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.

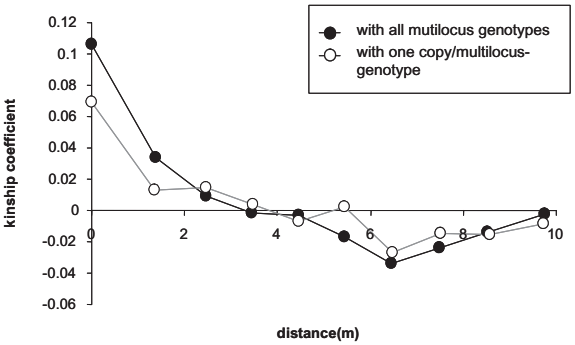

Fig. 1. Spatial autocorrelation of kinship for 241 oil spots over all microsatellite loci. Two data sets were analysed: in black including all sampled multilocus genotypes, in white including only one copy per multilocus genotypes.

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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.

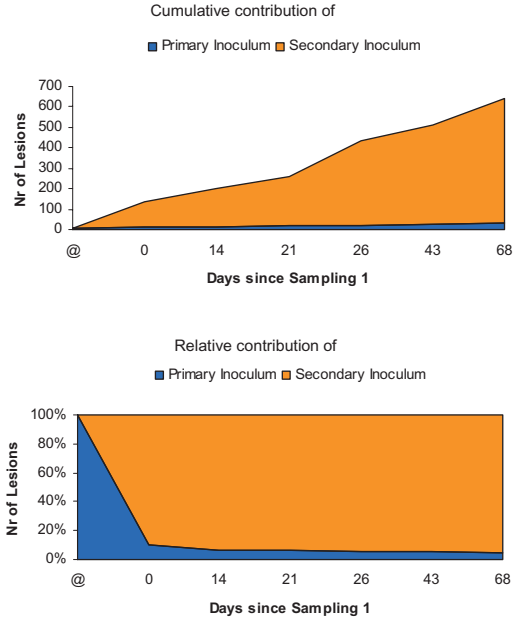


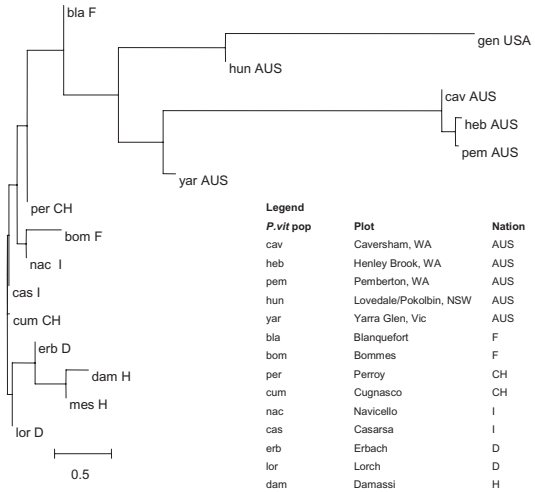
Fig. 1. Number of lesions (upper graph) and relative contribution of primary and secondary inoculum to the Caversham epidemic 2004-5 (lower graph). "@" indicates a date before the first sampling.

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.



Legend

P.vit pop	Plot	Nation
cav	Caversham, WA	AUS
heb	Henley Brook, WA	AUS
pem	Pemberton, WA	AUS
hun	Lovedale/Pokolbin, NSW	AUS
yar	Yarra Glen, Vic	AUS
bla	Blanquefort	F
bom	Bommes	F
per	Perroy	CH
cum	Cugnasco	CH
nac	Navicello	I
cas	Casarsa	I
erb	Erbach	D
lor	Lorch	D
dam	Damassi	H
mes	Messenikolas	H
gen	Geneva	USA

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.

