNA fragments from the infected samples have been identified, which are being purified, sequenced, and screened for their origin. All this information will hopefully be useful for a deeper understanding of plant reactions to the infection process and for the identification of *P. viticola* genes possibly involved in virulence.

As grapevine plants used for grape and wine production are susceptible to *P. viticola* and other specialized pathogens but resistant to many others, the expression of defence-related genes and the production of salicylic acid (SA) was investigated either after inoculation of *P. viticola* as a host pathogen and *Pseudoperonospora cubensis* as a non-host pathogen. Especially genes encoding enzymes involved in anthocyanin biosynthesis were affected. In Riesling, enhanced transcription of dihydroflavonol 4-reductase (DFR), flavanone-3-hydroxylase (F3H), leucoanthocyanidin dioxygenase (LDOX), and flavonol synthase (FS) occured after inoculation with *Ps. cubensis*. Interestingly, expression of phenylalanine ammonia lyase (PAL) seemed to be repressed during non-host interaction with *Ps. cubensis*. Induction of genes involved in the phenylpropanoid pathway led to an accumulation of the corresponding compounds at the site of inoculation. Furthermore, pretreatment with *Ps. cubensis* reduced disease severity after a subsequent infection with *P. viticola* compared to the non-treated controls.

Since salicylic acid (SA) is a potent inducer of local and systemic resistance, leading to a lower degree of susceptibility, the production of SA in leaves was monitored both after inoculation with the host pathogen and the non-host pathogen. In Riesling, both pathogens induced SA production during the first two days after inoculation. There was a further increase in SA production at day 4 and 6 after inoculation with *P. viticola* but not in the incompatible interaction. In the latter case, SA content in leaves decreases with time. The role of SA supplied to leaves before inoculation is now being investigated.

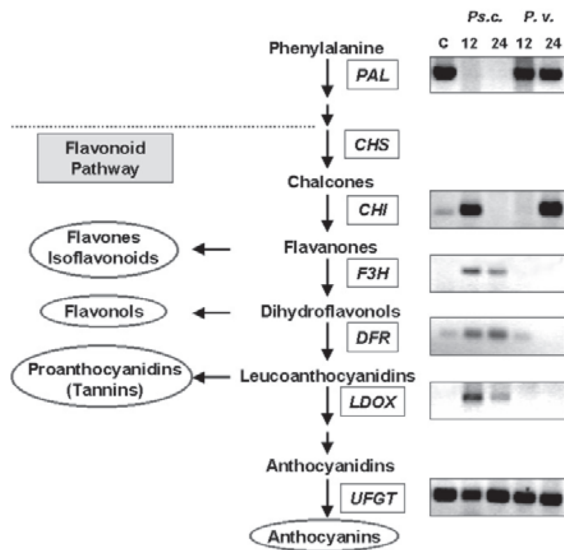

Fig. 2. Pattern of transcript accumulation of genes encoding enzymes involved in the phenylpropanoid pathway in the susceptible cv. Riesling 12 and 24 hours post inoculation with the host pathogen *P. viticola* and the non-host pathogen *Ps. cubensis*.

First approach of *Plasmopara viticola* population biology: merging epidemiology and population genetics

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The Oomycete *Plasmopara viticola* (Berk. et Curt.) Berl. et de Toni, the causal agent of grapevine downy mildew disease, constitutes the most destructive pathogen in viticulture regions with rainy springs/summers. The pathogen infects all the green tissues of the plant producing yellowish lesions. Losses are caused through weakening, fruit destruction and killing of leaf tissue. Current concepts regarding the pathogen's epidemiology postulate that the sexual spores (oospores) only play a role at the initiation of the disease early in the grapevine vegetative season. The explosive progress and the dispersal of the disease are attributed to the asexual spores (secondary sporangia) which are assumed to migrate in long distances within short time. The study conducted aimed to investigate the previous assumptions regarding the pathogen's epidemiology and, specifically, the qualitative and quantitative contribution of oosporic versus clonal infections and the mode of disease spread. Combining epidemiological and population genetics data, a broader and more encompassing perspective of the disease dynamics was finally obtained.

In five European countries (Switzerland, Germany, France, Italy and Greece) 32 plots were selected and natural downy mildew epidemics were tracked during the years 2000-2002 (1-22 samplings/plot). About 10,000 oilspots were collected and the oomycete strains were genotyped with the use of four specific microsatellite markers. Because *P. viticola* is diploid, every oilspot was characterized by a genetic profile consisting of eight microsatellite alleles. Genotypes presenting the same allele pattern were considered as clones (derived from the same oospore through asexual reproduction), while the ones presenting a different allele pattern were considered to have derived from different oospores. Following this principle, oosporic infections could be differentiated from the clonal ones. The population genetics analysis provided a description of the populations' structure (patterns of genotypic diversity, genetic variation, migration, genetic drift etc.), as well as assessment of genetic distances among populations. In parallel, spatial distribution analysis of the epidemics was conducted.

One of the main outcomes acquired is that the sexual spores constitute a major source of inoculum. The many genotypes identified and the high genotypic diversity estimated in most of the *P. viticola* populations studied, reflects the massive occurrence of oosporic infections and, consequently, a large pool of oospores in the soil. In contrast to the existing belief that primary infections occur only at early disease stages and in limited scale, it was shown that oosporic infections play a main role at the initiation of the disease in May and continue to occur throughout the epidemiological season. However, their quantitative contribution to the epidemic decreases with the progress of time. In Greece, they are also responsible for the disease regeneration in September, after the disease inhibition caused by heat and drought. Exception to the prior outcome consists in some island regions in Greece, where limited oosporic infections were observed. The climate in those regions is very dry and the disease usually appears with low severity, while occasionally bursts heavily. In conclusion, the genetically highly variable populations of the pathogen indicates high levels of sexual reproduction (Figure 1).

Considering the asexual spores, it was shown that their role for the epidemic was overestimated until now. The great majority of the genotypes in each population (85%, on average) were identified only once or twice throughout the survey period. The genotypes that underwent a relevant asexual reproduction (dominant genotypes, frequency <1%) were only one or two per epidemic. The contribution of dominant genotypes to the total disease severity ranged from 4.3 to 95%, depending on the epidemic surveyed, the macro- and microclimatic conditions and on the epidemic stage. The amount of clonal infections was low until the middle of July and increased only late in summer. The spatial distribution analysis showed that secondary infections were spatially localized in most cases (at leaf, branch or vine level). In one asexual cycle, the clones' dispersal usually did not cover an area wider than a few vines around the site where the oosporic infection was first identified. Migration distances were less than 20m per asexual cycle but larger distances could not be excluded. With an increasing number of asexual cycles, the clones spread throughout the plot following a stepwise migration pattern. Secondary infections played a leading role in epidemics where a small number of oosporic infections occurred; in some islands or isolated mountain regions, the lesions derived from the dominant genotypes represented until 95% of the sample size. Apart from the exceptions, the overall finding is that clonal infections in central Europe and Greece mainland play a relevant role in a small vineyard area (delimited for instance by a few vines) and a moderate role at plot size, in sharp contrast to previous beliefs about the major importance of asexual inoculum (Figure 1).

The contribution of primary versus secondary infections to epidemic development showed two general patterns. Most frequently, the role of primary infections at plot scale was major while the role of secondary

infections was minor throughout the growing season. This pattern was found in the majority of the central European populations and in the mainland populations in Greece. The other epidemic pattern was characterised by the predominance of one or a few clones and was found in low severity epidemics in central Europe as well as in Greek islands and coastal plots. The occurrence or absence of bottleneck events during the epidemic was another feature of the epidemics studied. In cases where a bottleneck did not occur (continuous epidemic), the disease started in spring, grew continuously triggered by both primary and secondary infections, and ended late in autumn. This pattern was found in central Europe and in one Greek plot. In contrast, in the remainder of the Greek plots, the disease started in spring and grew until mid-summer, and then stopped because both primary and secondary infections were not possible due to unfavorable climatic conditions. The disease started again in autumn (two-peak epidemic).

The different contributions of the primary versus secondary infections in combination with the presence or absence of bottlenecks during an epidemic led to different genetic substructures among samples within the same plot. During a single grape-growing season, either one (typical for 'continuous; epidemics in central Europe) or two *P. viticola* subpopulations were responsible for the epidemic (typical for 'two-peak' epidemics in Greece). Among samples of two or more consecutive grape-growing seasons either one or more subpopulations were responsible for the epidemics. The first case occurred only in two Greek plots characterized by the predominance of one clone and low disease severity, while the second case was more common and was found in all plots where the epidemic pattern was the 'two-peak' type.

Genetic subdivision among oosporic populations from different vineyards was very clear. Most pair-wise comparisons between populations from geographical sites more than 5 km away revealed a significant genetic differentiation. This means, in biological terms, that naturally occurring exchange of propagules (oospores or sporangia) is low. Furthermore, the widely held belief about long-distance secondary sporangia migration, which would cause the homogenisation of the *P. viticola* European population, is less relevant than assumed. Significant isolation by distance was found in central European *P. viticola* populations, suggesting a stepwise migration model. Conversely, in Greece no significant correlation was found between geographical distance and genetic divergence: population pairs separated by an increasing geographical distance may, but also may not, exhibit an increasing genetic divergence and geographically closer populations may have a higher genetic divergence than geographically distant populations This phenomenon is most probably due to the frequency of natural geographic barriers such as the sea and the mountains, as well as the frequent population bottlenecks occurring in these populations, preventing natural migration among populations and keeping them isolated from each other.

Another finding was the alternative overwintering of the pathogen in asexual form. This situation was observed in the Kephalonia island, presumably as mycelium in the buds or in leaves that did not fall during the winter and for first time it was proven by molecular genetic. Furthermore, two other biological phenomena were also recorded in this work. First, a "polyploid" genetic profile characterized some individuals (suggesting the existence of polyploidy in this fungus). Second, in all populations, we observed the regular appearance of clusters of similar genotypes, consistent with mutation events occurring during mitosis. These two mechanisms can contribute to the genotypic diversity of the pathogen and, possibly, also constitute adaptive mechanisms to unfavorable conditions for the survival of the population.

Our recent findings challenge the existing assumptions about *P. viticola* epidemics. The relative role of sexual and asexual spores on disease development was surprisingly misconceived. The new concept obtained after this survey is based on a site-related population structure and epidemic pattern. This means a leading or a minor role of oosporic infections depending on the micro- and macroclimatic conditions. The importance of those findings consists in their value for the design of control strategies under Integrated Pest Management and biological viticulture.

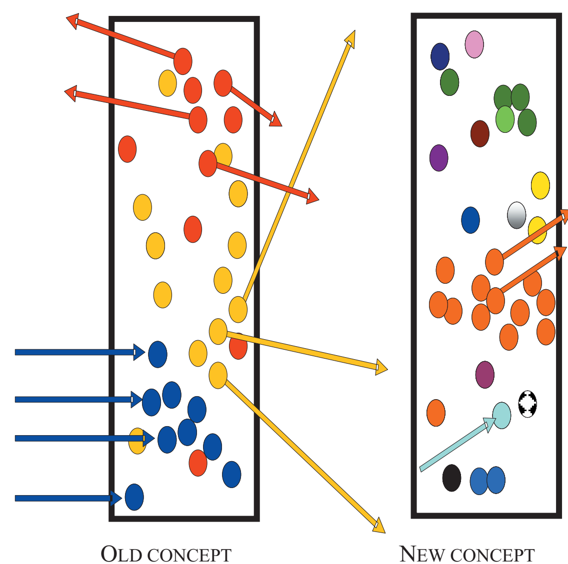

Fig. 1. A change in conception of grapevine downy mildew population structure. The traditional conception believes in genetically poor populations, where a few genotypes are present with thousand/millions of clones per population that easily migrate from plot to plot. The new conception contemplates genetically structured populations with the majority of genotypes represented one-two times and a dominant clone generating a very variable number of lesions. Clones are clustered and migration over long distance is rare. The vineyard is represented by a rectangle as seen from the top; *P. viticola* lesions are represented by circles; a different color or pattern means a different genotype. Migration of propagules is shown by arrows.

Assessment of *Plasmopara viticola* oospores germination by gene expression detection of a putative Puf Family member

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The casual agent of grapevine downy mildew, the oomycete *Plasmopara viticola* (Berk. *et* Curt.) Berl. *et* De Toni, is one of the most important grapevine pathogens worldwide.

In contrast to the existing beliefs, it was shown that oosporic infections play a main role at the initiation of the disease in May and continue to occur throughout the epidemiological season.

The first method used determine the timing of the germination of oospores, was the microscopical observation of leaf fragment on formation of primary sporangia. A second method developed by Hill, based on floating leaf disk, is today still in use. Both of these two methods are laborious and are of limited use to forecast oospores germination. The molecular biology of sporulation has been characterized in other oomycetes such as *Phytophthora infestans* and *Saprolegna parasitica* (Cvitanich *et al*. 2003, Su Kim *et al*. 2003, Andersson *at al*. 2002).These research works focuses on genes expression of members of Puf family of developmental regulators that bind and inhibit translation of specific mRNAs. In particular in *Phytophthora* was studied the M90 gene transcript that showed high levels in sporangia/conidia and zoospores but, once these spores were allowed to germinate and started to produce hyphae, the M90 signal fell >50-fold. The function of regulation of mitosis may be conserved between species, moreover particularly this family of RNA-binding proteins may play a universal role in oomycete sporulation. Therefore, the final objective of this work is to identify M90 like transcripts in *P. viticola* spores that can be used as oospores germination markers, so to obtain a faster and potentially predictive analysis.

Material and methods

Oospore of *P. viticola* can be formed in the vineyard at any time during the season but in autumn their presence increase. In order to collect a higher number of oospores, infected leaves with oil spots, sporulation and oospores, were collected in August from an untreated vineyard, air dried and blended by adding distilled water to obtain a homogeneous mass. The mixture was transferred in holed boxes and buried in soil to overwinter.

From March on, mixed sample of the buried mass were picked up and were frozen with liquid nitrogen.

Three different RNA extraction methods were compared: one published method (Moser *et al*. 2003) and two commercial kits (GeneElute Direct mRNA min prep Kit® Sigma Aldrich and Qiagen RNeasy Plant Mini Kit). Total RNA was isolated from oospores, previously extracted from the mass (Van Der Gaag *et al*., 1996) or directly from the mass. Two elution of RNA with 50 µl of RNasi-free water were made. The quality and quantity of RNA was evaluated by electrophoresis in TAE-agarose gel (1 %) stained with ethidium bromide, and afterwards by PCR analysis.

cDNA was synthesized by a reverse transcription polymerase chain reaction using the ThermoScript™ RT-PCR System (Invitrogen) with 6 µl of RNA using 2.5 µM oligo (dT)20. The cDNA controlled by electrophoresis (TAE-agarose gel 1.2%), purified with a Qiaquick PCR purification kit and eluted in water.

Specific primers (DOG1 and DOG2 forward and reverse) were designed using BioOligo™ software on conserved region after alignment of two sequences (NCBI accession number: AJ245441.1 and AF507056.1) related respectively to Puf1 and M90 genes of *S. parasitica* and *P. infestans*. Sequences and annealing temperature of the primers were as follows: DOG1, AACTACGTSATCCARAAGTTC and TTCTGCACCACGTAGTTG, 56°C; DOG2, GACCAGAACGGCAACCACGTG and CGCTGGATSACGCGGCAG, 60°C. The PCR specific reaction was performed a 20 µl mixture contained 10 µl of cDNA, , 1.5 mM $MgCl_2$, 0.20 mM concentration of each deoxynucleoside triphosphate, 0.2 µM of each primers and 1 U of Taq polymerase (Promega). Amplification were performed with the standard short cycling parameters (40 cycles of 94°C for 30 s, 50°C for 90 s and 72°C for 90 s). The PCR products were separate on TAE-agarose gel (1.5%) stained with ethidium bromide.

Results

We extracted total RNA with all three methods but the best quantity and quality of total RNA was obtained using Qiagen RNeasy Plant Mini Kit (data not shown). The total quantity of RNA per sample was quantified using GeneRuler™ 100 bp DNA Ladder Plus (Fermentas) and GelDoc software (Biorad).

Tab. 1. RNA extracted from two samples of the oospore overwintering mass with Qiagen RNeasy Plant Mini Kit

	Quantification of isolated RNA	
Lane	Sample/elution	µgRNA/100gLM*
A	Sample 1 elution 1	0.310
B	Sample 1 elution 2	0.256
C	Sample 2 elution 1	0.430
D	Sample 2 elution 2	0.413

*LM: leaf mass buried in soil

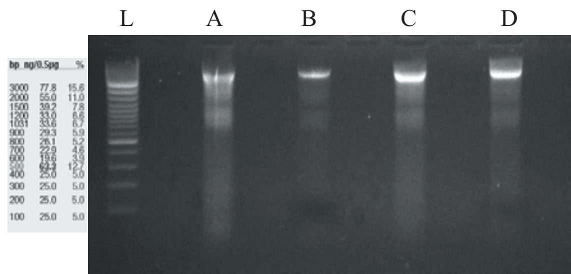

Fig. 1. Gel electrophoresis of total RNA samples, extract from leaves mass containing *P. viticola* oospores with Qiagen RNeasy Plant Mini Kit. RNA run on 1 % agarose gel stained with ethidium bromide. L: ladder, 1,3: samples 1,2 first elution, B,D: samples 1,2 second elution.

The RNA was successfully transcribed in cDNA, that is more stable, and a good amplification profile was observed with the two DOG primer pairs in particular with the RNA extracted with Qiagen kit (Figure 2).

Fig. 2. Gel electrophoresis of specific PCR primer products. Run on 1,5 % agarose gel stained with ethidium bromide. L: DNA ladder, A: products obtained from DOG1 primer amplification, B: products obtained from DOG2 primer amplification

Currently we are confirming the bands identity by cloning the two PCR products in the pGEMT easy vector and sequencing and, at the same time, directly by amplicons sequencing.

Discussion

These preliminary results show that, the same methodology applied to the other oomycetes could be a useful tool to detect germination of *P. viticola* oospores. Future works will focuses on the verification of the genetic identity of our transcript and on increasing the number of experimental data to support the goodness of this method. Moreover the diagnosis will be applied in vineyard to have a faster forecast method to asses the peremptory development of *P. viticola* primary infection.

The final step of this work will be to apply this method to oospores at different levels of maturation to confirm that this transcript can be used as germination markers. Oospore will be collected and divided in 10 samples. Five samples will be positioned in condition of silencing and five in optimal condition of temperature and humidity for germination. The extraction of RNA, RT-PCR and specific PCR will be performed every day in order to find a difference in the expression of the gene between dormant and germinating oospores.

Acknowledgement
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Genetic structure and epidemiology of *Plasmopara viticola* populations

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Genetic analysis of single lesions caused by *Plasmopara viticola* has yielded in the past few years, new contradictory results: various works have shown that the genetic diversity is high in most vineyards. The interpretation of the pattern resulting from the SSR analysis postulates that two lesions, which show a diverse SSR allele pattern originate from different oosporic infections and lesion presenting the same pattern should be considered to derive from the same oosporic infection by mean of secondary asexual zoospore infections. Under particular conditions or/and at the beginning of the season the downy mildew epidemics develop slowly and the lesions are few. In these conditions all lesions can be sampled and analyzed and therefore the true proportion of primary lesions contributing to the epidemic can be inferred (number of genotypes identified /number of lesions present). Whereas once a few hundred to thousands of lesions are present only a partial sampling is possible and the sampling strategy may determine the outcome. In this work we try to construct a picture of the downy mildew epidemics considering the possible biases using the available mostly published data.

Results

The most frequent pattern of any powdery mildew epidemic was a random distribution of genetically different genotypes with some localized clustered lesions derived from a single genotype. Overall contribution of primary infection to the total lesion present during the period in which the epidemics were followed, ranges from a few % to the exceptional high value of 83%. However the average European range seams to lay around 30-40% (Tabs. 1 and 2).

Often, after a first total sampling, the authors had to switch to a sampling pattern which considered only a fraction of the present lesions. In most cases the logic sample scheme was to collect every, or every few plants, a lesion, covering so the whole vineyard. Often the new lesions, which appeared between two sampling dates, were mostly new genotypes, sometime however 1-2 genotypes contributed to a large extent to the new lesions sampled (dominant genotypes). This confirms, in some way, the pattern detected by sampling all lesions. In the cases reported where all lesions could be sampled over a longer period, which corresponds to a rather slow epidemic increase, new, genetic different lesions, were randomly distributed over the sampled area, whereas lesions caused by dominant genotypes were clustered.

As the number of lesions increased, few or even a single genotype expanded from its original limited area contributing to a highly variable degree to the lesions sampled, in some less frequent cases contributing up to half of the sampled lesions (Hug et al., 2006). Over all the slowly increasing epidemic described by Hug et al. in Western Australia, where all present lesions could be sampled during a long period, only 5% of all present lesions can be attributed to primary infection, 95% must derive from less than half of those primary infections.

Tab. 1. Genotypic variability of *Plasmopara viticola* present during the initial 1.5 to two month from the discovery of the first lesions in the plot. Number of lesions exceeded the number analyzed and a partial sampling was done, picking from 1 to a maximum of 3 lesions per plant even if a high number was present on a single plant. Data from Gobbin et al. 2005, Rumbou and Gessler 2004, 2006, Hug et al. 2005. Les. anal: lesions analysed, N. gen: number genotypes identified, les/gen: avg number of lesions per genotypes, Prop pri les: proportion of primary lesions.

Plot location	Les. anal	N. gen	les/gen	Prop pri les
Biasca (CH)	314	190	1.7	0.61
Blanquefort (F)	557	363	1.5	0.65
Cugnasco 1 (CH)	178	69	2.6	0.39
Cugnasco 2 (CH)	484	193	2.5	0.40
Gaillac (F)	47	22	2.1	0.47
Navicello (I)	163	142	1.1	0.87
Perroy (CH)	325	108	3.0	0.33
Stäfa (CH)	328	129	2.5	0.39
Wädenswil (CH)	127	43	3.0	0.34
Bommes (F)	193	41	4.7	0.21
Zakynthos (GR) 2001	152	23	6.6	0.15
Zakynthos (GR) 2002	214	114	1.9	0.53
Lefkada (GR) 2001	430	129	3.3	0.30
Lefkada (GR) 2002	327	75	4.4	0.23

Mining the data published (Gobbin *et al*., 2003, 2005; Gobbin 2005, Rumbou and Gessler, 2004, 2006), it is relevant at which scale the sampling is done: on a plot scale including several dozen plants or even more than hundred or at level of a single plant or at a level of a single leaf. The few cases where data at plant level are available or on leaf level, show a reduced variability at leaf level compared to plant level (Figs. 1 and 2).

Comparing variability on the plant level to that of the surrounding plot we detect a similar pattern, the average number of lesions formed by a single genotype is higher on the small scale (Tab. 3).

Early in the epidemic, sampling all lesions, or later on, sampling a single lesion per plant, or even a lesion each 2-3 plants, the number of different genotypes is

high and the average number of lesions a single genotype produces is low (Tabs. 1, 2 and 3).

Partial sampling yields higher diversity, which is plausible if we accept that a single genotype spreads overwhelmingly, first on the leaf, shoot and plant and only slowly to the neighboring plants. However total analysis was only possible in conditions where relatively few lesions were present: early season or under conditions of slow epidemics. In all cases the number of genotypes contributing to the formation of secondary lesions was low, most genotypes were found once, and then disappeared.

Tab. 2. Genotypic variability of *Plasmopara viticola* present during the initial phase of the epidemics. Sampling covered a period ranging from a half to two months from the discovery of the first lesions in the plot. Epidemics were developing slow enough to allow a total sampling at several dates, e.g all lesions were sampled. Data from Gobbin et al. 2005, Rumbou and Gessler 2004, 2006. Lesion anal: lesions analysed, N. gen: number genotypes identified, les/gen: avg number of lesions per genotypes, Prop pri les: proportion of primary lesions.

Plot location	Lesion anal.	N. gen	les/gen	Prop pri les
Carpineta (I)	124	84	1.5	0.68
Erbach (D)	287	192	1.5	0.67
Geisenheim (D)	206	33	6.2	0.16
Lorch (D)	361	228	1.6	0.63
Navicello (I)	447	370	1.2	0.83
Tesero (I)	97	15	6.5	0.15
Vic (CH)	336	114	2.9	0.34
Wädenswil (CH)	111	6	18.5	0.05
Aghialos (GR) 2001	302	23	13.1	0.08
Aghialos (GR) 2002	330	54	6.1	0.16
Kephalonia (GR) 2001	127	18	7.1	0.14
Kephalonia (GR) 2002	72	18	4.0	0.25
Caversham	636	32	19.8	0.05

Tab. 3. Genotypic variability at plot level and single plant level. The single plant was always part of the above reported plot in which at most two lesions per plant were taken. On the single plant however all lesions were analyzed. Les: lesion, gen: genotypes identified

Plot location / Sampling date	N vines	N les	N gen	Avg les/gen
Caversham (AUS)	36	63	13	4.85
19.01.05	1	73	9	8.11
Cugansco (CH)	244	271	122	2.22
23.07.00	1	58	25	2.32
Stäfa (CH)	73	105	71	1.48
26.07.00	1	49	13	3.77
Geneva (USA)	108	263	129	2.04
28.07.03	1	50	18	2.78

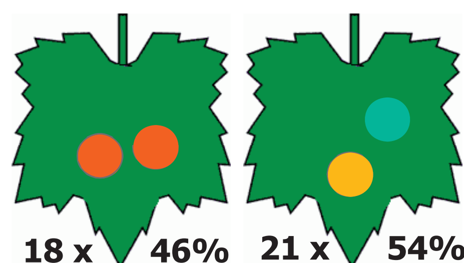

Fig. 1. In the data, we found 39 cases where two analysed lesions were present on a single leaf. In 18 cases a single genotype was present, in 21 cases two.

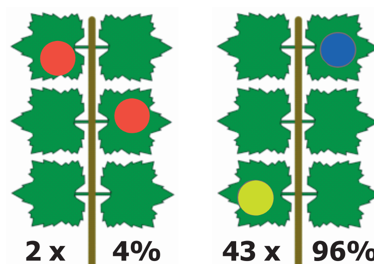

Fig. 2. On the plant scale in 45 cases where two lesions were present and analyzed, two cases a single genotype was present, in 43 two genotypes.

Conclusions

If this type of analysis could be extended to cover uncontrolled epidemics until fall or at least into the phase in which the number of lesions is sufficient to cause true damage (we assume that 50-70 lesions on a vigorous plant is still insignificant) we probably would find little genetic variability on the leaf level, some what more on the shoot and plant level. Average number of lesion per single genotype would drastically increase and most probably only a few genotypes would contribute. Under those conditions secondary infections are the cause of damage. However where the risk of such secondary

infections is high, epidemics are avoided by the use of “fungicides”.

The primary infections which occur randomly distributed and during a prolonged period are fundamental: between them the genotypes which will be able to spread and cause multiple secondary lesions are present; and if an unprotected time window overlapping with primary infection conditions opens at any moment of the growing season new infections occur. Generalized vineyard wide damaging epidemics are most probably the results of a large number of randomly distributed primary infections followed by unchecked secondary multiplication. From these considerations we may pose the questions - What effect would have a drastic reduction of overwintering oospores? Can we sanitize a vineyard by eliminating in fall leaf litter? Should we continue to control the disease also after harvest?

Acknowledgements
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Session 5:

Biological control and agronomical practices

Potential use and major constrains in grapevine powdery and downy mildew biocontrol

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The use of biocontrol agents (BCAs) against grapevine powdery mildew is more prevalent than against downy mildew, even though the European restrictions on the use of copper have fostered research to find new non chemical alternatives to it. Why has biological control become more integrated into management strategies against grapevine powdery mildew and have more experimental BCAs and commercial products been developed against it, compared to downy mildew?

Erysiphe necator (formerly *Uncinula necator*) presents unique biological and epidemiological characteristics that may make conditions more favourable for diseases biocontrol. The oldest commercial fungicide based on a microbial BCA is AQ10, containing the fungus *Ampelomyces quisqualis,* which is also the first organism reported to be a hyperparasite of powdery mildews. This early discovery can be related to the fact that it can be easily found associated with powdery mildew colonies and the successful commercial utilization is possibly due to its mode of action: *A. quisqualis* was shown to colonize hyphae, conidiophores and to parasitize cleistothecia of powdery mildews. Therefore it can easily survive if the host fungus is present on the crop. However the fact that products against powdery mildews are much more abundant than against downy mildew cannot be related only to the lucky presence of an easily growing hyperparasite.

The two diseases present differences that could make biocontrol easier on powdery than downy mildew. *E. necator* grows outside host tissues, produces many conidia that can survive for some days and can produce infections in a wide range of environmental conditions. *Plasmopara viticola* sporulates under suitable conditions, zoospores swim in water in order to reach the stomata and penetrate. After penetration the fungus grows inside the tissues and only after the incubation period, new sporangia exit from stomata to start others secondary cycles if favourable conditions. *P. viticola* infections can occur in a more narrow range of weather conditions (temperature, presence of water) compared to *E. necator* and plants tissues are susceptible only if stomata are differentiated, but once it reached the inside tissues, it cannot be controlled without a curative active ingredient.

Theoretically when a single *P. viticola* zoospore penetrates the stoma a new infection occurs. A single infection on a bunch can destroy it.

E. necator is an ectoparasite, which has all elements on the leaf surface except the haustoria. The process between conidia germination and haustoria formation during which the fungus is most susceptible to any adverse conditions, takes several hours, even in optimal conditions, allowing more time for control action. Moreover, contrary to the downy mildew which with a single zoospore can cause loss of a complete bunch, a single powdery mildew conidia will rarely lead to the loss of a bunch, most it will lead to the loss of few berries.

P. viticola oil spots evolve in host tissue necrosis more quickly than powdery mildew, which, conversely, slowly reduces tissues functionality.

Since rain and, even more, leaf wetness forecast, are not reliable, downy mildew disease control is based on continuously protecting leaves and bunches against pathogen penetration, with long lasting toxic compounds. Fungicides against downy mildew should have a long residual effect; they must be applied as preventive treatments if they are not absorbed by grape tissues. Once the infection has occurred, a curative product is needed to reduce damages. BCAs are usually short lasting, they do not penetrate the plant tissue and therefore they do not produce curative effect and, since they are living organisms, are greatly influenced by environment, antagonists and nutrient substrate availability.

As powdery mildew produces continuously conidia and infection conditions are almost daily given (high humidity), the timing of the fungicide application is less relevant, more important is that it can kill mycelium and conidia. Products that can constantly reduce the fungus on leaf surface or parasitize the reproductive structure (i.e. *A quisqualis*) can control the disease.

Most of the potential BCAs that can be found in the environment act by antibiosis or they compete for space and nutrients. Few of them can induce resistance or grow as hyperparasites. Competition for space or nutrients is not relevant to inhibit downy mildew as zoospore do not need nutrition nor occupy space as long as the oomycete has not penetrated. To be effective against downy mildew, a BCA must produce very active toxins against the pathogen, which has to be present in sufficient concentration on the host tissue during rain, and therefore has to be stable and not washed by rain, both requests contrasting with the nature of a BCA and its eventual toxin. Resistance inducers can decrease the plant susceptibility to infections, but since this mechanism usually is not absolute, they are not able to totally prevent infection. The exponential development of the disease can make biological control ineffective. Few periods of good infection conditions either for oospore (intensive rains) or zoospore infections with partial or no control can lead to high losses.

Conversely *E. necator* can be successfully hyperparasitizes, a toxic metabolite can inactivate the conidiophores or the conidia thus reducing the inoculum. If few infections start, they can be killed with a second repeated treatment. Space competitors can theoretically reduce the growth of the mycelium. The epidemic of *E. necator* has a more steady development than *P. viticola.*

There are, at least theoretically, several different mechanisms of actions that can be employed to fight

against *E. necator* compared to *P. viticola,* making the number of its potential antagonists larger than against downy mildew. These differences are reflected by the results obtained in the development of new commercial BCAs: AQ 10, Serenade and Sporodex for powdery mildews, none against downy mildews.

Most studies on plant pathogens biocontrol focus on a multitude of factors related to the behaviour of the microbial antagonists and the suitability of the pathogen to be control at satisfying economical threshold, has received little attention. A substantial progress could be made in the future if the biological characteristics of the pathogen and its epidemiology are taken in account.

Acknowledgements

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Efficacy evaluation of new control agents against grapevine powdery mildew under greenhouse conditions

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Fungicide resistance problems, concerns regarding pesticide residues and revocation of some widely used fungicides have increased research efforts for developing biocontrol agents (BCAs) of foliar fungal pathogens. In addition, the lack of good resistant cultivars has increased the interest in alternative methods to control powdery mildews (Kiss et al., 2004).

The efficacy of alternatives to chemical fungicides such as oils, salts, soluble silicon and plant extracts in powdery mildew control, have widely been studied, especially in greenhouse production (Belanger & Benyagoub, 1997; Pasini et al., 1997; McGrath & Shishkoff, 1999). Many potential BCAs have also been tested, but only few of them showed a good disease control under field conditions.

The aim of the present experiment was to evaluate the effectiveness of microorganisms and natural substances against grapevine powdery mildew under controlled greenhouse conditions, as a first step in the process of selecting new alternatives to chemical fungicides.

Material and methods

Several fungi, bacteria and yeasts isolated from natural environment and a plant extract (Timorex, Biomor, Israel) were tested in two greenhouse trials in order to control powdery mildew on grapevine. Sulphur, water and the commercial preparation AQ10 of the mycoparasitic fungus *Ampelomyces quisqualis* (Intrachem Bioitalia) were used as references. Five replicates, each consisting of one plant of the susceptible cultivar Pinot Gris having one shoot with 5-6 green and fully expanded leaves (at least 2.5 cm wide) were used. Plants were grown under controlled conditions (25°C and 60% R.H.) in a pathogen free greenhouse.

In the first trial (Tab. 1) the bacteria and yeasts strains that were isolated in Israel (Y89, Y16, Y13, S2, B71, B69, B6) and in Italy (F77, USB1, USB2) were tested. They were grown in nutrient broth at 25°C, for 48 hours, at 300 rpm. In the second trial (Tab 2) fifty unknown microrganisms (20 bacteria, 20 yeasts and 10 fungi) isolated from natural environment in Puglia (southern Italy) and in Trentino (northern Italy) were tested. All the microrganisms were grown in Petri dishes on Potato dextrose agar (Sigma) at 25°C for 48 hours and suspended and sprayed in distillate water plus an adjuvant (Tween 80). All the substances were applied 6 hours before powdery mildew inoculation whilst AQ10 was sprayed also seven days later. The spray suspensions contained $1x10^7$ conidia/ml and $1x10^8$ CFU/ml of bacteria and yeasts. For each treatment, 100 ml of solution with the microorganisms were used.

The treatments were sprayed by an air compressor system equipped with a spray gun working with a pressure of 2 bars.

Tab. 1. Tested products and references (*) used in the first greenhouse trial (2005)

Commercial name	Active ingredient	Dosage
Y89	Yeast	$1x10^8$CFU/ml
Y16	Yeast	$1x10^8$CFU/ml
Y13	Yeast	$1x10^8$CFU/ml
Y2	Yeast	$1x10^8$CFU/ml
B71	Bacterium	$1x10^8$CFU/ml
B69	Bacterium	$1x10^8$CFU/ml
B19	Bacterium	$1x10^8$CFU/ml
B6	Bacterium	$1x10^8$CFU/ml
F77	Bacterium	$1x10^8$CFU/ml
USB1	Bacterium	$1x10^8$CFU/ml
USB20	Bacterium	$1x10^8$CFU/ml
AQ10 +	*A.quisqualis*	0.12 g/l
Vaporgard *	Pinolene	1 g/l
Medium*	Nutrient broth	23g/l
Thiovit*	Sulphur	3g/l
Timorex	Tee tree extract	10 g/l

Tab. 2. Biological microrganisms and references (*) used in the second greenhouse trial (2006)

Commercial name	Active ingredient	Dosage
-	Fungi	$1x10^7$conidia/ml
-	Bacteria	$1x10^8$CFU/ml
-	Yeasts	$1x10^8$CFU/ml
Thiovit*	Sulphur	3g/l
Distillate water + Tween 80*	Distillate water + adjuvant	Drip off 0.1%

Infected leaves with fresh symptoms of powdery mildew (mycelium, conidia and cleistothecia) were gently rubbed on the healthy plants. Seven and fourteen days after inoculation, the percentage of infected leaf area (severity) and the percentage of infected leaves (incidence) were assessed on all leaves of each replicate. Replicates were arranged in a fully randomized block. For statistical analyses the treatments sprayed in the first trial were divided into three groups (microbials, natural products and EAW). Analysis of variance (ANOVA) was applied on "Arcsin" transformed data, using the software Statistica 7

(Statsoft, Italy). Significant differences among treatments were determined by Duncan's test.

Results and discussion

Here only results of the first trial are presented. Two yeasts (Y2 and Y16) and two bacteria (B19 and F77) were not effective compared to sulphur, whilst all other agents showed a partial reduction of the disease (Fig. 1).

Tea tree extract (Timorex) sprayed once 6 hours before powdery mildew inoculation seems to be not effective ingredient for powdery mildew control compared to sulphur reference (severity was respectively 12% and 2%).

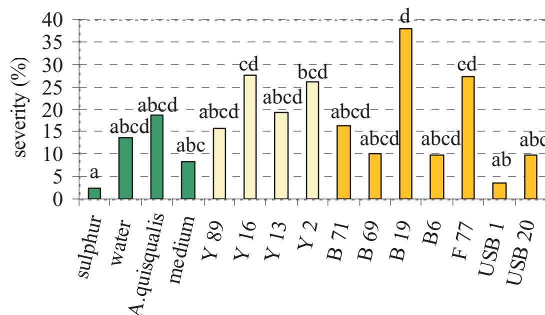

Fig. 1. Efficacy in reducing disease severity of bacteria and yeasts sprayed 6 hours before inoculation of grape leaves with *Erysiphe necator*. Columns with same letters do not significantly differ at $P \leq 0.05$ (Duncan's test).

Conclusion

In conclusion, some of the microbial control agents gave promising results in reducing the disease under controlled conditions. The organisms must be preliminary formulated to reduce their variability in disease control and increase their survival and efficacy before field applications. Tee tree extract tested here was not able to reduce infections but new plant extracts will be further studied in greenhouse experiments to evaluate their potential as alternatives to sulphur against powdery mildew in organic viticulture.

Acknowledgements

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Authors thank N.S. Iacobellis for kindly supplying the microrganisms USB 1 and USB 20.

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Timorex - a novel tea tree-based organic formulation developed for the control of grape powdery and downy mildews

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Powdery mildew (PM) caused by *Uncinula necator* and downy mildew (DM) caused by *Plasmopara viticola* are widely distributed destructive diseases of field-grown grapevines. Cluster and blossom infection with each fungus before or shortly after bloom may result in poor fruit set and considerable crop loss and a decrease in wine quality.

Disease control in conventional management is generally achieved by the use of fungicides. However, fungicide-resistant strains of the pathogens have developed on grapevines. Once resistant strains appear, most of them survive for several years, so that the risk of re-enforcing the resistant population through further applications of partially ineffective fungicides is very high.

Diseases control in organic growth is difficult and limited to spraying or dusting with preparations from mineral origin like sulfur (for PM), or copper (for DM), or preparations which are based on detergents, oils, or plant extractions (only in limited manners). Sulfur sprays must be limited, since they can scorch foliage (mainly flowers and young fruits) in warm and sunny conditions and harms natural enemies and other beneficial insects and copper application should be reduced due to its negative residual effects in the soil and on wine fermentation. This intensifies the need for new compounds with different modes of actions for disease control, and for their knowledgeable use in fungicide resistance avoidance strategies.

Tea tree oil is an essential oil steam distilled from the Australian plant *Melaleuca alternifolia*. This natural oil is an effective antiseptic, fungicide and bactericide, and has many safe and effective uses in the health and cosmetics industry. Its use against plant pathogens has not been investigated.
Recently,
Biomor Israel Ltd., has developed a new formulation Timorex containing 66% tea tree oil effective against broad spectrum of plant diseases in vegetables, herbs, grapevines and fruit trees, with no phytotoxicity to plant foliage.

Objectives: to evaluate the activity of the new tea tree-based formulation Timorex against *P. viticola* and *U. necator* in grapevines, and its efficacy in controlling downy and powdery mildews in field-grown grapevines.

Material and Methods

Timorex is a new organic bio-pesticide. The 66 EC formulation of Timorex (containing 66% tea tree oil) was used in all experiments. The concentrations of the laboratory experiments are presented as active ingredients (a.i.).

Effect of Timorex on germination of conidia of U. necator

Timorex was mixed with sterile distilled water to give a stock solution of a known concentration. Timorex was mixed with pre-autoclaved 1% water agar to give final concentrations of 0, 0.001, 0.01, 0.1 and 1.0%. Conidia were shaken onto glass slides previously coated with water agar containing Timorex. Slides were placed in Petri dishes containing wet filter paper and kept in the dark at 20°C for 16 h. The number of germinated conidia was counted under a microscope.

Growth chambers experiments

Grapevines of *Vitis vinifera* L. cv. ‘Emerald Riesling’ were used for studying the effect of Timorex on powdery and downy mildew under growth chamber conditions. Plants were grown from cuttings (one plant per 0.1-L pot) and grown in the growth room (22°C with a 12h photoperiod). Plants with at least four leaves fully developed were used.

Isolates of *U. necator* and *P. viticola,* obtained from infected plants in vineyards in the Golan region, Israel, were maintained on reinfected vines in separate growth chambers (23°C, 100-120 μE.m^{-2}.s^{-1}, 16h photoperiod).

Effect of prophylactic treatment on downy and powdery mildew development on potted plants

Grape plants were sprayed with Timorex at various concentrations (0, 0.25, 0.5 and 1%) on both surfaces, and 24 hours later were inoculated on the lower surface with sporangial suspension of *P. viticola*. The lower surface of each of six to eight attached leaves on each of six plants of each treatment was uniformly sprayed with 2 mL of a sporangial suspension of $4x10^4$ sporangia per mL, delivered from a glass chromatography sprayer. After inoculation, plants were covered with plastic bags, lightly sprayed on the inside with water, and were incubated at 19°C for 20 h in darkness. The plants were then uncovered and kept in a growth chamber (23°C, 100-120 μE.m^{-2}.s^{-1}, 16h photoperiod). For inoculation of U. necator, conidia were shaken onto the upper surfaces of the leaves and incubated in a separate growth chamber. Seven to nine days after inoculation, the percent leaf area infected with each fungus was visually estimated and recorded.

Field experiments

In field trials conducted in 2003-4 the efficacy of Timorex in controlling PM was evaluated in comparison to Helio-sulfur and the sterol inhibitor tebuconazole as

standards and to control non-treated vines. Various numbers of treatments for each trial were arranged in a randomized complete block design and replicated four or five times. Timorex was sprayed at 7 or 14-day intervals at various concentrations, and disease development on 40 clusters of each replicate was rated during the season.
The efficacy of Timorex in controlling PM was evaluated in comparison to Kocide 0.25% (53.8% copper hydroxide) as a standard and to control non-treated vines. Treatments for this trial were arranged in a randomized complete block design and replicated four times. Timorex was sprayed five times at 7-day intervals at 1% and disease development, as percent infected leaves, on each replicate was rated.

Sprays were applied to run-off (2500 L/ha) with a 100-L gun-sprayer (1400 KPa). Analysis of variance (ANOVA) using the SAS GLM (SAS Institute, Inc., Cary, NC) procedure was applied to the transformed data. Least Significant Difference Test was used to determine significant differences between treatments.

Results

In vitro tests showed that Timorex at concentrations of 0.001 and 0.01% provided 50 and 65% inhibition, respectively, in conidial germination of *Uncinula necato*r, relative to the control. A concentration of 0.1% completely inhibited germination.

Growth chambers tests on potted plants revealed that Timorex at a concentration of 0.05-0.1% effectively controlled powdery mildew when applied as a prophylactic treatment.

Timorex at a concentration of 0.75-1% suppressed the fungus, as indicated by reduction of visible colonies when sprayed on mildewed leaves bearing sporulating colonies of *U. necator*.

There was a reduction in the percentage of infected leaf area with downy mildew at concentration of 0.25% of Timorex, and total inhibition of disease development of plants treated with Timorex at 0.5%.

Field trials

Field trials conducted in 2003 revealed that Timorex at a concentration of 1% controlled powdery mildew and was as effective as sulfur or tebuconazole when applied at 14-d intervals.

