

Leaf morphological characteristics and stilbene production differently affect downy mildew resistance of *Vitis vinifera* varieties grown in Italy

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Summary

The degree of resistance to downy mildew of grape varieties belonging to the oenological tradition of Central Italy was evaluated by the analysis of plant responses to pathogen infections carried out in natural and controlled environments. Leaf morphological traits, such as hair and stomatal density, were determined for each variety, and the percentage of infected stomata and pathogen colonization of host mesophyll at 24, 48, and 72 hours post inoculation were assessed by epifluorescence microscopy. Furthermore, stilbene production at the site of *Plasmopara viticola* infection was analyzed at 72 hours post inoculation. Results indicate differences in resistance to downy mildew among selected varieties. Different significant values were detected among grapevines in the percentage of infected stomata and average number of successfully penetrated zoospores per stomata and per leaf surface unit. Differences also emerged in the rate of pathogen growth and stilbene production, signifying that defence mechanisms involved or induced during pathogen infection could be differentially effective among grapevine cultivars in limiting disease progression.

Key words: grapevine, sustainable viticulture, *Plasmopara viticola*, stomata, stilbenes.

Introduction

Since the second half of the twentieth century a wide effort aimed to the collection and conservation of grapevine genetic resources have been carried out in many European countries, leading to the establishment of national and regional germplasm collections including a large number of local and minor grape varieties. The need to preserve grapevine biodiversity derived from the severe reduction of cultivated varieties mainly due to the diffusion of pests and diseases and to the globalization of wine markets. The use of a restricted number of varieties in modern viticulture is not fully representative of the existing large phenotypic and genetic grape diversity. During the last years several researches and collaborative works have been focused on the description of preserved local varieties, leading to the

development of the European grapevine documentation systems (www.eu-vitis.de, www.ecpgr.cgiar.org/networks/fruit/vitis.html) which can also facilitate the study of grape varieties with adaptive traits responsible for natural resistance to diseases (<http://users.unimi.it/grapenet/>). The availability of *V. vinifera* disease resistant varieties and clones and their potential use in breeding projects have become key strategies aimed to reduce the use of plant protectants and the accumulation of pesticide derived molecules in soil, water and wine in many viticultural areas (OLESEN and BINDI 2002, MARACCHI *et al.* 2005, JIMENEZ *et al.* 2007, MATASCI *et al.* 2008, OLIVA *et al.* 2008, GONZALEZ ALVAREZ *et al.* 2012, PESCE *et al.* 2013, MONARD *et al.* 2013). Grapevine genetic resources for pathogen resistance are mainly found in American and Asian wild *Vitis* species (STAUDT and KASSEMAYER 1995, CADLE-DAVIDSON 2008). Nevertheless former agronomical and ampelographic studies emphasized differences in disease resistance among *V. vinifera* varieties and clones (VANNUCCINI 1892, BOSO *et al.* 2006, BOSO *et al.* 2011), encouraging researches on natural defence mechanisms within *V. vinifera* germplasm (ALONSO-VILLAVERDE *et al.* 2011a, VAN ZELLER DE MACEDO BASTO GONÇALVES *et al.* 2011, BOSO *et al.* 2012, MUGANU and PAOLOCCI 2013, VAN LEEUVEN *et al.* 2013).

Plants evolved constitutive and inducible natural defence mechanisms aimed at protection against biotic stresses. Among constitutive defences that are active in the plant before any pathogenic attack, the density of leaf prostrate and erect hairs of the abaxial leaf surface can lead to a reduction in water retention capacity that is fundamental during the infection caused by *Plasmopara viticola* (Berk. *et* Curt.) Berl. *et* de Toni, the causal agent of downy mildew (STAEDMAN and SHAIK 1988, KORTEKAMP *et al.* 1999). In addition, the number of leaf stomata can play an important role during *P. viticola* infection, considering that the penetration of zoospores occurs exclusively through stomata (GINDRO *et al.* 2003) and that the number of stomatal openings changes according to environmental conditions and genotype (PALLIOTTI *et al.* 2000, ROGIERS *et al.* 2011). Induced defences are the result of a plant reaction to pathogen attack and require the perception of plant tissue signals resulting from pathogen infection. Induced defence mechanisms can trigger the synthesis of anti-microbial compounds, among them the stilbenic phytoalexins, which are involved in grapevine resistance strategies (PEZET *et al.*

2004a, GINDRO *et al.* 2006). Stilbenes are induced in non-woody vine tissues, such as flowers, leaves, and berries, by different pathogen infections, including *P. viticola*. Stilbenes include resveratrol and different derived molecules, such as piceid and resveratrolsides (BAVARESCO *et al.* 2009, MATTIVI *et al.* 2011), and pterostilbene, δ - and ϵ -viniferins, which demonstrate toxicity against *P. viticola* (PEZET and PONT 1998, BAVARESCO 2003, PEZET *et al.* 2004b).