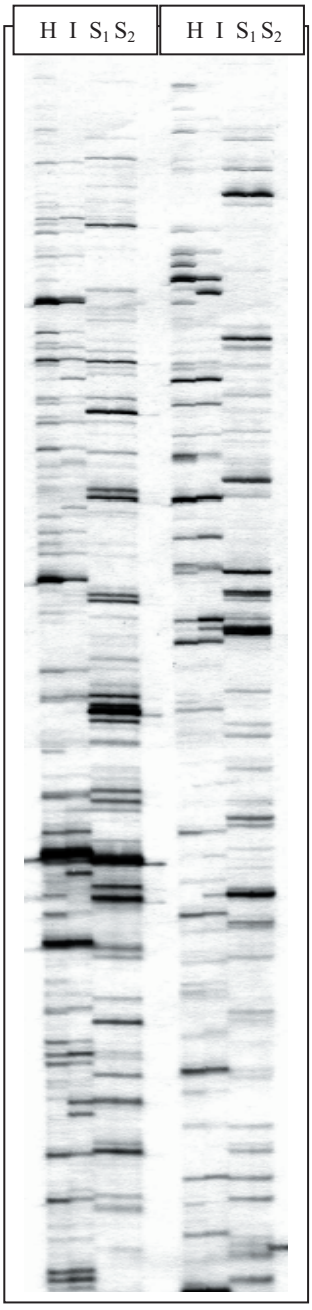

Fig. 1. The two groups of four lanes represent cDNA-AFLP amplifications with two different primer combinations on cDNA samples from healthy (H) and infected (I) grape leaves and on two replicas of cDNA from *P. viticola* sporangia (S_1 and S_2) as a control.

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.

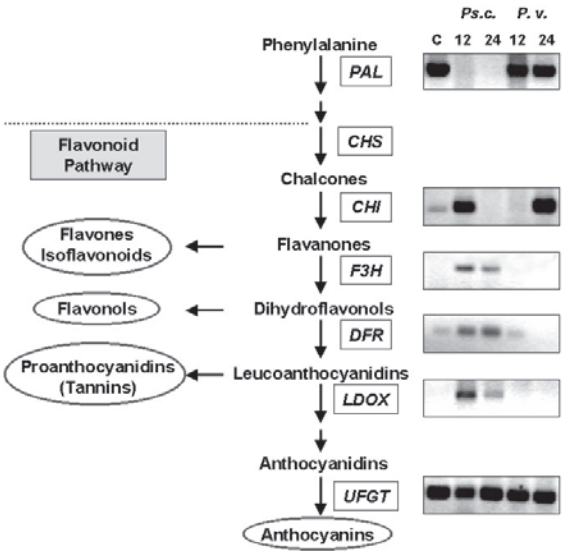

Fig. 2. Pattern of transcript accumulation of genes encoding enzymes involved in the phenylpropanoid pathway in the susceptible cv. Riesling 12 and 24 hours post inoculation with the host pathogen *P. viticola* and the non-host pathogen *Ps. cubensis*.

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.

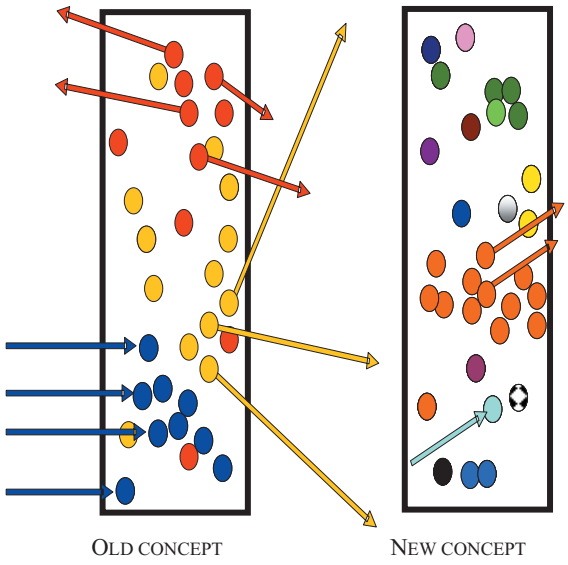

Fig. 1. A change in conception of grapevine downy mildew population structure. The traditional conception believes in genetically poor populations, where a few genotypes are present with thousand/millions of clones per population that easily migrate from plot to plot. The new conception contemplates 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

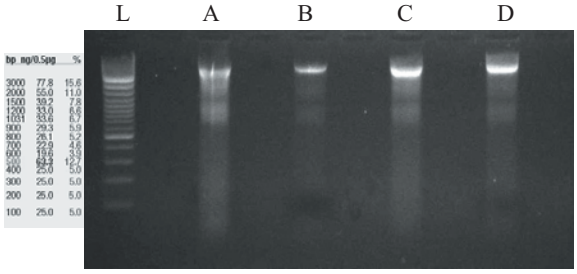

Fig. 1. Gel electrophoresis of total RNA samples, extract from leaves mass containing *P. viticola* oospores with Qiagen RNeasy Plant Mini Kit. RNA run on 1 % agarose gel stained with ethidium bromide. L: ladder, 1,3: samples 1,2 first elution, B,D: samples 1,2 second elution.