In 2004 when disease pressure was higher, Timorex was as effective as both standards on cv. 'Cabernet Sauvignon' and less effective than tebuconazole when applied to cv. 'Carignane'.

Timorex at a concentration of 1% controlled downy mildew and was as effective as Kocide, the copper treatment. The percent infected leaves on control non-treated vines, Timorex and Kocide treated vines was 62.5, 16.3 and 21.3%, respectively.

No phytotoxicity to the foliage was observed as a result of foliar applications of Timorex compound.

Conclusions

1. Timorex inhibited spore germination and had a prophylactic activity against powdery and downy mildews on young plants.
2. Foliar sprays of Timorex effectively inhibited powdery and downy mildews development on field-grown grapevines.
3. Timorex is safe to natural enemies and other beneficial insects and bees and can be used as a replacement of sulfur or copper in both organic and conventional growth.
4. Timorex acts as a protectant against wide range of fungi by inhibiting spore germination, mycelial growth and by suppression of the fungus on mildewed tissue.

The data in the present paper on control of grape powdery and downy mildews, as observed on potted and field-grown grapevines, makes Timorex an attractive compound for practical agronomic use against both diseases in vineyards.

Session 6:

Poster presentation

Genetic of the pathogens: population genetics, virulence-avirulence, variability and fungicide resistance

From molecular evolution to population genetics of strobilurin resistance in grapevine downy mildew populations

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Grape downy mildew is one of the most damaging fungal diseases of grapevine (*Vitis* spp) worldwide. It is caused by *Plasmopara viticola* (Berk. & Curt. ex. De Bary), a biotrophic Oomycetes (Stramenopiles) native of North America. In the late 1870s, *P. viticola* was accidentally introduced to Europe, probably when American vine stocks that were resistant to grape phylloxera were used to graft the European varieties. The solution to control this devastating pathogen was found by Alexis Millardet, who discovered in 1882 the prophylactic effect of lime and copper and developed the "Bordeaux mixture" which became the first successful fungicide to be used in vineyards. Today, grape downy mildew control in vineyards is ensured by effective fungicide spray program. However, grape downy mildew control by chemicals has led to fungicide resistance development in *P. viticola* populations. Managing fungicide resistance, *i.e.* controlling the occurrence and the spread of resistant strains in vineyards, requires a good knowledge of population genetics of the pathogen (reproductive mode, gene flow, effective population size). In order to provide a new basis for a sustainable management of this pathogen, we developed a research program to understand how resistance alleles appear, spread and are maintained in downy mildew population of grapes. We focused our study on strobilurines, one of the most widely employed fungicides against downy mildews because of their effective mode of action at a very low dose and the targeted-gene is well known. Moreover, the widespread use of these fungicides has resulted in the rapid adaptation of grape downy mildew populations.

For the first time, we have isolated the strobilurin fungicide-targeted gene (cytochrome *b*) and its neighbourhood regions in the mitochondrial genome of *P. viticola*. By combining phenotypic and genetic data, we found the existence of only one mutation at amino acid sequence position 143 of cytochrome *b* conferring resistance to strobilurins.

The variability of this mitochondrial marker was assessed by sequencing and/or genotyping more than one thousand samples collected in Europe. We found only four mitochondrial haplotypes in downy mildew population across European vineyards. Phylogenetic analyses demonstrated that alleles conferring resistance to strobilurins appeared independently several times (at least twice) in the field, i.e. they have evolved repeatedly in different places before spreading by gene flow across vineyards.

Finally, the prevalence of resistant alleles was assessed by characterising 600 strains collected in 20 different vineyards of Champagne (France). Resistant allele frequency ranged from low (0.01) to very high level (0.77) with an average value of 0.33. The level of population structure estimated with this selected marker was high and significant at the regional scale (F_{ST}=0.107) which indicates that selection resulting from chemical treatments is highly heterogeneous between vineyards.

The Role of Oosporic Infection in the Epidemiology of Downy Mildew in South Africa

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The asexual phase (sporangia) of *Plasmopara viticola* has long been viewed as the life cycle stage that is most important in causing expansion of epidemics. Contrarily, the role of the sexual phase (oospores) has been primarily viewed as only providing the initial primary inoculum of the epidemic (Lafon and Clerjeau 1988). However, population genetic studies in European *P. viticola* populations have challenged these long standing epidemiological views. These studies have shown that oosporic infections contribute to the epidemic throughout the season, with most genotypes having a limited ability to spread asexually. It has further been shown that only one or two genotypes are able to spread on a plot wide scale at a relative slow rate, and that long-distance migration of asexual spores are limited (Gobbin et al. 2003a, 2005, Rumbou and Gessler. 2004).

In South Africa the severity of downy mildew epidemics varies between years, due to substantial variations in climatic conditions that prevail among years. In most years, epidemics can be controlled readily due to low precipitation levels and high temperatures. However, in years with high rainfall and lower temperatures explosive epidemics occur that are difficult to control. The contribution of oosporic infections to the development of epidemics in South Africa is currently unknown. It is important to investigate this aspect since it holds important implications for managing strategies such as the use of specific forecasting models, as well as the importance of controlling soilborne inoculum. Aside from unique climatic conditions in South Africa, a different genetic makeup of South African populations compared to European populations, can all contribute to epidemiological differences in South Africa.

The aim of this study was to determine the importance of oosporic and asexual reproduction to the development of *P. viticola* populations in South African. *Plasmopara viticola* populations were investigated in one vineyard in two consecutive growing seasons. Genotyping of more populations in a second chemically sprayed vineyard over the same growing seasons is also underway.

Material and Methods

Plasmopara viticola populations were studied in an organically grown table grape vineyard (cultivar Red Globe), where only copper and compost teas are sprayed. The experimental block consisted of 360 vines spaced 2m within rows and 3m between rows. Downy mildew lesions were collected at either three or four sampling times in the 2004/5 or 2005/6 growing seasons respectively. In both growing seasons either the total number of lesions present within the block were sampled, or only a subset of lesions were sampled at each specific sampling date. During the first two sampling dates in both years all lesions were collected, except in the first sampling of the 2004/5 growing season where only 16 of the 30 lesions were genotyped due to a failure in DNA extraction from lesions. In the third and fourth sampling only a subset of lesions that were representative of the complete block were genotyped. A small section of each lesion was sampled, allowing genotypes to continue contributing to the epidemic.

Plasmopara viticola lesions were genotyped using four published multiallelic microsatellite markers (GOB, ISA, BER and CES) (Gobbin et al. 2003b). Populations were analysed for genotypic diversity (Shannon diversity index), gene diversity and Hardy Weinberg Equilibrium using the POPGENE software (Yeh et al. 1999).

Results and Discussion

The contribution of sexual (oospore derived) reproduction to the epidemic was evident in all of the sampled populations. At each sampling time in both growing seasons the Shannon diversity index was moderate to high and between 58 to 87% of the population of each sampling date consisted of new genotypes that were not detected in the previous samplings, even at the last sampling date at the end of the growing season (Table 1). All this suggest that a significant number of genotypes were produced by sexual reproduction throughout the growing season.

The majority of genotypes were not able to reproduce asexually, with only one to five genotypes at each of the sampling dates being able to produce more than five lesions (Table 1). However, the relative small sample sizes might have influenced these results as well as the detection of new genotypes. Therefore, more lesions that were collected in the third and fourth sampling times are currently in the process of being genotyped. In both growing seasons there were only two genotypes (predominant genotypes) that were able to produce more than ten lesions throughout the growing season (Table 1).

Tab. 1. Occurrence of *Plasmopara viticola* genotypes and genotypic diversity of populations collected at different sampling types in two consecutive growing seasons in a table grape vineyard

Growth season	Sampling date	No lesions analyzed	No lesions predominant genotypes[1]	No geno.	% new geno.	Geno. > 5 lesions	E_H^2
2004/5	1st 04/10/04	16	0 (1)	5	100	1	0.28
	2nd 08/12/04	77	14 (15)	32	88	2	0.29
	3rd 08/02/05	113	16 (13)	53	58	5	0.25
2005/6	1st 06/10/05	11	2 (0)	8	100	0	0.36
	2nd 14/11/05	74	34 (5)	25	84	2	0.27
	3rd 28/11/05	58	9 (9)	31	58	3	0.27
	4th 30/01/06	59	0 (10)	29	69	3	0.28

[1]Number of lesions containing the most dominant genotype (geno.), followed by the number of lesions caused by the second most dominant genotype in brackets
[2]Shannon's equitability calculated by H/H_{max}, where $H_{max} = \ln N$ (number of individuals in the sample)

Clone corrected populations sampled at different sampling times were all analyzed for Hardy-Weinberg Equilibrium. The analyses showed that all loci were in Hardy-Weinberg equilibrium, except for locus Ber at all sampling times and locus ISA at the last two sampling times in the 2005/6 growing season. In locus Ber only two alleles were observed, suggesting that the South African populations are fixed for these two alleles. The South African populations showed evidence of random mating throughout the growing season.

The level of heterozygosity varied between loci. Locus GOB had the highest level of heterozygosity followed by locus CES. Loci ISA and BER had medium to low levels of heterozygosity respectively (Table 2). In both growing seasons locus GOB had the highest number of alleles (22 alleles), followed by locus CES (10-12 alleles), locus ISA (2 alleles) and BER (2 alleles).

Tab. 2. Gene diversity at each microsatellite locus during different sampling times in two consecutive growth seasons

Growing Season	Sampling date	Locus			
		GOB	CES	ISA	BER
2004/5	1st 04/10/04	0.84	0.72	0.18	0.32
	2nd 08/12/04	0.90	0.79	0.29	0.37
	3rd 08/02/05	0.88	0.78	0.37	0.30
2005/6	1st 06/10/05	0.80	0.65	0.38	0.12
	2nd 14/11/05	0.88	0.73	0.36	0.11
	3rd 28/11/05	0.89	0.67	0.43	0.21
	4th 30/01/06	0.88	0.69	0.49	0.34

In each growth season, there were only two dominant genotypes that produced more than 10 lesions. The asexual spread of one of the predominant genotypes in each growth season was investigated by plotting their incidence at each sampling period (Fig. 1). Plotting of the asexual spread of the predominant genotype in the 2004/5 growing season showed that it had a plot-scale dispersion without previous clonal multiplication close to the source. The dominant genotype in the 2005/6 growing season also showed a plot-scale dispersion, but a significant decrease in lesions was detected after the second sampling, until no lesions were detected in the fourth sampling (Fig.1, Table 1). In both years aggregation of the predominant clones at certain positions in the plot would not have been evident due to the partial sampling strategy taken after the second sampling date (Gobbin et al. 2005).

Conclusion

In general the contribution of asexual and sexual genotypes to the development of epidemics in South Africa is similar to what was found in Europe (Gobbin et al. 2003a, 2005, Rumbou and Gessler. 2004).

In South Africa oosporic infections contribute between 58 to 87% of the epidemic throughout the growing season. Therefore, the control of oospore inoculum should have a significant contribution in controlling epidemics, especially in the absence of asexual spores traveling long distances. Additionally, forecasting models should be used that take into account the contribution of oosporic infections to epidemics.

Only a small percentage of genotypes were able to reproduce asexually. The failure of most genotypes to reproduce asexually from primary lesions can be ascribed to unfavorable environmental conditions such as UV-irradiation and lack of sufficient leaf wetness periods, which prevent asexual spores from causing new infections. Alternatively the asexual progeny arising from primary leaf lesions may be less virulent and capable of survival and infecting the host. Interesting in other oomycetes such as *Phytophthora infestans* and *Phytophthora cinnamomi* highly clonal populations exists in some locations even if ample opportunities exist for sexual reproduction and the generation of genetically diverse asexual populations (Dobrowski et al. 2003, Mayton et al. 2000). In one sexual cross that has been made in *P. infestans*, it has indeed been found that none of the progeny were able to cause a detectable epidemic under field conditions (Mayton et al. 2000).

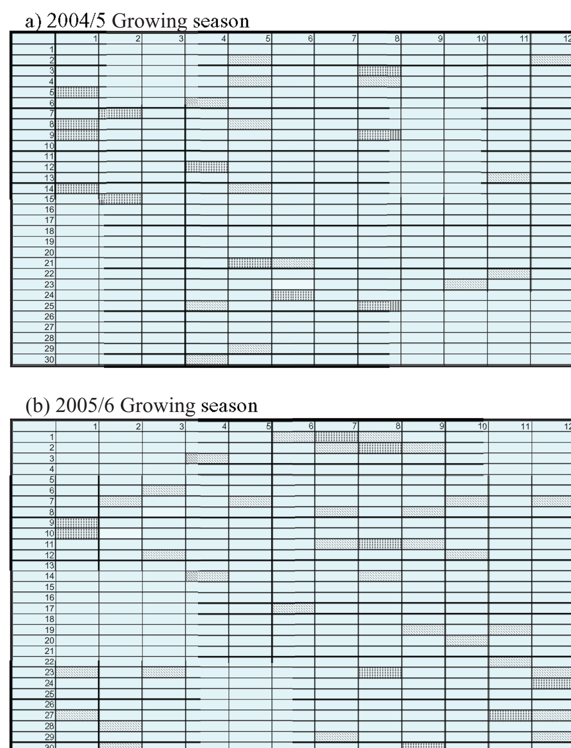

Fig. 1. Schematic representation of the vineyard where *P. viticola* populations were studied in the (a) 2004/5 and (b) 2005/6 growing seasons. Each square represents one vine, with vines being spaced 2m within rows and 3 m between rows. The dispersion pattern of one of the two most dominant *P. viticola* genotypes in each growing season is shown at three consecutive samplings times (1st = [pattern], 2nd = [pattern], 3rd = [pattern]). In the 2005/6 growing season a 4th sampling was also done, where the dominant genotype was absent from.

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Pathogenic and genetic variation among *Plasmopara viticola* isolates from different hosts

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Plasmopara viticola, an obligate parasite belonging to the class Oomycetes, has been reported on most grape species, including *Vitis vinifera* (European grape), *V. labrusca* (American grape), *V. aestivalis* (summer grape), *V. rotundifolia* (muscadine grape) and *Vitis* interspecific hybrids. The pathogen has also been found on Virginia creeper (*Parthenocissus quinquefolia)*, a perennial in the Vitaceae family. Since both Virginia creeper and *V. aestivalis*, are common in and around commercial vineyards in Michigan, the question arose whether these hosts could serve as a source of inoculum for commercial vineyards. Since physiological races or pathotypes have been identified in other downy mildew species, such as *P. halstedii* on sunflower, we decided to investigate host specificity in *P. viticola* as well.

Host specificity in *P. viticola* was studied by inoculating sporangial isolates onto healthy leaf disks of various hosts. Aqueous suspensions of *P. viticola* sporangia were placed on 2-cm-diameter leaf disks of each grape cultivar/species. Leaf disks were incubated in Petri dishes with moistened filter paper at 21ºC with 12 hours of light. After 7 days, newly formed sporangia were rinsed from the leaf disks and counted with a hemacytometer.

In 2003, four isolates of *P. viticola* from different hosts (*V. labrusca* 'Niagara' and 'Mars', an unidentified table grape, and *Vitis* interspecific hybrid 'Seyval') were used in replicated cross-infection studies. Most host-pathogen combinations resulted in infection and sporulation (Fig. 1). On 'Niagara' leaves, the highest sporangium production was by the Ni-03 isolate, which originated from 'Niagara'. This same isolate was not capable of sporulating on *Vitis riparia*, but isolates Ta-03 (from unidentified table grapes), Se-03 (from 'Seyval' grapes) and Ma-03 (from 'Mars' table grapes) all did. Sporangium production on *V. labrusca* 'Delaware' was generally low for all isolates. All isolates, including Ni-03, sporulated on *Vitis* interspecific hybrids 'Seyval' and 'Vignoles'.

In 2004, the Ni-04 isolate from 'Niagara' produced more sporangia on 'Niagara' leaf disks than any other host-isolate combination (Fig. 2), while producing moderate sporulation on 'Delaware' and little sporulation on the remaining hosts. Isolates Ri-04 (from *Vitis riparia*), Se-04 (from *Vitis* interspecific hybrid 'Seyval'), and Ta-04 (from an unidentified table grape) produced little sporulation on 'Niagara' leaves, and Ae-04 (from *Vitis aestivalis*) did not sporulate at all. The Ni-04 isolate produced over four times the number of sporangia in 2004 than Ni-03 did in 2003. Sporangia collected from *Parthenocissus quinquefolia* did not cause sporulation or visible infection on any host except for on leaves of *P. quinquefolia*, on which it sporulated readily. Isolate Ge-04 from *V. vinifera* 'Gewürtztraminer' did not cause any sporulation or sign of infection on any host in either replication, but a healthy leaf of *V. vinifera* could not be obtained for a positive control.

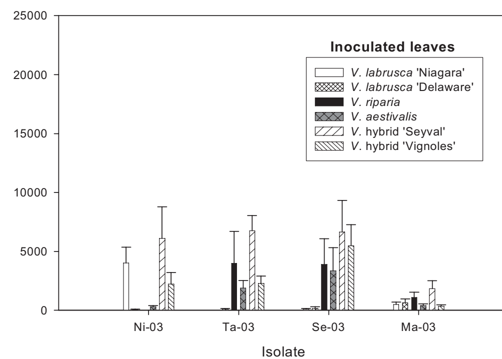

Fig. 1. Sporulation of *Plasmopara viticola* isolates from different hosts on leaf disks of various grape hosts in 2003. Isolate origin: Ni-03 ('Niagara' leaves), Ta-03 (unidentified table grape clusters), Se-03 ('Seyval' leaves), and Ma-03 ('Mars' table grape leaves). Error bars represent the standard error of the mean (n=5).

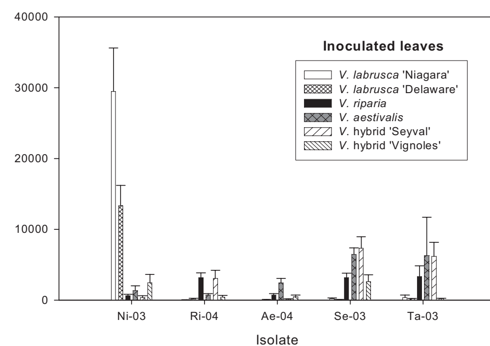

Fig. 2. Sporulation of *Plasmopara viticola* isolates from different hosts on leaf disks of various grape cultivars and species in 2004. Isolate origin: Ni-04 ('Niagara' leaves), Ri-04 (*Vitis riparia* leaves), Ae-04 (*V. aestivalis* leaves), Se-04 ('Seyval' leaves), and Ta-04 (unidentified table grape clusters). Error bars represent the standard error of the mean (n=5).

Analysis of sequences of the internal transcribed spacer (ITS) region of ribosomal DNA was used to study genetic relatedness of 57 *P. viticola* isolates collected from different hosts throughout the grape-growing areas of Michigan. Initial attempts to amplify the internal ITS region of *P. viticola* using universal primers ITS5 and ITS4 were not successful. Based on the full ITS sequence

of *Plasmopora halstedii* available in the Genbank, a primer (PV4) was designed from the 3' end of the ITS2 region. Amplification of *P. viticola isolates* with ITS5 and PV4 primers yielded amplicons of more than 2500 bp. ITS5 sequencing produced approximately 800-bp fragments that included ITS1 and partial sequences of ITS2, whereas PV4 sequencing also yielded 800-bp end fragments of ITS2. Additional primers were designed to sequence the rest of the ITS2 region. Amplification with these primers revealed the presence of multiple copies of tandemly arranged repetitive elements in the ITS2 region.

DNA sequences of the 800-bp amplicons were compared to each other and to ITS sequences of related organisms (*Peronospora*, *Pseudoperonospora*, *Pythium, Phytophthora*, and *Plasmopara* spp.) available in Genbank. Analysis revealed four distinct groups: Group 1 contained all isolates from *V. labrusca* 'Niagara', *V. labrusca* 'Concord', and *V. aestivalis* as well as a few others. Group 2 contained a mixture of isolates from *V. vinifera*, *Vitis* interspecific hybrids, and *V. riparia*. Group 3 contained only isolates from Virginia creeper, and Group 4 contained isolates from table grapes, *V. riparia* and *Vitis* interspecific hybrids.

The inoculation studies as well as sequencing data suggest that there is host specialization within *P. viticola*. 'Niagara' isolates may be adapted specifically to 'Niagara' vines or possibly to 'Concord' as well, since the ITS sequencing data placed all these isolates together in one group. Interestingly, both cultivars have hairy leaves, but 'Concord' grapes are resistant to downy mildew, whereas 'Niagara' is quite susceptible. In 'Niagara', infections can lead to premature defoliation and possibly reduced winterhardiness. The results may also help to explain the difficulties we experienced in inoculating 'Niagara' vines with field-collected isolates from wine and table grapes. Isolates taken from *P. quinquefolia* (Virginia creeper) seem to be very specific to the leaves of *P. quinquefolia*, suggesting a different biotype of even species on this host, but not much is known about downy mildew on *P. quinquefolia*. The results also suggest that Virginia creeper may not serve as a source of inoculum for epidemics in grapes. Furthermore, it appears that wild grapes are not much of a risk factor in initiating epidemics in 'Niagara' grapes, but potentially provide inoculum for infections of wine grapes. An increased understanding of host specificity of *P. viticola* isolates could ultimately lead to alternative and more efficient methods of disease control.

Trials testing the efficacy of alternative strategies for the control of powdery and downy mildews in organic vineyards on seven varieties in the Mediterranean environment

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The major difficulties in organic viticulture are ascribable to the control of some pathologies mainly powdery and downy mildew, the two most widespread fungal diseases throughout grapevine-growing regions.
In the areas where the powdery mildew pressure is high as in the Mediterranean basin, its control in organic vineyards is based on the use of sulphur although several researchers have recently substantiated the need to reduce its application due to its side effects (e.g., Bourbos *et al*., 2002). Numerous alternative products have been tested; notwithstanding, *Ampelomyces quisqualis* is the only biocontrol agent which has allowed to achieve promising results (e.g., Bourbos *et al*., 2002; Falk *et al*., 1995; Halleen *et al*., 2002). Another fungal disease frequently reported in the Mediterranean environment is Downy mildew which is controlled in organic vineyards with the use of copper. The European Commission Regulation N°473/2002 has reduced the amount of copper to a maximum of six kg/year/hectare as from 2006, due to its build up in the shallow soil layers which reduces the biological activity.

The present work is designed to evaluate the control of both fungal diseases in Mediterranean organic vineyards based on allowed control strategies with a view to (i) reducing the use of sulphur, (ii) integrating sulphur with *Ampelomyces quisqualis*-based treatments, and (iii) respecting the limits allowed for copper. The susceptibility of native and international varieties to both fungi has been tested in Apulia conditions.

The trial was carried out in 2005 in two organic vineyards of the Mediterranean Agronomic Institute of Bari (CIHEAM-MAIB); three table varieties (Victoria, Italia and Red Globe) and four wine varieties (Primitivo, Negroamaro, Uva di Troia and Chardonnay) were tested.
The control of downy mildew was achieved through the use of 50% copper oxychloride (Cuprocaffaro) applied when conditions were favourable to the fungus development.

As for powdery mildew, the control strategy was based on the combination of *Ampelomyces quisqualis* (AQ10) and sulphur. AQ10 was used at the start and end of the growing season in mixture with pine oil (Pinolene-Vapor Gard).

Samplings were performed on four plots per statistical treatment. For the control, which was the same for both diseases, six plants per plot were examined.
In the treated trials, only the plants in the middle of the plot were monitored; for each replication 100 leaves, 50 bunches and 25 shoots were sampled. Meteorological data were logged during the trial period. The results obtained were analyzed through the ANOVA test.
As for the control of powdery mildew, 18 treatments were done, five of them based on *A. quisqualis*. Nine copper-based treatments were carried out for the control of downy mildew.

The year at issue was quite favourable to the development of powdery mildew with summer climatic conditions characterized by high temperatures and low humidity. Only starting from the second decade of July, the rainfall originated some downy mildew foci prior to veraison.

The leaves of wine varieties were all susceptible to the ascomycete, with the highest incidence on Negroamaro and the lowest on cultivars (cvs) Uva di Troia and Primitivo. As for bunches those of cv. Uva di Troia were the most attacked ones while those of cv. Primitivo showed the lowest susceptibility. As regards attacks on shoots, Chardonnay proved to be the most susceptible whereas Primitivo the least sensitive.

Pertaining to table grapes, cv. Italia turned out to be more susceptible on leaves and bunches than Red Globe and Victoria. Half of the leaves on the cv. Italia control were attacked just before veraison whereas only one tenth showed symptoms in the case of Red Globe. The level of powdery mildew infection on bunches was higher than that on leaves reported for the three cultivars thereby corroborating their higher susceptibility to the fungus. Powdery mildew infections were very low during berries growth and started to rise after exceeding 70% of symptomatic bunches on cv. Italia control. Also in this case, bunches of cv. Italia were the most attacked whereas those of Victoria and Red Globe were less susceptible. On shoots, from bunch closing to veraison, symptoms were displayed only and exclusively by the control. Cultivar Italia showed a very high sensitivity and more than a half of its shoots were affected by the disease.

Both wine and table grape varieties gave very low levels of downy mildew infections on the three inspected organs. Higher incidence and severity were reported for cv. Negroamaro and cv. Italia; no downy mildew infections were observed on the shoots and bunches throughout the season.

The sulphur-*Ampelomyces* strategy enabled a good control of the attack on leaves, shoots and bunches although the latter showed the highest susceptibility. The only critical point is the interval between *Ampelomyces*-

and sulphur-based treatments, two weeks during which plants are not protected. It is therefore really needed to search for a product that might be compatible with *Ampelomyces* to be used in this interval.

For the control of downy mildew, the trial provides evidence that in low-pressure years, as in 2005 in Apulia, good results may be achieved with the allowed amount of copper. In the trial the amount of copper consumed equalled 7.5 kg/year/ha. The successful results have been attained through an in-depth monitoring of climatic data, a timely application of treatments, and through agricultural practices which made conditions unfavourable to the fungus development.

Further studies are needed in Apulia to evaluate the efficiency of cleistothecia parasitization by the mycoparasite given the low humidity in this environment. Additional investigations are to be promoted in order to estimate the susceptibility of these seven cultivars to both fungi.

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Sensitivity of *Plasmopara viticola* Italian populations to QoI fungicides

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Strobilurin and some non-strobilurin fungicides belong to a new cross-resistance group that has been designated as QoIs (Quinol outside inhibitors). They specifically inhibit fungal respiration by binding to the subunit protein of the cytochrome bc1 complex of the electron chain (Sauter *et al*., 1995). It is well known that site-specific fungicides generally possess a high risk of resistance development if resistant isolates of the pathogen are not impaired in their ability to survive and multiply in the agricultural environment.

A single point mutation (GGT to GCT) in the cytochrome b gene, that results in substitution of glycine by alanine at position 143 in the gene product, has been found in the most part of resistant isolates to QoI. To date, this particular mutation has been identified and characterized in sub-populations of pathogenic fungi resistant to QoIs under practical conditions of disease control such as *Venturia inaequalis*, (Zheng *et al.*, 2000), *Mycosphaerella fijiensis* (Sierotski *et al*., 2000), *Blumeria graminis* (Fraaije *et al.*, 2000), *Pseudoperonospora cubensis* and *Podosphaera fusca* (Ishii *et al*., 2001), *Plasmopara viticola* (Heaney *et al.*, 2000).

The sensitivity of pathogens to fungicides has been generally tested using biological assays, but this classical methodology can be now combined with a new molecular analysis relating to the specific G143A mutation from the DNA to screen for fungicide resistance. This can be sensitive and cost-effective, especially for obligate pathogens that are unable to grow on artificial media. Thus, to determine the frequency of the mutant allele, quantitative real-time PCR was reported the first time in *P. viticola* by Sirven *et al.* (2002).

A failure in grapevine downy mildew control with azoxystrobin occurred in 2000 in north-eastern Italy in some farms located in Emilia-Romagna Region (Ravenna area, where the fungicide had been largely and successfully applied through 1998 and 1999). In the experimental farm of Bologna University, where azoxystrobin was showing excellent results in field experiments since 1994, azoxystrobin failed to control downy mildew in an experiment carried out in 2000, with applications every 10 days. In the same trial, the activity of a famoxadone plus cymoxanil ready to use mixture was also unsuccessful. Laboratory and greenhouse sensitivity tests, carried out from 2000 with *P. viticola* populations collected from both commercial vineyards and trial plots, have clearly demonstrated the first occurrence of field resistance in Italy (Brunelli *et al*., 2001; 2002).

We report the results of our studies based on a wider monitoring of *P. viticola* sensitivity to QoIs in north-eastern Italy, through the use of biological assays and real-time PCR analysis to quantify the G143A mutation in each sampled population.

Material and methods

More than 200 *P. viticola* populations were collected in early growing seasons and summers from 2000 to 2005, in vineyards located in Emilia-Romagna, Friuli-Venezia Giulia and Lombardia Regions. Twenty to fifty infected vine leaves were randomly collected from field or trial plots and used as representative samples of each location. Sporangia obtained by washing the leaves with distilled water were used both in bioassays for the identification of the resistant/sensitive phenotype and in quantitative PCR analysis.

Three doses (30, 60 and 250 mg/l) of azoxystrobin (Quadris SC, 250 g/l, Syngenta) were applied in the bioassays. For each concentration (including an untreated control), a total of 15 discs (18 mm ø) or pieces of grape leaves were soaked in the different dilutions of formulated product then removed, blotted on dry paper towels and transferred to survival agar medium (1.5%) in Petri dishes in triplicates (Wong and Wilcox, 2000). The inoculation was done by depositing two 10 μl droplets of sporangial suspension ($1 \cdot 10^5$ sporangia/ml) onto the adaxial face of each disc/piece of leaf. Petri dishes were incubated under controlled conditions (20°C, 12-h photoperiod). The sporulation was assessed 8 days after the treatment and the phenotypes were defined as resistant or sensitive when sporangia were or were not formed (Latorse and Gonzales-Hilt,2003).

DNA extraction was performed from a part of sporangia prepared for biological assays according to Collina *et al*. (2005). DNA of each population was then analysed by quantitative PCR using the fluorescent dye SYBR Green I (Bio-Rad) with the ICycler iQ^{TM} Multi-Color Real-Time PCR Detection System (Bio-Rad) following a protocol referred in our previous article to construct the specific standard curves (Collina *et al*., 2005). The reactions were performed in a final volume of 22 μl, including 11 μl of 2x Syber Green I Bio-Rad, and 1 μl of primers (20 μM) designed by Sirven *et al.* (2002) under the following conditions: one cycle at 95°C for 8 min, and 50 cycles at 95°C for 12 s, 55°C for 8 s and 72°C for 15 s. Comparative Delta C_T method was used to analyse the real-time PCR data.

Results

P. viticola populations analysed by PCR Real-time showed the presence of G143A mutation with a extremely variable frequency, ranging from 0 to 97.1%.

Comparing the results obtained from bioassays and quantitative PCR analysis it was pointed out that also very low frequencies of mutant allele (0.97 %) caused sporulations on all pieces of leaves treated at different

active ingredient concentrations. No infection was noted in the bioassay leaf tests till to 0.3% of mutant allele allowing to detect less of ten sensitive populations in all monitoring.

Discussion and Conclusions

The molecular analysis has allowed the identification of the two allelic forms of cytochrome b resulting from the two different phenotypes (sensitive and resistant to QoI) shown by the bioassays. The frequencies of the mutant allele ranged from 0 to 0.3% for the QoI-sensitive populations, and between 0.97 and 97.1% for the QoI-resistant populations. This demonstrates that the QoI-sensitive populations have a very low or "null" frequency of mutant allele, while the resistant populations show a wide range of frequency. Our opinion is that quantitative PCR approach can be successfully combined with the biological tests to provide a reliable analysis of the evolution of fungicide resistance at the population level, through the monitoring of the *P. viticola* genotype.

Under a general point of view and according to data obtained till now, the frequencies of mutation detected in *P. viticola* populations collected in a wide area of north-eastern Italy were higher with a frequent use of QoI fungicides in vineyard. A reduced number (1-2) or no applications of these fungicides pointed out lower percentage of resistant phenotype. Furthermore the clearly poor activity showed in fields, characterized by the presence of populations with frequencies of mutant allele higher than 50%, could demonstrate as practical resistance occurs only with considerable high presence of mutation. The sporulation in bioassays, also in cases of remarkable low frequencies of mutant allele, shows that they overestimate the sensitivity decrease of QoI in field. Therefore this type of assay alone is not reliable when a practical response is requested.

Further investigations are in progress to contribute to a more suitable use of QoI fungicides towards grapevine downy mildew.

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Grapevine powdery mildew and the mycoparasite *Ampelomyces quisqualis* in Trentino vineyards (northern Italy)

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Powdery mildew, caused by *Erysiphe necator (*syn. *Uncinula necator*), is one of the most important grapevine diseases worldwide. At present grapevine protection against powdery mildew is mainly based on the use of chemical fungicides (Hewitt, 1998). To reduce the use of pesticides, several environmental friendly products have been evaluated for controlling this pathogen (Kiss, 2003). A natural occurring hyperparasite, *Ampelomyces quisqualis*, is considered one of the best alternatives to chemicals (Kiss et al, 2004). Several studies showed that *A. quisqualis* can parasitize *E. necator* cleistothecia overwintering on the bark (Falk et al, 2005). *E. necator* cleistothecia on the bark are the main source of inoculum for primary powdery mildew infections in Trentino vineyards. Until now, little is known about the natural occurrence of *A. quisqualis* in Trentino region.

The main objectives of the present work were to study the role of *E. necator* cleistothecia in the epidemiology of grape powdery mildew in Trentino region, to estimate the natural presence of *A. quisqualis* as mycoparasite in the vineyards, to isolate strains of *A. quisqualis* for a potential development as biocontrol agent.

Powdery mildew monitoring on leaves

To monitor the disease level in Trentino in the last stage of the season, disease incidence was assessed by counting the number of cleistothecia on leaves in autumn. The monitoring was carried out in three different vineyards during 2004 and fourteen during 2005. The results (Figures 1-2) showed, both in 2004 and 2005, a high presence of *E. necator* cleistothecia (respectively 31 to 45 and 0 to 53 cleistothecia/cm^2 of leaf), with a high percentage of mature (dark coloured) cleistothecia (respectively 70% and 50%). This is probably due to the high level of inoculum and the scarce rainfall during last three years.

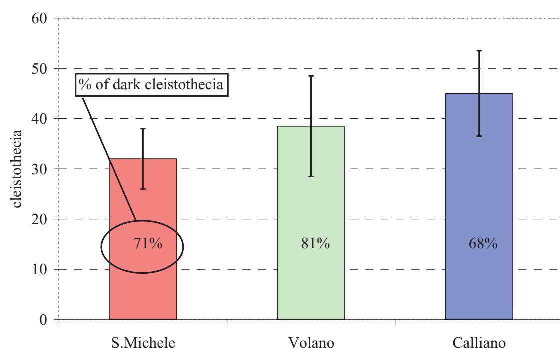

Fig. 1. Number of *Erysiphe necator* cleistothecia per square centimetre of leaf surface measured during 2004

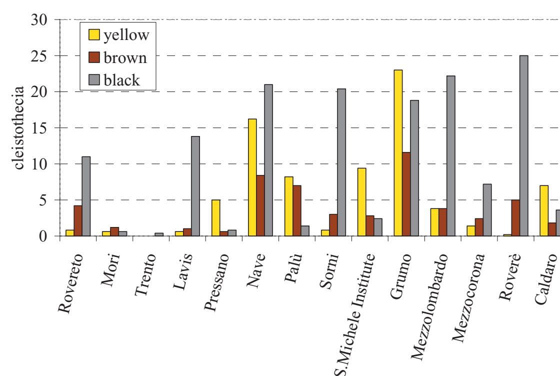

Fig. 2. Number of *Erysiphe necator* cleistothecia per square centimetre of leaf surface measured during 2005

Powdery mildew monitoring on the bark

The level of mature cleistothecia dispersal on the bark and their persistence throughout winter were measured using disk filter papers placed on the plant at three different levels counting the cleistothecia collected. The assessments were done every fifteen days all along winter.

The 2004 monitoring was carried out in five different vineyards. Among the sampled vineyards there was a high variation in cleistothecia presence (from 2 to 41 cleistothecia per disk of filter paper of 12 cm diameter).

During 2005 disk filter papers were attached at three levels on the plant and twelve different distances from untreated control. In 2005 we checked only one experimental vineyard and no cleistothecia were trapped from the bark. Moreover, we evaluated the relationship between the amount of cleistothecia on leaves and overwintering ones collected by filter papers since they are the responsible of the primary infections. No relation was found during 2005.

Natural presence of *Ampelomyces* spp.

We evaluated the natural presence of *A. quisqualis* in vineyards in Trentino with the aim of developing a strain with better adaptation to the local climatic conditions than AQ10. Wild strains were isolated to possibly develop a strain for disease control well adapted to local conditions.

To monitor the level of *Ampelomyces* spp., young and semimature cleistothecia were isolated from leaves and barks. *Ampelomyces* infects and only produces picnidia inside the cleistothecia, as reported in several studies of parasitism of *E. necator*.

Cleistothecia were classified in three levels according to the ripening stadium. The objective was to identify a

possible relationship between maturation of cleistothecia and level of parasitism. In the figure 3, young, semimature and mature cleistothecia on the upper leaf surface are shown. The presence of *Ampelomyces* spp. was evaluated in twenty vineyards of Trentino. A sample of 100 cleistothecia from each vineyard was collected and analysed for the presence of mycoparasites.

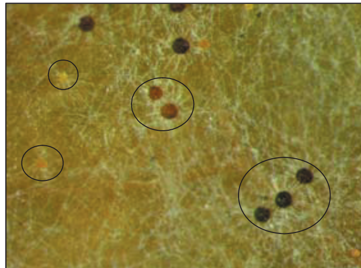

Fig. 3. Cleistothecia at different maturity stage, maturation increases with increased darkness.

The monitoring carried out during two years showed a low natural presence of the *A. quisqualis* (less than 1%). It was found as mycelium parasitizing *E. necator* cleistothecia and as conidia, both in conventionally treated and organic vineyards.

Among the isolated *Ampelomyces spp.* from the vineyards, a likely new strain was identified, having the same conidia size as the commercial strain (AQ10), but differing in the conidia shape. Conidia are more fusiform, compared to the ellipsoidal AQ10 ones (fig.4).

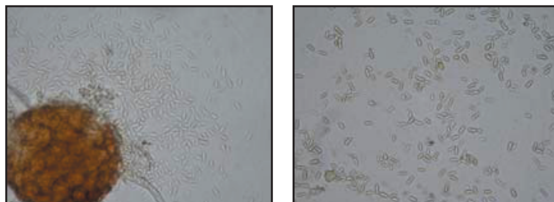

Fig. 4. Differences in shape of *Ampelomyces* spp. conidia between the wild strain (left) and the commercial AQ10 (right).

The wild types were isolated on a suitable agar medium. In vitro *Ampelomyces* spp. developed well in different nutrient substrates and the most vigorous growth was observed in malt extract agar and in potato dextrose agar. The mycoparasite formed mycelium within 5-7 days and produced both picnidia and conidia after 18-20 days.

Cleistothecia maturation curve in S.Michele

The ripening stadium of cleistothecia collected from untreated plants was measured during last Autumn by checking 100 cleistothecia per square centimetre of 25 leaves. According to this observation we established the timing of the *A. quisqualis* applications on the grapevine plants in order to reduce the powdery mildew infections.

The maturation of *E. necator* cleistothecia showed that *Ampelomyces* susceptibility started from August and finished at the beginning of October (Figure 5). The amount of dark mature cleistothecia strongly increased after middle of October.

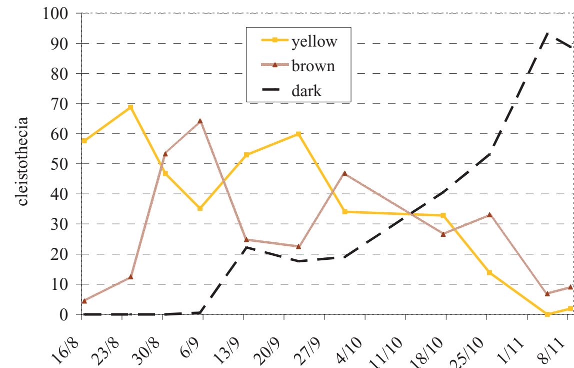

Fig. 5. Ripening stadium of cleistothecia: percentage of yellow, brown and dark cleistothecia per square centimetre of leaf surface.

Efficacy trial with a strain of *A. quisqualis*

To evaluate the effect of the *A. quisqualis* strain AQ10 (produced by Intrachem Bioitalia) in suppressing the pathogen by cleistothecia colonization, greenhouse and field trials were carried out during 2004 and 2005. In greenhouse controlled conditions ten plants were treated twice (every seven days) with a dosage ten times higher than the dose used in the field. In the vineyards two treatments of AQ10 (25-50 g/ha) were applied after harvest.

In both greenhouse and field trials no significant differences in mildew severity between AQ10 treated and untreated plants were registered. We hypothesise that the AQ10 application at the mature cleistothecia stage and *A. quisqualis* climatic requirements were the two major constrains that prevented *A. quisqualis* parasitizing activity in the tested conditions. Further experiments with different application timing will be tested in 2006 to improve *A. quisqualis* efficacy as powdery mildew hyperparasite during in the overwintering stage.

Conclusions

During last two years the presence of *E. necator* mature cleistothecia in Trentino was high. On the contrary the natural presence of *Ampelomyces* spp. was very low. The high presence of cleistothecia in Trentino's vineyards, associated with the low presence of "flag hoots" could signify a prevailing role of cleistothecia as primary inoculum. A wild strain of *Ampelomyces* spp. was isolated; it will be further studied to evaluate its potential development as biocontrol agent. The already mature stadium of cleistothecia at the time of application and climatic requirements of *Ampelomyces* could represent the reasons for the unsuccessful results of AQ10 in the tested vineyards.

Acknowledgements

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Observation of *Plasmopara viticola* development in grapevine leaves by microscopy and real-time PCR

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In the frame of breeding of grapevine for resistance to downy mildew (*Plasmopara viticola*), it is important to gain information about the adaptive potential of the pathogen against the major sources of resistance exploited in breeding schemes.

Here we describe an approach to study the plant-pathogen interactions using different combinations of *P. viticola* isolates and grapevine genotypes in semi-artificial infection conditions.

Different *Vitis* species (*V. vinifera*, *V. rupestris* and *V. riparia*) and *Muscadinia rotundifolia,* ranging from very susceptible to highly resistant to *P. viticola*, were chosen for this assay.

Two sources of inoculum were used and compared: isolate KobMt01 harvested in 2001 in Montpellier (Languedoc, South of France) on leaves of the rootstock *V. riparia* x *V. berlandieri* cv. Kober 5BB, and isolate FlRib03 recovered in 2003 on the susceptible *V. vinifera* cv. Muller Thurgau, in Ribeauvillé (Alsace, North-East of France) (Fig.1).

Fig. 1. Regions in France where the sources of *P. viticola* used in this work were harvested. Isolate FlRib03 in Alsace (North-East) and isolate KobMt01 in Languedoc (South).

The inoculation was performed by deposing 3 drops of a sporangia suspension in distilled water on leaf discs of 11 mm diameter. The inoculum was set to 5.10^3-10^4 sporangia/ml. Leaf discs were maintained in humid chamber in Petri dishes in a culture room at 21 °C, and a photoperiod of 16/8 hours (light/obscurity, respectively). Leaf discs of each genotype were sampled at fixed times (3, 6, 24, 48, 72 and 96 hours) after inoculation and frozen at -20 °C, to use them later in the different assays.

The capacity of the different grapevine genotypes to attract *P. viticola* zoospores to their stomata was studied using the staining agent Blankophor to observe the superficial structures under UV light. Blankophor is a brightener of the diaminostilbene disulfonic acid type (Rüchel and Schaffrinski, 1999) with high affinity for certain glycoside containing polysaccharides (Fig.2). The number of encysted zoospores and infected stomata in the infection area were counted at different times after the inoculation and reported to the number of stomata per area of the plant genotype.

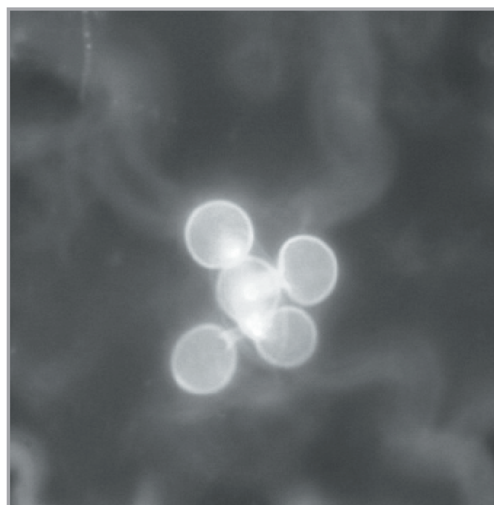

Fig. 2. Encysted zoospores of *P. viticola* over a grapevine stomata. Picture was taken using epifluorescence microscopy after Blankophor staining.

The development of internal structures of *P. viticola* was studied by aniline blue staining followed by fluorescence microscopy. Aniline blue has affinity to the -1,3-glucans (Hood and Shew, 1996). We observed the development of *P. viticola* in leaf discs at 3, 6, 24, 48, 72 and 96 hours after inoculation (Fig.3). A KOH-aniline blue fast staining technique for *P. viticola* detection was adapted in our lab to count the number of vesicles and structures at different developmental stages of the mycelium, in the same discs that had been previously observed by epifluorescence after blankophor staining.

On the basis of the microscopical observations, we could study if there were differences among the evolution of the two pathogen isolates in respect to their different geographic and host origins, mainly by following the mycelium shape and development speed of each isolate in the different grapevine genotypes used in our research.

The microscopy data collected from three replicates of each modality were statistically treated using the SAS® program package, to assess the observed differences for several parameters, including the number of encysted zoospores, their encystment speed after drop inoculation, and the frequency of formation of the different mycelial structures in the different inoculated grapevine genotypes.

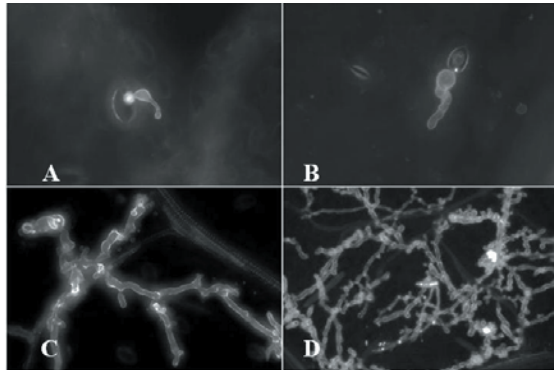

Fig. 3. Microscope images of *P. viticola* mycelium development in *V. vinifera* cv. Muscat Ottonel. A. 6 hours post inoculation (hpi)(x50). B. 24 hpi (x50). C. 48 hpi (x25). D. 72 hpi (x10), under UV light after KOH-aniline blue staining.

Real-time PCR was used to quantify the relative amount of *P. viticola* DNA accumulating in the infected grapevines tissues, at different times after the inoculation, using specific primers for the grapevine and for the pathogen (Valsesia et al, 2005). We used this method to verify the correlations between the quantified pathogen biomass and the microscopical observations on mycelium growth, in order to validate the Real-time PCR method and to propose this molecular assay as an alternative to follow *P. viticola* in grapevine leaf tissue in the early stages of infection cycle, relating to each plant-pathogen combination at different times after inoculation.

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Screening of new potential biocontrol agents against *Plasmopara viticola* using hightroughput method based on quantitative PCR

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Downy mildew, caused by the obligate oomycete *Plasmopara viticola* (Berk. and Curt.) Berl. and de Toni., is one of the most destructive grapevine diseases occurring worldwide. The pathogen attacks all green parts of the plant, flowers and bunches. Severe infections cause leaves and berries to shrivel and fall.

Control of downy mildew infection is currently based on application of chemical treatments. In organic viticulture the most effective agent is copper but, in order to reduce its accumulation in soil, EU has limited its use in organic agriculture. Alternative disease control strategies that focus on copper replacement are currently evaluated by several researchers. Biocontrol is a natural and less environmentally harmful alternative compared to chemical pesticides. Screening for biocontrol efficacy on plants is expensive and time consuming. Therefore the putative biocontrol activity of microorganisms was screened on grapevine leaf disks and indirectly assessed by estimating the *P. viticola* biomass by real-time quantitative polymerase chain reaction (RT-PCR).

Materials and methods

Two-hundred and fifty microorganisms were isolated from untreated grapevines or abandoned vineyards and tested for their potential preventive biocontrol activity against *P. viticola* on artificially inoculated grapevine leaf disks. Microorganisms were isolated and maintained on potato dextrose agar (PDA, Sigma) slants at 4°C. Mycelium agar plugs were transferred to PDA Petri dishes to prepare an inoculum and incubated for 15 days at 25°C. Suspension of *P. viticola* sporangia were prepared by washing sporangia from sporulating lesions on leaves with distilled water. The suspension of sporangia was adjusted to $4.8 \cdot 10^5$ sporangia/ml.

Leaf disks were cut from one-two week old grapevine leaves of susceptible cv. Pinot Noir. Leaf disks were kept in contact with suspensions of 250 selected microorganisms for five minutes and then sprayed with the *P. viticola* sporangia suspension. Leaf disks were incubated at 20°C in 24-well plates and 100% relative humidity for three days and freeze-dried. Four replicates (leaf disks) per microorganism (MO) were used.

DNA was extracted with NucleoSpin Multi-96 Plant Kit (Macherey-Nagel, Duren, Gremany). Instead of lysis buffer supplied, CTAB was used and instead of one final elution of 180 µl, two elution of 60 µl were made. The DNAs collected in the second elution were afterwards analysed with RT-PCR according to Valsesia *et al.* (2005). DNA of samples, pure *P. viticola* DNA, pure *Vitis vinifera* DNA and no DNA template control 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 IC). A high ratio (IC~1) between *P. viticola* DNA / *V. vinifera* DNA indicated a successful infection and tissue colonization by *P. viticola* and a low control activity. Conversely, a low IC (IC~0.5) indicated a good disease control.

Results

Untreated grapevine leaf disks (infection controls, NT) were successfully inoculated with *P. viticola*. RT-PCR of NT controls showed an average IC value of 0.86±0.09 (Fig. 1). Of the 250 microorganisms tested, 165 showed an IC< 0.8 and 85 showed an IC>0.8. The lowest amount of *P. viticola* DNA was assessed after treating the four leaf disk replicates with the microorganism coded by "1754" (IC= 0.71).

Sixty-nine microorganisms showed a significantly (T test ρ<0.05) smaller IC respect to the NT control (IC<0.79). One hundred and twenty microorganisms showed no significant difference (T test ρ>0.05) from the NT control (0.79<IC<0.93). The remaining 61 microorganisms showed a significantly higher IC. In figure 1 the results of 13 microorganisms are shown as an example.

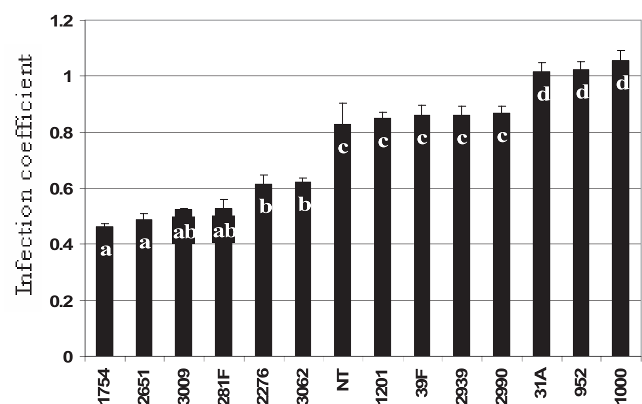

Fig. 1. Infection coefficient (IC = C_T *V. vinifera* / C_T *P. viticola*) after RT-PCR of the biocontrol assay. Of the 250 microorganisms tested, only 13 are shown. NT shows the non-treated control (inoculated with *P. viticola*). Standard deviation is indicated by error bars, significant difference with LSD test (p<0.05) is indicated by the letters a,b,c and d.

Discussion

The microorganisms tested in this assay could be classified in three main categories: potential BCAs, neutral MO and infection enhancers. This high-throughput screening allowed reducing 72.4% of them having no effect or even inducing, enhancing or favouring downy mildew infections. This quick and high-throughput method assured less time requirement and higher reliability and accuracy than the traditional screening technique (quantitative assessment of sporulation on treated leaf disks, by counting the number of sporangia/cm^2), but higher costs for equipment and consumables.

Acknowledgements

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Session 7:

Epidemiology, Disease forecasting models, Decision support system, Disease risk assessment

Dynamic simulation of grape downy mildew primary infections

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Plasmopara viticola (Berk *et* Curt.) Berlese *et* de Toni is the causal agent of grapevine downy mildew, an important disease in all grape-growing areas characterized by temperate climate and frequent rain during spring and summer.

Oospores represent the sexual stage of the fungus and are a key component in its life cycle: they are the only source of inoculum for primary infections. Oospores overwinter in the leaf litter above the vineyard soil; during this period they complete their morphological maturation and overcome dormancy. Dormancy is a physiological stage that does not allow oospores to germinate in winter even if climatic conditions are favorable. This is an ecological adjustment that prevents germination when there is not a susceptible host tissue to infect. As a further ecological advantage, oospores overcome dormancy gradually, at a rate which depends on meteorological conditions occurring during winter and early spring. Afterwards, oospores germinate in a macrosporangium that releases zoospores which are responsible for primary infections on grape leaves and clusters in spring.

The dynamics of oospores germination is a key factor for disease epidemics and, consequently, for scheduling fungicide sprays to control downy mildew. In order to increase the efficiency of the existing crop protection strategies, epidemiological models have been introduced to estimate disease risk and produce warnings for fungicide applications. Several models have been elaborated to describe the dynamics of downy mildew infection, with emphasis on primary infections. Despite this, none of these models has been demonstrated to be accurate or robust enough to be used satisfactorily in scheduling fungicide applications.

A new dynamic model based on ‘systems analysis’ was then elaborated for the sexual stage of *P. viticola*.

Model structure. The model simulates, with a time step of one hour, the entire process from oospore maturation and germination, to zoospore ejection and dispersal, and finally to infection establishment and disease symptom onset. This process was separated in different stages: the pathogen changes from one stage to another at different rates, depending on environmental conditions, as shown in the relational diagram of Fig. 1. Fungal stages are considered as state variables and their changes are regulated by rate variables; weather conditions influence rates acting as parameters or intermediate variables (Tab. 1). Equations linking the meteorological parameters to the rate variables were elaborated from both literature and data from specific studies.

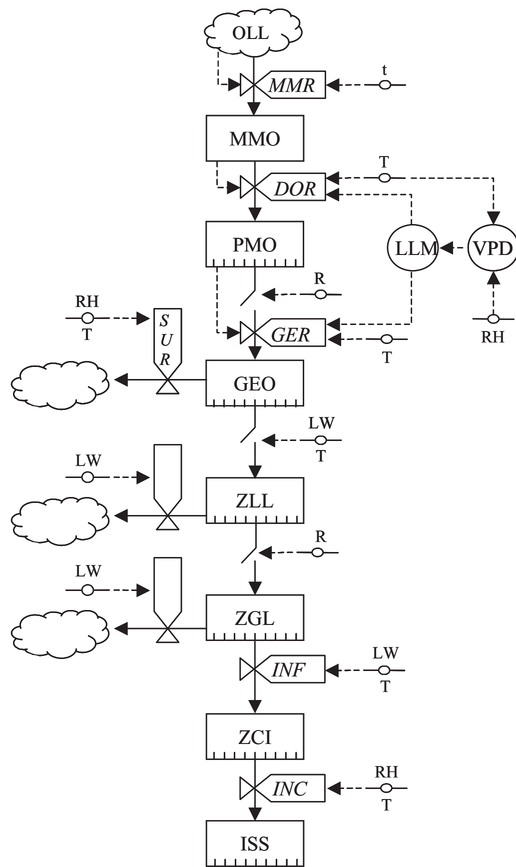
The first state variable is represented by the oospores produced in the leaf residues above the ground (OLL), the second is represented by the morphologically mature oospores (MMO). The rate of morphological maturation (MMR) depends on time elapsed after oospore formation (t).

The following state variables are the physiologically mature oospores (PMO), which are oospores that had overcome the dormancy, and the germinated oospores (GEO),

Tab. 1. List of variables used in the model.

State variables	
OLL	Oospores in the leaf litter
MMO	Morphologically mature oospores
PMO	Physiologically mature oospores
GEO	Germinated oospores
ZLL	Zoospores on the leaf litter
ZGL	Zoospores on the grape leaves
ZCI	Zoospores causing infection
ISS	Infection sites showing symptoms
Rate variables	
MMR	Progress of oospore maturation
DOR	Progress of dormancy overcoming
GER	Progress of oospore germination
SUR	Survival
INF	Progress of infection establishment
INC	Progress of incubation
Auxillary variables and parameters	
LLM	Leaf litter moisture (%)
VPD	Vapour pressure deficit (hPa)
t	Time (days)
T	Air temperature (°C)
RH	Relative humidity (%)
LW	Leaf wetness (yes or not)

Fig. 1. Relational diagram of the model simulating primary *P. viticola* infection on grape. See Tab. 1 for acronym explanation.



which are oospores that have completed the germination process and have produced a macrosporangium on the leaf litter surface. Oospores change from MMO to PMO at the end of dormancy, and from PMO to GEO when the germination process is completed; the corresponding rates (DOR and GER, respectively) both depend on air temperature (T) when leaf litter moisture (LLM) is not a limiting factor, but rainfall (R) is necessary to moisten the leaf litter and trigger germination. LLM depends on the balance between water absorption from and desorption to the atmosphere, measured by means of the vapour pressure deficit (VPD).

In the presence of a film of water (LW) macrosporangia release zoospores (ZLL); otherwise they can survive for a few days and then die: the survival rate (SUR) depends on T and relative humidity (RH).

These zoospores, swimming in the film of water covering the leaf litter, reach the grape leaves (ZGL) by splashes and aerosols triggered by rainfall. If the litter surface dries up before rainfall they do not survive; therefore the SUR of these zoospores depends on LW.

Zoospores in the ZGL stage go to the next stage of zoospores causing infection (ZCI) according to an infection rate (INF) which depends on LW and T during the wet period. During this period zoospores swim in the direction of stomata, form a cyst and produce germ tubes that penetrate the stomatal rimae. If the leaf surface dries before penetration, the zoospores dry out; therefore their survival depends on a combination of LW and T.

At the end of incubation, the infection sites become visible as disease symptoms (ISS); incubation progress (INC) is influenced by T and RH.

Model running. Considering that oospores usually reach the MMO stage in autumn, it is assumed that the population of oospores formed at the end of a downy mildew epidemic is all in the MMO stage on the 1st of January of the following year.

Since it is well known that oospore germination is a gradual process over the grape-growing season, the model considers that the oospore population of a vineyard overcomes dormancy in many subsequent groups (cohorts), and that the density of each cohort follows a normal distribution. Therefore, the model calculates the time when the first cohort of oospores enter the PMO stage at the end of its dormancy; further cohorts enter this stage progressively (Fig. 2). A measurable rainfall moistening the leaf litter is the event triggering germination of the oospore cohorts in the PMO stage at that time. Times required for completing both dormancy and germination are calculated by the variables DOR and GER, respectively, using two counters that increase according to T when LLM is not a limiting factor. When these counters reach a fixed threshold the model assumes that both dormancy and germination of an oospore cohort are finished.

Afterwards, the model calculates the time when the macrosporangia produced by the germinated cohort of oospores survive, the possibility that they release zoospores, that these oospores survive, reach the grape leaves and successfully infect the leaf tissue (Fig. 3). Finally the model calculates the incubation period and defines a time period when the downy mildew symptoms should appear on the affected grape organs.

Model outputs. The model provides tables showing the hourly progress of the main infection stages (Tab. 2), and graphs showing the state of the infection cycle on each day during the primary inoculum season (Fig. 3).

Tab. 2. Example of the tabular output of the model in a day with a successful infection.

Hours of the day	Oospore germination	Sporangium survival	Zoospore release	Zoospore dispersal	Infection progress	Incubation progress
2	0.99					
3	0.99					
4	0.99					
5	1.00	0.01				
6		0.01				
7		0.02				
8		0.03	yes			
9				yes	0.30	
10					0.77	
11					1.00	
12						0.01
13						0.02

Fig. 2. Example of model simulations. A: progress of DOR over time after 1st January and thresholds for the first and last cohorts of oospores overcoming dormancy. B: progress of GER for the cohorts of oospores able to start germination at each rainfall and density distribution of these cohorts.

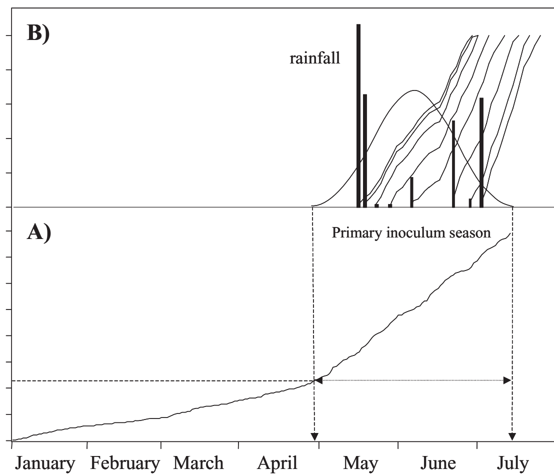
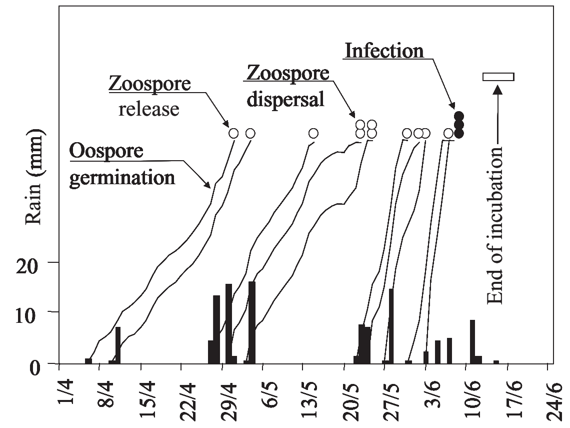


Fig. 3. Example of model outputs: bars represent the daily rainfall; lines show the progress of germination in different cohorts of *P. viticola* oospores; dots show the occurrence of the different stages of the infection cycle, from zoospore ejection to leaf infection (dots are black when the infection cycle is successfully completed); the box shows the period of the expected appearance of downy mildew symptoms.



Validation of a simulation model for *Plasmopara viticola* primary infections in different vine-growing areas across Italy

Tito Caffi, Vittorio Rossi, Riccardo Bugiani, Federico Spanna, Lucio Flamini, Antonello Cossu, Camilla Nigro

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Downy mildew of grape, caused by *Plasmopara viticola* (Berk *et* Curt.) Berlese *et* de Toni, is a disease of major importance in grape-growing areas with a temperate climate. It is a potentially destructive disease that requires repeated fungicide application during the growing season.

Some epidemiological models have been elaborated to support decisions about disease control but none are accurate or robust enough to be used for scheduling fungicide application. Consequently, warning systems are still based on the empirical rule called "three tens" even if it is frequently unreliable.

A new model has recently been elaborated, which can simulate the dynamics of primary inoculum and infection during the season. This model uses meteorological data (air temperature, relative humidity, rainfall, leaf wetness) to simulate, with a time step of one hour, the infection chain from oospore germination to the onset of disease symptoms, including the germination progress, survival of sporangia, zoospore ejection and survival, zoospore dispersal, infection and incubation. The model performs several simulation runs per season, considering that the overwintering oospore population overcomes dormancy gradually. In particular, the oospore population of a vineyard is composed of different cohorts that become able to germinate according to a normal distribution. When a measurable rainfall wets the leaf litter containing these oospores a simulation run starts with the beginning of oospore germination. This simulation run can be interrupted at any stage of the infection chain if the environmental conditions do not favour the fungus, or can complete the infection chain until the appearance of the disease. The model provides both tables showing the hourly progress of each simulation run and graphs showing the state of the infection cycle for each day during the primary inoculum season (Fig. 1).

Validations were performed in 77 commercial vineyards throughout five regions of Italy, between 1995 and 2005 (Fig .1). Some data were provided by historical series available at the local services delegate to producing disease warnings for vine-growers in the different areas. Other, more recent data, were specifically collected for validation. In both cases, vineyards can be considered representative of the different vine-growing areas, for soil type, varieties, training systems and cropping regimes. They also contained a representative dose of overwintering inoculum because a regular fungicide schedule was applied the previous season. During winter, a plot which included several rows of vines was set apart in each vineyard and not sprayed with fungicides against downy mildew till the time of first disease onset. Starting from bud burst, plots were carefully inspected at least once per week, to detect the time of appearance of the first disease symptoms such as "oil spots" on leaves.

Data collection was coordinated by the team working on this paper, from the regional phytosanitary services of Emila-Romagna (R. Bugiani) and Piedmont (F. Spanna), SAR (regional agrometeorological service) of Sardinia (A. Cossu), Assam in Marche (L. Flamini), and Alsia in Basilicata (C. Nigro). In Oltrepò Pavese (Lombardy) data were collected by the first author of this work.

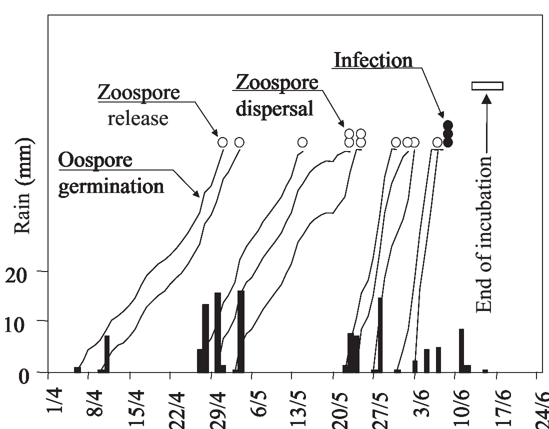

Fig. 1. Model output showing 10 simulation runs triggered by rainfall (bars). Lines show the germination course for different *P. viticola* oospore cohorts; dots show progress over time of the infection process, stage by stage: they are white when the infection chain aborts and black when it is successfully completed; the box shows the period of expected downy mildew appearance.

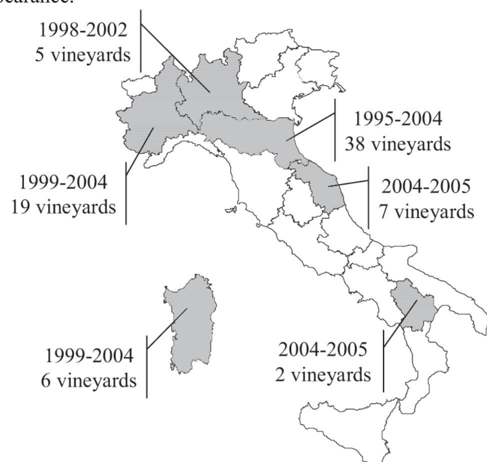

Fig. 2. Distribution of the vineyards used in model validation.

Hourly meteorological data of air temperature, relative humidity, rainfall, and leaf wetness were collected to be used as model inputs. In Piedmont and Oltrepò Pavese, data were measured by automatic and mechanic weather stations, respectively, installed in the vineyards. In the other regions data were supplied by the regional networks for the nearest automatic station. In Emilia-Romagna, after the year 2000, the meteorological data were extracted from a data-base containing data interpolated on a grid of 5x5 km.

The model was used to simulate, for each vineyard, the infection process in each cohort of oospores between the end of dormancy and the time of first disease onset in the vineyard. Total simulations were first classified as aborted or successful. A simulation was considered aborted when the infection process stopped at any stage before infection, while it was considered successful when all the stages progressed until infection was established. Both aborted and successful simulations were then classified as: i) accurate, when the model produced a successful simulated infection that actually produced symptom appearance in the vineyard, or when an aborted simulated infection did not correspond to an actual symptom onset; ii) overestimated, when the model produced a successful simulated infection but the disease did not appear; iii) underestimated, when the model did not simulate an infection that actually occurred. A possible criticism of the above mentioned classification is that there is no proof that an aborted simulation process actually occurred in the vineyard; nevertheless, from a practical point of view, the model produces accurate information in such a case, because it indicates that there is no risk of infection occurring.

The model produced 736 simulations in total: the χ^2 test showed a significant correlation between model simulations and actual observations (Tab. 1). The model was very accurate in simulating successful infections, because all the observed disease appearances in the vineyards were correctly simulated by the model; this happened 122 times (16.6% of total simulations). In 543 simulations (73.8%) the model rightly interrupted the simulation runs because the environmental conditions did not permit the infection process to proceed; no disease symptoms were actually observed in the vineyards as a consequence of these possible infection events. In these cases, the infection process was aborted because sporangia died before zoospore release (14% of cases), zoospores died before dispersal (75%) or before infection establishment (8%) (data not shown). Considering both successful and aborted infections, there were 665 accurate simulations out of 736 (90.4%) (Tab. 1). In 71 simulation runs the model simulated an infection event that did not actually occur (9.6% of cases overestimated). On the contrary, the model never failed to simulate actual infections.

Model outputs were validated in different epidemiological conditions (areas x vineyards x years) (Tab. 2). In Piedmont the model was validated in 19 different situations; it provided 153 simulations with only 4% of cases overestimated. In Oltrepò Pavese, the model was validated for a 5-year period in the same vineyard providing 26 simulations; only in one case (4%) infection was overestimated. In Emilia-Romagna, the model was validated in 38 cases that provided 365 simulations in aggregate; 7% of them were overestimated while the remaining 93% were accurate. In the Marche region the model provided 104 simulations, under 7 different epidemiological conditions; it produced 88 accurate simulations, with 15% overestimation. In Basilicata the model produced 34 simulations in two different vine-growing areas: in 91% of cases the model correctly simulated the primary infection process, with 3 (9%) unjustified alarms. In Sardinia the model was validated in the same vineyard over a six-year period; it provided 54 simulations. Also in this area the model never failed to signal an actual infection, but 19 simulations (35%) signalled an infection that actually did not occur.

In conclusion, this dynamic model for primary *P. viticola* infections was accurate and robust: in more than 90% of the simulations performed the model correctly simulated either aborted or successful infections, under different epidemiological conditions and over a wide seasonal period, from early to late disease onsets (Fig. 3). In the remaining cases, the model overestimated the actual situation because it simulated an infection that did not actually produce disease symptoms; these cases frequently occurred in early spring, at the beginning of the primary inoculum season (data not shown). Moreover, the model never failed to simulate actual infections.

Inaccuracies produced by the model were always due to false positive infections, which have a low negative impact on model performances because it simply produced unjustified warnings. Nevertheless, the model can be improved to reduce false positive prognoses. Considering that this model runs using only meteorological data and does not include information about the growth stage of the host at the time of a possible infection, there are good reasons to believe that unjustified alarm could be avoided by ignoring infections which occur before the beginning of host susceptibility.

Tab. 1. Comparison between model simulations and observed occurrence of *P. viticola* primary infections (χ^2 = 411.4 calculated using the Yate's correction, significant at P<0.001)

		Observed	
		No	Yes
Simulated	No	543 *73.8%*	0 *0%*
	Yes	71 *9.6%*	122 *16.6%*

Tab. 2. Results obtained by model simulations for 77 vineyards in different vine-growing areas and years (see Fig. 1). Total simulations are distinguished in accurate (rightly aborted and with an actual successful infection) and overestimated.

Areas	Total n	Rightly aborted %	Actual infection %	Over estimated %
Piedmont	153	71.2	24.8	3.9
Oltrepò Pavese	26	50.0	46.2	3.8
Emilia-Romagna	365	80.0	12.9	7.1
Marche	104	70.2	14.4	15.4

Basilicata	34	79.4	11.8	8.8
Sardinia	54	53.7	11.1	35.2

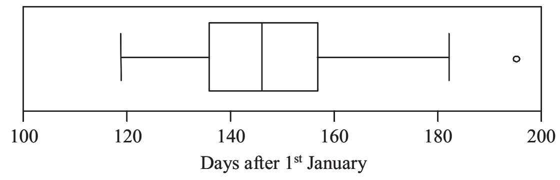

Fig. 3. Box plot representing distribution over time of the downy mildew appearance for the 122 infections observed in 77 vineyards.

Modelling leaf wetness duration for downy mildew simulation

Anna Dalla Marta, Simone Orlandini, Luca Martinelli, Roger D. Magarey

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Agrometeorological variables play an important role in the development of plant diseases. In particular, leaf wetness duration (LWD) is directly involved in the infective processes of grapevine downy mildew (*Plasmopara viticola*). In fact, the spores of *P. viticola* move on a layer of free water to reach the stoma and to enter the host tissue.

Despite the key role of LWD, the lack of standards for its measurement gives rise to a series of problems not only for the choice of sensors and the interpretation of their output, but also for their positioning in field. For these reasons, the use of agrometeorological models for the simulation of surface wetness duration represents an interesting alternative to field monitoring. A lot of models have been developed for the simulation of LWD both with empirical and physical approaches. Their operative application is a very important tool for the planning of field interventions in order to reduce the use of pesticides for environmental safeguarding, for reducing the waste of resources and financial losses and for producing with high quality standards.

In this work a physical model based on the energy balance was applied for the simulation of LWD on grapevine leaves. The model (SWEB), developed in the United States, was adapted for the Sangiovese variety and for use with agrometeorological data easily available from standard weather stations. The model outputs were compared both with data measured by sensors in 2002 and 2003 and with visual inspections conducted during 2003. Following, all LWD data were used to feed PLASMO, an agrometeorological model for the simulation of grapevine downy mildew. The results were compared with epidemiological data observed in non treated plots to establish the impact of different LWD data on the quality of model simulations.

The field research was conducted in the experimental farm Mondeggi Lappeggi, located in the northern part of the Chianti region in Tuscany (Central Italy). Close to the experimental field, a complete agrometeorological station was installed over turf just for the measurement of air temperature (°C), relative humidity (%), precipitation (mm), wind speed and direction (m s^{-1}; degrees), global radiation (W m^{-2}) and leaf wetness (0/1).

The occurrence of downy mildew was monitored through visual surveys made during the vegetative season. For SWEB verification, LWD visual observations were made in the vineyard close to the weather station during 2003. The surveys were conducted for 12 rainless nights on 40 leaves chosen randomly in the canopy. Two observers made observations of the percentage of wet leaf area aided by electric torches. Visual observations were compared with recorded data and SWEB simulations, and the errors were calculated to test the sensor performances, both as leaf wetness occurrence (0-1) and duration (minutes).

Finally, PLASMO was run using LWD data simulated by SWEB and recorded by sensors for the same period. The two outputs were compared with epidemiological observations collected during the corresponding years.

To compare the number of hours correctly classified as wet and dry, a contingency table was used. In particular the indices analysed were percentage correct (PC), critical success index (CSI), and BIAS. At the same time, the measured and the observed hours of onset, depletion and duration of leaf wetness were compared and root mean square error (RMSE) and mean real error (MRE) were calculated. Comparing observations and simulations of LWD, the statistical indices of PC and CSI provided values of 84% and 0.55 respectively, while the analysis of BIAS showed that the model has a tendency to under-estimate the number of leaf wetness hours (Table 1). On the other hand, the comparison between measurements and simulations led to a PC of 76% and a CSI of 0.48. The under-estimation is confirmed also in this case (BIAS 0.59). This analysis showed that SWEB simulation had a better correspondence with the observations matching the wet events in the 84% of the cases. The CSI is lower in both cases because it doesn't take into account the dry events, which are the majority. The CSI is higher comparing SWEB with observations. In other words it means that the model seems to be more precise than sensors used in this experiment in the detection of wetness.

To analyse the differences between observations and sensor records, a further comparison was made in terms of duration. The results confirmed that the data collected by sensors are subjected to errors and uncertainties. The error for sensors was on average two hours, with an overestimation of both condensation and depletion time (Table 2).

Tab. 1. Comparison between leaf wetness duration visually observed (OBS), simulated with SWEB (SIM) and measured by sensor (MEAS) during 12 rainless nights. PC = Percentage correct; CSI = Critical Success Index.

	OBS-MEAS	MEAS-SIM
PC	83.77%	76.00%
CSI	0.55	0.48
BIAS	0.85	0.59

Tab. 2. Comparison between visual observation and sensor. Values are expressed in hours. RMSE = root mean squared error; MRE = mean real error.

	START	END	DURATION
RMSE	2.00	1.10	3.03
MRE	-1.05	-2.11	-2.15

The influence of simulated and measured LWD on downy mildew simulation was analysed by using the two data sets as input for PLASMO and the results were compared with epidemiological observations (Table 3) (Fig. 1).

In both years, PLASMO run with sensor LWD data led to a higher number of total infections. This difference obviously affected the disease intensity that in 2002 was more over-estimated by the use of sensor LWD data. In 2003 errors were lower with a slight better performance with the use of sensor data (Table 4) (Fig. 1). Nevertheless, statistical analysis clearly showed that in both cases the simulation results are very similar to the real situation.

Tab. 3. Number of *P. viticola* infective cycles simulated using LWD recorded by sensors and simulated by SWEB.

	NUMBER OF INFECTIONS	
	SENSOR	SWEB
2002	5	3
2003	21	20

Tab. 4. Comparison of observed (OBS) and simulated disease intensity during 2002 and 2003, using LWD data measured (MEAS) and simulated with SWEB (SIM). MRE = mean real error; MR%E = mean real percentage error; RMSE = root mean square error.

		MRE	MR%E	RMSE
2002	OBS-SIM	-1.51	-0.04	2.57
	OBS-MEAS	-2.90	-0.07	4.96
2003	OBS-SIM	0.49	0.10	0.88
	OBS-MEAS	-0.01	0.00	0.79

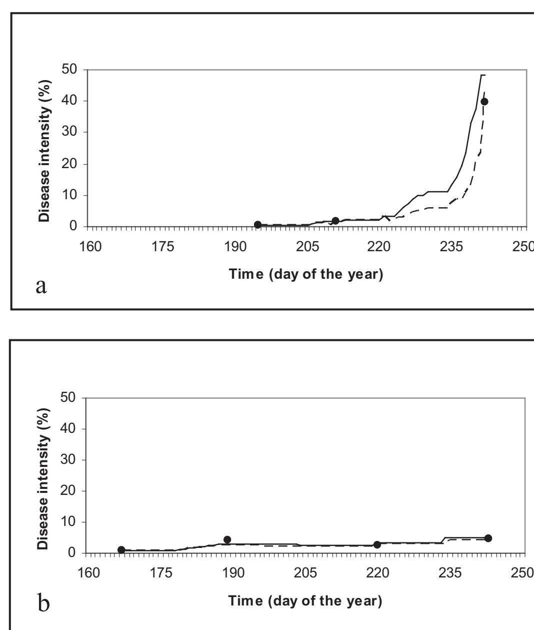

Fig. 1. Severity (%) of downy mildew in 2002 (a) and 2003 (b), observed (points), simulated with LWD sensors (continuous line) and with SWEB output (hatched line).

The results demonstrated that, for epidemiological applications, the use of SWEB model can be considered as an alternative to field sensors. In fact, the simulation of LWD led to a precise simulation of the disease severity and for this reason it could be an important tool for operational applications.

Recent Investigations of the Biology of *Plasmopara viticola*: Considerations for Forecasting and Management of Grapevine Downy Mildew

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Over the last 25 years, our ability to control downy mildew has improved due to two principle factors: (i) new fungicide chemistry, and (ii) increased knowledge of pathogen biology and ecology. Contributions from the former can flow and ebb as new materials are deployed and then lost due to resistance or cancelled registrations. In contrast, new knowledge of pathogen biology and ecology provides improvements that are stable. Our goal has been to improve the biological base underlying forecast models for grapevine downy mildew by investigating a number of poorly understood aspects of the pathosystem.

Ontogenic resistance. Clusters of *Vitis vinifera* and *V. labrusca* are reported to become resistant to *Plasmopara viticola* at stages of development ranging from 1 to 6 weeks postbloom. It has been suggested that resistance is associated with loss of the infection court as stomata are converted to lenticels, but the time of onset, cultivar variation, and seasonal variation in ontogenic resistance has remained uncertain, as has the comparative susceptibility of stem tissue within the fruit cluster. In New York, we inoculated clusters of *V. vinifera* Chardonnay and Riesling and *V. labrusca* Concord and Niagara at stages from prebloom until 5 to 6 weeks postbloom.

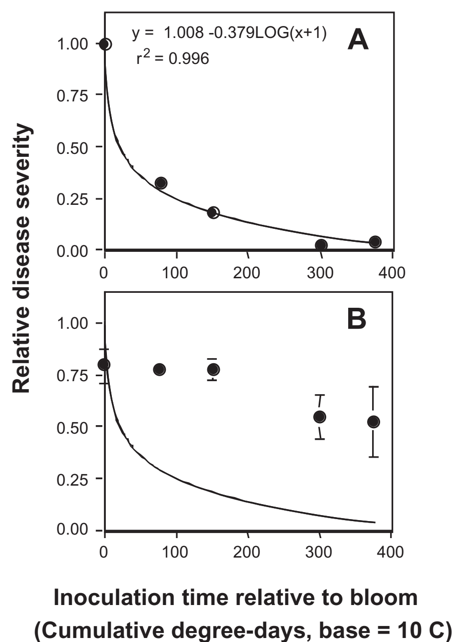

Fig. 1. Relative severity of downy mildew on (A) *V. vinifera* Chardonnay and Rielsing and *V. labrusca* Concord and Niagara clusters in NY, USA, and (B) Chardonnay and Riesling clusters in Loxton, SA, Australia, inoculated at various degree-days (base = 10C) after bloom. Model fitted to NY data (A) is superimposed on data from Australia (B).

Berries were infected and supported profuse sporulation until 2 weeks postbloom, and pedicel tissue remained susceptible until 4 weeks postbloom (Fig. 1A). Although berries on later-inoculated clusters failed to support sporulation, discoloration and necrosis of berry tissues was often noted, and necrosis of the pedicel within such clusters often led to further discoloration, shriveling, reduced size, or loss of berries. We repeated the study on Chardonnay and Riesling vines in South Australia and found that the period of berry and rachis susceptibility was greatly increased (Fig. 1B). The protracted susceptibility of the host was related to the increased duration and phenological heterogeneity of bloom and berry development in the warmer climate of South Australia. The time of onset and subsequent expression of ontogenic resistance to *P. viticola* may thus be modified by climate, and should be weighed in transposing results from one climatic area to another.

When the epidermis of discolored berries that initially failed to support sporulation was cut, the pathogen emerged and sporulated through incisions (Fig. 2), indicating that lack of sporulation on older symptomatic berries was due to infection at an early stage of berry development, followed by conversion of functional stomata to lenticels during latency.

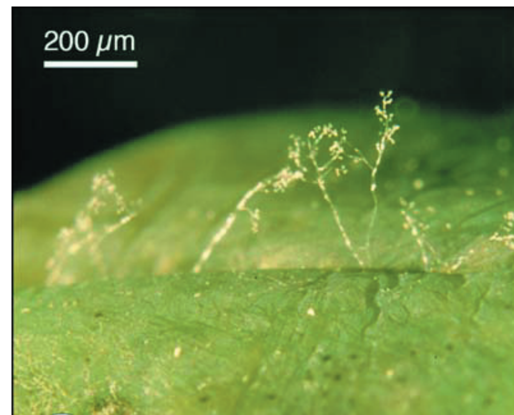

Fig. 2. Emergence of sporangiophores through incisions made in berry skin of symptomatic post-veraison berries.

Our results can be used to refine forecast models for grapevine downy mildew to account for changes in berry

and rachis susceptibility, and to focus fungicide applications schedules upon the most critical periods for protection of fruit.

Primary and secondary infection. Several other aspects of grapevine downy mildew epidemiology that were fundamental to model predictions were investigated. We evaluated simple rainfall-, temperature-, and phenology-based thresholds (rain > 2.5 mm; temperature > 11C; and phenology > Eichorn and Lorenz [E&L] growth stage 12) to forecast primary (oosporic) infection by *Plasmopara viticola.* The threshold was consistent across 15 years of historical data on the highly susceptible cultivar Chancellor at one site, and successfully predicted the initial outbreak of downy mildew for 2 of 3 years at three additional sites.

Field inoculations demonstrated that shoot tissue was susceptible to infection as early as E&L stage 5, suggesting that initial germination of oospores, rather than acquisition of host susceptibility, was the limiting factor in the initiation of disease outbreaks.

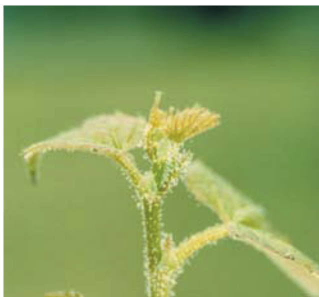

Fig. 3. Sporulation of *Plasmopara viticola* on a grapevine shoot inoculated at E&L stage 7 (one leaf unfolded) at two weeks after inoculation.

We also found that oospores may continue to germinate and cause infections throughout the growing season, in contrast to the widely-held assumption that the supply of oospores is depleted shortly after bloom. Furthermore, oospores persisted for more than one growing season and subsequently germinated to cause infection. Thus, the total oospore load in vineyard soil may include cohorts of oospores from more than one growing season.

Microsatellite analyses indicated that a high proportion of lesions in an unsprayed vineyard were derived from unique genotypes (presumably from oosporic inoculum) throughout the course of the epidemic. The most common genotypes were those detected earliest in the epidemic, and their frequency was highly correlated with time available for secondary reproduction (Figure 4).

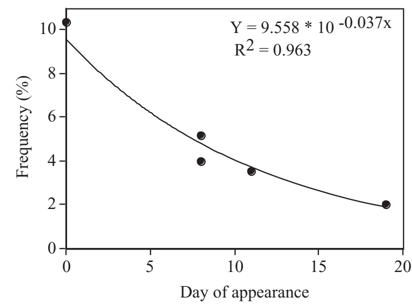

Fig. 4. Frequency of the 5 most-common isolates in a collection of *P. viticola* from 849 lesions collected 40 days after initial detection of disease vs date of initial appearance of an isolate. Early establishment was highly correlated with frequency of collection on day 40. There were no significant differences in rates of disease increase among the above five isolates.

Lesion productivity and survival of sporangia. Lesion productivity (sporangia/lesion) did not decline with age of a lesion in the absence of suitable weather to induce sporulation. However, the productivity of all lesions declined rapidly through repeated cycles of sporulation. Extremely high temperatures (i.e., one day reaching 42.8° C) had an eradicative effect under vineyard conditions, and permanently reduced sporulation from existing (but not incubating) lesions to trace levels, despite a later return to weather conducive to sporulation. In fair weather, most sporangia died sometime during the daylight period immediately following their production. However, over 50% of sporangia still released zoospores after 12 to 24 hours of exposure to overcast conditions.

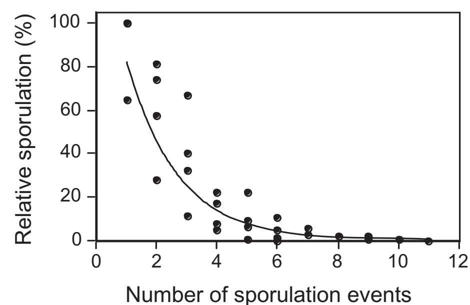

Fig. 5. Decline of sporangial production with successive sporulation events. Production of sporangia was assessed on cohorts of lesions induced to sporulate repeatedly. Curve fitted to the observations was generated by equation 1 ($Y = 4.757 - 0.496X$) after ln+1 transformation of the response variable.

Summary and Conclusions

At the producer level, outputs from forecast models are primarily useful in decisions to either deploy or withhold a fungicide spray. The end product of the model, from the producer's perspective, is relevant in how well it can assist the foregoing decision. Current forecast models for grapevine downy mildew focus on discrete weather-driven infection events. While an admittedly complex sequence of environmental circumstances is required for infection, disease forecasting based upon weather alone is still overly simplified. Biological studies described herein were

designed to provide information from which we could design a more comprehensive forecast model. We can now refine output of DMCast with an improved understanding of (i) development of ontogenic resistance in fruit, (ii) timing of initial oosporic infection, (iii) seasonal duraiton of oospore infection, (iv) initial shoot susceptibility, (v) longevity of lesions through cycles of sporulation, and (vi) mortality of sporangia after production. Certain aspects of the foregoing may also apply to other models of grapevine downy mildew.