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).

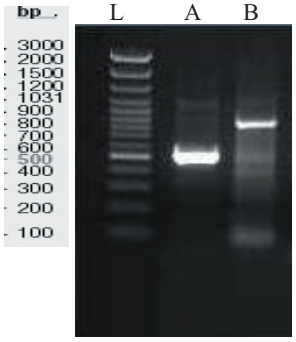

Fig. 2. Gel electrophoresis of specific PCR primer products. Run on 1,5 % agarose gel stained with ethidium bromide. L: DNA ladder, A: products obtained from DOG1 primer amplification, B: products obtained from DOG2 primer amplification

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

This research was supported by SafeCrop Centre, funded by Fondo per la Ricerca, Autonomous Province of Trento.

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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

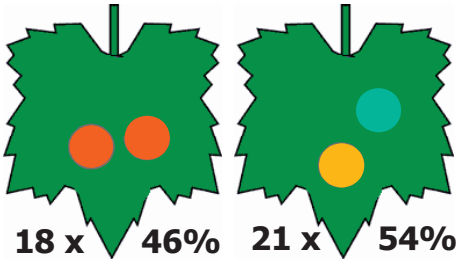

Fig. 1. In the data, we found 39 cases where two analysed lesions were present on a single leaf. In 18 cases a single genotype was present, in 21 cases two.

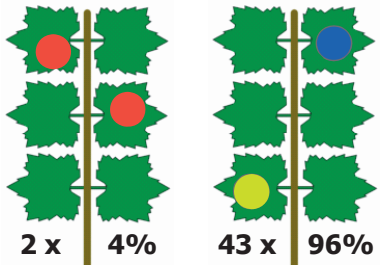

Fig. 2. On the plant scale in 45 cases where two lesions were present and analyzed, two cases a single genotype was present, in 43 two genotypes.

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

This work was supported by SafeCrop Centre funded by Fondo per la Ricerca, Autonomous Province of Trento.

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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

This paper was supported by SafeCrop Centre funded by Fondo per la Ricerca, Autonomous Province of Trento.

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%).

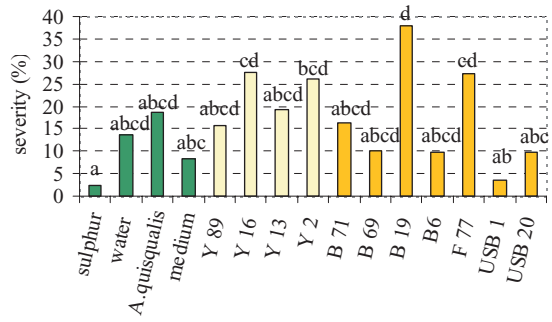

Fig. 1. Efficacy in reducing disease severity of bacteria and yeasts sprayed 6 hours before inoculation of grape leaves with *Erysiphe necator*. Columns with same letters do not significantly differ at $P \leq 0.05$ (Duncan's test).

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

This research was supported by SafeCrop Centre and Syrtox project (for the organisms USB 1 and USB 20) funded by Fondo per la ricerca, Autonomous Province of Trento.

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^{o}C, 100-120 $\mu E.m^{-2}.s^{-1}$, 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^{o}C for 20 h in darkness. The plants were then uncovered and kept in a growth chamber (23^{o}C, 100-120 $\mu E.m^{-2}.s^{-1}$, 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).