The relative contribution of oosporic vs sporangial inoculum is an area that awaits further clarification. Recent microsatellite-based studies, as well as the discovery of a protracted season of oospore survival and germination will require revision of some models. Specifically, the simultaneous and season-long presence of both oosporic and sporangial inoculum may require, under certain circumstances, that primary and secondary infection models run in tandem for an entire growing season.

The comparative importance of the two inoculum source is likely to vary according to the absolute dose of oospores (*e.g.*, oospores/m^2 of vineyard floor) and prevalence of weather conducive to secondary infection. As a practical matter, this would require investigation of the relative contribution of primary (oosporic) vs secondary (lesion-derived sporangia) across a range of oosporic inoculum levels that would be reasonably expected in commercial vineyards, and across a range of environmental conditions that might favor infection by one source of inoculum over another.

Subsequent to model refinement, the greatest need remains the meaningful validation of model forecasts. Performance comparisons of model-timed sprays vs intensive calendar-based spray programs may be useful to promote a particular model, but are not designed to show a correspondence between model-predicted events and the magnitude (or even occurrence) of stepwise increases of disease in the vineyard.

Statistical Relations between Monthly Means of Temperature and the Sum of Rainfall on Powdery and Downy Mildew

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Air temperature and atmospheric water are main components for the spread of fungus diseases. In lab-experiments, the effect of nature-like weather parameters can only be partly simulated. Ex-post data analysis of covariance may close this gap and verity the lab-evaluated data. But ex-post analysis often give doubtful results because the biological relations are more complex than the model applied (Sokal and Rohlf, 2003). This contribution analyses carefully ancient data for weather conditions and the disease incidence of downy mildew (Plasmopara viticola) and powdery mildew recorded at Weinsberg, Germany.

Data base and Evaluation.

Since 1953, diseases have been recorded at the Weinsberg research station by experienced viticulturists using the same scale:

0 = no disease
1 = very low disease (< 5 % disease incidence)
2 = low (5 - 10 % disease incidence)
3 = medium (10 - 25 % disease incidence)
4 = strong (25 - 50 % disease incidence)
5 = extreme (> 50 % disease incidence)

The records were made in November after harvesting each year. Eight different persons dealt with the recording.

Fourty nine years of data from powdery mildew and 43 years of downy mildew were used for calculations. Weather data were recorded since 1947, but only monthly means of temperature and the sum of rainfall per month were available. Simple and multiple regression models have been calculated using the correlated features and excluding the character with the lowest contribution step by step until all remaining characters had an error level < 5 %.

Results and Discussion

Data base and evaluation. Some significant trends over the 50 years period were found. Temperatures increased in January, March, June, August and September. A significant trend to higher rainfall was calculated for March, October and November and lower rainfall for August. These weather trends made the data analysis much harder. No significant trend was found for the disease incidence of both fungus. Because the staff that recorded the data changed several times the last 50 years, there undoubtedly are some imponderabilities there is no doubt, that diseases in spite of much better fungicides, better technique and increasing knowledge the fungus diseases still cause the same problems.

Tab. 1. Weather trends (correlations between years and weather data recoded at Weinsberg, Germany 1953-2003)

month	Mean t temperature	Sum of rainfall
January	0,37**§	0,10 n.s.
February	0,26 n.s.	0,07 n.s.
March	0,29*	0,34*
April	0,26 n.s.	0,07 n.s.
May	0,16 n.s.	0,02 n.s.
June	0,35*	-0,14 n.s.
July	0,13 n. s.	0,14 n. s.
August	0,35*	-0,32*
September	0,68**	0,15 n. s.
October	0,07 n.s.	0,28*
November	0,25 n.s.	0,31*
December	0,16 n.s.	0,19 n.s.
Minimum temperature	0.37**§	

§: *, ** n.s.: Error level 5 %, 1 %, or. not significant

Powdery Mildew.

The disease incidence of Uncinula necator correlated significantly to the mean temperature in December and to temperatures one year ago in January and February (0.42**, 0.28*) and to the absolute minimum of the two preceding winters (0.20 ,0.40**). The step by step regression analysis rejected these monthly means and indicated a significant influence of the absolute minimum of the two preceding years.

Hill (1990) reported a similarity in relation to the influence of winter conditions on disease severity of powdery mildew in the Rheinhessen-region in Germany. He suggested a threshold of -13°C for the survival of mycelium in buds. A biological explanation of this relation is still lacking. Hill and Hermann (1995) could not prove the effect of deep temperatures on the mycelium in lab experiments or differences between infected and healthy buds.

Because the disease incidence fluctuates in periods of several years, the high correlation value for the minimum of the year before should be reliable.

Tab. 2. Results of the step by step elimination of variables for powdery mildew

	Variable	Regression coefficient ± standard deviation	Standard regression coefficient
y = 1	Disease incidence	88,94 ± 0,15	-
x = 2	Years	-0,05 ± 0,02	-0,44
x = 3	Min. temperature	0,09 ± 0,04	0,27
x = 4	Min. temperature preceding year.	0,15 ± 0,04	0,44
x = 7	Tempera. August preceding year.	0,29 ± 0,17	0,28
x = 8	Temperature July	0,26 ± 0,09	0,30
x =10	Rainfall January	-0,02 ± 0,01	-0,37
Coefficient of determination: 60 %			

Mean temperatures in July and August are correlated (0.35*/0.28*), too describing the well known effect of temperature on sporulation and mycelium growth (Delp, 1953). A correlation with the temperature in August of the preceding year was found, too. A biological effect is only possible for the overwintering cleistothecia, because bud infections set earlier in the season. Up to now cleistothecia in Southern Germany are not regarded as a relevant source of primary inoculum. Cleistothecia should have much more relevance than assumed for Southern Germany, if this correlation is not an artefact.

The monthly sum of rain was negatively correlated for January. A biological explanation was given by Hill et al. (1995), who reported about a 49 % reduction of infected buds after immersing canes for 16 h in water.

Downy Mildew

Strongest correlations were found in the weather data of August (-0,37 % for temperature and + 0,40** for rainfall). Because these features were closely correlated among themselves, the step by step analysis excluded temperature and focused on rainfall in August. For infections coming from Oospores in May, June and early July the amount of rainfall should theoretically be much more relevant than for the spread of the disease by sporulation on leaves in August. Yet, the correlation to May, June and July gave lower values. Our results (Kast + Borowka 2000) indicate for high infection potential of rain in August resulting from spores of far away landscapes in the West of our region. We found a lot of spores during rainfall without preceding sporulation condition in the vine sites nearby.

Positive correlations were found for rainfall in April, too. Weather conditions in this period perhaps may trigger the Oospore germination.

Two further correlations that gave significant results after the step by step analysis were found with temperatures of March and September of the preceding year. For these correlations we could not find any biological explanation. Perhaps it is an artefact due to intercorrelations.

Tab. 3. Results of the step by step elimination of variables for downy mildew

Variable		Regression coefficient ± standard deviation	Standard regression coefficient
y = 1	Disease incidence	6,157 ± 0,174	-
x = 3	Temperature March preceding year.	-0,160 ± 0,077	-0,26
x = 4	Temper. September preceding year	-0,291 ± 0,117	-0,31
x = 6	Temperature August	-0,014 ± 0,006	0,28
x = 7	Rainfall August	-0,013 ± 0,005	0,32
Coefficient of determination: 75 %			

Refereces

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Effect of temperature and leaf-wetness-duration on the infection severity of the grapevine downy mildew *Plasmopara viticola* (Berk. *et* Curtis *ex.* de Bary) Berl. *et* de Toni

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The obligate biotrophic oomycete *Plasmopara viticola* (Berk. *et* Curtis *ex.* de Bary) Berl. *et* de Toni is the causal agent of grapevine downy mildew and can cause severe losses if treatments are not accurately timed. Several prognostic models based on weather data are already established (Vitimeteo etc.) providing a reliable predication as to whether an infection took place in a certain area or not. But in spite of the basic factors of the pathogen's lifecycle as well as for dissemination being well known, it is not yet possible to predict the intensity of an infection event. Beside other epidemiological relevant parameters being determined and characterized in our studies the effect of temperature and leaf-wetness-duration on the infection severity was researched and quantified.

In this study the impact of infections set at temperatures ranging from 5 to 30 °C was tested for periods of leaf-wetness from 1 to 24 h.

In order to get a quantifiable parameter describing the intensity of an infection, the amount of encysted zoospores at the stomata of the host (*Vitis vinifera L.* cv. Müller-Thurgau) was set in relation to the estimated total stomata in an intercostal field. The enumeration was performed via fluorescence microscopy after staining the pathogen (see figure 1).

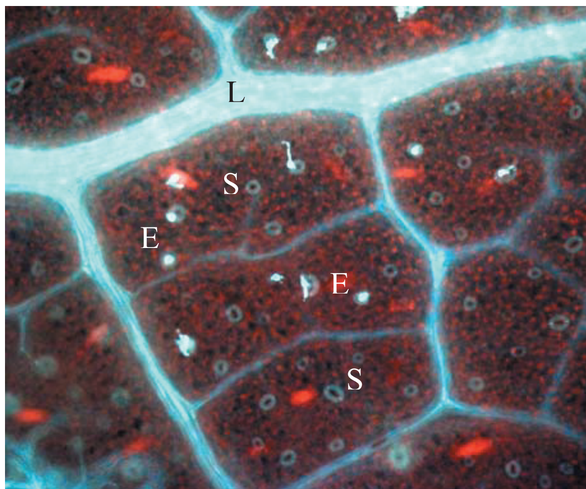

Fig. 1. Fluorescence image of an infected leaf (underside surface). E: encysted zoospore, S: stoma, L: leaf vein.

The results show that the influence of leaf-wetness-duration is only of importance in a certain time-frame, depending on the prevailing temperature (see table 1).

Tab. 1. Overview of the time-frames within an infection event leading to an significant increase in infection-severity depending on the temperature.

Range of temperature	Period of leaf-wetness leading to an significant increase in infection-severity
5 – 10 °C	3 – 6 hours past inoculation
15 – 20 °C	3 – 10 hours past inoculation
25 – 30 °C	Within first hour past inoculation

Besides the time-frame also the maximum obtainable severity of infection depends on the prevailing temperature during an infection event as shown in figure 2.

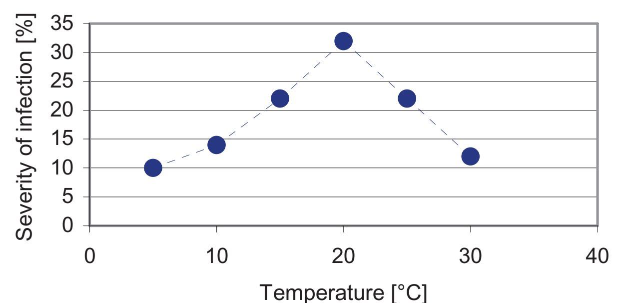

Fig. 2. Maximum obtainable infection severity depending on temperature.

The acquired data were transferred into an algorithm making it possible to calculate the infection-severity based on weather data within already existing prognosis models.

Acknowledgements

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Linear vs. Logarithmic Disease Assessment Scales: The Repeal of the Weber-Fechner Law

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The accuracy and precision of disease severity assessment data might be improved if there was a better understanding of how the laws of psychophysics actually relate to the theory and practice of phytopathometry. Horsfall and Cowling (1978) suggested that the eye is an objective photocell for measuring disease severity, but it is well established in the fields of psychophysics and plant pathology that individuals vary greatly in their ability to discriminate among visual stimuli. Psychophysics is the study of the relationship between a physical stimulus (e.g. actual disease severity of a sampling unit) and the observer's perceived (estimated) response to that stimulus (e.g. estimating percentage disease severity of a sampling unit).

The difference threshold or "Just Noticeable Difference". When quantifying a difference threshold, the reason for doing so is to determine the minimum difference between two stimuli that can be detected. As plant pathologists, we would like to know the minimum difference required for a rater to perceive that two disease severity levels are actually different. Researchers in the field of psychophysics would ask "what is the "just noticeable difference (JND)" required to perceive that a comparison stimulus is different from a standard (or reference) stimulus? For example, if the standard or "reference" stimulus represents a disease severity level of 25%, what level of disease severity is needed in a comparison stimulus for a rater to perceive that there is a difference between the two disease severity levels? Horsfall and Barratt (1945) have argued that raters cannot accurately discriminate disease severity levels between 25 and 50% because "visual acuity in the human eye is proportional to the logarithm of the stimulus". Horsfall and Barratt assumed this to be true by invoking what they referred to as the "Weber-Fechner law". Actually, the Weber-Fechner law is not one, but two separate laws: Weber's and Fechner's. Furthermore, not all human senses (including vision) perceive stimuli in logarithms. For example, Stevens (1957) showed that an audio signal at 20 JND units above a threshold is perceived as being much more than twice as loud as a sound 10 JND's above threshold.

Weber's Law. Weber found that the difference threshold (or JND) was dependent upon the weight of the standard (reference) stimulus. For example, an observer can just notice the difference between a 100g standard weight and a 103g comparison weight, so the JND in this example is 3g. Weber found, however, that if the weight of the standard was increased to 1,000g, the JND was no longer 3g but increase to 30g (i.e. the comparison weight must be heavier than 1,030g to perceive a difference. Weber investigated further and found that the size of the JND for most human senses (e.g. sight, sound, taste, touch, etc.) is a constant fraction of the size of the standard stimulus. Expressed mathematically, this is known as Weber's law: JND=kS, where, k is a constant called the Weber fraction and S is the value of the standard stimulus. This equation is usually expressed in the form: $k=JND/S$. The JND concept could also be applied to assessing disease severity, however, to date, the JND for assessing percentage disease severity has not been quantified, nor has Weber's law been tested and verified in plant pathology.

Fechner's Law. Gustav Fechner (1860) derived a relationship between stimulus intensity and perceived magnitude (Baird and Noma, 1978; Goldstein, 1989) by making two important assumptions: (i) Weber's law holds that the JND is a constant fraction of the stimulus, and (ii) the JND is the basic unit of perceived magnitude, so that one JND is perceptually equal to another JND. Fechner accepted these assumptions and then hypothesized that the mathematical relationship between perceived magnitude (P) and stimulus intensity (I) was: $P=k \log I$ where, k is a constant. Fechner's assumptions have never been tested with regards to the estimation of percentage disease severity as a distal stimulus, yet the so-called Weber-Fechner law is often invoked to legitimize logarithm-based disease assessment scales. Since Horsfall and Barratt (1945) published their abstract, it has been assumed for 60 years (but not yet proven) that a log increase in the stimulus (disease severity) is required to perceive an increase in the estimated magnitude of the stimulus (estimated disease severity).

Objective*s*. The purpose of this study was to: (i) employ the method of comparison stimuli to test Weber's law by quantifying the JND threshold for percentage disease severity in downy mildew of grape pathosystems, and (ii) determine the JND, Weber's fraction, and crossover points for three reference (standard) levels of disease severity (25, 37, and 50%) in the grapevine downy mildew pathosystems.

Method of comparison stimuli. The method of comparison stimuli (Baird and Noma, 1978; Goldstein, 1989) was used to determine the JND of the downy mildew of grape pathosystem. A computer program "Comparison Stimuli" was written (Nutter et al., 2001), and all disease severity images for this program were generated using the computer program "Severity.Pro" (Nutter and Litwiller, 1998).
Using the method of comparison stimuli, a reference stimulus was presented along side of a "comparison stimulus" to each rater as shown in Figure 1. Each rater was then asked to determine if the comparison stimulus (on the left) was less than, greater than, or equal to the

reference stimulus displayed to the right. Once each response (<, >, =) was recorded, a new pair of comparison and reference stimuli would be displayed on the computer screen. This process was repeated for a wide range of comparison stimuli, in random order. In this study, three reference stimuli (25, 37, and 50%) were selected for study??), and comparison stimuli varied plus/minus 25% in 1% increments. Each comparison stimulus was randomly paired with one of the reference stimuli (either 25%, 37%, or 50%) on a computer screen. Therefore, for each reference stimulus, there were 50 comparison stimuli, plus one comparison stimulus that was equal to the reference stimulus in percentage disease severity (i.e., 51 comparison images x 3 reference stimuli = 153 comparison images per disease pathosystem). Ten raters participated in each pathosystem study and each study was repeated 3 times. To avoid rater fatigue, each rater was provided two 5-minute rest periods, one after the 51st comparison and the second after the 102nd comparison.

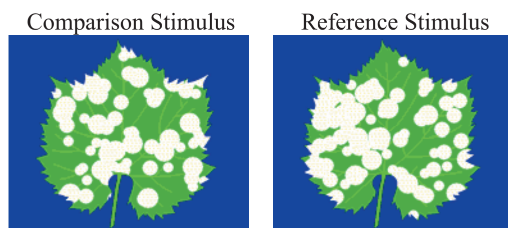

Fig. 1.

The JND and Weber's fraction. The JND was operationally defined as the absolute difference between the disease severity percentage values beyond which all rater responses were correct, minus the percentage disease severity of the reference stimulus. Weber's fraction was calculated by dividing each JND by the reference stimulus (for this example, 7% / 25% = 0.28). For the JND below the 25% reference stimulus, the JND for Rater #10 was 4 % (|21% minus 25%| = 4%), and the Weber fraction was 0.16??).

Crossover Points. Crossover points were defined as the mid-point between the last incorrect response and the first response beyond which subsequent comparison responses were correct These data were then used to determine if visual acuity was proportional to the logarithm of the intensity of the stimulus, i.e. to test Horsfall and Barratt's assumption that raters could not accurately discriminate among percentage disease severity levels in the 25 to 50% range.

The JND, Weber's Law, and crossover points in the grape downy mildew pathosystem. The Weber fractions above (0.164, 0.219, and 0.144) and below (0.264, 0.115, and 0.144) the 25%, 37%, and 50% reference stimuli (respectively) were not significantly different from one another ($P \leq 0.05$)??). Raters could accurately discriminate between 25 % and 50% disease severity, with a mean crossover point above the 25% reference stimulus of 30.0 ± 3.66% and a mean crossover point below the 50% reference stimulus of 44.2 ± 2.75% (Figure 2).As with leaf rust of wheat, another disease level that lies between 33.6 and 41.5% (~ 37%) could be added to the Horsfall-Barratt scale, thus making this scale linear between 25 and 50% disease severity for grapevine downy mildew. Thus, for grapevine downy mildew, Weber's law also holds for reference stimuli of 25, 37, and 50%, but Fechner's law does not hold because a doubling of the intensity of the stimulus (i.e., a log increase in disease severity) was not needed for raters to perceive a difference in the estimated response between 25 and 50% disease severity.

For 60 years now, plant pathologists have been told that the Laws of Psychophysics dictate that rating scales used to visually assess disease severity should be logarithmic (Horsfall and Barrett, 1945). No supporting data from the field of psychophysics was presented by Horsfall and Barratt (or has yet been presented) to support their conclusion that raters perceive increasing disease severity in logarithmic steps (according to the Weber-Fechner law). Moreover, there is no Weber-Fechner law *per se*, but two separate laws, Webers's and Fechner's. Horsfall and Barratt did not test Fechner's two assumptions needed to derive Fechner' law. Using the classical psychophysics method of comparison stimuli, it was shown here that Weber's law holds true for reference stimuli of 25, 37, and 50% disease severity, but Fechner's law does not. This study is also the first to demonstrate that raters can discriminate another level of disease severity between 25% and 50%, thus, calling into question the validly of employing logarithmic scales while assessing disease severity.

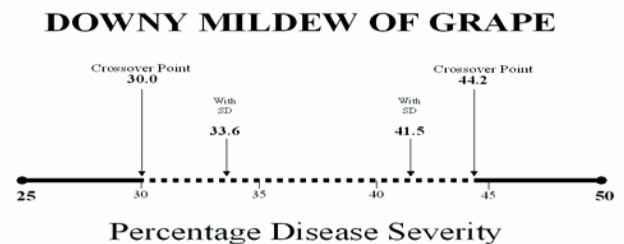

Fig. 2. Use of the method of comparison stimuli to determine the "Just Noticeable Difference (JND) and cross-over points for disease severity levels between 25 and 50%

Epicure, a geographic information system applied on downy and powdery mildew risks of epidemics on the Bordeaux vineyard

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Since more than a century, cryptogrammic diseases oblige European winegrower to protect its vineyard against downy and powdery mildews. Modelling of these diseases offers the possibility to evaluate the risks of epidemics, in order to improve the strategy of treatments. ITV France uses in Bordeaux "Potential System" (PS) models developed by the private society SESMA directed by S. STRIZYK.

Since 2000, a Geographic Information System (GIS) named Epicure, is developed on the Bordeaux vineyard. Epicure is requested for gathering a ten years data base to develop a decision tool for winegrowers : since 2003, a cartography of weekly forecasted risks is available for the Bordeaux winegrowers on their professional Website (CIVB). This experience is now extended to other French vineyards (Cognac, Toulouse, Angers, Nîmes) using Web interfaces.

Epicure works on different networks and is based on specific software developments :

Modelling network:
40 meteorological stations (MS) are distributed on the Bordeaux vineyard. They register hourly temperature and rainfall.
50 untreated parcels are distributed near the MS. They are weekly controlled by technicians and winegrowers.
Downy and powdery mildews as well as Black rot behaviours are simulated by the PS models delivering data on each MS (figure 1)

Fig. 1. Theoretical Frequency of Attack (TFA) simulated by the downy mildew PS model on the Bordeaux vineyard (2005).

The Epicure data base is developed with the Relationship Data Base Management Software Oracle. It is adapted to our observation protocols and gathers as well level of attacks registered on untreated plots, climatic, and simulated data.

A GIS software (Arcview ESRI) coupled with specific ITV France development is used in between Epicure and PS models to produce automatically a great number of thematic maps.

A geostatistical module, based on krieging method and Isatis software is set up for each disease, parameter and vine stage, as shown in figure n° 2.

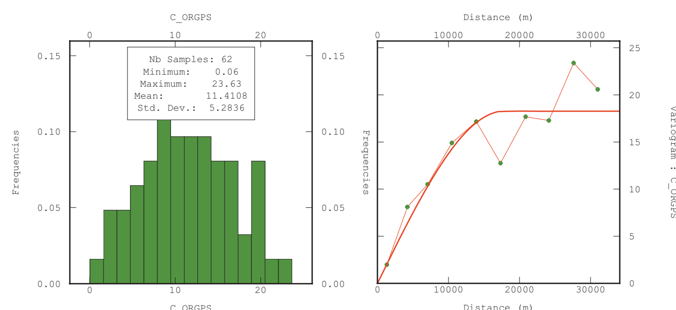

Fig. 2. statistical distribution of the downy mildew TFA simulated by the PS model, (left) and distribution of these values according to the distance between each couples of MS (right).

The observed differences of TFA values according to distance between couples of MS allows to define a specific variogram as a model of interpolation. This method allows to deliver an information on each point of the vineyard (figure 3).

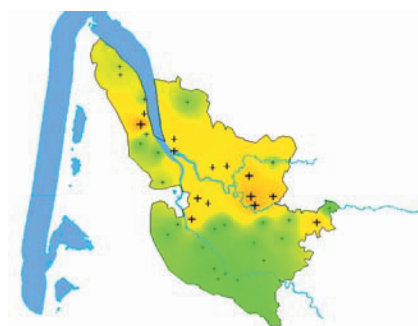

Fig. 3. krieged representation of the TFA on the Bordeaux vineyard (2005).

This krieging method also allows to evaluate the standard deviation and thus gives an appreciation of uncertainness around the studied MS or untreated plots.

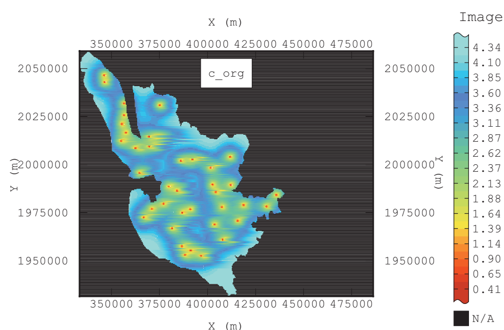

Fig. 4. uncertainness map of the TFA based on standard deviation of the krieged values.

Client applications :

Animation of the growing Epicure network needs Web applications. The Bordeaux ITV France station allows to its partners to access to these informations by means of an Intranet Website based on ArcIMS (ESRI) and Php technologies.

This Website allows to technicians of other distant vineyards to fill Epicure with their data gained on their own untreated network. They so can get back valuable informations via maps of simulated or observed attacks, as shown on figure n°5 for the Cognac Vineyard.

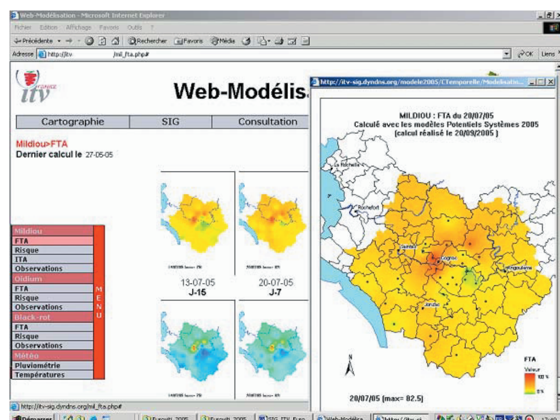

Fig. 5. example of the intranet map viewer on the Cognac Vineyard

Conclusion and perspectives

ITV France is now going to develop nomad systems using differential geographic positioning systems (DGPS) to get directly data from field to the core Epicure information system.

We then plan to extend the system to the vineyard area of the south east of France (Nîmes).

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A powdery mildew/grapevine simulation model for the understanding and management of epidemics

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The grape-powdery mildew pathosystem is characterised by: i) a polycyclic pathogen capable of explosive multiplication, ii) a host population with a high degree of spatial structure at the field level but with a complex architecture of leaf and berry structure at the plant level iii) rapid changes in susceptibility to disease over time (Gadoury *et al*., 2003; Ficke *et al*., 2003), iv) high levels of human interference. The resulting dynamical interactions are poorly understood. A rational approach for reducing fungicide treatments will almost certainly depend on an improved understanding of the interactions that trigger early infection and the most invasive spread of powdery mildew.

We developed a spatially explicit epidemiological simulation model that couples vinestock growth, with the spread of powdery mildew. This model will be further used as a research tool to (i) grade and quantify the most important factors, which modulate the dynamical interactions (ii) to simulate spatio-temporal dynamics starting from various climatic conditions, production system and pathogen initiation, (iii) to identify the lack of knowledge, (iv) to assess the relevance of variables particularly difficult to measure or to conduct experiment on, (v) to test some strategies of vine management to control invasive spread.

In this paper, we compare simulations of epidemics from different environmental data that reflect contrasting yet representative seasons of vine growth. We examine how small differences in the onset of an epidemic affects changes in host and pathogen factors that lead, in turn, to large differences in disease levels at the time of highest berry susceptibility.

Model derivation. The model simulates the development of a single vine stock during a single season. This is coupled with the simulation of inoculum and disease dynamics from primary infection of susceptible leaves and secondary infection as disease spreads from sporulating lesions. Functions, parameterised from literature or experimental data, are used to describe growth of the host and spread of the pathogen. The model input parameters characterise the crop system (number of buds, training system), and conditions of growth for the vine and the pathogen. Input variables are environmental (temperature, wind speed and direction) or are related to the pathogen (location and onset of primary infection). The environmental input variables dictate growth of the crop (appearance and growth of organs) and spread of the pathogen (latent period, infection, lesion growth, spore production and release). Infection and lesion growth are also function of leaf age. Spore dispersal is described as the motion and short-range dispersal of a large sample of particles released from each colony. The quantity of spores captured by a leaf depends upon its distance to the source and is proportional to its surface. The coupling between the model of growth of the host and that of the development of the pathogen occurs at this main step.

Model output. The model describes over time

i) changes in inoculum (density of spores over time in the region surrounding the vine or total density of spores produced by the vine),

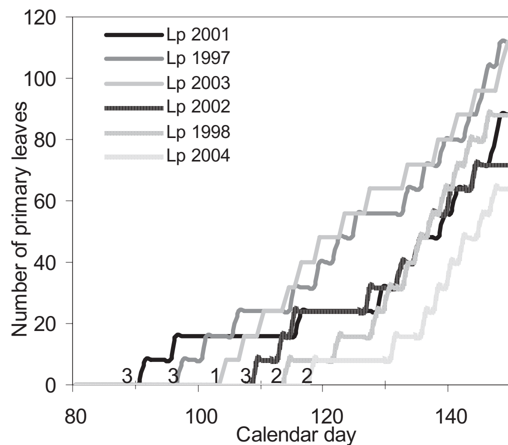
ii) the number and location of healthy and infected (latent or infectious) host organs (including primary and secondary leaves),

iii) the leaf age classes. Based on our data of ontogenic resistance, leaves are classified in 4 susceptibility classes: s1, leaves younger than 5 days (highly susceptible), s2, leaves older than 5 and younger than 10 days (susceptible), s3, leaves older than 10 and younger than 20 days (low susceptibility), s4, leaves older than 20 days.

iv) a visual representation of plant organs that provides a more accurate perception of the development of a single vine stock over time. This output consists of files representing the state of the vine stock at each time step together with all other necessary information: coordinates, size, age, health state of all stored elements.

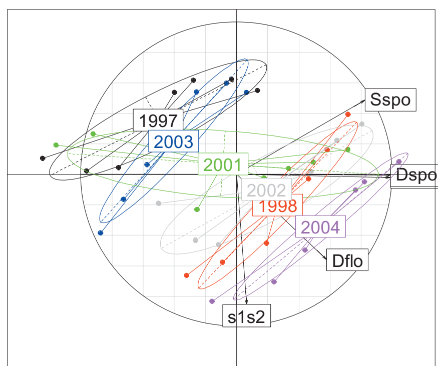
Model behaviour**.** To illustrate the model behaviour, we simulated epidemics using environmental data that reflect different contrasting seasons of vine growth: (1) 2003 characterized by an early bud break (day 104) and an early flowering (day 152), (2) 1998 or 2004 a late bud break (day 114 and 118), late flowering (day 159 and 163), and other conditions with a cool period after bud break (3) 1997 and 2001 very early bud break (day 97 and 92) with respectively early (day 150) or later flowering (day 158), and 2002, a normal bud break (day 109) with late flowering (day 165) (Fig. 1). For simulations, the day of bud break is achieved when the accumulated sum of the mean daily temperature above 10 °C reaches 90 starting from day 1, and 380 for the day of flowering.

Fig. 1. Number of primary leaves simulated from different environmental data.



We use the model to test the sensitivity of an epidemic to plant phenology during the time lag of primary infections (from a stage 1 to a stage 7 expended leaves, later called L1 to L7) and to explain differences in epidemic development according to differences in the development of host (leaf age structure, phenological stage at inoculation) and pathogen components (sporulation events, spores captured).

Fig. 2. Description of the different years according to a principal component analysis based on: day at bud break (Dbud), day at flowering (Dflo), day at first sporulation event (Dspo), phenological stage at 1rst sporulation event (Sspo), average percentage of <10 days leaves during first sporulation event (s1s2).



Results

Simulated epidemics were characterized by an increasing rate of disease progression on leaves until the last shoot topping. For 2003, 1998 and 2004, disease was most severe for an epidemic initiated at the first leave stage (day 105, 115 and 119), whereas for 1997, 2001 and 2002, characterized by a cooler period after bud breaks, epidemics were most severe when initialized at L2 to L3 stage. In relation to the critical time for berry infection (flowering) a significant reduction in disease severity was detected when initial infection was delayed after these stages and may be highly variable depending on the year (Fig. 3).

When comparing instead of stages, the dates of primary contamination, difference between years can be enhanced. For example, for primary contaminations occurring at day 115, a vine with late bud break (like 1998) could present 56% of diseased primary leaves at flowering against 18% for a vine with early bud break as in 2003. To contrast, for early primary contaminations at day 105 and only one event of ascospore release, the year 1998 (or a variety having a late bud break) can escape from the epidemic.

Growth of the vine was characterized by a progressive change from a leaf population of age classes corresponding to very high susceptible leaves (s1 less or equal to 5 day old) to a leaf population of age classes corresponding to low susceptible leaves to infection by powdery mildew. Profiles were different depending on the year (Fig. 4).

A principal component analysis performed on different host components showed that the number of leaves infected at flowering was negatively correlated with the phenological stage at the beginning of the first sporulation event, and that the number of infected leaves during first sporulation was positively correlated with the average percentage of susceptible leaves at this time. Variations between years comes from differences in host growth during cool period (2001-2002) modifying the landscape of susceptible leaves at early infection. A cool period after bud break and during the first fungus cycle, increase the latency. A consequence of this is a release of spores when leaves are not susceptible anymore. However we do not have any data to support this result. The effect of phenological stage of the primary infection on disease severity probably result on both a dilution of susceptible leaves (s1 + s2) with older leaves combined to the increase in distance between primary infected leaves and these susceptible leaves.

Conclusion

By examining the behaviour of a characteristic feature of powdery mildew epidemics, the link between disease severity at flowering and the time of initial contamination, for contrasting environmental regimes, we have used a relatively simple but very important example for demonstrating the potential of the model as a research tool. Experimentation to examine other epidemiological components of the system is ongoing, in particular, different component of the pathogen aggressiveness as well as the influence of vine management and vine vigor on the leaf susceptibility.

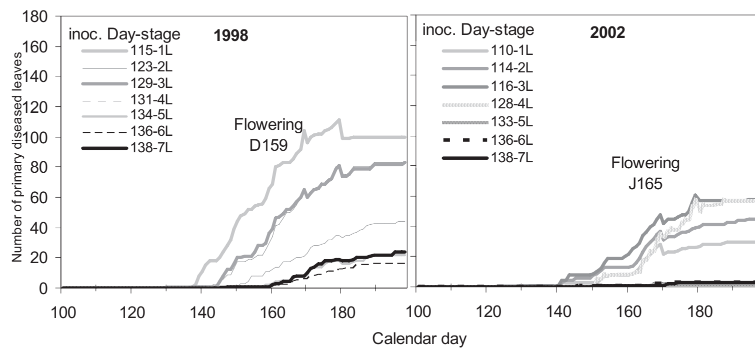


Fig. 3. Time evolution of the number of primary diseased leaves for several stage of primary inoculation (one leaf – L1, to 7 expended leaves – L7) and two sets of environmental data (1998 and 2002). Inoculations were located on the first primary leaf.

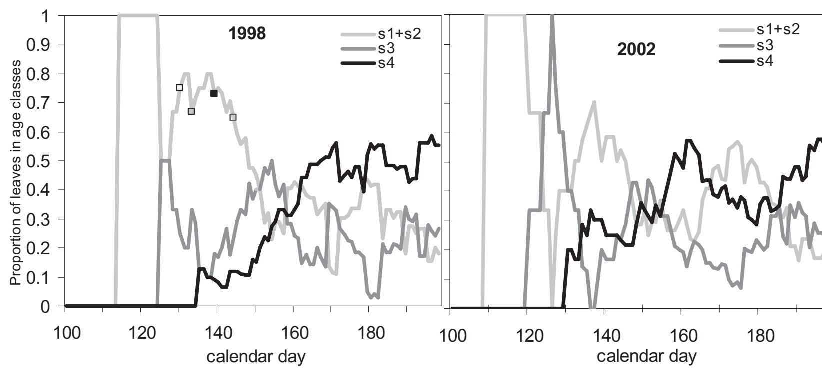


Fig. **4.** Time evolution of the leaves in age classes according to simulation for the environmental data of 1998 and 2002; S1=leaves younger than 5 days; S2=leaves older than 5 and younger than 10 days; S3= leaves older than 10 and younger than 20 days; S4=leaves older than 20 days.

Downy mildew forecast regarding primary and further soil borne infections based on a splash algorithm and a microclimate model

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Since the first downy mildew forecast models have been published it was difficult to predict the date of primary infection. The year 2005 can serve as current and serious example for this fact: Based on appearing oil spots and the local weather record the date of primary infection was antedated on May 7, 2005, in several German vine growing regions. Cold weather conditions until May 20, 2005, led to an extremely long incubation period of about 14 days. Over and above, leaves seemed to be too small for successful infection in the first decade of May. Due to the camouflage of the disease start and the epidemics progress, primary infection was not observed by the local advisory service as well as by most of the regional vine growers. They started fungicide applications at the end of May. This was rather too late in 2005, regarding the strong primary infection and severe secondary infections. In some vineyards of the Rheingau region, Germany, this underestimation of the epidemic led to an economic loss of more than 80 %.

Miscarriage of disease development like in 2005 is unacceptable and requires effective research programmes aimed at optimised disease management recommendations of the advisory service. Current downy mildew forecast systems used for more than ten years by the German advisory service for viticulture mainly consider the secondary disease cycle of *Plasmopara viticola*. Genetic studies of Loskill et al. (2003) and Loskill (2004) indicated, that primary infection could be followed by several soil borne infections causing superimposition of primary and secondary cycles. Regarding the fact, that soil borne infections may occur not only at the beginning of the epidemic but also in June and July, research should also focus on this part of the disease cycle.

Actually, the estimated dates of the primary infection and further soil borne infections, respectively, are only based on a critical rain rate in the period of oospore maturation. A rain rate that exceeds this threshold is assumed to induce a splash of sufficient height to transport the inoculum from the soil surface into the vine canopy. Therefore, the aim of a three year project was to develop a disease model that estimates the dates of primary and further soil borne infections.

In the first part of the project a splash algorithm was developed in order to describe conditions for inoculum transportation. Within this project, the splash height was calculated by type and rate of precipitation for the first time. Splash reaches highest levels in the canopy during thunderstorms, lowest levels during widespread rain and intermediate levels during showers. In general, higher splash levels occur within vine rows without cover crops compared to bare soil without any crop. So-called canopy drip drops are responsible for this phenomenon. They are larger than typical rain drops and therefore have a higher kinetic energy although their fall velocity may be less. Bare soil induces higher splash levels than grass vegetation.

In the second part of the project microclimatic conditions in the upper soil layers were calculated with view on maturing oospores. The existing microclimate model AMBETI/BEKLIMA (Braden, 1995) was adapted to viticulture. This model calculates the temperature and the water content of the upper soil layer of 5 cm depth, which contains most of the overwintered inoculum.

Based on a redundant approach the developed primary infection model enables the user to evaluate the risk of soil borne infections. The program includes the following items:

- days required for germination of oospores;
- splash height;
- primary infection index.

For practical purposes of the advisory service the model was implemented into the "Agrometeorological Advisory Software" package AMBER (in German: AgrarMeteorologische BERatungssoftware). AMBER also includes an optimised model of the secondary disease cycle and a growth model regarding vine phenology. The validation of the complete model will be conducted in the following growing periods (2006, 2007).

Acknowledgement

We thank the Federal Ministry for Food, Agriculture and Consumer Protection (Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz, BMELV, Bonn, Germany) for financial support.

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Session 8:

Economical and technological aspects of disease management

Juice Composition and Yield from Ontario *Vitis vinifera,* cultivar Chardonnay Grapes are moderated by severity of powdery mildew infection

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Powdery mildew (PM) diseases are common in most fruit-growing areas of the world and under optimum conditions for disease development can result in significant yield losses and poor juice quality. In Canada, Ontario is the largest grape-growing area, and is a region prone to PM infection from *Erysphe necator.*

Previous studies seeking to determine the impact of PM on grape and juice quality have tended to describe PM infections merely as "severe". That is, detail on the nature of the infection was not given, and description of the severity of fruit infection (when specified) was based on assessment of the area of the cluster affected rather than that of individual berries. While it can be assumed that berries with severe symptoms at harvest may have adverse effects on juice quality, it has not been demonstrated whether less severely affected fruit may be used successfully for the production of quality juice or juice-based products, such as wine.

Whole clusters of *Vitis vinifera,* cultivar Chardonnay grapes were hand-harvested from a commercial vineyard in the Niagara Peninsula, Ontario, Canada. Individual grapes were removed from their clusters and sorted according to degree of PM infection into five categories (treatments). The following visual assessment guide was used, with categorization based on the percentage of the berry surface showing scarring: '*Absent'*: 'Clean' fruit - 0% berry scarring; *'Low'*: More than 0% but less than 25% berry scarring; *'Moderate'*: Greater than 25% but less than 100% berry scarring; '*Severe'*: 100% berry scarring; '*Vineyard sample'*: Grape bunches were randomly selected from harvested fruit. The weight of fruit per treatment varied but a minimum of 2 kg fruit per treatment was used for vinification After sorting, the grapes were crushed, de-stemmed and pressed. Basic chemical and physical measurements were performed on the juice, and fruit:juice yields were calculated. Descriptive analysis techniques were used to evaluate wines produced from the various juice treatments.

The volume of juice extracted from the grapes decreased with increasing degree of PM infection. This result would likely have significant commercial implications; in particular in the case of the severe infection (*Severe*), where a 44% loss in juice yield was observed compared to no infection (*Absent*). The reduction in yield is attributable to berry dehydration in the vineyard.

After pressing, a trend of increasing ºBrix as infection severity increases was observed ($F = 64.66$, $p<0.001$). In the case of the severe infection, a 16% increase occurred relative to no infection. It remains to be determined whether the higher ºBrix in infected grapes is solely due to an increase in sugar concentration, or whether other extracted components – such as pectins, glucans, or phenolic compounds – may be contributing to the soluble solids content.

Juice pH increased significantly across the *Low, Moderate*, and *Severe* treatments ($F = 16.75$, $p<0.001$). The pH of all treatments, and particularly *Moderate* and *Severe*, would be of potential concern for juice producers. At these elevated pH's, oxidative reactions normally detrimental to juice quality proceed more rapidly, and growth of common spoilage microbes are favored.

Titratable acidity (TA) was highest in the *Absent* and *Severe* treatments ($F = 87.17$, $p<0.001$). Low to moderate levels of PM infection appeared to reduce TA. In the *Severe* treatment, the higher TA may be due to concentration of acids from berry dehydration. Visually, the pressed juice became darker and more turbid as the severity of PM infection increased

The data for the 0, >25 and 100% infected treatments is presented below: only sensory attributes that differed significantly are displayed. As extent of grape infection increases, tropical flavour intensity decreases in the resultant wines. Citrus aroma and flavour is also significantly reduced in the 100% treatments compared with both clean and >25% wines. Unique and less desirable sensory attributes are introduced in wines made from infected fruit; specifically, caramel flavour, earthy aroma and earthy flavour. The identity of the aroma-active compound(s) responsible for these characteristics is currently under investigation. Viscosity and bitterness intensities were also higher in the two infected treatments. Interestingly, perceived acidity was highest in the >25% treatments, and lowest in the 100% wines. Taken overall, these data demonstrate the capacity for powdery mildew-affected fruit to affect taste, tactile, aroma and flavour characteristics in the resultant wines, and that these effects are moderated by the degree of infection.

Production of quality juice and juice-based products is dependent on healthy, disease-free fruit. Previous research on PM in grape has examined the effects of severity as measured by the area of the cluster infected rather than with consideration to the nature of the infection. Grading of fruit according to the proportion of the berry infected offers a more precise measure of PM severity, and therefore of how it may impact juice quality. Chardonnay grapes from the Niagara region were thus graded, and the resulting juices analyzed. Overall, the data confirm that PM has considerable impact on juice composition and yield, although this is moderated by the degree of infection. This

suggests commercial and processing implications for grape growers and juice producers.

Tab. 1. Influence of severity of powdery mildew infection on selected juice quality parameters

	Severity of Powdery Mildew Infection[1]				
	Vineyard sample	Absent	Low	Moderate	Severe
Juice yield (L/kg)[2]	0.34	0.48	0.44	0.39	0.27
°Brix - after crushing	24.1 a[3]	23.4 a	23.6 a	23.6 a	26.2 b
(n,sd)	(2, 0.14)	(2, 0.00)	(2, 0.00)	(4, 1.20)	(3, 0.20)
- after pressing	24.1 a	23.8 a	24.2 ab	24.8 b	27.5 c
(n, sd)	(5, 0.52)	(3, 0.00)	(3, 0.35)	(3, 0.20)	(4, 0.35)
pH - after crushing	3.39 a	3.36 a	3.38 a	3.48 b	3.64 c
(n, sd)	(3, 0.03)	(3, 0.05)	(4, 0.01)	(4, 0.07)	(3, 0.01)
- after pressing	3.46 a	3.50 ab	3.47 a	3.55 b	3.67 c
(n, sd)	(6, 0.06)	(7, 0.05)	(6, 0.04)	(5, 0.02)	(4, 0.04)
Titratable acidity (g/L)	8.30 b	8.50 a	7.65 d	7.95 c	8.45 a
	(2, 0.00)	(2, 0.00)	(2, 0.07)	(2, 0.07)	(2, 0.07)

[1] Absent, low, moderate and severe categories based on area of individual grapes showing visual scarring: 0, 1-24, 25-99, and 100%, respectively

[2] Volume juice extracted after pressing L/ weight of fruit (kg)

[3] Within each row, means followed by the same letter do not differ significantly (Fisher's Protected $LSD_{0.05}$).