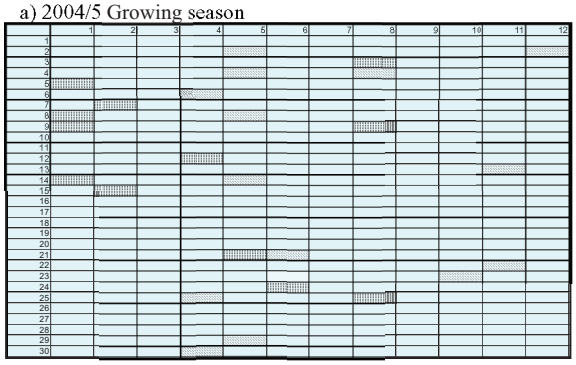
Conclusion

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).

a) 2004/5 Growing season



(b) 2005/6 Growing season

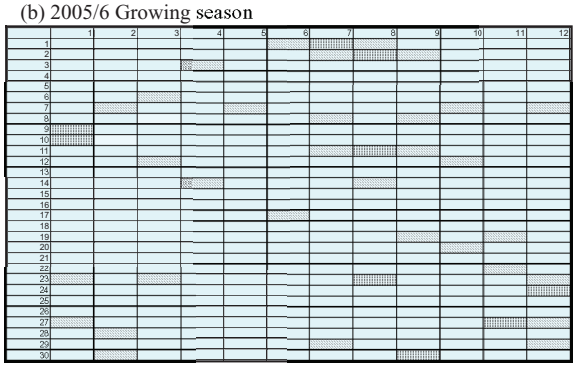


Fig. 1. Schematic representation of the vineyard where *P. viticola* populations were studied in the (a) 2004/5 and (b) 2005/6 growing seasons. Each square represents one vine, with vines being spaced 2m within rows and 3 m between rows. The dispersion pattern of one of the two most dominant *P. viticola*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

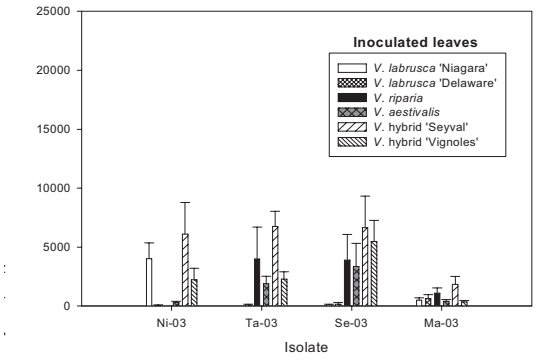

Fig. 1. Sporulation of *Plasmopara viticola* isolates from different hosts on leaf disks of various grape hosts in 2003. Isolate origin: Ni-03 ('Niagara' leaves), Ta-03 (unidentified table grape clusters), Se-03 ('Seyval' leaves), and Ma-03 ('Mars' table grape leaves). Error bars represent the standard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

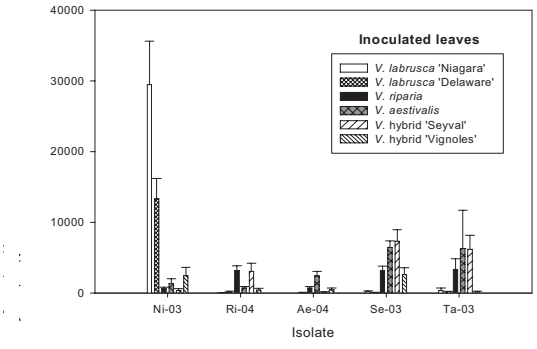

Fig. 2. Sporulation of *Plasmopara viticola* isolates from different hosts on leaf disks of various grape cultivars and species in 2004. Isolate origin: Ni-04 ('Niagara' leaves), Ri-04 (*Vitis riparia* leaves), Ae-04 (*V. aestivalis* leaves), Se-04 ('Seyval' leaves), and Ta-04 (unidentified table grape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

Acknowledgments

The present work has been carried out within the project Interreg III A Italy-Albania 2000-2006 "Integrated project for the diffusion and technical assistance in the implementation of methodologies for the production of organic products (PAB)".

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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™ 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.

Acknowledgement

This research was supported by Emilia Romagna (L.R. 28/98) and Lombardia (Piano per la Ricerca e lo Sviluppo 2004) Regions.

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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.