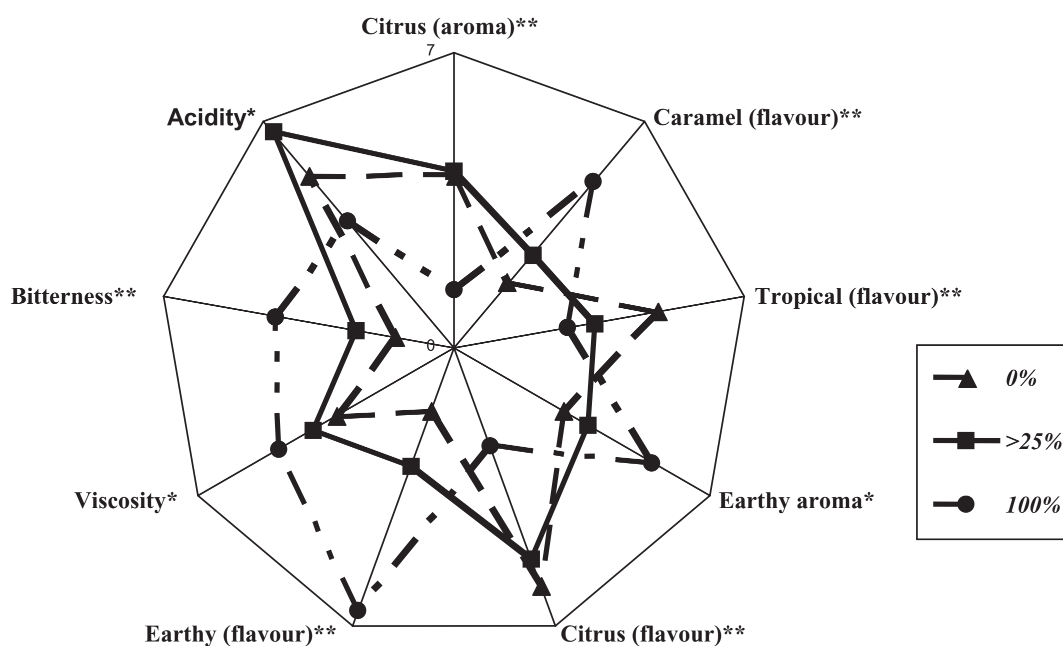

Fig. 1. Effects of powdery mildew infection on final wine quality

Detection of Powdery Mildew in Grapes using a DNA Assay and Near Infrared Reflectance Spectroscopy, and Assessment of Chardonnay Wine Quality

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Wines made from powdery mildew-affected grapes have reduced quality and negative sensory attributes, although data for the effects of quantifiable amounts of powdery mildew on wine quality for cultivars commonly grown in Australia are scarce (Stummer et al. 2005). Estimating disease severity by visual assessment is difficult in large consignments of fruit, particularly after machine-harvesting. Accurate measurement of disease severity is required to assess and grade fruit at the weighbridge, as such information would enable wineries to make more informed decisions about the utilisation of fruit and must. The objectives of this work were to assess the effects of known amounts of powdery mildew on wine quality and to develop DNA-based tools and near infrared reflectance (NIR) spectroscopy for detection and quantification of powdery mildew in grape samples at the winery.

Chardonnay bunches were inspected in the vineyard and sorted into disease severity categories based on a bunch assessment key designed by R. Emmett and T. Wicks (pers. com.) over five vintages (2001-2005). Infection categories were later confirmed by microscopic observation.

Four batches of grapes representing each of four disease severity categories (0, 1-5%, 10-30%, 31-100% of the bunch with sporulating powdery mildew) were crushed and vinified in 2001 and 2002 (1). Grapes were harvested at commercial maturity on one day in 2001 and over 2 weeks as they reached a standard sugar ripeness in 2002. Juices and wines were subjected to standard chemical analysis. Sensory analysis comprised duo/trio tests to identify differences and descriptive analysis to describe differences in aroma and palate attributes (Stummer et al. 2005).

Southern blots and slot blots were hybridised with the *E. necator*-specific clone, pEnA1, obtained from a plasmid library (Stummer et al. 2000). *E. necator* DNA was quantified by slot blot hybridization using DNA extracted from grapes, must, juice, clarified juice and wine. The amount of DNA per sample was estimated by comparing the hybridization signal with known amounts of *E. necator* DNA.

Chardonnay grapes with various degrees of powdery mildew were homogenized and scanned in reflectance mode with a FOSS *NIRSystems 6500*. The homogenates were also analysed for total soluble solids (TSS, °Brix), pH and *E. necator* DNA content.

Grapes with powdery mildew generally matured earlier than healthy grapes and were smaller and lighter. Titrable acidity, total phenolic content, hydroxycinnamates, flavonoids and brown pigments in juice increased with increasing infection. Similar trends were observed for the resulting wines.

Wines made in 2002 were similar in alcohol concentration, which facilitated the perception of sensory differences among treatments. Wines made from grapes with powdery mildew were perceived as having pronounced viscosity/oiliness compared to the control, and this was correlated with the phenolic composition of the wines. Wines made from diseased grapes, especially the 31-100% infected category, exhibited pronounced fungal, earthy and cooked tomato aroma attributes compared to those made from uninfected grapes.

Clone pEnA1 hybridised to *E. necator* DNA but not to DNA extracted from grapevine or from a diverse range of microbes associated with grapevines. In addition, the probe was sensitive enough to detect 50 pg of DNA, which equates to less than 1% of the bunch infected or fewer than 100 conidia.

E. necator DNA was detected in grapes, juice and must but not in clarified juice and wine. There was a strong positive relationship between the amount of *E. necator* DNA detected in must and juice samples and the infection category assigned to the corresponding bunches following visual and microscopic assessment (2004: must; $r = 0.85$ and juice; $r = 0.94$). To determine the relationship between the amount of *E. necator* DNA in juice and disease severity, a linear regression model was assessed was made for the 18 grape samples of varying disease severity obtained in vintage 2005. The equation of $y = 1.552(x) + 3.289$ and $r^2 = 0.955$, $P < 0.001$ was obtained for these juice samples, where y = disease severity (%) and x = *E. necator* DNA content (ng/100 ng total DNA).

Strong spectral correlations with disease severity were observed over the 400-2500 nm wavelength range, including spectral changes not related to differences in pH and TSS. Principal component analysis (PCA) of spectral data showed distinct clustering of samples based on disease severity. The PCA scores were used to prepare a discriminant analysis algorithm to classify disease severity category and although a small number of infected samples were predicted in adjacent categories, all non-infected samples were classified correctly and no infected samples were classified as non-infected.

Conclusions

The *E. necator*-specific DNA clone detected the pathogen in grapes, must and juice. Compared with visual assessment, the *E. necator*-specific DNA probe provides a reliable and objective means of detecting and quantifying powdery mildew in grapes, juice and must. The detection threshold is approximately 100 conidia. NIR could discriminate the least infection category from uninfected samples, but these samples were from a small number of trial plots. To ensure a robust calibration, further material representing more diversity must be scanned.

Even small amounts of powdery mildew, as little as 1-5% of the bunch infected, resulted in increased oily, viscous mouth-feel characters. The detection tools described here offer the grape industry a means of obtaining objective, quantitative data on disease severity in grapes, must and juice samples at the winery, particularly when disease is slight and visual assessment is difficult. Such information can then be used to inform decisions about the use of grapes and must to achieve desired outcomes.

This project was supported by the Commonwealth Cooperative Research Centres Program and conducted by the CRC for Viticulture. This work was financially supported by Australia's grapegrowers and winemakers through their investment body the Grape and Wine Research and Development Cooperation, with matching funds from the Australian Government. Support from Hickinbotham Vineyard, Clarendon, SA is gratefully acknowledged.

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Session 9:

Synthetic and natural fungicides

Systemic Activity of Phosphorous Acid against Grapevine Downy Mildew

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Phosphorous acid (H_3PO_3) is effective in controlling many diseases caused by Oomycete fungi, including grapevine downy mildew. It has systemic activity but the extent of this activity in the *Plasmopara viticola*-*Vitis vinifera* system has never been documented. The purpose of this study was to explore the systemic activity of phosphorous acid used as a pre- or post-inoculation treatment.

To investigate systemic pre-inoculation activity of phosphorous acid, a 3% solution of Agri-Fos 400 (mono- and dipotassium salts of phosphorous acid) was applied to run-off to the 3rd oldest leaf of potted *Vitis vinifera*, cv. Riesling, rooted cuttings in a replicated greenhouse study. At intervals of 24 or 48 hr or 1 or 2 weeks after treatment, all leaves on the vine were inoculated with 5 x 10^4 *Plasmopara viticola* sporangia mL^{-1} and maintained in the greenhouse for 10 days when oil spots developed on untreated control plants. Digital images of each leaf were recorded and analyzed to determine the severity of downy mildew (% area) using Assess Image Analysis Software for Plant Disease. Sporangia were washed off each leaf and counted and the number of sporangia per mm leaf area was calculated.

The protectant effect of phosphorous acid treatment appears to be distributed acropetally to non-treated leaves over time (Fig. 1). This redistribution did not significantly reduce the degree of protection afforded to the treated leaf, although some of this could have been due to ontogenic resistance as the treated leaf aged through the course of the experiment.

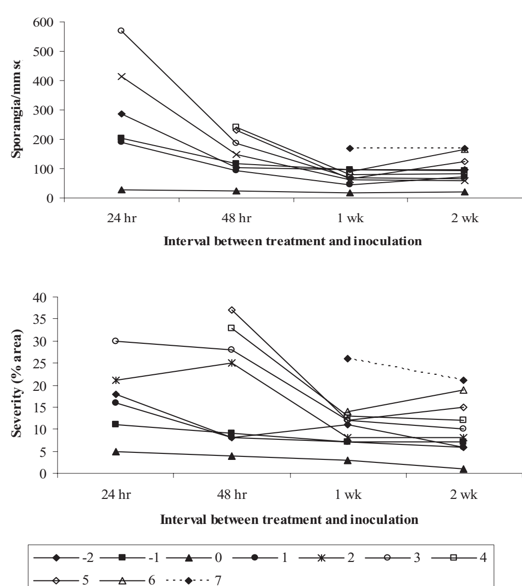

Fig. 1. Pre-infection activity of phosphorous acid: Sporangial production and disease severity on different leaves with increasing interval between treatment of leaf 0 with Agri-Fos and inoculation with *P. viticola*

To explore systemic post-infection activity, all leaves on rooted cuttings of *V. vinifera*, cv. Riesling, were inoculated with 5 x 10^4 *Plasmopara viticola* sporangia mL^{-1} and incubated under high humidity for 24 hours. At intervals of 24, 48 hr or 1 week after inoculation, a 3% solution of Agri-Fos was applied to run-off to the 3rd oldest leaf of each plant in a replicated greenhouse study. After 10 days plants were incubated under high humidity to stimulate sporulation. Disease severity and sporangial production were quantified as described above.

Phosphorous acid reduced disease severity and sporulation when applied up to 1 week after inoculation on leaf 0 (Figure 2). There was some systemic acropetal post-infection activity; however, this was much more limited than was observed in the pre-inoculation treatment.

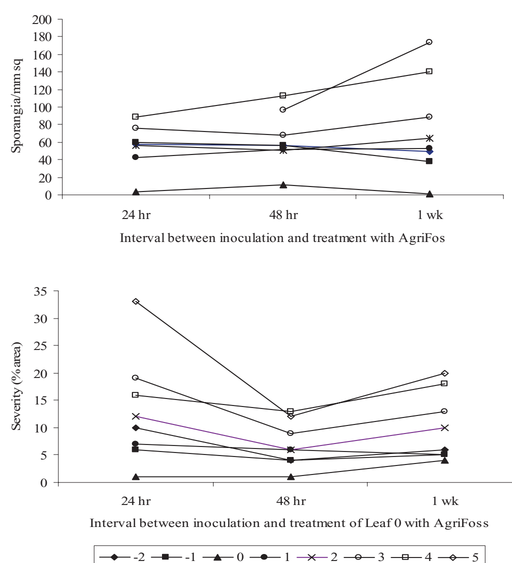

Fig. 2. Post-infection activity of phosphorous acid: Sporangial production and disease severity on different leaves with increasing interval between inoculation with *P. viticola* and treatment of leaf 0 with Agri-Fos.

The phosphorous acid treatment reduced infection on the treated leaf equally well when applied pre- or post-infection. The systemic activity was not as notable as expected. This may be attributable to the limited sink (single leaf) for phosphorous acid.

Biological control of powdery mildew – controlled conditions and field experience

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Powdery mildew of grapes as powdery mildews of other crops, are important targets for the development of control agents. We developed a biocontrol system that is capable of controlling various powdery mildew pathogens, including grape powdery mildew. In a process of development of a biocontrol system for powdery mildews, various microorganism strains that were isolated from the canopy of wild plants and commercially grown crops were grown in liquid culture, collected and sprayed on plants such as grape vines, cucumber, pepper, tomato, strawberry and barley. The treated plants were challenged by their respective powdery mildew pathogens. Plants were incubated under disease promoting conditions. Severity and incidence of powdery mildew symptoms were evaluated frequently.

The objectives of the presented work were to evaluate the effectiveness under commercial vineyard conditions compared with available chemical fungicides and to study various means of improving the biocontrol activity.

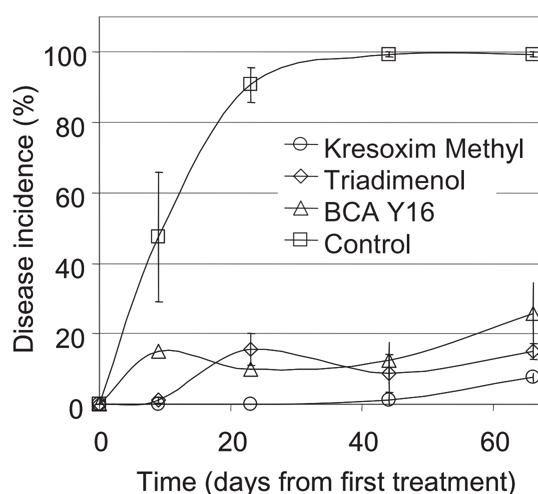

Fig. 1. Frequency of grape bunches cv. Carignane infected by powdery mildew in a vineyard experiment. Plots were treated twice a week by the biocontrol agent and weekly with the commercial fungicides starting on day 0.

In order to develop a biocontrol program for grape powdery mildew, selected bacteria and yeast isolates were tested as potential biocontrol agents of powdery mildews. The yeast isolate 16 was chosen for further testing in a commercial vineyard at the end of an intensive screening program. The biocontrol agent was compared with effective chemical fungicides in the vineyard during May-July, 2005.

Plots of the powdery mildew susceptible cv. Carignane consisted of six vines each and agents were applied each in four replicates for six weeks. The biocontrol agent Y16 was sprayed at the rate of 10^7 twice a week whereas the chemical fungicides Kresoxim methyl (Stroby at 0.02%) and Triadimenol (Shavit at 0.01%) were sprayed 6 times.

It may be important that the biocontrol agent will survive between applications on the target plant surfaces in order that it will be effective. Due to the biocontrol agent expected population decline, after treatment it was applied every 3-4 days. A comparison between the initial yeast level immediately after ninth application on the berries in the vineyard and its population immediately before the following application showed that within four days it declined from $5.43 \pm 0.19 * 10^5$ only to $2.36 \pm 0.16 * 10^5$ CFU/g berries ($\pm$SE). Thus, an effective control of the disease may have been expected in the vineyard.

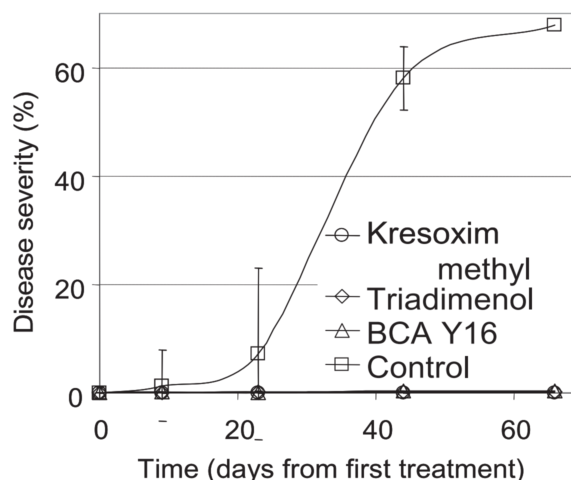

Fig. 2. Severity of powdery mildew on grape bunches cv. Carignane naturally infected in a vineyard experiment. Plots were treated twice a week by the biocontrol agent and weekly with the commercial fungicides starting on day 0.

Incidence of powdery mildew increased dramatically on bunches of the untreated vines. It reached more than 90% twenty three days after initiation of the experiment (Fig. 1). The incidence of infected bunches in plots of the two chemical treatments and the biocontrol treatment was 0-15% during six weeks after the initiation of the treatments. Three weeks later (four weeks after the last treatment) disease slightly increased and was c. 25% in the biocontrol plots and significantly less in the chemically treated plots (Fig. 1). The severity of powdery mildew reached c. 70% in the untreated control

whereas the different treatments showed minor symptoms (Fig. 2).

Concluding remarks

The biocontrol agent and the chemical fungicides were found effective in powdery mildew suppression on grapes. In other works (data not shown), we also were successful in prolonging the survival of the biocontrol agent on plant surfaces and in improving their efficacy by adding some substances to the suspension, by combining them with chemical fungicides and by changing their concentration and frequency of application.

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Systems for testing the efficacy of biofungicides and resistance inducers against grapevine downy mildew (REPCO project)

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At present grapevine downy mildew is controlled by fungicides mostly not suitable for organic viticulture, so copper is used to control the disease. Permanent applications lead to an accumulation of copper in the soil. The objective of REPCO (Replacement of Copper Fungicides in Organic Production of Grapevine and Apple in Europe) is to find new copper replacing products. Screening for new organically based fungicides as well as activators of the plants innate defence response by resistance inducers was used as a two-fold strategy. First, relatively quick and easy to handle test systems have been established to assess the efficacy of substances on the control of grapevine downy mildew and specify substances which worth further investigations. The efficacy of potential biofungicides against grapevine downy mildew was tested on leaf discs. The induction of resistance in V*itis* was tested on cell suspension culture. Potential fungicides and resistance inducers were obtained from animal and plant extracts and purified microbial compounds. Substances showing a positive effect on the control of *P. viticola* or causing a shift in pH in the cell suspension culture system are worth further investigation on plant level.

New copper replacing products studied and developed within REPCO were tested on leaf discs first. The tested products were applied in aqueous solution of various concentrations to define the range of the dosage preventing the sporulation of *P. viticola* but not harming the plant tissue. Several substances inhibited sporulation. Surfactants and stickers were used in order to improve the uptake of active compounds into the leaf and their rain fastness. The influence of several additives on the leaf disc tissue and sporulation of *P. viticola* without and with active compounds were tested.

Products studied and developed within REPCO were also tested for potentiators capacity of resistance in *Vitis*. Plants are able to defend themselves against the attack of fungi, oomycetes and bacteria with a set of defence mechanisms. This innate resistance is induced by elicitors from the surface of the pathogens and by breakdown products of the plant's cell wall. These elicitors comprise oligosaccharides, peptides and lipids.

An early cellular reaction on pathogen attack is the transport of H+- ions through the plasmalemma. In cell suspension cultures the influx of H+- ions results in an alkalinisation of the culture medium. *Vitis vinifera* cv Pinot noir cell culture was used to measure this alkalinisation as an indicative of increased plant defence (Fig. 1).

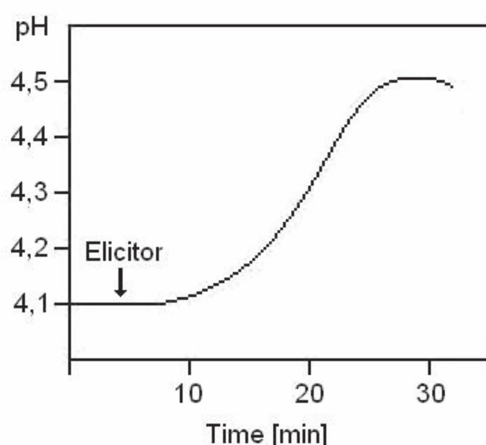

Fig. 1. Changes of the pH measured in the medium of the cell suspension culture from *V. vinifera* after addition of an elicitor.

Molecules from microbes, products from the secondary plant metabolism and oligosaccharides from animals and plants showed an efficacy against the pathogen and induced resistance response. They are tested further on plant level.

Acknowledgement

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Efficacy of KBV 99-01 against *Erysiphe necator* and *Plasmopara viticola*

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Despite the choice of effective fungicides available, new antifungal products with low toxicity for the environment and consumers are still needed to cope with fungicide resistant pathogens and deliver improved yield and quality products. In addition, the lack of good resistant cultivars has increased the interest in alternative methods and/or products to control grapevine powdery and downy mildew. KBV 99-01 is a new experimental fungicide base on the lactoperoxidase system (LPS) developed by Koppert (The Netherlands).

The aim of this work was to evaluate the efficacy of KBV 99-01 against grapevine downy and powdery mildew under controlled greenhouse conditions, in order to estimate its potential application as a new alternative to chemical fungicides.

The experimental product KBV 99-01 was tested on potted grapevine plants in two different trials against *Erysiphe necator* (syn. *Uncinula necator*) and *Plasmopara viticola*. Sulphur (Tiovit jet, Syngenta crop protection), copper hydroxide (Kocide 2000, Du Pont de Nemours) and water were used as references. Five replicates, each consisted of one plant of the susceptible cultivar Pinot Gris having 8-10 green and fully expanded leaves (at least 2.5 cm wide) were used. Plants were grown under controlled conditions (20°C and 60% relative humidity) in a pathogen free greenhouse. The products (Table 1) were applied by an air compressor system equipped with a spray gun working with a pressure of 4 bars.

Tab. 1. Products, dosages and time of application vs. inoculation used in the trials against *Plasmopara viticola* and *Erysiphe necator* under greenhouse conditions.

Commercial product	Active ingredient	Dosage	Application vs. inoculation (I)
KBV 99-01[a]	Confidential	1.5 g/l	1day after I
KBV 99-01[a]	Confidential	1.5 g/l	3 days after I
KBV 99-01[a]	Confidential	1.5 g/l	7 days after I
KBV 99-01[b]	Confidential	1.5 g/l	6 hours before and 1 day after I
Thiovit jet[a]	Sulphur	3g/l	6 hours before I
Kocide 2000[b]	Copper hydroxide	1.3 g/l	6 hours before I
Untreated[ab]	Water	-	6 hours before I

[a] used in the trial against powdery mildew, [b] used in the trial against downy mildew.

Due to the different characteristics of the two pathogens, KBV 99-01 was applied twice just before and after inoculation time in the case of *P. viticola* and in the case of *E. necator* three different application times were compared (table 1).

Replicates were arranged in a fully randomized block. Infected leaves with fresh symptoms of powdery mildew (mycelium, conidia and cleistothecia) were collected in an untreated vineyard. The artificial inoculation was done shaking the infected leaves on the plants. A water suspension of fresh sporangia of *P. viticola* was used as inoculum, with a concentration of $5*10^5$ sporangia/ml. *P. viticola* sporangia suspension was sprayed on the underside of each fully expanded wet leaves. Infected plants were incubated for 12 hours in darkness, 20 °C temperature at RH 80% for *E. necator* and 95% for *P.viticola*. Ten days after inoculation, downy mildew infected plants were placed again in 95% R.H. and 20°C in darkness overnight to promote sporulation. Eleven (*P. viticola*) and fourteen days (*E. necator*) after inoculation, the percentage of infected leaf area (severity) and the percentage of infected leaves (incidence) of both diseases were assessed on all leaves of each replicate. Analysis of variance (ANOVA) was applied on "Arcsin" transformed data, using the software Statistica 7 (Statsoft, Italy). Significant differences among treatments were determined by Tukey's test.

KBV 99-01 when applied 3 or 7 days after *E. necator* inoculation inhibited powdery mildew to the same extent as sulphur; severity was not significantly different among the three treatments. On the contrary, application just one day after inoculation allowed a disease severity as in the untreated control. Disease incidence was never significantly reduced by KBV 99-01 (fig. 1).

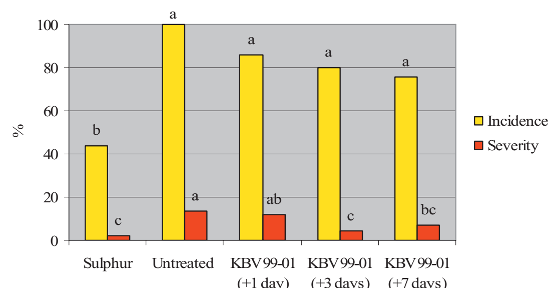

Fig. 1. Incidence and severity of powdery mildew on potted grapevine plants treated with the experimental fungicide KBV 99-01 at various times after artificial inoculation. Means followed by different letters are significantly different (Tukey's test, $P \leq 0.05$).

Similar results were obtained against *P. viticola*, where KBV 99-01 significantly reduced disease severity, but not incidence (fig. 2).

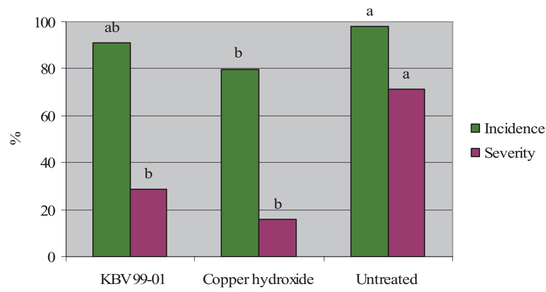

Fig. 2. Incidence and severity of downy mildew on potted grapevine plants treated with the experimental fungicide KBV 99-01 6 h before and 1 d after artificial inoculation. Means followed by different letters are significantly different (Tukey's test, $P \leq 0.05$).

In conclusion KBV 99-01 showed promising results against powdery and downy mildew of grapevine, even if not as good as the references if disease incidence is considered. KBV 99-01 thus presents good potential alternative to the chemical fungicides traditionally used to control powdery and downy mildew of grapevine, nevertheless, these trials represent only a preliminary efficacy evaluation and further studies are needed before envisaging any field application.

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Efficacy of electrolysed acid water against *Plasmopara viticola* and *Erysiphe necator*

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Electrolyzed water is produced by electrolyzing purified tap water with the addition of a small quantity of saline solution (most usually NaCl or KCl). Anode and cathode are separated by a cation exchange membrane (fig. 1). At the anode, Cl_2, O_2 and H^+ are produced. Cl_2 then reacts with water and produces HClO and HCl. Small amount of H_2 and OH^- are produced at the cathode on the other side of the membrane. The cation exchange membrane between the electrodes allows movement of cations such as Na^+, but not anions, as OH^- (Tsuji et al., 1999). The water in the cell around the anode is acid, while the water in the cathode cell is basic. The electrolyzed acid water (EAW) has a pH lower than 2.7, mostly due to the HCl; and due to the HClO, which is oxidative, the redox potential (ORP) is over 1000 mV. The electrolyzed basic water (EBW) has a pH over 11 and a redox potential around -800mV.

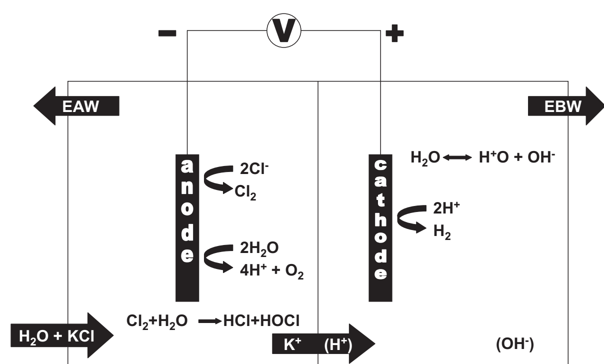

Fig. 1. The system for producing EAW and EWB

EAW has been used for over ten years as a disinfectant. It is widely used in Japanese hospitals for endoscope disinfection (Lee et al., 2004). The activity of EAW is based on its high redox potential (Tsuji et al., 1999), which causes damages on microorganism membrane (Kiura et al., 2001). The free-chlorine content of the EAW, enhances breaks and blebs on the membrane. Some studies on *Blumeria graminis* (Mueller et al., 2003) showed that EAW could have a possible uses in agriculture as a fungicide.

The aim of this research is to evaluate the potential use of EAW against the two main grapevine pathogens.

EAW was produced using Oxylyzer Ox 01 (CBCE, Italy). EAW was stabilised with the addition of EBW reaching pH 6±0.5 and an ORP of 650 mV. Deionised water was used as untreated control; copper hydroxide (Kocide 2000, Du Pont de Nemours) and wettable sulphur (Tiovit jet, Syngenta Crop Protection) were used as fungicide treated references respectively for downy and powdery mildew. The effect of experimental wetting agent X22 (Shin-Etzu, Japan) was tested, adding it (0.05 %) to water and to EAW.

Downy mildew

Trials were carried out on Pinot gris potted plants (5 plants/treatment). Plants were maintained under greenhouse controlled conditions (20° C), artificially inoculated with a water suspension of *Plasmopara viticola* (Berk. et Curt) Berl. and De Toni sporangia ($1x10^6$ sporangia/ml) and overnight incubated at 20° C and 95% relative humidity (RH). Since the efficacy of EAW last only few seconds, plants were treated with 100 ml of water, EAW with and without X22, 1, 3 or 7 hours after artificial inoculation, to see if EAW can interfere with the first stages of infection (stoma penetration and colonization). Copper and water controls were applied 6 hours before inoculation. Ten days afters inoculation plants were incubated overnight at 20°C and 95% RH to induce sporulation; afterwards severity (percentage of infected leaf surface) and incidence (percentage of infected leaves) were assessed.

Powdery mildew

Trials were carried out on Pinot gris potted plants (5 plants/treatment). Artificial inoculation was done shaking powdery mildew *Erysiphe necator (*syn.*Uncinula necator)* sporulating leaves. Plants were then incubated overnight at 20° C with high RH (80%). Daily treatments with water, EAW with and without X22 were applied for one week after inoculation. Sulphur was applied six hours before infection.

Statistical analyses of data were performed using the Statistica 6.0 software (Statsoft, Italy). Incidence and severity were respectively arcsin transformed to obtain constant variance, before analysis of variance. Duncan's test at $P<0.05$ was used to compare treatments.

As shown in table 1, EAW was not able to significantly reduce *P. viticola* incidence, probably because of the high concentration of inoculum applied. A reduction in disease severity on leaves was seen only when X22 (in water and EAW) or copper were applied. The results show that as soon as the pathogen has entered the stoma, X22 is not able to affect it.

Tab. 1. Results obtained against downy mildew with artificial infections under greenhouse controlled conditions

Products	Time (hours)[1]	Dosage (ml/l)	Severity[2] (%)	Incidence[2] (%)
$Cu(OH)_2$	6 before	0.5[3]	16.5 a	68.7 a
X22	1 after	0.5	23.4 a	60.2 a
EAW+X 22	1 after	999.5+0.5	23.6 a	76.7 a
EAW	1 after	1000	40.5 ab	80.6 a
EAW+X 22	7 after	999.5+0.5	48.5 b	81.0 a
EAW+X 22	3 after	999.5+0.5	51.6 bc	75.3 a
X22	7 after	0.5	52.7 bc	65.4 a
EAW	3 after	1000	57.6 bc	77.0 a
X22	3 after	0.5	57.6 bc	77.1 a
Water	6 before	1000	61.6 bc	86.8 a
EAW	7 after	1000	65.3 c	78.1 a

[1]Time from inoculation; [2]Means in the same column follower by different letters significantly differ (Duncan's test with $P \leq 0.05$); [3] Expressed as g/l of copper .

Regarding powdery mildew (table 2) EAW and X22 were able to reduce disease severity and incidence, compared to water applied once before or daily after inoculation. Water daily applied was able to reduce the area of powdery mildew colonization on leaves (severity), but not the incidence.

Tab. 2. Results obtained against powdery mildew with artificial infections under greenhouse controlled conditions

Products	Time [1]	Dosage (ml/l)	Severity[2] (%)	Incidence[2] (%)
Sulphur	6 hours before	3[3]	2.3 a	44.0 a
Water	6 hours before	1000	13.5 d	100c
Water	1,2,3,4,5,6,7 days after	1000	6.1 c	100 c
X22	1,2,3,4,5,6,7 days after	0.5	3.6 ab	70.0 b
EAW	1,2,3,4,5,6,7 days after	1000	4.6 b	75.0 b
EAW+X22	1,2,3,4,5,6,7 days after	999.5+0.5	3.0 ab	53.3 ab

[1]Time from inoculation; [2]Means in the same column follower by different letters significantly differ (Duncan's test with $P \leq 0.05$); [3] Expressed as g/l of commercial product (Thiovit).

The results confirm what obtained by Mueller et al. (2003). EAW to be effective should be applied during inoculation. This is quite difficult with *P. viticola* whose infections occurs during rain, because EAW quickly loose its effect if applied on wet leaves. Practical applications could be very difficult since treatments one hour after inoculation are already ineffective against the disease. On powdery mildew the efficacy of EAW is higher compared to downy mildew. This is probably due to the external growth of the fungus on leaves that can easily affected by EAW. The wetting agent X22 is fungitoxic by itself on the two pathogens, but also showed a high risk of phytotoxicity on grapevine.

The use of EAW could be an additional tool in IPM to reduce the fungicides against powdery mildew, but seems not practicable against downy mildew. Further studies will be necessary to define the right application timing on powdery mildew on grapevine.

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Properties of Sulfur in Control of Grapevine Powdery Mildew

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Sulfur has been used to provide control of powdery mildew for over 150 years, but many properties that impact its efficacy under commercial use conditions remain poorly characterized. Therefore, we sought to clarify the purported, but unsubstantiated, reduction of sulfur's activity at temperatures <18°C; to characterize the protective and curative activities of this material; and to examine the effects of rainfall and various interactive factors on disease control.

Protective and curative activities. A series of five repeated experiments was conducted in the greenhouse on single-shoot seedlings of cv. Riesling, which were sprayed to runoff with a solution containing 6 g/L of a "micronized" formulation of sulfur (or water, for the check) at eight different timings relative to inoculation with *U. necator* conidia. Sprays were applied 3 to 14 days before inoculation to assay protective activity, and 1 to 10 days after inoculation to assay curative and eradicative activities. In each experiment, all seedlings were inoculated on a single date. The youngest open leaf at the time of inoculation was marked, seedlings were incubated in the greenhouse at 24 to 28°C for 2 weeks, and disease severity (% leaf area infected) was assessed separately on each of the three leaves beneath the marked one for each of five replicate plants per treatment. After this rating, sporulation was assessed by washing the entire leaf and collecting the rinsate; conidia were then quantified with the aid of a hemacytometer. To facilitate comparisons among experiments and leaf positions, data were standardized to reflect the percent control relative to the appropriate check.

Control provided by the protective (pre-inoculation) sprays was strongly influenced by leaf position, as indicated in Figure 1. As shoots continued to elongate rapidly, new leaves were produced that were free of sulfur deposits and at increasing distance from the sprayed tissues. By 14 days after the spray application, the youngest inoculated leaf (#1) was, on average, removed by five nodes from the nearest sprayed leaf and received almost no control from that application; the oldest inoculated leaf (#3) was removed by three nodes, and received only modest control. This effect of "outgrowing" the spray coverage can be seen in the progressively improved control on leaf #3 vs. #2 vs. #1 for the various timings, e.g., 71, 60, and 31% control, respectively, when seedlings were inoculated 7 days after spraying. Apparently, vapors emanating from the sprayed leaves were insufficient to effect meaningful control on the nearby unsprayed leaves. Measured effects of the various treatments on spore production were very similar to those on estimated disease severity.

Curative sprays provided complete control when applied 1 or 3 days after inoculation. By 7 and 10 days post-inoculation, colonies of moderate and heavy densities, respectively, had developed before sprays were applied, and it was sometimes difficult to estimate the degree of remaining viability following the spray treatment; thus, we report reductions in sporulation as the more dependable measure of treatment effects. As shown in Fig. 2, conidium production 2 weeks post-inoculation was reduced by 91 and 84% following the 7- and10-day curative treatments, respectively.

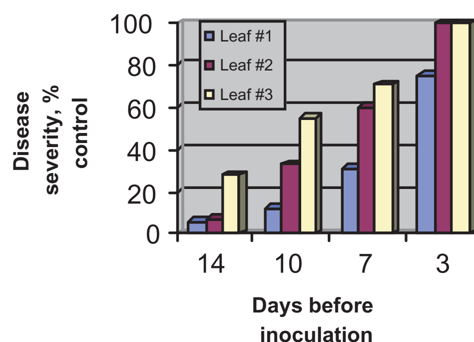

Fig. 1. Protective activity of sulfur (6 g/L) applied 3 to 14 days before inoculation with *U. necator* conidia, expressed as % control of leaf area diseased 14 days after inoculation. Leaf position #1 = 1st leaf below the youngest unfolded leaf at the time of inoculation, #2 = 2nd leaf below, etc. Data represent the mean for 25 leaves per position (5 replicate plants per experiment x 5 experiments).

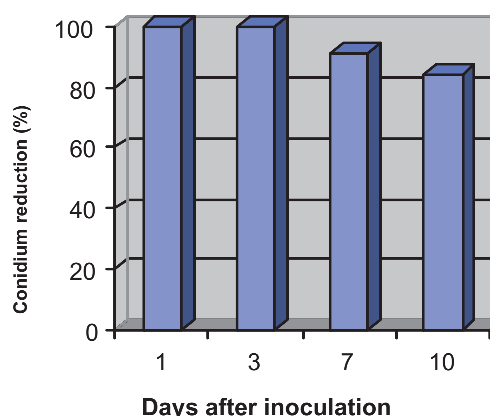

Fig. 2. Curative activity of sulfur (6 g/L) applied 1 to 10 days after inoculation with *U. necator* conidia, expressed as % reduction in conidum production (relative to the water check) 14 days after inoculation. Leaf position #1 = 1st leaf below the youngest unfolded leaf at the time of inoculation, #2 = 2nd leaf

below, etc. Data represent the means for 25 leaves per position (5 replicate plants per experiment x 5 experiments).

Effects of temperature on activity.

To determine temp effects on vapor activity, Riesling seedlings grown in paper cups were inoculated with 5-µl droplets of a *U. necator* spore suspension and incubated overnight at room temperature. The next morning, 5.5-cm-diameter filter paper disks were soaked in a 6-g/L sulfur solution and placed singly into the cups, which then were sealed and incubated for 8 days at either 14° or 28°C with a 12-hr photoperiod. Colony diameters and sporulation were determined at 20X magnification at each of three inoculation points per leaf. In a related experiment, heavily-infected Chardonnay leaves were excised from vines, existing condia were washed off, and the leaves were sealed within double petri dishes containing filter paper disks soaked in a 6-g/L sulfur solution. After 1 wk incubation at either 14° or 28°C, resporulation (condia per chain) and germination frequency of new conidia at room temperature were determined. All treatments were replicated five times and both experiments were repeated.

In these experiments, saturation vapor levels of sulfur had no effect on growth or sporulation of newly-developing colonies nor on resporulation or germination of new condia from existing colonies at either 14° or 28°C, i.e. all responses were equal to those for the check treatment at the same temperature.

In another experiment, detached Chardonnay leaves bearing 5- to 7-day old mildew colonies were placed in double petri plates and each colony was treated with a single 3-µl droplet containing 6g/L of a micronized sulfur product. The droplet dried within 30 min of application. Mildew colonies were incubated at 14 or 28°C, and were then observed 4 and 24 hr post-application. A subset of leaves was rewetted by spraying with distilled water 4 hr post-application, and treated with the fluorescent vital stain fluorescein diacetate to assess colony mortality under fluorescence microscopy 4 and 24 hr post-application, by measuring zones of mortality (non-fluorescing hyphae, conidia, and conidiophores). Treatments were replicated 5 times, and the experiment was repeated.

As shown in Table 1, temperature had no effect (P = 0.05) on the size of the zone of mortality surrounding the sulfur deposit, at either temperature. However, the size of the zone 24 hr post-application was increased 13- to 14-fold (P = 0.01) by rewetting the dried sulfur deposit 20 hr earlier. There was no evidence of vapor activity at either temperature: mildew colonies often grew around the dried sulfur deposit during the 2-3 days after application, and often sporulated within 100µm of the margin of the sulfur deposit at both 14° and 28°C.

Tab. 1. Effect of temperature and rewetting of sulfur deposits on the viability of treated powdery mildew colonies

Temp (°C)	Rewetting	Zone of mortality (mm) 4 hr	24 hr
14	-	0.18	0.19
28	-	0.16	0.21
14	+	nd	2.52
28	+	nd	2.90

To further examine the effect of temperature on curative activity, single-shoot Riesling seedlings (approx. 30 cm tall) were inoculated with *U. necator* conidia at 25°C and sprayed with 6 g/L of a micronized sulfur formulation (or water) 5 days later, just before symptom appearance. Sprays were allowed to dry, then all seedlings were immediately transferred to growth chambers maintained at either 15 or 28°C. After 9 days at these temps, disease severity (% area infected) and sporulation were evaluated on the four youngest leaves for each of five replicate seedlings per treatment, as described in the curative assays above. A third set of seedlings, also held initially at 15°C for 9 days after the spray date, was then transferred to the greenhouse and incubated for an additional week at ambient temperatures ranging from 25 to 35°C before disease severity and sporulation were similarly assessed. The purpose of moving these plants to warmer conditions was to determine whether any effect initially apparent after incubation at 15°C was the result of fungistasis or colony mortality. The experiment was conducted three times.

Powdery mildew was much more severe on check plants incubated at 28° rather than at 15°C (means of 67% versus 38% leaf area diseased, respectively). However, sulfur sprays provided virtually complete control under all three post-application temperature regimes. No mildew was macroscopically visible among the spray residues on treated plants. Although a few conidia were detected in the rinsate from treated leaves, their numbers were reduced by 87 to 100% relative to the water check, depending upon treatment and experiment, with an average 94 to 96% reduction for the three temperature regimes across all experiments.

Effect of rainfall on activity.

The interaction of rainfall with sulfur rate and addition of an alkanolamide ("spreader-sticker") surfactant was investigated under both greenhouse and field conditions. Riesling seedlings were sprayed to runoff with suspensions of a micronized sulfur product containing either (i) 6 g/L, without surfactant; (ii) 6 g/L plus 0.03% (v:v) surfactant; or (iii) 12 g/L. After sprays had dried, seedlings were subjected to artificial rainfalls totaling either (a) 25 mm; (b) 50 mm; or (c) no rain. Three days later, they were inoculated with a spore suspension of *U. necator*, and disease severity (% leaf area infected) was assessed 14 days after inoculation.

As shown in Fig. 3, a single 50-mm rainfall decreased the protective control provided by the 6-g/L rate by 35% relative to no rain. This effect was somewhat ameliorated by the addition of a bonding surfactant, and even more so by doubling the rate without surfactant. In a field trial, where these same treatments were applied to vines at 14-day intervals throughout the season, 6 g/L provided 67% control of foliar disease severity (relative to unsprayed checks) without the surfactant and 80% control with it, versus 89% control for the 12 g/L rate. The following season, these treatments provided 68, 84, and 87% control, respectively (all differences were significant at P = 0.05).

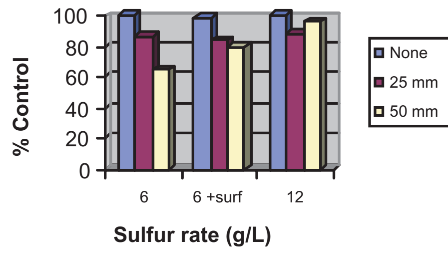

Fig. 3. Effect of artificial rainfall on the protective control provided by micronized sulfur at two rates, and the interactive effect of an added surfactant. Data are expressed as percent control relative to the water-sprayed check, and represent the means from 3 runs of the experiment (5 plants/treatment/run).