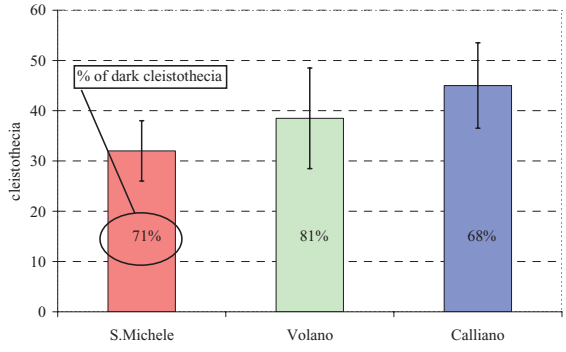

Fig. 1. Number of *Erysiphe necator* cleistothecia per square centimetre of leaf surface measured during 2004

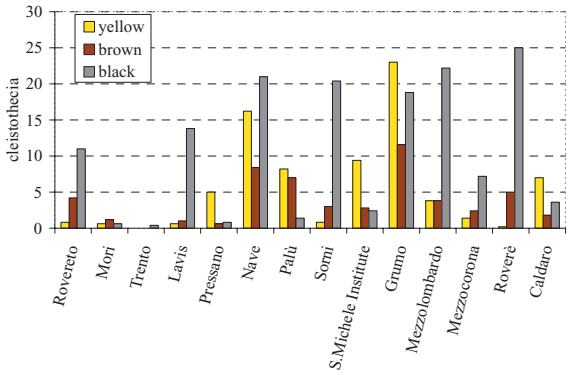

Fig. 2. Number of *Erysiphe necator* cleistothecia per square centimetre of leaf surface measured during 2005

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.

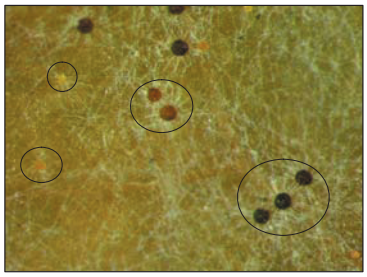

Fig. 3. Cleistothecia at different maturity stage, maturation increases with increased darkness.

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).

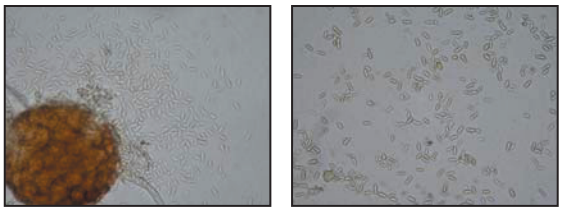

Fig. 4. Differences in shape of *Ampelomyces* spp. conidia between the wild strain (left) and the commercial AQ10 (right).

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.

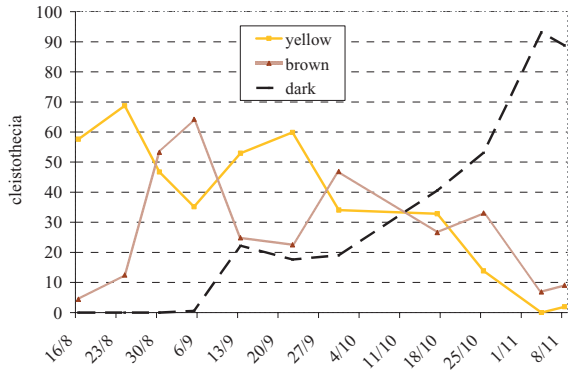

Fig. 5. Ripening stadium of cleistothecia: percentage of yellow, brown and dark cleistothecia per square centimetre of leaf surface.

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

This research was supported by SafeCrop Centre funded by Fondo per la Ricerca, Autonomous Province of Trento.

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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).

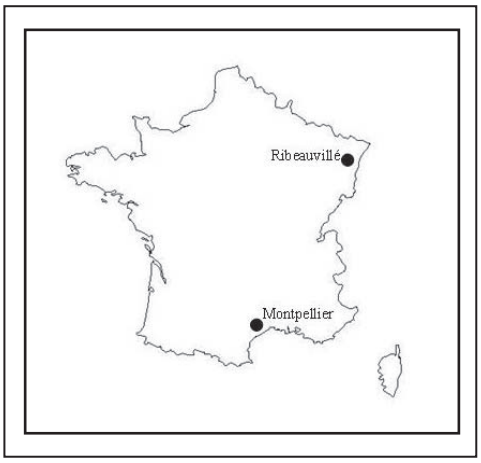

Fig. 1. Regions in France where the sources of *P. viticola* used in this work were harvested. Isolate FlRib03 in Alsace (North-East) and isolate KobMt01 in Languedoc (South).

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.

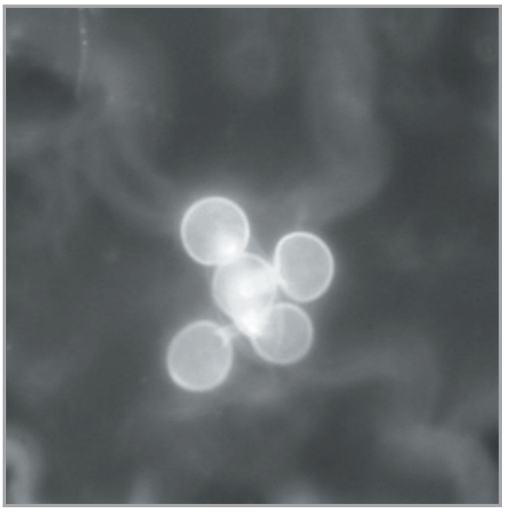

Fig. 2. Encysted zoospores of *P. viticola* over a grapevine stomata. Picture was taken using epifluorescence microscopy after Blankophor staining.

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.

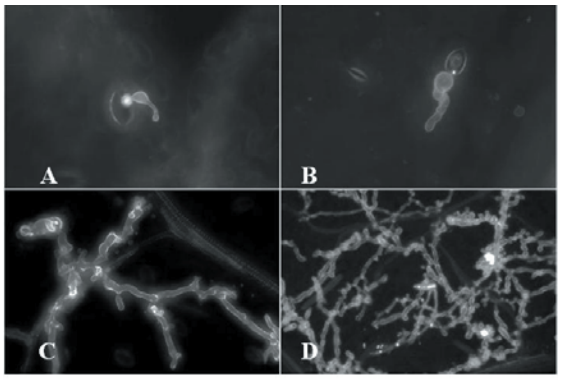

Fig. 3. Microscope images of *P. viticola* mycelium development in *V. vinifera* cv. Muscat Ottonel. A. 6 hours post inoculation (hpi)(x50). B. 24 hpi (x50). C. 48 hpi (x25). D. 72 hpi (x10), under UV light after KOH-aniline blue staining.

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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Acknowledgements

Ana M. Díez is supported by a postdoctoral fellowship from the program of researchers' formation, from the Departamento de Educación, Universidades e Investigación of the Gobierno Vasco – Eusko Jaurlaritza.

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 $\rho<0.05$) smaller IC respect to the NT control (IC<0.79). One hundred and twenty microorganisms showed no significant difference (T test $\rho>0.05$) 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.

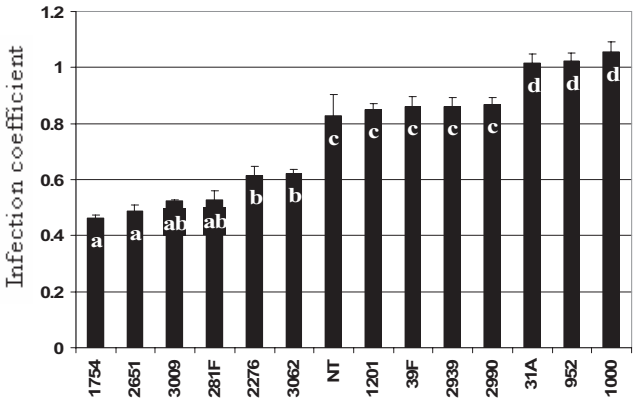

Fig. 1. Infection coefficient (IC = C_T *V. vinifera* / C_T *P. viticola*) after RT-PCR of the biocontrol assay. Of the 250 microorganisms tested, only 13 are shown. NT shows the non-treated control (inoculated with *P. viticola*). Standard deviation is indicated by error bars, significant difference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

This research was supported by the project "Studi finalizzati ad ottemperare alle limitazioni dei quantitativi di rame o mediante l'impiego di formulazioni a basso dosaggio o con l'adozione di mezzi alternativi" funded by MiPAF, Italy.

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