Additional experiments suggested that the reduced control following rainfall was more pronounced with wettable powders than with the smaller-particled micronized formulations.

Session 10:

Poster presentation

Epidemiology, Disease forecasting models, Decision support system, Disease risk assessment

Synthetic and natural fungicides

Evaluation of fungicide strategies designed to reduce the number of treatments against Grapevine Powdery Mildew

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The objective of an integrated plant protection program in vineyards is not to prevent the development of pests and diseases at any price but to limit their potential damage to the crop and the yield. The use of such a threshold concept should allow a more efficient use of fungicides, in particular limiting their number of applications whilst still ensuring the objectives of high quality production and adequate yield.

Two criteria must be taken into consideration to improve the performance of treatment strategies:

1) the positioning of the applications
2) the choice of the fungicides depending on their type of action towards the pathogen (protectant, curative or anti-sporulant).

In the case of grape powdery mildew *(Erysiphe necator)*, the main damage is caused on bunches. The period during which bunches are most sensible is relatively short and lasts approximately two weeks from flowering onwards depending on the cultivars (Gadoury *et a*l, 2003). This corresponds to the period of bunch set.

A study conducted in 2004 had the objective to:

1) investigate the possibility to limit the number of applications against grape powdery mildew to three during the period when bunches are most sensitive (from flowering onwards)
2) evaluate the interest of an additional application at pre-flowering for its disease control on leaves and to strengthen the protection of the grapes.

Several spray programs based on grape powdery mildew fungicides representing the major chemical groups (except the non-organic products based on sulphur) were tested in a series of trials carried out in the major vine growing areas in Southern Europe under situations with natural or artificial infection.

Material and methods

Within a European based network of trials (Table 1), two strategies were compared: one with 4 treatments starting at stage 57 (according to BBCH scale) and one with 3 treatments starting at stage 69. In both cases, fungicides were applied every 14 days with the last application performed at the stage 77/79. One DMI fungicide *tebuconazole (*Corail EW*,* Bayer Germany*),* one strobilurin, *trifloxystrobin* (Flint WG, Bayer Germany) and an association of *myclobutanil and quinoxyfen* (GF-1160, DowAgroSciences USA) were compared.

These strategies were tested under natural disease infestation conditions. A split-plot design with two factors (strategy, fungicide) with 4 replicates was used. The size of the plots were sufficiently large to ensure at least 100 bunches for assessment.

In addition to the trials run under natural infestation conditions the same treatments were also compared in 2 trials performed under artificial inoculation (table 1). A randomized design with complete blocks and 4 replicates (with control) was used. The unit plot consisted of 3 plants on a single row. The inoculation was performed on the central plant of each plot, at the stage 14/15, according to the method developed at the INRA centre of Bordeaux (Delière *et al*, 2002). The treatments started at stage 57 and only the strategy with 4 applications was performed.

Tab. 1. Characteristics of the trials network

Name	situation	cultivar	infection
F302	France	Carignan	natural
F303	France	Carignan	natural
I302	Italy	Aglianico	natural
S301	Spain	Carignan	natural
P301	Portugal	*unknown*	natural
OG3	France	Cabernet sauvignon	artificial
F301	France	Cabernet sauvignon	artificial

Tab. 2. Rate of fungicides used in the trials

Fungicide	Active ingredient	Rate of product/ ha
GF-1160	myclobutanil + quinoxyfen(45+45 g/l)	1.0 L
Flint	50 % trifloxystrobin	0.125 kg
Corail	250 g/l tebuconazole	0.4 L

Incidence and severity on bunches were assessed at the stage 77/79.

Results

For all trials run under natural infection, the program of 4 applications showed significantly better mean efficacy than that obtained with the program of 3 applications (Table 3).

Tab. 3. Disease severity (%) on bunches at stage 79 (on untreated plots and mean value for each strategy) Two ways ANOVA (split plot) and Newman Keuls test - Comparison between 4 and 3 treatments strategies on natural infection trials.

Treatment strategy	trial				
	F302	F303	S301	I302	P301
Untreated	97	78	68	80	90
4 treatments	6.6 a	5.6 a	6.0 a	3.2 a	0.5 a
3 treatments	34.6 b	22.2 b	28.8 b	7.0 b	1.8 b
Probability	<0.00001	0.0017	0.00036	0.00242	0.006

All fungicides delivered equally good efficacy within the 4 treatment program. Trifloxystrobin showed the least variable level of efficacy between the individual trial sites. Tebuconazole and the myclobutanil + quinoxyfen combination showed more variable results, though being equivalent (table. 4).

Tab. 4. Disease incidence and severity on bunches at stage 79 (mean value for 7 trials) ANOVA grouping trials with Newman & Keuls test (performed without untreated plot).

Treatment	Incidence		Severity	
	mean	SD	mean	SD
Untreated	98.7	2.9	71.1	17.1
myclobutanil + quinoxyfen	41.2 ns	30.0	6.2 ab	5.1
trifloxystrobine	29.2 ns	23.0	2.2 b	2.1
tébuconazole	42.1 ns	35.5	7.2 a	7.6
P (treatment)	0.17		0.034	
p(treatment x trial)	0.019		0.218	
SE	9.5		4.1	

Discussion

The degree of damage observed in the non treated plots reveals very high disease levels at the various sites. In this context, the programs based on 3 applications starting at flowering do not achieve satisfactory disease control on bunches since a level of 2 % - 35 % infection was observed depending on trial site. However, the overall efficacy of the programs – close to 77 % - is still considerable. This result confirms that the protection of the newly formed berries during their stage of their highest sensibility is important but insufficient in situations with high disease pressure.

Overall, the strategy with 4 applications starting at B 57 ensures a better level of protection in all trials (mean efficacy of 95 %) and demonstrates the interest of a treatment at pre-flowering to limit the infection on bunches. These results confirm the importance of an early disease epidemic on leaves as a source of inoculum for the infection of the young berries at flowering. The damage on bunches is even more severe if the disease has started to develop on leaves situated nearby (Calonnec *et al*, 2005, Peyrard *et al*, 2005). The application at pre-flowering appears to be necessary to limit early disease spread on leaves, thus reducing the build up of stock inoculum responsible for infection on young bunches.

Although not perfect, the level of bunch protection obtained with only 4 fungicide applications is significant and consistent in different vineyards, and at the boundary of what is being considered as acceptable for production of good quality wine grapes. In fact, berries infected by late and superficial symptoms of powdery mildew (= not completely destroyed by the pathogen) and present at a proportion of < 5 % at harvest have no significant qualitative impact. They do not cause any aromatic defect "of a fungal type" which is eliminated at the first alcoholic fermentation steps (Darriet *et al*, 2002) and have only little effect on the organoleptic qualities of the produced wines with the cultivars Sauvignon and Cabernet – Sauvignon (Calonnec *et a*l, 2004). Other authors indicate that very low levels of powdery mildew influence the quality of the grape (Gadoury *et a*l, 2001) and mention lower acceptable thresholds of 1- 5 % (Stummer *et a*l, 2005) for cultivars Concord and Chardonnay.

The performance of this type of program depends very much on the type of fungicide used. Products demonstrating curative and good protectant activity appear to be the most effective. The combination with quinoxyfen, a purely protectant fungicide (Green *et al*, 1998) and the curative molecule myclobutanil gave excellent performance equivalent to the level of tebuconazole and trifloxystrobin in these studies.

In practice, spray programs combine several fungicides of various modes of actions to limit the risk of development of resistant strains. A judicious alternation of fungicides depending on their intrinsic properties is therefore necessary in order to optimise the efficacy of strategies with a reduced number of treatments.

These results show that it is possible to reduce the number of fungicidal applications necessary to ensure the protection of the grapes against powdery mildew. The measure proposed can be optimised by improved management of protection at pre-flowering in relation to the disease pressure prevailing in the vineyard. This requires the availability of objective information on the start and the early development of the powdery mildew epidemic in the plots. The reinforcement or suppression of treatments in the case of high or very low disease pressure could be adjusted according to ability to improve protection at pre-flowering. The choice of fungicides would also be of importance in this optimisation.

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Development of non linear forecasting model of *Plasmopara viticola* infections using Artificial Neural Networks

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In order to verify the possibility of creating a new forecasting model of *Plasmopara viticola* infections, we have decided to set a RTU (Remote Terminal Units) network spread all over the Italian national territory.
Any unit sends, every 15 minutes, meteo and podologic data (table 1) via GPRS to a centralized Data Base in which converge also field data collected by the use of PDA (Personal Digital Assistant).

Tab. 1. Parameters collected from each RTU

primary	secondary
soil moisture - 20 cm dept.	soil moisture - 20 cm dept.
soil moisture - 40 cm dept.	leaf wetness over the canopy
rain	leaf wetness under the canopy
leaf wetness	
solar radiation	
relative humidity	
temperature	
soil temperature	
wind direction	
wind speed	

The information are pre-elaborated and subsequently analyzed with an Artificial Neural Networks (ANN).

We hope to develop a new forecasting model based on non linear model of a complex parameters context and compare it with traditional empiric-based models.

The aim of this work, that will last for three years, is to avoid ineffective treatment on biological and conventional vineyard and to reduce the risk of pesticide contamination on products and environment.

During the first year the units have been set in an organic vineyard (Ariccia – Rome) and in two conventional vineyards (Aprilia – Latina, Rovereto – Trento) in which trial thesis were compared with untreated test and with standard strategy usually applied in the respective farms (table 2).

Tab. 2. Treatments against grape downy mildew

Location	Thesis		
	Deca	Standard	Test
Ariccia	2	3	0
Aprilia	1	5	0
Rovereto	7	12	0

In Rovereto even with a high infection pressure five treatments were avoided in comparison with standard thesis, reducing the input of pesticide on the vineyard.

In Latium only a few suspected infection events were reported, however they did not lead spread of the pathogen in test thesis. Downy mildew was detected only near the harvest phase.

In Aprilia four treatments were avoided while in Ariccia only could be avoided in comparison with test thesis.

This was our first approach to the target of recognizing environmental conditions that precede the downy mildew infection with a high level of reliability, and further surveys will be conducted in the next two years.

Modeling and Mapping the Relationship Between Climate and Ontogenic Resistance to the Major Fungal Diseases of Grapevine

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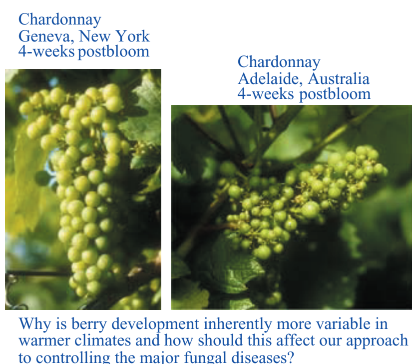
Most cultivars of the world's most widely planted wine grape species *(Vitis vinifera)* are highly susceptible to powdery mildew (*Uncinula necator*), downy mildew (*Plasmopara viticola*), black rot (*Guignardia bidwellii*), *Phomopsis* fruit rot (*Phomopsis viticola*), and bunch rot (*Botrytis cinerea*). Research in relatively cold climates has shown that grape berries are most susceptible to infection by the above pathogens during a critical period in their development. This critical period is not the same for each disease, but the development of ontogenic (or age-related) resistance can nonetheless be precisely defined for each disease. With respect to fruit infection, actions taken during the critical periods largely determine success or failure of management programs. Disease on the fruit can be relatively easy to control if everything is done "correctly" during the critical periods, which are generally the bloom and early postbloom stages of berry development.



Our research indicates that the critical period of susceptibility is substantially affected by climate. In the northeastern US, bloom of grapevine is more or less synchronous, and it is not unusual for an entire Chardonnay vineyard to begin and complete bloom in a 2-day period. Contrast this situation with that of a warmer climate, such as Adelaide, Australia where bloom can take 2 weeks or more to complete. The warmer the climate, the longer and more variable is bloom, and therefore the longer and more variable is the period of fruit susceptibility. Thus, phenology and climate are inseparably linked to ontogenic resistance, and consequently to intelligent deployment of fungicides. Our objective is to adapt our findings on the duration of the critical periods of susceptibility to the major grape diseases to warmer areas based upon local climate.

Development of ontogenic resistance under synchronous flowering. Berry age has a marked, but predictable effect on susceptibility to powdery mildew, downy mildew, and black rot in the relatively cold climate of New York State. Similar relationships between berry age and susceptibility have been described for *Phomopsis* fruit rot and latent infections of berries by *Botrytis cinerea*. We developed three simple regression models to describe the onset of ontogenic resistance to powdery mildew, downy mildew, and black rot under the synchronous host development observed in cold climates (Fig. 1). These models generate distributions that can be adjusted based upon the degree of asynchrony and variation in host development observed in warmer climates.

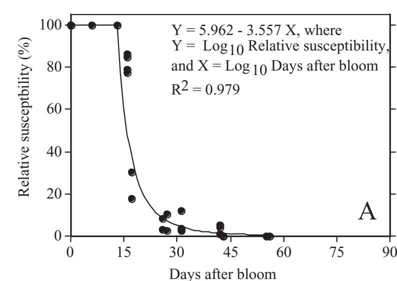


Fig. 1. Development of ontogenic resistance to powdery mildew in berries of *Vitis vinifera*. Data are from Gadoury, et al (Phytopathology 93:547-555). Similar models were developed for downy mildew (*Plasmopara viticola*) and black rot (*Guignardia bidwellii*) using data from Kennelly, et al (Phytopathology 92:S47), and Hoffman, et al (Phytopathology 92:1068-1076), respectively.

Monitoring vine development in different climates. Vineyards of the cultivars Chardonnay and Riesling were located in the US, Germany, and Australia. Five vines were selected at each site, and bloom (% anthesis or "capfall") of basal and second clusters on proximal, medial, and distal shoots was monitored daily. Climatic

temperatures were obtained from various databases, and on-site instrumentation provided records of weather during the bloom period. Monitoring began in 2002. The mean temperatures of the 3 mid-winter months ranged from a low of –4.1 C (Geneva, NY, USA) to a high of 11.8 C (Adeldaide, SA, Australia) among the sites.

The magnitude and nature of asynchrony in bloom. In warmer climates, temporal heterogeneity in bloom was hierarchical. Variance in bloom was increased within individual clusters, between shoots at different positions on the vine, from vine to vine, and between closely-related cultivars within the same vineyards. The same forms of variation are seen in cold climates (Fig. 2A) and warm climates (Fig. 2B), but the scale of time is greatly compressed in cold climates, resulting in apparently synchronous host development.

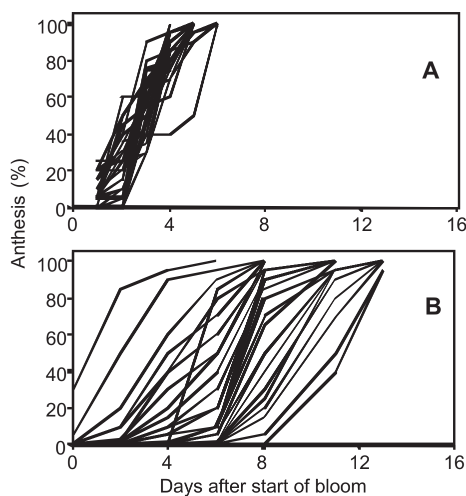


Fig. 2. Variance at the population level. Cumulative bloom of 30 individual fruit clusters on *Vitis vinifera* 'Chardonnay' in (A) Wooster, Ohio, USA,and (B) Adelaide, South Australia. Each line represents the cumulative bloom of a single cluster.

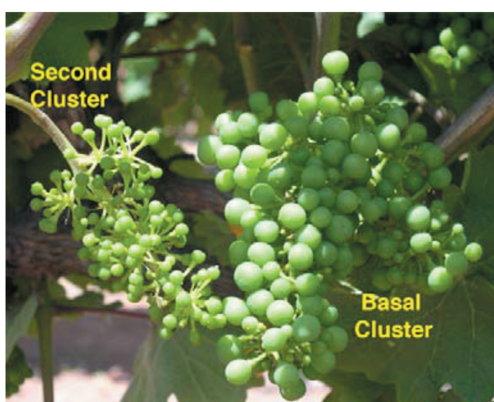


Fig. 3. Asynchrony between basal and second clusters of the same shoot is increased in warmer climates. In warmer climates, a basal cluster may complete bloom more than a week before the second cluster on the same shoot. This asynchrony is reflected in subsequent developmental stages and in the acquisition of ontogenic resistance

How is increased asynchrony related to increased severity of disease? Synchronous host development in cold climates compresses heterogeneity in phenology and creates a single cohort which acquires ontogenic resistance synchronously. Warmer climates create multiple, successively-aged cohorts, which acquire ontogenic resistance successively. In the case of a disease such as black rot, where fruit acquire ontogenic resistance over a period of several weeks, expanding the bloom period from 2 days to 2 weeks adds relatively little time to the broad window of critical host susceptibility. However, in the case of powdery mildew and downy mildew, or any other disease in which the host acquires ontogenic resistance in a relatively brief period, this can more than double the time during which fruit are highly susceptible to infection.

How can models of synchronous development of ontogenic resistance be adapted for climate-based asynchrony? A number of approaches can be suggested. In the example below (Fig. 4), we created a hypothetical population of 10 cohorts of fruit clusters, each offset in age by 1 day (similar to the distribution seen in Fig. 2B). Each cohort then acquired ontogenic resistance to powdery mildew at the rate predicted by the equation shown in Fig. 1. The acquisition of ontogenic resistance by the population was estimated as the mean susceptibility of all cohorts on any given day after the start of bloom in the first (oldest) cohort.

The left curve (Fig. 4) shows the development of ontogenic resistance by the first cohort, and is identical to that observed under synchronous bloom (Fig. 1). The central curve (Fig. 4) shows the mean susceptibility of the hypothetical population in which 10% of the population initiates blooms on 10 successive days. The right curve (Fig. 4) shows the development of ontogenic resistance by the last (youngest) cohort.

A perhaps simplistic, but reasonable assumption would be that the increased duration of susceptibility, and increased area under the curve could result in an equivalent increase of disease severity, selection for fungicide resistance, loss of crop, or all of the foregoing.

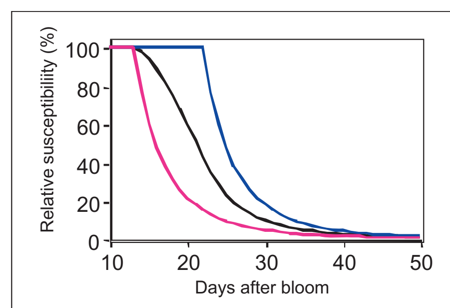


Fig. 4. Estimated development of ontogenic to powdery mildew under synchronous bloom (left curve) or asynchronous bloom (central curve). Right curve depicts the estimated development of ontogenic resistance by the youngest 10% of clusters under conditions of asynchronous bloom observed in Adelaide, Australia.

Conclusions

Duration and variability of bloom appear to be related to mid-winter temperatures.

Warmer climates may satisfy minimal chilling requirements to break dormancy, but additional chilling is required for synchronous host growth.

Asynchrony of grapevine development in warmer climates is seen at multiple levels: within individual clusters, within shoots, between shoots, between vines, and between cultivars.

Asynchronous host development has the greatest potential to impact severity of those diseases in which the acquisition of ontogenic resistance is most rapid under synchronous host development.

Models of ontogenic resistance should be adjusted for climate before testing or deployment. The increased efficacy of control observed in cold climates due to consideration of ontogenic resistance is more likely to be realized in warmer climates if models are adapted to reflect asynchronous host growth.

Asynchronous development of deciduous perennial hosts in warmer climates is a general phenomenon, and may have important effects in diverse pathosystems (e.g., *Erwinia amylovora* and *Malus/Pyrus*, *Monilinia fructicola* and *Prunus*, and *Venturia inaequalis* and *Malus*).

Potentials and drawbacks of grapevine downy mildew control with Tecnobiol®, a product based on fatty acids.

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The use of copper, a traditional element extensively used in organic viticulture to control downy mildew, is matter of concern due to its ecotoxicological long term consequences. Therefore the EU directive 473/2002 limited its use. However under downy mildew favourable climatic conditions the allowed quantities are insufficient to control the disease in organic vineyards. Therefore there is an increasing interest in alternatives to copper.

During 2005, greenhouse and field trials were carried out to evaluate the efficacy against downy mildew of Tecnobiol® (Tecnotrea S.r.l., Crema, Italy) a new fungicide based on fatty acids. The formulation contains 25-35% of fatty acids, mainly oleic acid (>75%), a smaller amount of linoleic acid (>10%) and others (to 100%). Fatty acids are anphipatic molecules with a hydrophilic and a hydrophobic functional group. Their mode of action is related to the negative effect on functionality of pathogen's membrane due to the intercalation of fatty acids between phospholipidic molecules of the cell membrane.

Tecnobiol® is a quickly biodegradable biocide with preventive activity.

Material and Methods

Efficacy trials were carried out under greenhouse controlled condition on cv. Pinot-gris potted plants. Each treatment was applied on five replicated (plants with two shoots and at least four well developed leaves each). Tecnobiol® was applied at concentration of 10 g/l. Copper hydroxide (Kocide 2000®, Dupont De Nemours, used at 1.33 g/l) and water were used as references. Products were sprayed by an air compressor system working at 4 bar. Six hours after treatments, plants were artificially inoculated spraying a *Plasmopara viticola* water suspension ($1 \cdot 10^5$ sporangia/ml) on the lower side of leaves. Plants were then overnight incubated at 20°C with high relative humidity (RH>95%). When firsts oil spots appeared on plants they were incubated overnight at 20°C and RH>95%. Disease incidence (percentage of leaves with disease symptoms) and severity (percentage of diseased leaf area) were recorded. This experiment was repeated twice.

Field trials were performed according EPPO guidelines, on cv. Cabernet sauvignon grafted on Kober 5BB plants in an experimental vineyard of the Istituto Agrario di San Michele all'Adige, located in Rovereto, Northern Italy. A complete randomised blocks design was used with four replicates of 83 plants each. Products were weekly applied during the growing season by an atomiser using a volume of 12 hl/ha. Tecnobiol® was applied at 1000 g/hl and copper hydroxide (Kocide 2000®) at a concentration that varied between 75 and 175 g/hl, according the period (more concentrated when disease pressure was higher: during raining periods and highly susceptible plants). Untreated plots were used as control. The first and the last treatments were carried out with copper hydroxide on the entire vineyard, excluding the untreated control.

Severity and incidence were weekly evaluated on 50 leaves and 50 bunches in each replicate. Area under disease progress curve (AUDPC) of severity and incidence was calculated using the following formula:

$$Y = \sum[(X_i + X_{i+1})/2](t_{i+1} - t_i)$$

where Y is AUDPC X_i is the severity at i-th evaluation, X_{i+1} is severity at i+1-th evaluation, and (t_{i+1} - t_i) is days number between the two evaluations.

Statistical analyses were done on arcsin transformed data and means were separated by HSD Tukey's test using Statistica software (Statsoft). In addition the T-test was used to analyse the difference between severity (AUDPC) of untreated and Tecnobiol®.

Results

Tecnobiol® provided a good control of downy mildew under controlled conditions, not significantly different from copper treated plants (Tab. 1).

Tab. 1. Greenhouse trials: Incidence (Inc.) and severity (Sev.), in percent, of downy mildew on leaves of cv. Pinot-gris plants treated with plant protection agents. Each value represents the mean of five replicate plants.

Product	I trial Inc.	I trial Sev.	II trial Inc.	II trial Sev.
Tecnobiol®	81.7 a	9.8 a	4 a	0.8 a
Kocide2000®	79.0 a	15.4 a	23.3 a	8.5 a
Water	98.0 b	71.6 b	84.3 b	48.7 b

Data in the same column followed by the same letter are not statistically different by HSD Tukey's test (P<0,05).

Regarding field trial, data on leaves acquired on the last assessment at harvest (Tab. 2), showed that both Kocide 2000® and Tecnobiol® significantly decrease downy mildew incidence and severity compared to the untreated control.

Tab. 2. Field trial: downy mildew incidence and severity on leaves at harvest.

Product	Incidence (%)	Severity (%)
Tecnobiol®	57.0 a	21.2 a
Kocide 2000®	40.0 a	10.2 a
Untreated	99.0 b	59.3 b

Data in the same column followed by the same letter are not statistically different by HSD Tukey's test (P<0.05);

Analysing the entire season (AUDPC) leaf incidence was not significantly different among the different modalities if Tukey's test is used (Tab. 3). The T-test analysis between Tecnobiol® and untreated did not reveal any difference (P value = 0.089). Conversely the analysis of downy mildew severity (Tab. 3) showed that only Kocide 2000® significantly reduces AUDPC using Tukey's test. However T-test shows a significant difference between Tecnobiol® and the untreated control with a value of P= 0.042

Tab. 3. Field trial: incidence and severity (AUDPC) of downy mildew on leaves.

Product	Incidence (AUDPC)	Severity (AUDPC)
Tecnobiol®	1606.0 a	409.2 ab*
Kocide® 2000	1651.0 a	217.7 a
Untreated	2659.0 a	1020.9 b

Data in the same column followed by the same letter are not statistically different by HSD Tukey's test (P<0.05); * T-test Tecnobiol®-Untreated P = 0.042.

The application of Tecnobiol® resulted in a significant disease reduction on bunches, both at harvest (Tab. 4) and over the whole season (Tab. 5).

Tab. 4. Field trial: downy mildew incidence and severity at harvest on bunches.

Product	Incidence (%)	Severity (%)
Tecnobiol®	11.5 a	0.1 a
Kocide® 2000	13.5 a	0.2 ab
Untreated	45.0 b	5.5 b

Data in the same column followed by the same letter are not statistically different by HSD Tukey's test (P<0.05)

Tab. 5. Field trial: downy mildew incidence and severity (AUDPC) on bunches.

Product	Incidence (AUDPC)	Severity (AUDPC)
Tecnobiol®	512.3 a	30.9 a
Kocide 2000®	469.5 a	15.8 a
Untreated	1214.8 b	272.0 b

Data in the same column followed by the same letter are not statistically different by HSD Tukey's test (P<0.05)

Discussion

Tecnobiol® showed a good efficacy controlling the pathogen under greenhouse condition and artificial infection made 6 hours after the application. Tecnobiol® could be a potential alternative to copper. The results

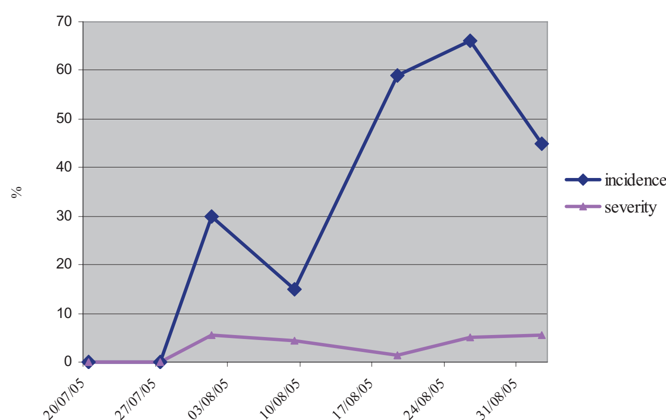

Fig. 1. Downy mildew development on bunches in 2005 on untreated Cabernet sauvignon vines in Navesel experimental vineyard of Istituto

The hypothesis that can be formulated to explain the reduced efficacy under field conditions of Tecnobiol® is based on the composition of the product: light, temperature and rain have a detrimental effect on linoleic and oleic acids stability. The effect of temperature and light intensity will be studied in future experiments.

Acknowledgements

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Dipeptides secreted by the grapevine endophyte *Alternaria alternata* cause structural damages to *Plasmopara viticola*

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Higher plants are commonly colonized by endophytes, microorganisms such as fungi, bacteria or actinomycetes, which can form communities specific to a certain host/environment. The precise biological meaning of this plant inhabitants is not known. Endophytic fungi include weakly parasitic strains living in the host tissues which may become pathogenic as the plant's physiological state deteriorates. Interestingly, endophytic and pathogenic fungi can coexist in plant tissues, although the nature of their interactions is unknown in most cases. It is reliable that during the course of their coevolution, plants and endophytes have developed an intimate relationship that has probably resulted from an extensive exchange of information at cellular and molecular level.

Endophytes that colonize host tissues before the pathogens may produce pathogen-inhibiting metabolites and other defence-related compounds that hinder expression of the disease.

Moreover, they demonstrated, in many cases, the capacity in promoting plant disease resistance. The great variability of the metabolites produced by endophytes suggested the possibility to use them as natural antagonists, thus indicating their employment as alternative means for the control of plant diseases.

We recently reported the isolation and identification of grapevine fungal endophytes. We tested them for their potential as biocontrol agents against *P. viticola.* Among these, only the endophyte *A. alternata* demonstrated a complete effectiveness in inhibiting *P. viticola* sporulation on grapevine leaf disks.

Ultrastructural analysis of grapevine tissues revealed that *A. alternata* did not cause leaf tissue damages. By contrast, *P. viticola*, inoculated on leaf disks previously treated with *A. alternata*, exhibited marked structural changes in the presence of the endophyte, even without close contact between the two fungi. They consisted of abnormal vacuolization, accumulation of electron-dense material in the vacuoles and appearance of necrotic haustoria. A real contact between the two organisms was never observed. A mechanism of direct hyperparasitism of *P. viticola* by *A. alternata* can therefore be excluded. The production of toxic compounds can be hypothesized.

Three low-molecular weight dipeptides, belonging to the family of diketopiperazines (DKPs), were extracted from *A. alternata* broth culture. They reduced *P. viticola* sporulation in grapevine leaf disks as well as in grape plants in the greenhouse. The antifungal activity resulted not concentration-dependent; at least with the concentrations used in our experiments (0.33 and 2 mM). Several DKPs and other antimicrobial peptides are reported to be active even at a higher concentrations.

In our experiments the DKPs were active against the pathogen only in the treated areas of grapevine leaves, which could be explained by the poor translocation capacity of DKPs, as already reported from literature. Moreover, in our experiments no necrotic lesions or other phytotoxicicity symptoms were observed on DKP-treated grapevine leaf tissues.

Ultrastructural analysis performed on grapevine leaf tissues revealed that the mixture of the three DKPs at above reported concentrations, did not cause leaf tissue damages. By contrast, hyphae of *P. viticola* exhibited marked structural changes correlated with the presence of *A. alternata*/or or its toxic metabolites. This fact demonstrates the involvement of these metabolites in the relationship of these two fungi. From our first results it seams that DKPs are not or poorly systemic in grapevine tissues. This behaviour must be ascertained after longer periods besides plant treatments. But this experiments need first to know the activity and persistence of the DKP molecules in grapevine. It is interesting that pathogenic *A. alternata* isolated from symptomatic tomato acts as non-pathogenic fungus when inoculated in grapevine. Still, it produces active DKPs against *P. viticola* in grapevine plants.

In conclusion, our results demonstrate that the grapevine endophyte *A. alternaria* induces ultrastructural alterations in *P. viticola* mycelium and inhibits its sporulation. Furthermore, DKPs extracted from *A. alternaria* broth culture showed a marked ability to prevent *P. viticola* sporulation, thus representing a promising means to control the pathogen. Last but not least, because of their low molecular weight, DKPs are easy to synthesize, since there are many different strategies by which these dipeptides may be obtained. Further investigations are in progress to verify the effectiveness of DKPs in reducing *P. viticola* sporulation in grapevines grown in the field and to clarify the mechanism by which *A. alternata* and these molecules affect *P. viticola*. Not less important is to ascertain the ability of *A. alternata* and its DKPs to control oidium and *Botrytis* besides *Plasmopara* in grapevine.

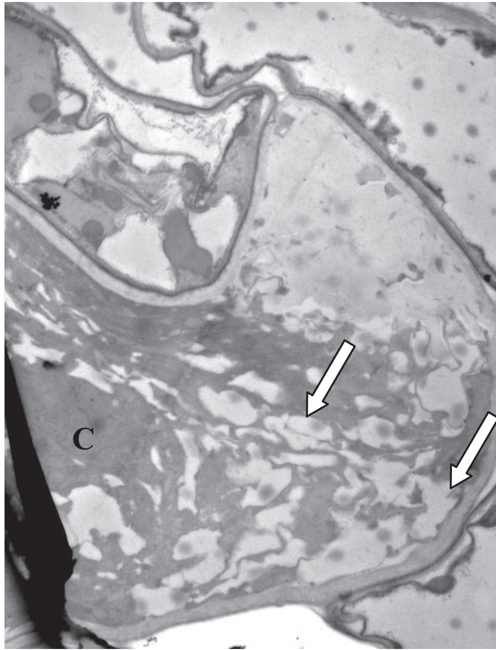

Fig. 1. Particular of *P. viticola* mycelium treated with DKPs extracted from *A. alternata*: anomalous vacuolisations (arrows) and cytoplasmic addensations (C) are evident. Bar corresponds to 2.9 μm.

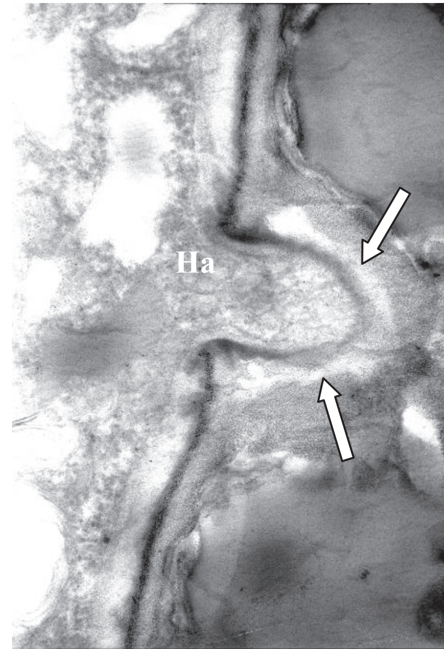

Fig. 2. Initial stage of a *P. viticola* haustorium completely surrounded by callose. Bar corresponds to 0.53 μm.

Physical Modes of Action of Phosphites in Control of Grapevine Downy Mildew

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Phosphites provide control of a number of oomycetous plant pathogens, including *Plasmopara viticola*, the causal agent of grapevine downy mildew. These compounds are inexpensive to produce, have no significant negative toxicological or environmental effects (e.g., they are exempt from residue tolerances by the United States Environmental Protection Agency), and have shown good efficacy under commercial conditions. Until recently, their use in the United States was limited due to legal (patent) issues, and use in Europe has been relatively limited as well. We undertook this study in order to better understand the fundamental physical modes of activity of these compounds, specifically their protective, curative, and eradicative capabilities with respect to control of *P. viticola*.

Trials were conducted over a 3-yr period on unsprayed vines, prior to the observation of natural downy mildew epidemics each year, using variations on a basic protocol. All foliage of the treated vines was sprayed to runoff with commercial phosphite products using a back-pack sprayer, at different times according to the intended treatment, and all vines in an experiment were inoculated on a single date. Inoculations were performed on the youngest flat leaf >25 mm in diameter (which was marked at the time to facilitate subsequent assessments), and the next four leaves beneath it, on each of seven to 10 shoots in each of four replicate plots per treatment. Leaves were inoculated in the evening with a suspension containing 10^5 sporangia/ml, and the inoculated shoots were bagged overnight to promote infection.

Two weeks after inoculation, each leaf was rated by position (#1 = youngest, #5 = oldest) for the percentage of the leaf area appearing diseased. Then, each leaf was removed from the vine and the sampled leaves were bulked by leaf position for each plot (all leaves from the #1 position, all from the #2 position, etc.). Leaves were incubated overnight in the laboratory to induce sporulation, then sporangia were rinsed from the leaves, quantified under the microscope using a hemacytometer, and the number of sporangia per leaf was determined. To facilitate comparisons among treatments for leaves that varied in size and levels of susceptibility according to their age (position), data were ultimately expressed as a percentage reduction (% control) relative to the untreated check for that position.

In 2003, vines of the interspecific hybrid cv. Lakemont were treated with a 0.3% solution of a potassium phosphite product (providing 1 g/L phosphorous acid equivalent), either 7 days before, 3 days before, or 3 days after inoculation. In this trial, control of disease severity was strongly influenced by leaf position (Fig. 1). For example, all treatments provided 94 to 100% control on the two youngest leaves.

However, the protective activity declined significantly in the oldest inoculated leaves (#4 and #5) for sprays applied 7 versus 3 days before infection, perhaps due to translocation of the chemical towards the shoot tips. The leaf position effect was much less pronounced in the 3-day protective and 3-day curative applications, only declining on the oldest inoculated leaf to levels of 81 and 74% control, respectively. In contrast, sporulation was strongly inhibited at all leaf positions, even when lesions did develop. That is, sporangium production was reduced by 84 to 85% at the two oldest leaf positions in the 7-day protective treatment and by 94 to 100% for all other leaf positions and treatments (*data not shown*).

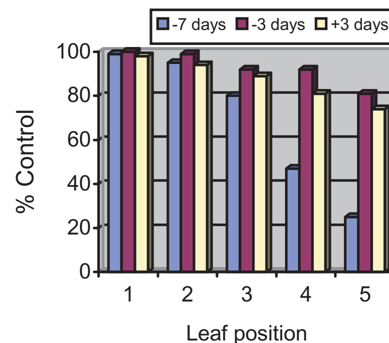

Fig. 1. Control of downy mildew on cv. Lakemont with a solution of 0.3% potassium phosphite (1 g/L phosphorous acid). Sprays were applied either 7 or 3 days before inoculation protective (assay) or 3 days after (curative assay). Data are expressed as % control of disease severity relative to the unsprayed check treatment for each leaf position.

The eradicative activity of this same product and rate was examined in two additional experiments on vines of cvs. Chardonnay and Lakemont. Sporangia were rinsed from the leaves of infected vines in the field, and the phosphite treatment (or water) was applied. Leaves were harvested 6 days later and assessed for new sporulation as before. Although the phosphite spray did not eradicate the lesions, sporangium production was reduced by 70 and 82%, respectively, relative to the water check.

The following year, similar trials were conducted on cv. Chardonnay and *Vitis* x *labruscana* 'Delaware'. In the Chardonnay experiment, we again used a rate of 1 g/L of phosphorous acid equivalent, either 7 days before, 3 days before, or 5 days after inoculation. The results are provided below in Table 1.

Tab. 1. Effect of spray timing on control of downy mildew with phosphorous acid (1 g/L) on cv. Chardonnay

Spray timing	Leaf	Percent control	
(days vs. infection)	position	Lesion area	Sporulation
-7	1-5[a]	91	84
-3	1-5[a]	97	97
+5	1	100	100
	2	92	95
	3	86	91
	4	81	97
	5	74	86

[a] Mean values for leaf positions 1 through 5.

Sprays applied either 3 or 7 days before infection provided very good to excellent control, with no effect of leaf position, whereas control was reduced moderately with respect to leaf position in the 5-day curative treatment.

In the experiment on cv. Delaware, we examined the interaction of spray timing with rate (1 vs. 2 g/L phosphorous acid). We also tested to see whether curative activities could be increased by applying a second spray 5 days after the first. In this trial, there was little to no effect of leaf position on the level of control provided, hence all data reported are means for the five leaf positions per inoculated shoot.

As shown inn Table 2, both rates of phosphite provided excellent protective activity on all inoculated leaves, giving virtually complete control when applied either 4 or 8 days before inoculation.

When the first application was made 4 days after infection (about 1 day before symptoms started to appear), the sprays provided 80 to 90% control of lesion area and virtually complete control of sporulation. Both rates appeared to be equally effective. When the first spray was applied 6 days after infection, small non-sporulating leaf lesions were just starting to become visible. These lesions continued to expand after treatment, although they did not attain the final size of those on unsprayed leaves. More importantly, these lesions produced very few sporangia, with an average of 86 to 98% reduction in spore formation relative to the unsprayed check. Control of both lesion expansion and spore formation appeared to improve when the rate was increased from 1 to 2 g/L or when the initial application of the lower rate was repeated 5 days later.

Tab. 2. Effect of spray timing and rate on the protective and curative activites of phosphorous acid in control of downy mildew on cv. Delaware

Spray timing		Percent control	
(days vs. infection)	Rate (g/L)	Lesion area	Sporulation
-8	1	95	99
	2	98	99
-4	1	99	99
	2	99	100
+4	1	90	100
	2	80	99
+6	1	27	86
	2	46	98
+6, +11	1	59	98
	2	72	98

In 2005, a similar experiment was conducted in the same Delaware vineyard, employing 19 different treatments to compare (i) three different phosphite products; (ii) rate (1 vs. 0.5 g/L phosphorous acid equivalent); and (iii) application timing (7 days before, 4 days and 7 days after infection). Treatment effects were determined by calculating the percent reduction in sporangium formation for each leaf position, as before.

When used at the higher rate of 1 g/L, there were no consistent differences among products and there was no effect of leaf position. Inhibition of sporulation was strong but not as complete as in other trials, with mean reductions of 80, 86, and 73% across all three products when applied 7 days pre-infection, 4 days post-infection, and 7 days post-infection, respectively. However, phosphite provided significantly less inhibition of sporulation on the oldest inoculated leaf (#5) than the two youngest (#1 and 2), when the rate was reduced to 0.5 g/L.

Conclusions

Phosphites control downy mildew through a combination of preventive, post-infection, and antisporulant activities.

Phosphites do not eradicate established infections, but strongly inhibit the formation of additional sporangia from them.

Activity is affected by rate. In the United States, the minimum rate of products labeled for disease control is generally about 1 g/L of phosphorous acid, and the use of rates below this minimum dosage may result in some loss of control. Higher rates may improve the extent and/or duration of post-infection activity.

Post-infection activity may be increased by a second application of the materials, particularly at minimal rates.

The well-established mobility of these materials in plant tissues appears to limit their persistence (hence, activity) in older leaves, at least under some conditions. This effect is likely to be particularly pronounced at lower use rates.

Interpretation of correct fungicide dose in commercial vineyards: a global review

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Powdery and downy mildew require particular attention to adequate coverage and chemical dose.

Fungicides are first screened under controlled laboratory conditions, a range of rates are then tested in the field under standard conditions often with small hand operated spraying systems. Development trials are also conducted to show efficacy, crop safety and residues, using small 'model' spraying systems. Prior to commercial release an effective use rate or range of rates is tested under normal commercial field conditions, often utilising a range of dilution rates to simulate a range of water volumes and mixing regimes which may occur amongst the large numbers of growers.

Traditionally labels were written as rate of product (grams or mls) per unit area of vineyard eg hectare or acre. However as it is the vine rows only which are sprayed, a hectare could contain vastly different amounts of crop target depending upon:

- Crop growth stage
- Canopy size
- Canopy density
- Row spacing
- Pruning regime
- Trellis design
- Vine vigour.

In Australia it was agreed in about the year 2000 that labels would express the rate of product per 100 litres of water, assuming that spraying is conducted 'to the point of run off' (dilute spraying), this more closely reflects the way the research trials are conducted and also potentially allows for the large range of vineyard variables mentioned above.

Growers who are not spraying 'to the point of run off' (dilute spraying) are using less water but still need to use the correct amount of product for the appropriate canopy size. This type of practice is called 'concentrate spraying' as the label rate of the product needs to be concentrated to the same degree as the water rate reduction. Growers then calculate a new mixing rate depending on their concentration factor (Dilute water volume/Actual water volume). See the standard label instructions attached. Concentrate spraying is the usual practice in winegrapes particularly later in the season. Spray coverage may still be adequate to maintain control.

The questions and problems:

- Many growers are still struggling with the concepts of dilute and concentrate spraying and what these mean at the time of mixing the product into the tank.

- Spray diaries now require the rate calculations to be documented in a standard format.

- The industry guidelines for indicative water volumes have not been scientifically validated.

- Most growers need to calculate a new mixing rate for each spray timing and canopy change, manually.

- Growers need a method to estimate the 'dilute volume', eg by using further calculations (Unit Canopy Row), using industry charts or by trial and error using spray machinery.

- There is a risk that growers could be overdosing early in the season and underdosing later.

- Modern spray machinery and it's manufacturers are promoting low water volumes to improve spraying efficiency, some are also claiming reduced chemical rates through superior coverage.

- On some vineyards there is the chance that many different rates will be required to apply in one spraying cycle if the different vine blocks have a range of canopy sizes.

- Australia has an enormous range of vineyard management systems and subsequent canopy sizes.

A Global Consultation Approach

A survey has been conducted across the major grapegrowing countries to assess the extent of these problems and also to examine the best available solutions from regulatory, manufacturer and practitioner. The paper will compare the approaches from the countries surveyed and propose possible solutions to enhance the management of these diseases and ensure efficient use of fungicides and spraying resources.

Session 11:

Disease management (organic and IPM)

Applying Spatial Information in a Whole-of-block Experiment to Evaluate Spray Programs for Powdery Mildew in Organic Viticulture

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The Tasmanian wine industry increased from 507 ha of bearing vines in 2000 to 961 ha in 2005. With a climate more like New Zealand, adoption of recommendations from mainland Australia for powdery mildew management has resulted in poor spray timing in relation to disease progression (Fig. 1). The problem is compounded when the flowering period is extended, often beyond 14 days. Cool conditions at other times lead to the grower practice of applying non-label (high) rates of sulfur.

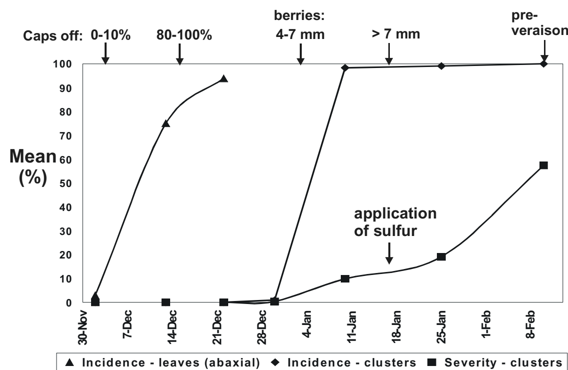

Fig. 1. Progression of powdery mildew in small plots (n = 6, randomised blocks) of Chardonnay vines that were not treated with fungicide until pea size berries, Frogmore Creek, Coal River Valley, 2004/05.

In Tasmania, powdery mildew is managed in organic vineyards with spray programs based on sulfur, bicarbonates and canola-oil based sprays. A major limitation of sulfur application in organic viticulture is its negative impact on beneficial arthropods. There is also the threat that sulfur will be restricted as an acceptable input in organic viticulture. In the search for alternatives to sulfur, Crisp *et al.* (2003) demonstrated the potential of contact materials, such as diluted pasteurised milk, whey or mixed programs involving bicarbonates and oils, in reducing the severity of powdery mildew in a South Australian vineyard. Since then, these materials have been tested in a range of environments, including several grapevine varieties and trellis systems in southern Tasmania.

We report results of small plot trials to evaluate alternatives to sulfur and introduce a 'whole-of-block' approach to experimentation (Bramley *et al.* 2005) for identifying a 'best bet' spray program for organic viticulture that minimises the input of sulfur. The development of compost extract technology, as presented by Palmer, Evans and Metcalf in these Proceedings, is another step towards minimising the use of sulfur in organic viticulture.

Small plot trials

Methods

Four trials over two growing seasons were designed as a randomized complete block with six replicates of 5-9 vines per plot. In 2003/04, Chardonnay and Cabernet Sauvignon vines in different vineyards were used. In 2004/05, Chardonnay and Pinot noir were in close proximity.

Materials tested included pasteurized 4% bovine milk (1 in 5 or 10 dilution), cheese whey (25 g/L), Synertrol Horti-oil® plus Ecocarb® (activated potassium bicarbonate, 4-6 g/L, Organic Crop Protectants P/L), sulfur (6-12 g/L), or programs involving various materials applied at different times during the growing season. In 2003/04, materials were applied eleven times with a hand-held sprayer (1,500 kPa) at intervals of 9-15 days from Eichhorn-Lorenz (E-L) stages 9 to 34. In 2004/05, materials were applied nine times with a small-plot air blast sprayer (550 kPa) at intervals of 8-15 days from E-L stages 12 to 30. The entire trial site was sprayed with sulfur at E-L stage 34.

Disease incidence and severity were assessed, with the aid of standard area diagrams, for 40 leaves or 20-30 bunches selected randomly per plot.

Results

Both growing seasons were conducive to the development of severe powdery mildew. The main findings were:

- All treatments reduced mean disease severity when compared with the untreated control
- Milk or whey resulted in disease control that was equivalent to the standard sulfur treatment in one trial out of four (Fig. 2)
- Season-long programs of milk or whey provided equal or greater disease control than milk or whey programs incorporating the bicarbonate/oil mixture before flowering
- In 2004/05, programs of alternative materials that included applications of sulfur during flowering were not as effective as a season-long program of sulfur

Summary of small plot trials

Alternatives to sulfur, such as whey, have potential to control grapevine powdery mildew for varieties that are not highly susceptible to disease, or when shoot vigour is low. Good coverage is essential for disease control.

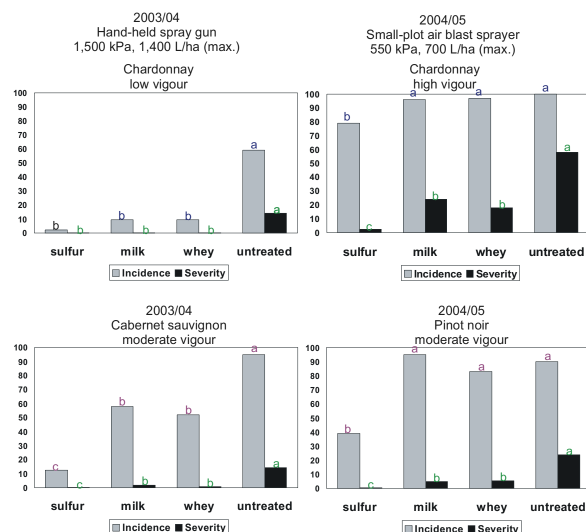

Fig. 2. Mean incidence and severity (%) of powdery mildew in four trials conducted over two growing seasons. Means followed by the same letter are not significantly different at P = 0.05.

‘Whole-of-block’ experiment

Background

In 2005/06, a ‘whole-of-block’ approach is being evaluated for overcoming limitations associated with small plot experiments. A major limitation is interpreting the gap between the effective dose, as determined by hand spraying single panels of vines, and the actual dose achieved over a large and variable area using commercial equipment. While randomisation of small plots attempts to accommodate the effects of underlying vine variation, it is assumed that the variation is also distributed randomly. However, variation in vine vigour, for example, is unlikely to be distributed randomly within rows that constitute the ‘blocks’ of small plot trials. Powdery mildew is often severe in dense, shaded canopies. Vine vigour is likely to be correlated positively with disease severity, although this phenomenon does not appear to have been quantified scientifically.

Objective

Our grower cooperator was aware of the results of our small plot trials, yet he wanted to reduce sulfur applications with commercial equipment and available materials. Through consultation, the whole-of-block experiment was developed with the aim of investigating if application of only one or two sulfur sprays during the critical flowering/fruit set period would provide commercially acceptable control of powdery mildew. The experiment was designed specifically to evaluate the *magnitude* of treatment effects accounting for variation in inherent vine vigour.

Methods and preliminary results

Each of two spray programs was applied using commercial equipment to a block of six rows, with each program applied in an alternating pattern across a 4.5 ha block of Pinot noir with 92 rows at Frogmore Creek (organic) vineyard. A mixture of Synertrol Horti-oil® (250 ml/100 L) plus Ecocarb® (400 g/100 L) was applied to the whole block from E-L stage 16 until the beginning of capfall. The next two applications of fungicide comprised a) Program A: two applications of sulfur (800 g/100 L) or b) Program B: one application of sulfur followed by an application of the Ecocarb/oil mixture. The Ecocarb/oil mixture was then applied to the whole block for the remainder of the season until veraison.

Powdery mildew was assessed multiple times for 116 vines per spray program. Single vines were sampled in the middle two rows of the six row ‘block’, using a regular grid but with sampling points removed at random from 15% of the grid nodes and reallocated to vines adjacent to other grid nodes (Bramley 2005).

Images of the ‘plant cell density’ (PCD) index were obtained at veraison by aerial remote sensing for mapping canopy vigour across the block. At veraison, clusters of Program A vines had a mean disease severity of 1.5%, which was significantly lower than the mean of 3.1% recorded for clusters of Program B vines ($P<0.001$, df = 230, one-sided *t* test of angular transformed data). At the time of writing, harvest was imminent and our grower cooperator was pleased that the low level of powdery mildew across the block was commercially acceptable.

When the nets are removed after harvest (late April, 2006), each of the sample vines will be geo-referenced using a differentially corrected global positioning system (dGPS). We shall present progress on the application of geostatistical methods (Bramley 2005) to compare spatial patterns (variograms) in powdery mildew severity with patterns of variation in vine vigour across the 4.5 ha block. Development of the powdery mildew epidemic across the block will be illustrated and the standard *t* test will be applied for testing the significance of treatment differences for zones of low or high vine vigour.

Discussion

The use of spatial information allows performance of the test material to be evaluated over a range in conditions of vine vigour. In addition, the ‘whole-of-block’ approach aids development of a more effective relationship between the grower cooperator and researcher for the direct transfer of ‘commercial ready’ knowledge. The grower has control over decisions about timing fungicide applications and the results relate directly to the capability of his or her commercial equipment. Indeed, the grower had no problem with giving the whole block over to experimentation and the ease of implementation meant that the grower did not need to understand the complex nature of the geostatistical design.

Acknowledgements

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Efficacy evaluation of integrated strategies for powdery and downy mildew control in organic viticulture

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Application of copper and sulphur is currently the main practice in organic viticulture to control fungal diseases. The products based on sulphur can cause the high level of residues in the wine and skin and eye irritation for the workers. Furthermore, sulphur is toxic to beneficials and predator mites. Copper accumulates in soils where is toxic to macro and microorganisms and its use will be strongly limited in the future.

All these constraints have encouraged the search of alternatives. The most promising and commercially available alternatives to sulphur and copper are respectively *Ampelomyces quisqualis* and Mycosin. The fungus *A. quisqualis* is a naturally occurring hyperparasite of powdery mildews. An *Ampelomyces* strain has already been formulated, registered and commercialized (AQ10 Biofungicide, produced by Intrachem Bioitalia) in several countries. Mycosin is a biological compound based on sulphuric acidic clay minerals and deactivated yeasts cell membranes that promote disease resistance in vines.

The objective of the present work was to reduce the copper and sulphur by using alternatives maintaining satisfactory diseases control. Therefore, control strategies based on the integration of AQ10 and Mycosin were compared with a reduced number of copper and sulphur treatments against powdery and downy mildew.

Material and methods

Climatic conditions**.** The study was done in 2005 in a temperate climate (S. Michele all'Adige, Trentino Region) and Mediterranean climate (Andria, near Bari, Apulia Region).

The varieties Schiava and Uva di Troia, which are highly susceptible to the diseases, were respectively used in S. Michele and Andria. The climatic conditions usually are more suitable to *Erysiphe necator* (formely *Uncinula necator)* than to *Plasmopara viticola,* both in Andria and in S. Michele all'Adige. Meteorological data were recorded during the whole season.

Tested products. AQ10, new sulphur and copper formulations (produced by Intrachem-Italy) and Mycosin (produced by Biogro-New Zealand) were used (Tab. 1). Application were done using an atomizer and a spraying volume 10-12 hl/ha, were applied at 8-10 days interval starting two weeks after bud-break (shoots length = 5-10 cm) and continued until veraison.

Tab. 1. Products used in the strategies in Andria and S. Michele

Active ingredient	Commercial name	Dosage
A.quisqualis + Pinolene	AQ10Biofungicide + VaporGard	6 g/hl 100 ml/hl
Sulphur	Heliosoufre	300/400 ml/hl
Acidic clay minerals	Mycosin	500 g/hl
Copper hydroxide	Heliocuivre	130/150 ml/hl

Strategies. In the two experimental vineyards, five different strategies were compared (fig. 1).

AQ10, the BCA-product was used when temperature and relative humidity were suitable to the organism (beginning and end of the growing season) and sulphur in the remaining periods. Heliocuivre, the new copper formulation was used in combination with AQ10, whilst Mycosin was applied in combination with the new sulphur formulation (Heliosoufre). A strategy (2), based only on copper and sulphur, used during the whole cropping season, was used as reference. In strategy 3, 4 and 5 three AQ10 treatments were sprayed later in the season in order to additionally reduce the source of inoculum for the next growing season (overwintering cleistothecia).

<table>
<tr><th>Strategy</th><th>May</th><th>June</th><th>July</th><th>Aug.</th><th>Sept.</th><th>Oct.</th></tr>
<tr><td rowspan="2">1</td><td colspan="6">untreated</td></tr>
<tr><td colspan="6">untreated</td></tr>
<tr><td rowspan="2">2</td><td colspan="6">sulphur</td></tr>
<tr><td colspan="6">copper</td></tr>
<tr><td rowspan="2">3</td><td colspan="2">AQ10</td><td colspan="2">sulphur</td><td colspan="2">AQ10</td></tr>
<tr><td colspan="2">copper</td><td colspan="2">Mycosin</td><td colspan="2">copper</td></tr>
<tr><td rowspan="2">4</td><td colspan="4">sulphur</td><td colspan="2">AQ10</td></tr>
<tr><td colspan="4">Mycosin</td><td colspan="2">copper</td></tr>
<tr><td rowspan="2">5</td><td colspan="3">sulphur</td><td colspan="3">AQ10</td></tr>
<tr><td colspan="3">Mycosin</td><td colspan="3">copper</td></tr>
</table>

Fig. 1. Strategies to control powdery and downy mildew

Experimental design. Each strategy was applied in one of the four big blocks. The untreated block (strategy

1) was placed longitudinally at one side of the vineyard opposite to the wind direction. The assessments were made weekly by checking random selected 100 leaves and 50 bunches in five sampling plots (10 plants) per each block for incidence % leaves or bunches with symptoms) and severity (% area with symptoms). Analysis of variance (ANOVA) was applied on AUDPC (Area Under the Disease Progress Curve) "arcsin" transformed data, using the software Statistica 7 (Statsoft, Italy). Significant differences among treatments were determined by Duncan's test.

Results and discussion

During the growing season weather conditions were more suitable to *E. necator* than to *P. viticola* both in S. Michele and in Andria. In 2005 downy mildew infections were extremely weak in San Michele and not detected in Andria, so the tested strategies do not differ from each other.

In Andria. Powdery mildew infections were extremely severe. On 28th of June in all the strategies, included the untreated control, specific chemical fungicides were applied in order to limit E. *necator* damages. At the end of August the planned strategies were applied again. The strategy that uses AQ10 treatments early in the season (strategy 3) showed lower efficacy against powdery mildew especially on bunches, compared to the reference (strategy 2). The reference strategy based on sulphur and copper gave the best results (fig. 2).

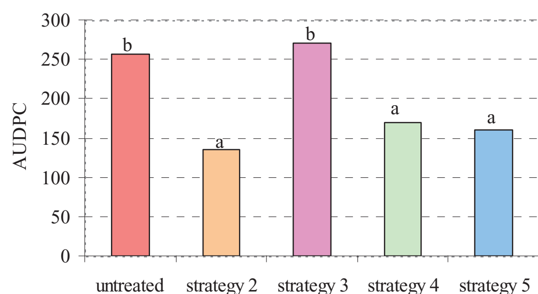

Fig. 2. Powdery. mildew severity on bunches in Andria. Columns with same letter do not significantly differ with $P \leq 0.05$ (Duncan's test)

Untreated control (23%) and strategy 3 (24%) with AQ10 sprayed early that had a high level of powdery mildew, had a comparable high incidence of *Botrytis cinerea* on bunches. The higher incidence is due to the presence of cracks and wounds on berries caused by *U. E. necator,* throughout *B. cinerea* can easily penetrate. No differences were observed between late AQ10 treatments (10%) and sulphur reference (10%).

In S. Michele. We observed the first symptoms on bunches on 9th of June and disease increased faster than on leaves. The incidence on untreated bunches was 100% at the end of June. Under high disease pressure, all treatments significantly reduced powdery mildew infection on bunches compared to untreated control. Using sulphur (strategy 2) during the whole season powdery mildew infections were strongly reduced. Strategy using *A. quisqualis* early during spring (strategy 3) led to higher powdery mildew symptoms than sulphur control. There is no statistically significance difference between strategy 5 and 4 (where AQ10 was sprayed respectively starting from August and September) and sulphur reference (fig. 3)

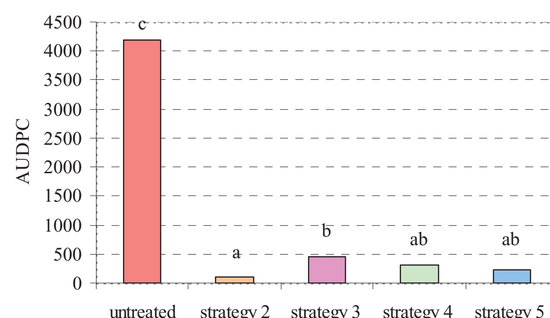

Fig. 3. Powdery mildew severity on bunches in S. Michele. Columns with same letter do not differ with $P \leq 0.05$ (Duncan's test)

The presence of powdery mildew on untreated bunches increased also the presence of Grey mould. There is no significant significance difference in Grey mould severity between AQ10 sprayed in autumn (4) and the reference strategy (2). AQ10 sprayed in spring (3) and summer (5) increases the number of damaged bunches than sulphur reference (Fig. 4).

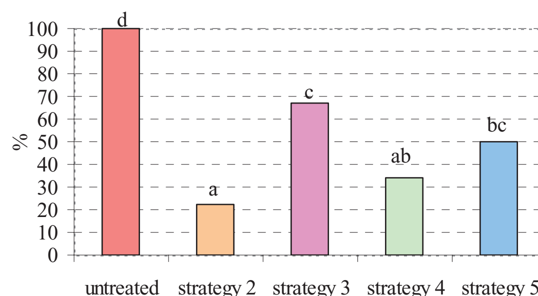

Fig. 4. Percentage of damaged bunches two days before harvest time in S. Michele. Columns with same letter do not significantly differ with $P \leq 0.05$ (Duncan's test)

Conclusion

During 2005 powdery mildew pressure was high in Andria and the infections were reduced only using sulphur, but not with AQ10 in spring. On bunches the use of AQ10 at the beginning of the season was insufficient and had a low effectiveness, similarly as on leaves when it was sprayed at the beginning and in the middle of the season. AQ10 sprayed early increased also the presence of cracks and wounds on berries compared to sulphur. With high disease pressure, the reduction in the use of sulphur cannot be achieved by the use of AQ10. In S. Michele with lower disease pressure, the strategies did not differ from each other.

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We thank Intrachem Bio Italia for kindly supplying products.

Grapevine bud infection by powdery mildew (*Erysiphe necator*): Varietal susceptibility and the evaluation of fungicide treatments to reduce flag shoot development

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Diseased shoots (flag shoots) produced in spring from over-wintered infected grapevine buds are an important source of primary infection for epidemics of powdery mildew (*Erysiphe necator*) in many viticultural regions. Levels of over-wintering inoculum can vary from season to season. Studies of the spread of powdery mildew in vineyards (Emmet et al, 1997) have shown that losses caused by the disease are more likely to occur when levels of over-wintering inoculum are high and disease spread occurs early in the growing season, especially before flowering. In inland warm climate regions of Australia, substantial spread of conidial inoculum from flag shoots occurs from 2-4 weeks after bud break in most seasons.

Previous investigations of the susceptibility of grapevine buds to infection by *E. necator* in Germany (Rügner et al, 2002) and USA (Gubler and Rademacher, 2002; Rumbolz and Gubler, 2005) indicated that buds are often infected before flowering. Inoculated buds on shoots of *Vitis vinifera* cv. Carignane with 3 and 6 unfolded leaves had the highest levels of external infection (Rumbolz and Gubler, 2005). External bud infection in the first season was correlated with internal bud infection and flag shoot incidence in the following season. Histological studies showed that infection is established in the bud interior within 3 weeks of inoculation.

Within buds, haustoria and hyphae colonised the inner tissues and produced conidiophores bearing conidia (Gubler and Rademacher, 2002; Rumbolz and Gubler, 2005). As buds aged, physiological changes to the outer bud scales prevented hyphal entry and infection of inner bud tissues.

In Australian vineyards, fungicide sprays are mostly applied from 2 weeks after bud break to 2-4 weeks after bloom to prevent disease development on vine foliage and bunches. In most districts, fungicides with systemic activity are applied at 4-6 weeks after bud break, pre-bloom and/or post bloom for optimum bunch disease control. Further studies were needed to assess the effects of early season fungicide sprays on bud infection and flag shoot development in the following season.

The aim of this research was to examine bud susceptibility to infection by *E. necator* in some grapevine varieties grown in Australian vineyards, and determine the effects of selected fungicide treatments on bud infection and the development of flag shoots.

Bud infection and flag shoot development on different vine varieties. In glasshouse studies, buds on shoots with 0-1, 3, 6 and 9 leaves unfolded on potted grapevines (*Vitis vinifera* cv. Verdelho, Chardonnay and Sultana) were inoculated with conidial suspensions of *E. necator*. Treatments (inoculated shoots at each growth stage) were arranged in randomised block designs and replicated 140 times. After 3-4 weeks under optimal conditions for the pathogen, the inoculated shoots were sprayed with mineral oil and later with sulphur to prevent further infection. After the vines had over-wintered in a shade house, the proportion of buds of different age that produced flag shoots in spring or died during winter were assessed.

On inoculated Verdelho vines, where 1-2% of over-wintered buds produced flag shoots in the following spring, most flag shoots were produced from buds that were aged 1-18 days at the time of inoculation. Flag shoots developed from 0.4%, 0.9% and 0.6% of over-wintered buds on shoots with 3, 6 and 9 leaves, respectively, at the time of inoculation. Depending on the age of buds when they were inoculated, from 6-19% of over-wintered buds died. Histological studies to determine the proportion of inoculated buds with internal infection are continuing.

Fig. 1. A flag shoot produced from an over-wintered bud on a cane of a Verdelho vine inoculated with *E. necator* in the previous season.

In different experiments conducted under controlled conditions, numbers of flag shoots produced per 1000 buds (on Nodes 1-6) exposed to infection during similar post-inoculation powdery mildew epidemics on Verdelho, Chardonnay and Sultana vines were 108, 33 and 1.4, respectively. The percentages of nodes

producing secondary flag shoots after the removal of primary flag shoots were 0.4 and 0.03 on Verdelho and Chardonnay vines, respectively. After the removal of secondary flag shoots, 0.03% of nodes on Verdelho vines also produced tertiary flag shoots.

These observations confirm that buds at an early stage of development are infected by *E. necator*. Our studies also indicate that there are differences in flag shoot production between vine cultivars. In vineyards of cultivars with a high potential for flag shoot production, crop loss is more likely to occur in the absence of adequate control measures because of the higher likelihood of early season powdery mildew epidemics.

Evaluation of fungicide treatments to reduce flag shoot development. The effects of fungicide treatments on the survival of buds and the development of flag shoots on potted Verdelho vines infected with *E. necator* were investigated in glasshouse studies over two seasons, from spring 2004 (Season 1) to early summer 2005 (Season 2). On each vine, buds on a shoot with 5-6 unfolded leaves were inoculated with a suspension of *E. necator* conidia at 3 weeks after bud break. Fungicide treatments were applied to plots of 20 vines arranged in a randomised block design and replicated 10 times. Shoots were thoroughly sprayed to the point of run-off with a hand-held applicator at 2 or 4 weeks after inoculation in Season 1, or just after bud break (when shoots had 1-2 leaves) in Season 2. Fungicides used in the spray programs included penconazole (Topas®, 12.5mL/100L) or a tank mixture of spiroxamine (Prosper®, 40mL/100L) and tebuconazole (Folicur®, 11.6mL/100L).

The incidence and severity of powdery mildew on the leaves, stems and buds of randomly selected shoots in each plot were assessed just before the first fungicide sprays were applied in Season 1, and scarring on stems was assessed during dormancy. The incidence of dead buds and buds that produced flag shoots was assessed 1-9 weeks after bud break in Season 2.

In relation to the untreated control (Treatment 6, T6), penconazole applied at 2 weeks after inoculation (T1) in Season 1 or a mixture of spiroxamine and tebuconazole applied at 4 weeks after inoculation (T4) in Season 1 reduced flag shoot incidence at 7 and 9 weeks after bud break in Season 2 (Fig. 2, $P < 0.05$). Penconazole applied just after bud break in Season 2 (T5) subsequently reduced flag shoot incidence at 9 weeks after bud break. The proportion of inoculated buds that died during winter (1.9-2.9%) was not affected by fungicide treatment.

The results indicate that correctly timed fungicide sprays can reduce flag shoot development. Furthermore, fungicides or fungicide mixtures with different chemistry may have different effects and may be used at different times after bud infection. For example, an application of penconazole when shoots have 9-10 leaves and/or an application of a mixture of spiroxamine and tebuconazole two weeks later, when shoots have 12-15 leaves, could reduce flag shoot expression on vines and as a result, levels of primary inoculum in the following season. Further glasshouse and field studies are required to clarify these effects and determine the timing of sprays of different fungicides for optimum control of bud infection, flag shoot expression and disease on vine foliage and bunches. In preliminary field evaluations of spray programs on inoculated Chardonnay and Sultana vines, only low numbers of flag shoots developed in Season 2, apparently because of adverse weather conditions, and there were no differences between treatments. Further field evaluations have been established for assessment in spring 2006.

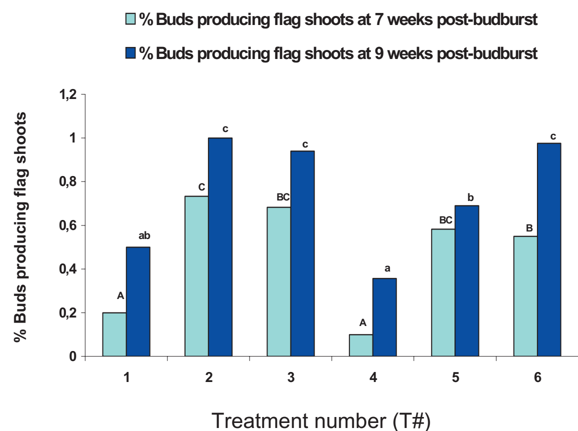

Fig. 2. Effects of chemical treatments on the development of flag shoots on potted Verdelho grapevines.

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Impact of four years application of the Minimal Fungicide Strategy for downy mildew control on the plant recovering capacities of *Vitis vinifera* cv Merlot

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Prior studies of the interactions between downy mildew (*Plasmopara viticola*) and the grapevine has permitted to propose a minimal fungicide strategy (MFS) based on a first treatment at the apparition of the first symptoms (to avoid yield quantity losses), followed by one or two additional treatments during the early epidemic phase to delay and keep the epidemic under the Economic Injury Level (EIL), that goes from 5% severity at the beginning of the veraison to 10% severity at the end of the first ripening phase, to limit the plant stress during the ripening and therefore yield quality losses. During the period 1999-2002 the impact of the MFS on epidemic, yield quantity and quality was studied in comparison with a standard schedule (SS) and an untreated plot.

Tab. 1. Results of the most important yield components at harvest (September 29 for 1999, September 22, September 26 and October 2 for 2000, 2001 and respectively 2002) MFS = Minimal Fungicide Strategy; SS = Standard Schedule; Av = Average; s = standard deviation

	Plot	Yield (kg/m^2)		Must soluble contents (°Brix)		Total acidity (g/l)	
		Av.	s	Av.	s	Av.	s
1999	MFS	0.820	0.08	18.08	0.19	6.01	0.20
	SS	1.320	0.07	19.04	0.66	5.71	0.15
		$P = < 0.001$		$P = 0.03$		$P = 0.04$	
2000	MFS	1.160	0.19	19.29	0.14	5.58	0.12
	SS	1.112	0.26	19.79	0.26	5.21	0.21
		$P = 0.44$		$P = < 0.001$		$P = 0.04$	
2001	MFS	0.793	0.21	19.44	0.21	7.35	0.23
	SS	0.926	0.26	19.94	0.17	7.29	0.08
		$P = 0.03$		$P = < 0.001$		$P = 0.39$	
2002	MFS	0.865	0.10	19.70	0.12	7.11	0.18
	SS	1.016	0.19	19.79	0.12	6.94	0.22
		$P = 0.067$		$P = 0.155$		$P = 0.04$	

The MFS application has permitted to reduce between 43% and 67% the number of fungicide applications in comparison with the SS under downy mildew conducive conditions. The disease severity measured on the leaf canopy was, at the middle of August, limited between 13.1% and 2.0% indicated a delay of the epidemic phase. In the untreated plots the severity was at the same period greater than 30% with a total yield loss. At harvest, the yield in the MFS plot did not differ statistically, with the exception of 1999 and 2001, from that of the normally treated plot. The must soluble solids contents were lower of 0.96°Brix for 1999, 0.5°Brix for 2000 and 2001 and 0.1°Brix for 2002 (table 1). In according with our prior results, we assumed that the plant compensated partially the leaf damage with the mobilisation of the reserves stored in the root. Consequently, the for 4 years application of the MFS permanently in the same plot permitted to evaluate the potential negative effects of the reserves reduction during the following seasons on plant growth. We observed a significantly reduction of the shoot length only after 3-years stress and the tendency to reduce the main and lateral leaf surface after 2-years stress. The most important influence was a significant decrease in the potential yield quantity, which was evaluated in the late July, but the productivity in MFS plot was still higher than the production limit of 1 kg/m^2 valid in our country.

Despite the risk due to the application of the MFS over several years was minimal, we assumed that a year with a low disease impact or without disease is enough to reconstitute the reserve pool in the roots and trunk and to eliminate the stress situation and therefore the differences in plant growth and production. Consequently, we applied in the 2003, the recovering year, a normally spray schedule in the MFS plot from June 6 after the apparition of the first downy mildew sporulations. The measurements, performed during this recovering year, confirmed the cumulated stress effects observed on plants, with significantly decrease in the shoot length, in the potential production and, for the first time, in the leaf number/plant which was related to a decrease of the number of lateral shoots and leaf/lateral shoots. The unfavourable climatic conditions for downy mildew and the spray schedule applied avoided leaf damages in the MFS plot, but the reduced leaf area in the MFS was probably responsible for the significantly decrease of the yield quantity of -0.114 kg/m^2, and most soluble contents of -0.39°Brix (1.8%) at harvest.

In 2004, after the recovering year 2003, no differences were found in the growth parameters (tab 2)

Tab. 2. Results of the most important growth parameters at the last control of June 17 2004 before topping. MFS = Minimal Fungicide Strategy; SS = Standard Schedule; Av = Average; s = standard deviation.

Plot	Shoot length (cm)		Lateral shoots/main shoot		Total leaf/shoot	
	Av.	s	Av.	s	Av.	s
MFS	79.4	3.23	6.8	0.56	25.79	1.31
SS	82.7	5.95	7.4	1.09	27.32	2.39
	$P = 0.19$		$P = 0.21$		$P = 0.13$	

and we found only a significantly higher potential production of 0.152 kg/m^2 in the SS plot.

We found the same situation at the harvest (tab 3) but without differences in must sugar contents and, contrary to the stress years, a significantly higher total acidity content of the must obtained from the SS production. These differences were therefore due to the different yield level between the two plots and not to an effect of the cumulated stress.

Tab. 3. Results of the most important yield components at harvest (September 28) MFS = Minimal Fungicide Strategy; SS = Standard Schedule; Av = Average; s = standard deviation.

Plot	Yield (kg/m^2)		Must soluble contents (°Brix)		Total acidity (g/l)	
	Av.	s	Av.	s	Av.	S
MFS	0.775	0.11	20.52	0.15	7.02	0.19
SS	0.960	0.05	20.55	0.12	7.21	0.12
	P = < 0.001		P = 0.72		P = 0.04	

To understand the physiology of the plant during the recovering year, we performed measurements of the chlorophyll *a* fluorescence with the help of the JIP-test, which is a tool to analyze the polyphasic rise of the Chl *a* fluorescence transient (OJIP labeled phases) and has been developed to investigate *in vivo* the plant vitality and the adaptive behavior of the photosynthetic apparatus to different stressors. The analysis of Chl *a* fluorescence transient has been largely studied and allows to evidence abiotic stresses, like light intensity, drought, temperature, nutrient deficiency, and heavy metals, herbicides or air pollutants toxicity. Contrary, the use of fluorescence monitoring to reveal biotic stresses, like nematode or fungal pathogen effects is less common.

The measurements have been carried out at the 10th of June and at the 11th of August comparing MFS and SS plots with an untreated control plot. This approach has the advantage to give a measure of the photosynthetic efficiency and to provide information on the relationship between the structure and the function of the photosystem II (PSII) reaction centers and core complexes. At 10th of June little differences were evidenced, two months later, at the 11th of August, differentiated data were detectable (fig 1).

Plants of untreated and of the MFS plots showed significant differences to normally treated plot used as reference. In the MFS plot, the vitality of the plants was higher than the normally treated and the untreated plants and a higher photosynthetic activity was revealed. On the contrary, the plants of the untreated plot presented a large decrease of phenomenological fluxes (per cross section; DIo/CSm, TRo/CSm, ABS/CSm and RC/CSm) and higher dissipation (DIo/RC) through heat. The stress situation caused by downy mildew epidemics disturbed the stressed leaves on the biochemical side (effect on the J to P phase of the transient).

Therefore, the vines of the MFS plots stimulated the photosynthetic productivity of the leaves more than the plants of normally treated plot. These results confirms:

- that a recovering year is enough for the plant to compensate the cumulated stress induced from the partial control during 4 years of the downy mildew epidemics and confirms our preview observations made by comparing treated and untreated plot;

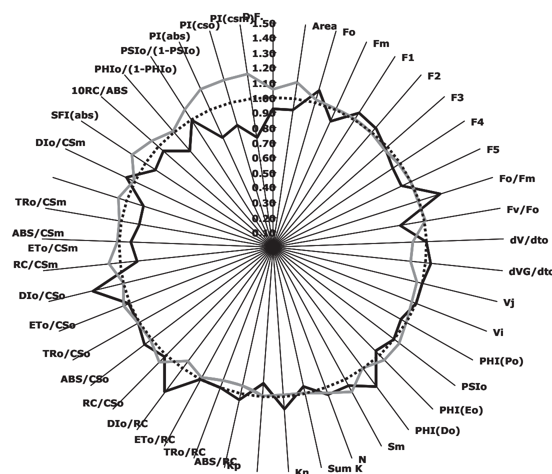

Fig. 1. Influence of the MFS (Minimal Fungicide Strategy, gray line) and of an untreated plot (black line) on PSII biophysical parameters of leaves of the cv. Merlot. Dotted line represents normal fungicide treated plants and it set as reference sample in the radar plot.

- that a downy mildew stress situation induces in Merlot grapevines an important change on the biochemical side with an increase of the efficiency in the use of the photosynthetic energy. This stress was not present in a normally or in an untreated plant with low infection level, like in 2003. This can probably explain the strong compensation capacities of the vine (particularly in the Merlot) to compensate a stress situation.

Nevertheless, we should consider that each growing season is independently from the previous one, because the reconstitution or depletion of the reserve pool depends on the plant stress during ripening period (e.g. years with a high downy mildew severity versus years without relevant down mildew). It is also sure that no downy mildew epidemic models are able to give an adequate answer on the seasonal epidemic development and it is impossible to forecast the potential stress situation of the grapevine. Consequently, repeated applications of the MFS need at least every each 4-5 year a normally treated year to promote the recovering capacities of the plants.

CropWatchOnline.com: A Website for Growers and Researchers to Improve Vineyard Management of Grapevine Downy and Powdery Mildew

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In recent years, a number of useful services have been available in Australian viticulture to assist vineyard management operations for the control of the mildews *viz. Plasmopara viticola* (downy mildew) and *Erisyphe necator* (powdery mildew), and other diseases. In this paper we describe a new on-line service that integrates some of these services for easy access by growers, technicians and researchers.

Existing Services

Use of the following technologies is designed to assist Australian grapegrowers achieve more efficient and effective control of the mildews and other diseases in Australian viticulture. Some growers find it easier to use routine (calendar-based) spray strategies whereas it is intended to encourage use of proactive strategies that use optimum spray timing (either pre-infection or post-infection spraying, or a combination of both), the most effective fungicide products, and the most efficient spray application methods.

There are four services discussed in this paper.

1. Diagnosing vineyard symptoms. A number of aids to correct diagnosis of the mildews and other symptoms in the vineyard are available. One of these is a *Field Guide* with colour photos and simple diagrammatic keys that help growers confidently and quickly make the right diagnoses (Magarey *et al.* 1999).

2. Weather services. The Australian Bureau of Meteorology (BoM) provides regional weather forecasts and a variety of data from past events. Since the late 1980's, BoM has linked with the first and fifth authors to provide grapegrowers in all South Australian viticultural regions and more recently, Sunraysia (Mildura, Vic), with forecasts of downy mildew weather events during the growing season. BoM staff assess forecast events 3-5 times daily for risk of the mildew. When the perceived risk exceeds 75% following consultation with the pathologist, downy mildew alerts are issued immediately and accompany weather forecasts to all media outlets. This rapid distribution allows maximum warning for the application of pre-infection (protectant) fungicides. The service also alerts growers to evaluate the need for post-infection (eradicant) fungicides if preventative sprays were not applied and the forecast event eventuates.

3. CropWatch SA®. A vineyard disease and pest management service known as CropWatch SA® has been operating especially for the management of downy and powdery mildew in the Riverland wine region (near Loxton, SA) since 1995-96, and in McLaren Vale (SA) since 2003-04. A network of automatic weather stations provides microclimatic data for the decision-support software, AusVit™, to determine the risk of downy mildew primary and secondary infection events and calculates date of oilspot appearance. With vineyard monitoring data to assist, the predictions are reviewed and interpreted to develop specific management options for both mildews, and for bunch rots and pests such as mites and light brown apple moth. CropWatch is sponsored and managed locally by the respective regional grape-industry bodies. In an industry-funded service for growers and vineyard managers, a 1-2 page bulletin is faxed or e-mailed weekly (or more often) through the growing season. A similar service, Horticulture Hotline®, has been provided by Department of Primary Industries in Sunraysia since 1988.

4. Grape-industry information services. For most viticultural regions of Australia, a rapidly broadening array of industry-based newsletters (printed and e-mail versions) and similar information services (including communal noticeboards), provide a diversity of R&D findings, news and other information to growers. Though the delivery (on a daily, weekly or monthly basis) and the uptake of these and other services is a matter of grower and/or industry choice, few of these services are available on-line.

CropWatchOnline.com is a web-site that provides disease management information on the mildews and other diseases and pests of viticulture and incorporates the above services. With an emphasis on the timely control of the mildew diseases, the site offers users assistance in swift and accurate diagnosis, advice and options on the type and timeliness of disease management strategies.

Fig. 1. CropWatch Online is designed for easy access by grapegrowers and others unfamiliar with electronic media. Users select their location of interest – they are then taken to specially tailored e-products for that region.

It also provides information on the selection and application of fungicides, and offers other management tools, all of which are needed for efficient and effective outcomes.

There are four main modules within CropWatch Online.

1) Disease Diagnosis**.** The contents of the *Field Guide* (Magarey *et al.* 1999) have been formatted to provide users with rapid electronic access to a web-based tool that allows users to successfully diagnose vineyard symptoms. [See accompanying paper on Disease Diagnosis in these proceedings].

2) Weather services. Targeted links to BoM present regionally formatted forecasts and tables of useful weather data. The forecasts are focussed for relevance to the region which the user nominates, to provide optimum information for managing downy mildew. Where available, data from local weather station networks are also accessible.

3) CropWatch SA®. Management advice from the CropWatch service (as above) is provided *via* the web-site to complement the delivery system previously available *via* the weekly information 'push' system *eg* fax or e-mail. The web-site provides current messages and past records of CropWatch SA® and Horticulture Hotline® in a regionally specific format *via* an information 'pull' system that is available at any time.

4) Local industry noticeboards. Bulletin boards tailored by local industry groups, present news and information targeted to growers in their region *eg* advising of coming grower meetings and field days.

In addition, CropWatch Online provides e-links to a number of viticulturally useful web-sites for sources of other information. CropWatch Online gives users swift access to and return from the external sites - it functions like a central railway station terminus with (free) delivery and return to external locations at will.

The website provides information and resources of relevance to Australian and New Zealand users but has facilities that may be beneficial to others internationally.

CropWatch Online has been formatted for access from standard computers and from modern mobile phones to allow users to access the resources within the site from locations other than homes and offices.

Discussion

CropWatch Online is an e-tool for cheap and rapid flexibility in delivering the latest vineyard information with special reference to users seeking disease management information for the mildew diseases of grapes. Each of its four main modules is designed to encourage growers and others, though perhaps unfamiliar with the web, to have confidence in making better disease management decisions that are biologically sound and economically productive.

Estimated costs of disease management are a guide to the economic benefit of this technology. For instance, using values in AUD$ from 2004, a routine spray schedule will cost $456/ha/year (where Cost/ha = Cost of chemicals + Cost of application at $53/ha). Similarly, a strategic spray schedule encouraged through use of CropWatch services will cost $259/ha/year, a saving of around $197/ha/year.

Thus, total savings in costs of disease controls alone for the Riverland-Sunraysia districts (at a 30% adoption rate) would be near AUD$3.6m./year. More effective disease control and, as a result, reduced crop loss is an additional gain.

Fig. 2 and 3. Having clicked on the region of relevance, *CropWatchOnline.com* delivers the user to the latest mildew management advice for that region. In the above example, growers from McLaren Vale, SA, can read a summary of the latest CropWatch message with options to access further detail as required (see below).

The website is 'free to air' and can be accessed at www.cropwatchonline.com.

Acknowledgement

For their assistance with the initial establishment of the concept we thank the late Don Stephenson of the Riverland Winegrape Industry Development Council, Ashley Lipman from Rural Solutions SA at Loxton, the Grape and Wine Research and Development Corporation, and the Murray Pioneer and Winetitles, both of whom have generously donated web-server space for the CropWatch Online system.

Reference

Magarey P.A., MacGregor A.M., Wachtel M.F. and Kelly M.C. 1999. (Editors). *The Australian and New Zealand Field Guide to Diseases, Pests and Disorders of Grapes.* A companion to 'Diseases and Pests', Grape production Series No.1., Winetitles, Adelaide, South Australia. 108 pp.

Disease Diagnosis: A Website for Swift Identification of Grapevine Downy and Powdery Mildew and Other Diseases and Pests from the Tractor-Seat!

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For maximum efficiency in managing the mildew diseases *Plasmopara viticola* (downy mildew) and *Erisyphe necator* (powdery mildew), and other diseases, pests and disorders of grapevines, a thorough understanding of factors such as disease epidemiology, crop growth and vineyard microclimate is needed. However, correct identification of field symptoms is crucial to effectiveness of this knowledge in application of the most appropriate controls.

Since 1999, the pocket-sized book, The Australian and New Zealand Field Guide to Diseases, Pests and Disorders of Grapes (Magarey et al. 1999, Winetitles, Adelaide, SA) has provided access to colour photos of typical symptoms of most diseases, to images of pests and useful insects, and of weedicide, nutritional and other disorders in Australasian vineyards. The Field Guide was designed to make the identification of symptoms easy and to give the user confidence in that process. This was done in part through an easy-to-use 'Looks Like' cross-reference system that assists correct diagnosis.

In recent years, the IT industry has made significant progress in the speed and accessibility of web-based information. Increasingly, grapegrowers are seeking to utilise the advantages that online facilities provide eg low-cost and immediate access to comprehensive sources of information that can be up-dated with ease. The Field Guide has proven popular but is not available on-line. In this paper we report on the development of an electronic system designed to assist growers diagnose vineyard symptoms.

A Website

More than 300 images from the Field Guide have been scanned and uploaded in a simple, easy-to-operate database-driven website. The database provides ease of maintenance and a high level of flexibility to expand and alter the website as needed.

Called *Disease Diagnosis*®, the system presents the user with a simple system to search for a particular disease, pest or disorder by image (Fig. 1).

The user is then asked to choose which image best describes the symptoms in hand eg a leaf with yellow spots, white spots, etc. (Figs. 2-4).

Fig. 1. *Disease Diagnosis* provides the user with a simple key to select the type of grapevine material bearing the symptoms. Selecting then clicking on the relevant image sends the user to the next decision key (see Fig. 2).

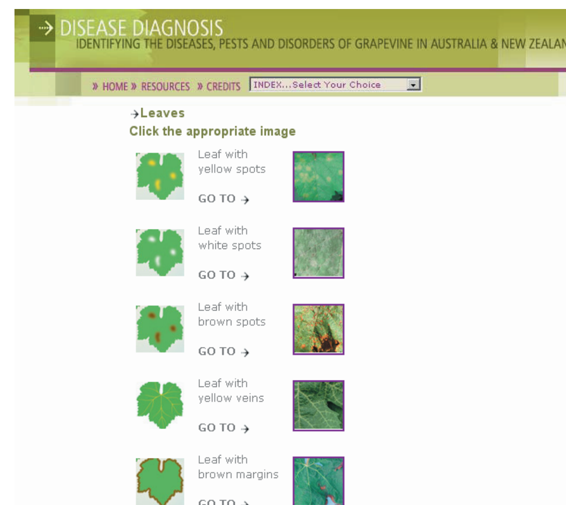

Fig. 2. Users are asked to click on the thumbnail image that best suits the type of symptom to be diagnosed. This opens the photo library which provides more detail (see Fig. 3).

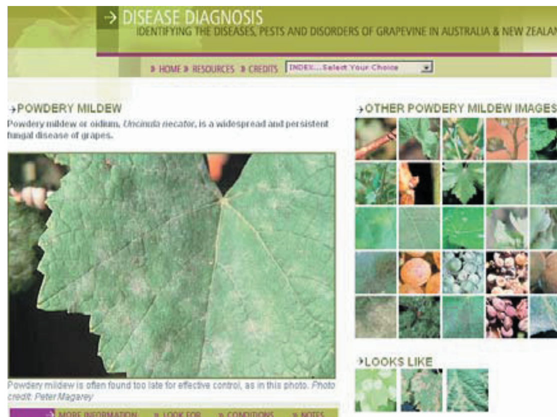

Fig. 3. The expanded image presents the user with a photo illustrating the symptoms of the type selected in Fig. 2 and most likely to be found in Australasian vineyards. In this case, the white leaf spots may be powdery mildew but check more information before concluding the diagnosis (see Fig. 4).

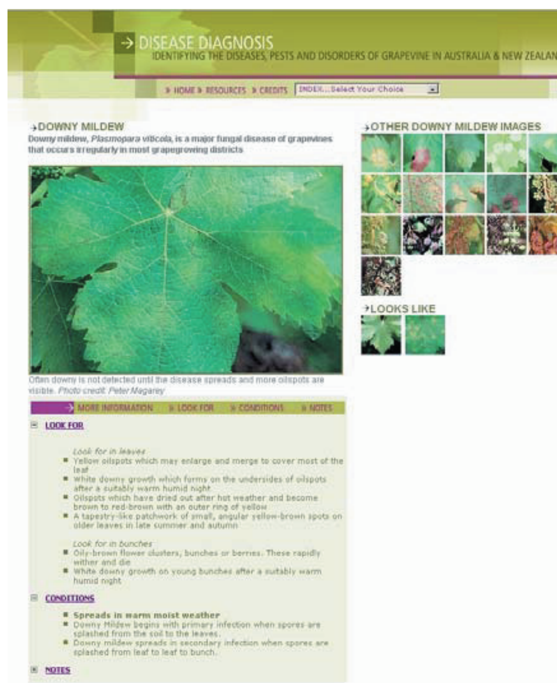

Fig. 4. Users can select and click to enlarge the thumbnail photos. of the 'family' of Other Images - this illustrates the array of symptoms caused by the pathogen or pest. A second series entitled, Looks Like, are images that can be confused with the family of images for the main photo. By selecting the relevant thumbnails, users are led through the library of photos in a 'self-guided tour' of likely symptoms

Associated with each enlarged image is a series (or 'family') of '*Other Images*' that illustrate the array of symptoms for that particular disease.

Below the family of images is '*Looks Like'*, a second series of images. These present alternate families of photos for each cause of disease that produce similar symptoms.

By scanning and enlarging these images where necessary, the user can follow a self-guided tour of the website and can read detailed information in text that accompanies each image under tabbed headings of '*Look For'*, '*Conditions*' and '*Notes*'.

By clicking on these tabs below, the user can read notes about what symptoms to look for and about the conditions that favour that disease, pest or disorder and other notes of significance.

Fig. 5. Modern technology such as CropWatch Online allows growers to access valuable disease diagnostic information while on their tractor seat. This is especially important for some growers who find it confusing to distinguish between the two mildew diseases.

Discussion

Disease Diagnosis is a website for easy identification of vineyard symptoms. Installed within CropWatch Online, a website for grapegrowers], it is accessed at www.cropwatchonline.com. [For more details See accompanying paper on CropWatchOnline.com in these proceedings]

The website has been formatted for mobile-phone users to access Disease Diagnosis whilst away from their office – even from within the vineyard, on their tractor seat!

It is expected that *Disease Diagnosis* will encourage those unfamiliar with websites to gain confidence in their use.

Acknowledgements

We thank the Grape and Wine Research and Development Corporation for financial assistance and Winetitles for the scanned the images from the *Field Guide*.

Results from Two Years of Field Studies to Determine Mancozeb-based Spray Programmes with Minimal Impact on Predatory Mites in European Vine Cultivation

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Mancozeb is a dithiocarbamate fungicide with contact activity against a wide range of economically important fungal diseases. Its multi-site mode of action means that resistance is much less likely to develop and to date there have been no recorded incidences of field resistance developing to mancozeb despite many years of use on high risk diseases. One such disease, grape downy mildew (*Plasmopara viticola*) has developed resistance to a wide range of important oomycete specific fungicides following their introduction onto the market. The role of mancozeb either as a mixing or alternation partner in helping to manage these resistance situations remains critically important.

Historical use patterns for mancozeb in vine crops involved many applications of product at high use rates. Although this gave excellent disease control, a negative impact on predatory mites was often reported by researchers. Following inclusion of mancozeb onto the Annex I listing, future use patterns for mancozeb in vines in the EU are likely to involve lower use rates and number of maximum permissible sprays per season. This has led to the development of mancozeb spray programmes in vines with a much reduced impact on predatory mites.

A range of field studies were conducted over the 2004/5 season in France, Germany, Italy, Portugal and Spain where either 2, 3 or 4 applications of mancozeb at 1.6 kg a.i./ha were made per season at different spray timings (pre-flowering, flowering and over fruit set. A total of 24 trials were conducted to GEP standard and covered a representative range of agronomic practices, mite species and geographical locations in Europe. Using the findings of these trials we look at year to year variation and recommend application scenarios with mancozeb which are compatible with Integrated Pest Management (IPM) programmes and the conservation of predatory mites.

The experimental design of the trials followed the standard field method of Blümel et al (2000). Assessments of mite population were made prior to treatment, approximately 7 days after each application and at the end of the trial around 30 days after the last application. Safe and toxic references were included in each trial for comparative purposes. All data were converted into mobile forms per leaf per trial and the impact of treatments was expressed as % Abbot mortality.

Data were analyzed by ANOVA and also by a more novel cumulative probability approach which was used to estimate the probability of 50% or less impact on mites (IOBC class 1 or 2) with 95% confidence limits using linear regression analysis.

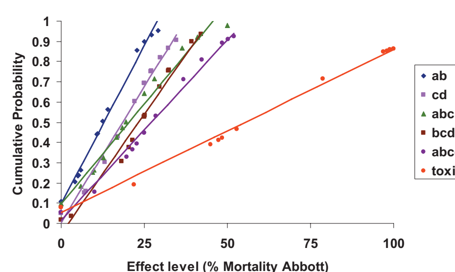

Fig. 1. Cumulative probability analysis of effect (% Abbot mortality) of 2, (ab, cd) , 3 (abc, bcd) and 4 (abcd) applications of 1.6kg ai/ha of mancozeb on predatory mite populations.

Of the 3 mite species detected across trials (*Typhlodromus pyri, Kampimodromus aberrans* and *Euseius stipulatus*) all were observed to exhibit similar sensitivity to mancozeb under field conditions. As expected, impact on mites increased with increasing numbers of applications however 2, 3 or 4 applications were still all IPM compatible. Simple probabilistic modelling indicated that 2 applications are virtually always below 50% effect (IOBC class 1 or 2), 3 applications rarely exceed 50%, and 4 applications occasionally exceed 50% effect. In addition all mite populations had fully recovered by end of the trials irrespective of the number of applications made.

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Aerated compost extract: standardising a new approach for integrated management of powdery mildew

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An integrated approach to disease management is becoming essential due to the potential for resistance to synthetic fungicides, non-target impacts associated with vine protection and market demands for no or low fungicide residues in wine. Aerated compost extract is a relatively new concept for disease management, with potential for incorporation into conventional strategies for powdery mildew management.

Briefly, aerated compost extract, also known as compost tea, is a watery extract of compost containing a diversity of beneficial aerobic microorganisms and nutrients. When applied as a fruit and foliar spray, aerated compost extract has been shown to suppress several horticultural diseases (Scheuerell and Mahaffee, 2002). However, the effectiveness and mode of action of aerated compost extract has not been studied comprehensively.

The primary objective of our research is to standardise production of aerated compost extract, using compost produced by Soil First Pty Ltd, for maximum suppression of *Botrytis* bunch rot and powdery mildew on grapevine. Soil First maintain a target carbon:nitrogen ratio for their compost in a market where the type of raw ingredients for compost production can vary.

Aerated compost extract was prepared on a small scale by placing 10 kg of compost in a polypropylene bag and suspending the bag in dechlorinated water (1 part compost: 3 parts water). Aerobic conditions were maintained by circulating water with a pond pump (1,500 L/hr). Parameters of compost extract such as pH, temperature, conductivity and nutrient analyses were recorded in all experiments.

Determining parameters for compost extraction

A detached bean leaf bioassay was used to investigate the impact of selected production variables on growth and reproduction of *Botrytis cinerea*. The variables evaluated were compost sampled from different positions on the windrow, compost sampled at different times after windrow initiation (5 weeks to 13 weeks; compost cooling phase to compost maturation, respectively) and the duration of extraction (0, 24, 48, 72 hours).

Four leaflets of *Vicia faba* (beans) were placed in the designated compost extract for 1 min. prior to placing each leaflet on sterile paper towels moistened with sterile water in a 9 cm-diameter Petri plate. The adaxial surface of each leaflet was inoculated with six 10 µl drops of a suspension containing 1 x 10^5 *B. cinerea* conidia per ml. Petri plates were sealed with Parafilm and incubated at room temperature in the dark. The number of necrotic lesions on four leaflets was counted 2 days post inoculation, the area of necrosis was assessed 5 days post inoculation and the number of lesions producing conidia of *B. cinerea* was assessed 9 days post inoculation. The experiment was repeated and the number of colony forming units of bacteria and fungi per ml of compost extract was counted for each extract. Data were analysed by repeated measures analysis of variance.

Treatment of bean leaflets with various compost extracts prior to inoculation with *B. cinerea* reduced the mean number of necrotic lesions and lesions with sporulating *B. cinerea* significantly (Fig. 1, Fig. 2A, P<0.001). The extent of pathogen colonisation was similar when extraction times 24, 48 and 72 h were compared (Fig. 2B). Aerated compost extract produced from 6-week old compost resulted in significantly fewer lesions with sporulating *B. cinerea* than extract produced from 5 or 8-13 week old compost (Fig. 2A). When the experiment was repeated, pathogen suppression correlated positively with the number of bacteria and fungi per ml of extract (data not shown).

Fig. 1. Bean leaflet 5 days after treatment and inoculation with six 10 µl drops of *Botrytis cinerea* conidia. Treatments were dechlorinated water (left) or aerated compost extract (right, 48 h extraction of 6-week old compost).

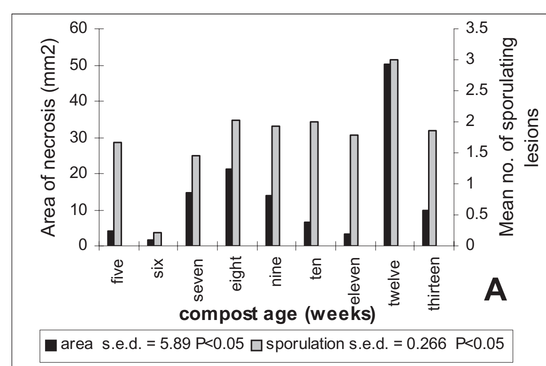

Fig. 2A (legend on next page)

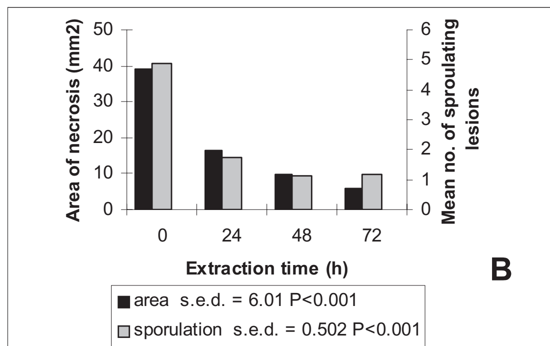

Fig. 2. Effect of (A) compost age on the mean area of necrosis and mean number of sporulating lesions on bean leaflets inoculated with *B. cinerea* and (B) extraction time (across various ages of compost). Data for 0 h extraction time omitted for analysis of compost age.

The detached bean leaf assay was useful for selecting parameters for extract production that were then applied for a study of effectiveness in the field. This efficient assay is now being used to assess consistency in results among batches of compost extract, especially following a significant change in the raw ingredients used for compost production.

Field trial

In 2005/06, the effectiveness of aerated compost extract in suppressing a natural epidemic of powdery mildew (*Erisyphe necator*) was evaluated. Aerated compost extract was prepared from six week-old compost in a 48 hour extraction followed by application to Chardonnay vines at 10-14 day intervals in a randomised block design (n=5). The addition of Foundation Fish (Soil First Pty Ltd) and kelp to aerated compost extract was also evaluated.

All treatments reduced the mean incidence and severity of powdery mildew on the underside of leaves when compared to the untreated control (Fig. 3). Powdery mildew was not detected on pea-size berries of treated vines, whereas the incidence and severity of disease on berries of untreated vines was 100% and 70%, respectively. At E-L stage 35-36 (veraison), all treatments had significantly less powdery mildew on bunches when compared with the untreated control (Fig. 4). The aerated compost extract treatments were equivalent to the standard organic spray program and resulted in commercially acceptable control of powdery mildew on bunches (Figure 4).

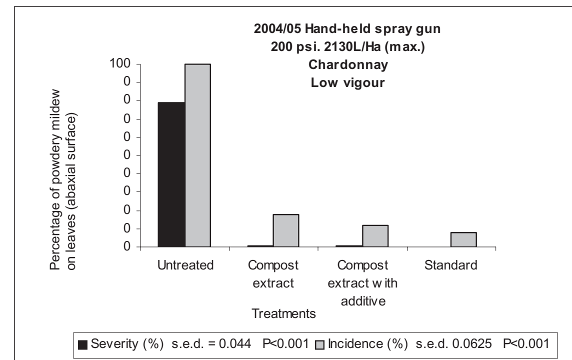

Fig. 3. Mean incidence and severity (%) of powdery mildew on underside of leaves on Chardonnay vines at E-L 31 (pea-size berries). Standard = organic program based on Synetrol-Hortioil® and sulfur. Standard errors of the difference of the mean calculated following arcsin transformation of the data.

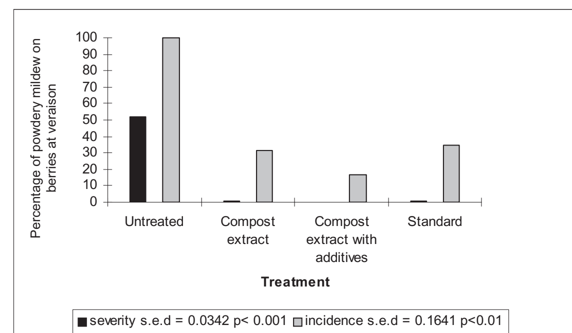

Fig. 4. Mean incidence and severity (%) of powdery mildew on grape bunches on Chardonnay vines at E-L 35-36 (berries beginning to colour and enlarge). Standard = organic program based on Synetrol-Hortioil® and sulfur. Standard error of the difference of the mean calculated following arcsin transformation of the data.

Conclusion and future research

Aerated compost extract can reduce infection and colonisation of two diverse pathogens: *Botrytis cinerea,* which causes bunch rot and *Erisyphe necator,* the cause of powdery mildew in grapevines.

Based on results of one field trial, aerated compost extract has the potential to be incorporated into an integrated disease management program for grapevine powdery mildew. Disease suppression in the field, including the impact of compost extract on *Botrytis* bunch rot, will be evaluated further. We plan to investigate the mechanism of disease suppression, the molecular ecology of compost extract and the possibility for human pathogen re-establishment. These studies will allow specification of the level and maximum variation in each production variable for achieving the desired level of disease suppression and product safety.

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Novel Control of Grapevine Powdery Mildew on a Commercial Vineyard in South Australia: Effects on Disease and Quality

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Powdery mildew (*Erysiphe* (*Uncinula*) *necator*) is generally controlled by the application of sulphur and synthetic fungicides in conventional vineyards, and by sulphur and vegetable oils in organic vineyards. However, sulphur can be toxic to agricultural workers (1) and beneficial mites and insects, including natural antagonists of *E. necator* (2). In a study of alternatives to sulphur for the control of powdery mildew, Crisp *et al*. (3) showed that *Bacillus subtilis*, whey, milk, potassium bicarbonate (Ecocarb®) and canola-based oils reduced disease severity on Viognier and Cabernet Sauvignon vines in the greenhouse. Preliminary field trials in 2000-2003 were used to evaluate the products selected from the greenhouse experiments. Materials that reduced the severity of powdery mildew in greenhouse experiments and preliminary field trials (3) were evaluated in the vineyard to assess efficacy in a commercial environment. Although effective in greenhouse trials, *B. subtilis* performed poorly in the field in 2000/2001 and was not included in subsequent experiments.

All trials were conducted on a commercial organic vineyard at Langhorne Creek in South Australia, where conditions were generally warm and dry with an average annual rainfall of 600 mm. In 2003/2004, 2004/2005 and 2005/2006, mature vines of cvs Verdelho and Shiraz were sprayed with milk, whey, Ecocarb® 3-5 g L^{-1} plus Synertrol Horti-Oil® 2-3 mL L^{-1} or a mixed program of Ecocarb® plus Synertrol® (Organic Crop Protectants) in rotation with whey. Treated vines were compared to untreated vines and vines sprayed with sulphur (3-6 gL^{-1}, Yates Pty Ltd).

Treatments were first applied when shoots were approximately 15 cm long and continued until veraison, a total of 5-8 sprays per season depending on the level of disease. In 2003/2004, treatments were applied using a Solo 475 backpack spray with a hand-held wand, and in the other two seasons, a 50-L Silvan sprayer with a hand-held wand was used.

Vines were monitored throughout the season and disease severity on leaves and bunches assessed as area affected by powdery mildew. The presence of flagshoots was recorded in spring each season. At harvest, yield was assessed for each treatment, except in 2005/2006 when only yield from Verdelho was assessed, and pH, titrable acidity (TA) and °Brix were assessed for representative bunches. Juice samples obtained in 2003/2004 and 2004/2005 were assessed for differences using duo/trio tests and, in 2005, Verdelho juice was also assessed by a tasting panel of five people.

In 2003/2004 untreated Verdelho vines were severely affected by powdery mildew by early December, with approx 90% of the leaf area affected, and were sprayed with sulphur to reduce the risk of inoculum spreading to the remainder of the vineyard. The severity of powdery mildew on milk and whey treated-vines (both 0.3%) was not significantly different from that on sulphur-treated vines (0.2%). Powdery mildew was not observed on the Shiraz vines. No significant differences were detected for pH, TA and °Brix of juice or yield at harvest. The duo/trio tests revealed a difference between juices from Verdelho vines sprayed with sulphur and those sprayed with whey or left untreated until sprayed with sulphur in December. For Shiraz, differences were detected between the juices from vines treated with sulphur and those sprayed with milk.

In 2004/2005 powdery mildew was not detected on either Verdelho or Shiraz vines, including untreated vines. Microscopic examination revealed powdery mildew on approximately 5% of aborted berries and on the rachis only. In 2005/2006 only trace amounts of powdery mildew were detected. There were no significant differences in yield, pH, TA and °Brix among treatments in either season. Duo/trio testing in 2005 revealed a difference between juices from Verdelho vines sprayed with sulphur and those sprayed with the Ecocarb/Synertrol Horti-Oil mixture. Sensory evaluation showed that juice from Verdelho grapes was suitable for winemaking regardless of treatment and that any differences detected were likely to reflect pressing characters or ripeness.

In October 2003, approximately 30 flagshoots per row were detected in the Verdelho vines and were removed and destroyed immediately. No flagshoots were found at the trial site in 2004 and 2005.

Regular application of milk, whey and Ecocarb® plus Synertrol Horti-Oil® controlled powdery mildew to acceptable levels in 2003/2004, provided that application was regular and coverage was maintained at near 100%. This supported findings of previous vineyard experiments (3). It is not clear why there was little of no disease on either cultivar in 2004/2005 and 2005/2006, as in both seasons, the Gubler-Thomas powdery mildew risk index (4) predicted a higher disease pressure in both seasons than 2003/2004 when disease was severe. The lack of flagshoots in these years may have reduced the amount of inoculum, however, widespread, low-level powdery mildew was detected in other parts of the vineyard.

The test materials appeared to have little or no effect on pH, TA and °Brix or yield, irrespective of the presence (2003/2004) or absence (2004-2006) of powdery mildew in the trial sites. There were some differences in taste of juices each year, possibly due to differences in ripeness of fruit or in pressing characters. However, sensory evaluation of juices obtained in 2005 showed that such differences were minor and unlikely to affect quality.

While flagshoot numbers reflected disease severity in the season in which they occurred, whether they are the source of inoculum for the outbreak or an indicator of the suitability of conditions for powdery mildew is not known. The differences in powdery mildew severity between cultivars and seasons highlight the need for flexible spray programs based on monitoring and the susceptibility of cultivars.

Acknowledgements

This research was conducted with support from the Grape and Wine Research Development Corporation, Temple Bruer Wines, Glenara Wines and Organic Crop Protectants. We thank David Bruer and Leigh Verrall for support, encouragement and advice.

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Session 12:

Poster presentation
Disease management (organic and IPM)

Organic Control of Grapevine Powdery Mildew in Eastern Australia

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Successful control of powdery mildew requires repeated application of fungicides throughout the growing season. Sulphur is commonly used alongside synthetic fungicides in conventionally managed vineyards in Australia. Organic viticulture also relies on the use of sulphur, however, concerns have been raised with regard to the negative effects it may have on beneficial arthropods and microorganisms. 'Alternative' fungicides were tested in glasshouse and preliminary field trials in South Australia (SA) for their efficacy in controlling powdery mildew (1). Milk, whey, potassium bicarbonate and canola oil-based sprays were identified as promising 'alternatives' to sulphur. To test these 'alternatives' in different climatic regions of Australia, field trials were established in vineyards in SA, Tasmania and New South Wales. This paper reports results of field trials conducted in the Charles Sturt University commercial vineyard Wagga Wagga (approximately 452 km SW of Sydney). Wagga Wagga has a warm and dry climate with an annual mean rainfall of 572 mm. Maximum temperatures in summer average between 29ºC and 32ºC with a 3-pm average relative humidity of approximately 30% (Australian Bureau of Meteorology, www.bom.gov.au). Powdery mildew occurs every season when control measures are less than optimal.

Field trials were conducted during three growing seasons (2003-06) using adjacent blocks planted to Chardonnay and Cabernet Sauvignon. The canopy structure for both varieties is "Aussie Sprawl" (single-wire, spur-pruned). The trial was established as a randomized complete block design. In 2003-04 there were six replicates of six vines per treatment for Chardonnay and four replicates of six vines per treatment for Cabernet Sauvignon. In 2004-05 there were four replicates of six vines per treatment for both cultivars. In 2005-06 there were five replicates of six vines per treatment for Chardonnay. The Cabernet Sauvignon vines were removed at the end of the 2005 so were not included in the 2005-06 trial. Treatments included pasteurized bovine milk (~4% fat, 1:5 dilution during flowering and 1:10 at all other times), spray dried non-hygroscopic whey powder (25 g/L; Bonlac Foods Ltd, Victoria), Ecocarb® (4 g/L; activated potassium bicarbonate; Organic Crop Protectants P/L), a mixture of Ecocarb® and Synertrol Horti-oil® (3 ml/L; blend of Australian essential botanical oils and canola oil; Organic Crop Protectants P/L), and sulphur (3-6 g/L; Thiovit® Jet; Syngenta Australia). In 2003-04 and 2004-05 these materials were applied individually or in a program. Unsprayed vines acted as controls in 2003-04 and sulphur as a control in 2004-05 and 2005-06. In 2005-06 materials were applied in programs targeted at the different phenological stages of the vine (Table 1). Sprays were applied at 7-14 day intervals when possible. Initial sprays were applied with a hand held sprayer and, as the canopy increased in size, further sprays were applied using a Solo® 450 motorised mist blower (applications rates of 300-900 L/ha). Water sensitive papers (TeeJet®, Wheaton, IL USA) were placed throughout the trial sites to monitor spray distribution.

Tab. 1. Treatments applied during the 2005-06 growing season.

Treatment	Phenological Stage		
	1st leaf unfolded to flowering	Flowering to fruit-set	Fruit-set to veraison
1	Sulphur[a]	Sulphur	Sulphur
2	Sulphur	SH/E[b]	Milk
3	Sulphur	Milk	Milk
4	Sulphur	Sulphur	Milk
5	SH/E	Milk	Sulphur
6	SH/E	Sulphur	Milk

[a]Sulphur applied at 6 g/L
[b]SH/E; Synertrol Horti-oil® (3 ml/L)/Ecocarb® (4 g/L)

During 2003-04 and 2005-06 the incidence and severity of powdery mildew for 40 randomly selected leaves and bunches in each plot were assessed on a 1 to 10 scale (based on percentage leaf or bunch/stem area infected). In 2004-05, 60 leaves and bunches were examined. Where possible, assessments were made fortnightly, 2 to 3 days prior to each spray application. During 2004-05 and 2005-06 humidity, temperature and dew point were recorded every 20 min using Hobo® U12 data loggers. The temperature data were used to calculate the Gubler-Thomas (G-T) powdery mildew risk index (2). To measure canopy density a plant canopy analyzer (LAI-2000, LI-COR®, NE, USA) was used to determine the leaf area index from inflorescences visible to veraison during 2004-05. At harvest for 2003-04 and 2004-05, 50 berry samples (25 from each side of the vine row) were taken from each treatment and the mean berry weight, pH, total soluble solids (TSS) and titratable acidity (TA) of juices measured. Chardonnay berries were not tested in 2004-05.

Powdery mildew occurred in all seasons and was first observed between three leaves unfolded to inflorescences visible in both Chardonnay and Cabernet Sauvignon. No flag shoots were observed. Water sensitive papers indicated satisfactory spray coverage and no significant differences in canopy density (P = 0.528) were observed for the two cultivars. During 2004-05, the G-T index reached 60 points in early October (3 leaves unfolded) and remained at this level until early January (veraison). The same trend was recorded for 2005-06. In both seasons a total of 70-74 days above 60 points was recorded.

In 2003-04 control efficiency of powdery mildew on Chardonnay was not commercially acceptable. Sulphur provided the best control with an incidence of 34.2% and severity of 4.2% (Table 2). There were no significant differences between the remaining treatments for incidence, however milk and whey provided reduced severity of disease on bunches. In contrast, acceptable control for Cabernet Sauvignon was provided by all treatments. The 2004-05 season was particularly conducive to infection by powdery mildew. Incidence of infection reached 100% for Chardonnay with most treatments (Table 2). To minimize the inoculum from spreading to neighbouring grapes used for commercial winemaking, the Chardonnay was sprayed with a systemic fungicide on 21/12/04. At harvest, there were no significant differences between treatments in the incidence of powdery mildew. However, the severity was less for the sulphur treatment (14%). For all other treatments the severity of powdery mildew ranged between 66% and 75%. As in 2003-04, acceptable levels of control were achieved for Cabernet Sauvignon with no significant differences recorded between treatments for severity.

Tab. 2. Mean percentage incidence (I) and severity (S) of powdery mildew on bunches at harvest[a].

2003-2004	Chardonnay		Cabernet Sauvignon	
Treatment	I	S	I	S
Unsprayed	93.3 (11.8) a	21.5 (2.5) a	1.3 (2.5)	0.13 (0.1)
Ecocarb® 4g/L	80.4 (29.3) a	18.6 (2.1) b	0 (0)	0 (0)
Milk	80 (22.6) a	12.9 (1.7) c	0 (0)	0 (0)
Whey 25g/L	76.3 (19.4) a	9.8 (0.9) d	0.6 (1.3)	0.06 (0.1)
Program 1[b]	79.6 (17.8) a	10.3 (0.8) cd	0 (0)	0 (0)
Sulphur 3 g/L	34.2 (22) b	4.2 (0.6) e	0 (0)	0 (0)
P value	< 0.001	< 0.001	0.520	0.220
2004-2005	**Chardonnay**		**Cabernet Sauvignon**	
Treatment	I	S	I	S
SH/E[c]	100 (0)	67.9 (18.2) a	31.3 (35.9)	3.3 (3.8)
Milk	98.8 (1.8)	66.1 (5.5) a	19.4 (25.7)	2.3 (3.3)
Whey 25g/L	100 (0)	69.6 (11.3) a	22.5 (19)	2.5 (2.2)
Program 1	100 (0)	74.8 (12.9) a	28.1 (20.7)	2.9 (2.2)
Sulphur 6g/L	100 (34)	14 (10.6) b	4.4 (5.5)	0.44 (0.6)
P value	0.358	0.003	0.293	0.408

[a]Standard deviations presented in parentheses. Means followed by different letters are significantly different ($P = 0.05$) based on l.s.d.
[b]2 initial sprays of Synertrol Horti-oil®/Ecocarb®(SH/E) followed by whey and SH/E alternated for the rest of the sprays.
[c]Synertrol Horti-oil® (3 ml/L)/Ecocarb® (4 g/L).

Due to the unacceptable control of powdery mildew on Chardonnay from 2003-05, it was decided to incorporate the test materials into programs rather than spraying one material continuously. The aim was to reduce the overall sprays of sulphur during the 2005-06 season. Sprays were targeted at the most mildew-susceptible stages of vine growth. The sulphur only treatment produced the best results, however it did not significantly reduce the incidence of powdery mildew when compared to treatment 3 (sulphur, milk, milk) and treatment 4 (sulphur, sulphur, milk). For severity, sulphur only was not significantly different from programs using sulphur from flowering to fruit-set and then combinations of SH/E and milk from flowering to fruit-set and fruit-set to veraison (Table 3).

Tab. 3. Mean percentage incidence and severity of powdery mildew on Chardonnay bunches at harvest for 2005-06[a].

Treatment[b]	Incidence	Severity
1	49.0 (23.3) b	5.9 (3.2) b
2	65.5 (21.6) ab	7.9 (2.7) b
3	51.0 (20.0) b	7.4 (3.5) b
4	53.0 (29.1) b	6.6 (4.1) b
5	90.5 (8.7) a	21.7 (10.0) a
6	87.0 (10.2) a	22.3 (16.9) a
P value	0.014	0.004

[a]Standard deviations are presented in parentheses. Means followed by different letters are significantly different ($P = 0.05$) based on least significant difference.
[b]Treatments are according to Table 1.

Analysis of juice from Cabernet Sauvignon and Chardonnay from 2003-05 revealed no significant differences ($P = 0.477$) between berry weight, pH, TSS and TA for all treatments. Significant differences were observed for Chardonnay TSS from 2003-04, however when the unsprayed treatment was removed from the analysis, no significant differences ($P = 0.06$) were observed.

Results of trials during three grape growing seasons suggest that 'alternative' materials to sulphur would be suited to controlling powdery mildew on Cabernet Sauvignon. Early control of powdery mildew with an effective fungicide is crucial in warm, dry regions, after which time 'alternative' materials such as milk and whey may be used. The use of 'alternatives' early season appears to provide sub-optimal control powdery mildew. The G-T index to predict powdery mildew infections correlated well with the onset of disease in this vineyard and could therefore be used to inform spray timing. This aspect requires further investigation over a number of seasons.

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Copper reduction, a successful approach to control downy mildew in organic viticulture

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Recently, copper applications are under consideration for harmful effects on natural environment. In response, the Council Regulation (EEC) 2092/91 Annex II limits the use of copper compounds to 6 kg/ha∗a from 2006 onwards. Moreover, German associations for organic farming concerted to limit the use of copper products to 3 kg copper/ha∗a. With respect to organic viticulture the aim of this project is to develop sustainable and economically feasible strategies to get a sufficient control of downy mildew on grapes (*Plasmopara viticola*) with less than 3 kg/ha∗a to avoid further environmental risks. Further more, investigations on disease reduction of grey mould (*Botrytis cinerea*) by selected plant resistance improvers are part of the project.

The project started in April 2004 and will be finished in December 2007. It consists of three parts: Screening under greenhouse conditions with potted vines (part one), field studies in organically managed test vineyards (part two) as well as tests on organically managed vineyards of four winegrowers (part three). SME partners will assure a close link between wineries and research.

In part one, four potted vines (cvs. 'Riesling' or 'Mueller-Thurgau'; BBCH 16-18) were used for each treatment. Vines sprayed with plant protection products (Folpan 80 WDG (a.i. Folpet) and Cuprozin Flüssig (a.i. copper hydroxide)) and with distilled water alone served as controls. Greenhouse trials are accomplished with new copper formulations with reduced copper concentration, plant resistance improvers, plant extracts and stone meals. Disease severity (defined as % infection per leaf area) of the lower leaf surface was recorded.

In part two, selected test agents from part one were tested in organically managed test vineyards at six different research stations for viticulture and plant protection in Germany. The control plots of the greenhouse and field tests were comparable: (i) untreated control, (ii) Folpan 80 WDG, (iii) Cuprozin Flüssig. Moreover, applications of sulphur were used as additional plant protection measure to prevent powdery mildew infestation. The spraying interval was about ten days. Disease incidence (in %) and disease severity (in %) of the lower leaf surface and of the berries per bunch, respectively, were recorded three times (BBCH 71, 79 and 81). Additionally, the following trials were conducted in the Geisenheim Research Center: micro vinification of all musts, analyses of musts and wines and finally sensoric evaluation by a professional test panel. Moreover, an assessment of possible side effects on beneficial organisms (*Typhlodromus pyri*) as well as additional rating of *B. cinerea* have been included.

Part three combines the results of part one and two and examines the applicability under practical conditions on four German organic pilot sites. Additionally, gained knowledge is presented to wineries by seminars and publications in magazines regarding more practical aspects of viticulture.

The year 2004 was characterised by a low natural infestation level of downy mildew in the wine-growing district Rheingau, Germany. Therefore, the trial could not be evaluated. In contrast to this, an early primary infection (7 May 2005; growth stage: BBCH 13/14) and a high infestation level of *P. viticola* was designative for the year 2005 (fig. 1). In addition, secondary infection cyles started on 21 May 2005. However, applications started not before 25 May 2005.

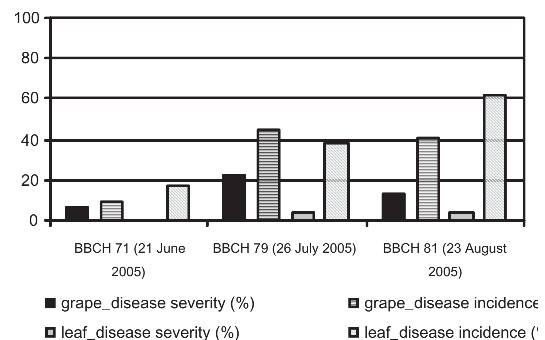

Fig. 1. Epidemiological data of the test-vineyard at Geisenheim; disease severity (in %) and disease incidence (in %) on grapes (control plot; cv. 'Riesling', Geisenheim)

After two years of experiments under greenhouse and field conditions the cluster project concludes that phosphonate applications, stone meals and new copper formulations are comparable concerning their efficiency to the reference treatments Folpan 80 WDG and Cuprozin Flüssig as well as "organic standard" (mixture of copper, sulphur and stone meal), respectively. Considering the low amount of total copper of less than 2 kg/ha∗a the results are satisfactory (fig. 2).

Finally, the results indicate that extracts of algae (part one), stone meals and new copper formulations could probably serve as plant resistance improvers and plant protection products, respectively, to prevent attacks of *P. viticola*.

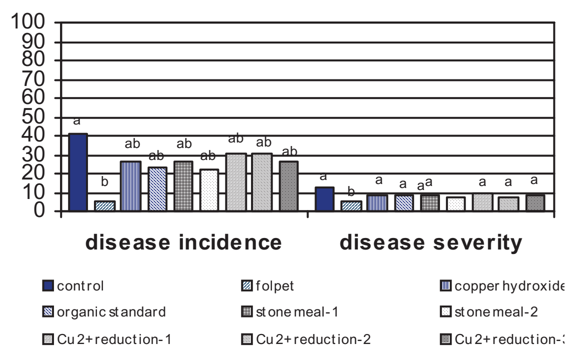

Fig. 2. Disease severity (in %) and disease incidence (in %) on grapes (cv. 'Riesling', Geisenheim, 27 July 2005, BBCH 79); significant differences between the treatments are indicated by different letters (Tukey HSD test (α= 0,05)).

Moreover, it was possible to select prosperous agents and strategies for the growing period 2006. Agents of the substance categories "new copper formulations", "plant extracts", "stone meals" and "algae extracts" could be selected for further trials in 2006 in test vineyards. Regarding the results of two growing periods, the project will provide effective and economically feasible alternatives for copper applications in order to deliver sustainable approaches for *P. viticola* control in practise.

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Evaluation of Novel Controls of Grapevine Downy Mildew, *Plasmopara viticola*

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Downy mildew of grapevines, caused by *Plasmopara viticola,* can reduce grape yield and the quality of fruit and wine produced from affected vines. In years with wet spring weather, downy mildew can cause widespread devastation, such as occurred in South Australia in 1992-93. The most common preventative treatment for downy mildew in organic vineyards is copper; however, copper can be phytotoxic, accumulate in vineyard soils (Hoffman, 2000) and contaminate ground water (Kauer *et al.,* 2002). The potential withdrawal of copper from the acceptable inputs in organic viticulture (EC regulation 2092/91; International Federation of Organic Agriculture Movements, 1998), and the need to reduce the environmental and ecological impact in conventional viticulture provide incentives to develop environmentally friendly controls for downy mildew.

The following materials were evaluated in greenhouse experiments in 2003 and 2004; $FeSO_4$ applied at 3, 6 and 9 g L^{-1}, and at 3 g L^{-1} mixed with Synerscreen® (Agricultural Research Service, USA), Ecocarb® plus Synertrol Horti-Oil® (Organic Crop Protectants), a chitosan/oil mixture and Vermiboost® (MR & EA Edwards), a compost tea made from worm casts. Vines sprayed with copper oxychloride (4 g L^{-1}) and untreated vines were used as controls. Cabernet Sauvignon vines, approx 75 cm tall, grown in 25-cm pots were sprayed with test materials using a hand-held spray, with six replicate vines per treatment. Five leaves on each vine were inoculated with a sporangial suspension of *P. viticola* (10^6-10^7 mL^{-1}) and covered with plastic bags for 15 hours, 24 hours after the treatments were applied. After 10 days, the inoculated leaves were removed from the vines and incubated in moist plastic bags overnight. Disease severity and phytotoxicity were evaluated with the aid of a dissecting microscope; severity was assessed as the percentage of the leaf surface affected.

In 2004/2005 and 2005/2006 a range of materials was evaluated in field trials at the Lenswood Centre, Adelaide Hills, South Australia. Treatments included $FeSO_4$ alone (6 g L^{-1}) and at 3 g L^{-1} mixed with Synerscreen® or 2 mL L^{-1} molasses, Ecocarb® plus Synertrol Horti-Oil® (3 g L^{-1}), Vermiboost® (1:10 dilution), tea tree oil-based products Timor® and Timorex® (0.5, 1 and 1.5 mL L^{-1}, Biomor, Israel), Brotomax® (1 mL L^{-1}), and Acadian® (Acadian Seaplants Ltd). Vines sprayed with copper oxychloride (4 g L^{-1}) and untreated vines were used as controls. Mature Nebbiolo vines were sprayed once with the test materials using a 15-L Solo backpack with a hand-held wand. Each treatment was applied to plots of four vines in each of four blocks. Eight shoots with five fully expanded leaves were tagged in each plot and inoculated with a suspension of *P. viticola* sporangia (10^6-10^7 mL^{-1}) in the evening 48 hours after treatment and the shoots were bagged overnight to maintain leaf wetness. After 12 days, six shoots from each plot were removed from the vines and incubated overnight in moist conditions to induce sporulation. Disease severity and phytotoxicity were evaluated as described above.

In 2003, 20% of the leaf area on untreated vines in the greenhouse was diseased. All materials tested, except Vermiboost® (23%), significantly reduced the severity of downy mildew on vines compared with the untreated control (Fig. 1). Disease on all vines treated with $FeSO_4$ was not significantly different from that on vines sprayed with copper. However, $FeSO_4$ was phytotoxic, with severity increasing as the concentration increased, with up to 35% of the leaf area damaged on shoots that received 9 g L^{-1}. Leaves sprayed with copper also had black/brown lesions on 1% of the surface.

In the vineyard trial in 2004/2005 the severity of downy mildew on vines sprayed with all treatments, except the lower rate of Acadian®, was significantly less than that on untreated vines (28% of leaf area diseased, Fig. 2). The severity of downy mildew on vines sprayed with $FeSO_4$, Timor®, Timorex®, Brotomax® and Ecocarb® plus Synertrol was not significantly different from that on vines sprayed with copper. However, none of the treatments, including copper, provided commercially acceptable control.

In 2005/2006 results were compromised by the late arrival of materials and downy mildew was well established at the start of the experiment. Only copper oxychloride and Timor at 1 mL L^{-1} reduced the severity of downy mildew compared to the untreated control. Leaves on vines sprayed with Brotomax had scattered, small, dark lesions. The phytotoxicity of $FeSO_4$ varied relative to concentration and was least on vines where molasses had been included.

$FeSO_4$ and Ecocarb® plus Synertrol Horti-Oil® reduced the severity of downy mildew in the greenhouse and also in the field to levels not significantly different from copper. Brotomax®, Timor® and Timorex® also provided similar results in the field. However, the amount of disease was not commercially acceptable, possibly due to incomplete coverage of the leaves in 2004/2005 and to application of treatments after downy mildew was well established in 2005/2006.

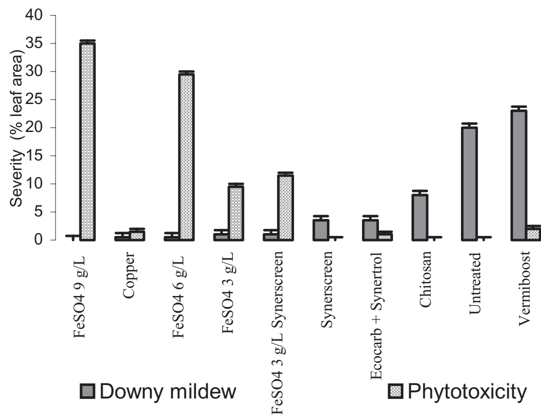

Fig. 1. Downy mildew severity and phytotoxicity on grapevine leaves sprayed with potential novel materials for downy mildew in the glasshouse experiment. Bars = LSD (5%).

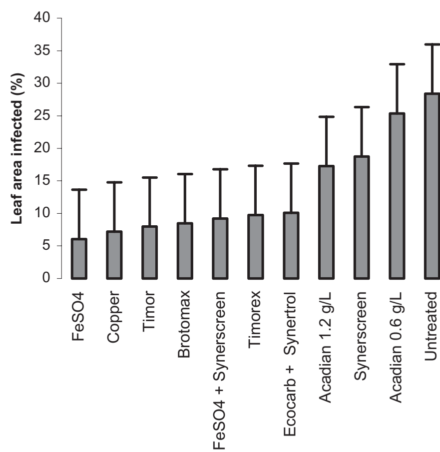

Fig. 2. Severity of downy mildew on grapevine leaves sprayed with novel materials for downy mildew control 24 hours prior to inoculation with *P. viticola* in the field in 2004/5. Bars = LSD (5%).

Phytotoxicity on leaves sprayed with $FeSO_4$ was most severe on those that had been inoculated and kept moist overnight, whereas damage to uninoculated leaves was limited to occasional black lesions usually less than 2 mm in diameter. These observations suggest that $FeSO_4$, at least in the formulation used here, was not a suitable replacement for copper in the control of downy mildew.

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