Considering the old wine-making tradition of the central part of Italy, founded on the use of different grapevines (<http://www.vitisdb.it>), and the risk that grape varieties with potential environmental adaptations may be lost altogether, since 1987 at University of Tuscia there has been a concerted effort to recover local and minor varieties of the area (NATALI and BIGNAMI 1988), aimed to carry out genetic, ampelographic and agronomic characterization of preserved accessions (BIGNAMI and FILIPPETTI 2002, MUGANU *et al.* 2006a, 2006b, 2007a, 2008, 2009, 2011), and to the evaluation of the degree of resistance to downy mildew among collected grape varieties (MUGANU *et al.* 2007b). Because the study of *V. vinifera* germplasm with different susceptibility to *P. viticola* can improve our knowledge of plant/pathogen interaction, the aim of the present paper was to evaluate the influence of some leaf morphological traits and of stilbene production on downy mildew infection in different grape varieties.

Material and Methods

Plant material: Five grapevines that contribute to the production in Central Italy of protected designation of origin (PDO) and protected geographical indication (PGI) wines, were selected: 'Aleatico' and 'Canaiolo nero' (black berry), 'Romanesco', 'Trebiano giallo' and 'Trebiano toscano' (white berry). Previous ampelographic description and DNA analysis confirmed the correct identification of the five varieties and highlighted the different level of in-field resistance to downy mildew of selected vines (MUGANU *et al.* 2007b, 2009, MUGANU, unpubl. data 2009). Downy mildew-susceptible grape variety 'Chasselas' and the resistant 'Solaris' ['Merzling' \times ('Saperavi severneyi' \times 'Muscat Ottonel')] were included as control plants in analysis carried out under controlled conditions.

Evaluation of resistance under natural conditions: The trials were carried out during 2009, 2010, and 2011. The degree of resistance to *P. viticola* was evaluated in five vines of each selected variety. Disease incidence (percentage of symptomatic leaves) and disease severity (percentage of leaf surface with symptoms) were observed from berry-set to veraison. The vines were grown in the vineyard at the experimental farm of the University of Tuscia (42°25'21"N; 12°04'45"E), grafted on '420A Millardet et de Grasset', Guyot-pruned and spaced at 3 m \times 1.5 m, with a north-south orientation of the rows; all plants were subject to the same climatic and agronomic conditions. Data were collected considering rainfall and temperature trends monitored daily by a Campbell Mod CR 10X1 data logger (Campbell Scientific Inc., United Kingdom). Analysis of variance (ANOVA) and Tukey's test were performed to

identify significant differences among data at $p \leq 0.05$.

Determination of leaf hair density: For each variety, the density of hairs of the abaxial surface was evaluated in field-grown young and mature leaves. Evaluations were performed according to OIV descriptors n. 053 and n. 084, respectively (OIV 2007).

Inoculation under controlled conditions: Two-year-old potted plants of each variety obtained from woody cuttings were grown at 25 ± 1 °C under a natural photoperiod and sprayed weekly with sulphur. Whole leaves with pedicel were detached from the 5th to 6th nodes from the apex of potted vines, rinsed with sterile distilled water, and infected with an aqueous suspension of 5×10^5 zoosporangia \cdot mL⁻¹. The inoculum was derived from naturally infected plants showing symptoms of the disease and maintained on the susceptible grape variety 'Malvasia del Lazio'. Three different infections of five leaves per variety were performed after inoculum standardization with a Thoma counting chamber by spraying the abaxial leaf surface until runoff. After each inoculation, single leaves were laid in covered glass 20-cm Petri dishes, lined with wet filter paper, and incubated in a growth chamber at 24 °C \pm 2 °C with a 16-h photoperiod. Control leaves were sprayed with distilled water. Seven days post inoculation, disease symptoms were evaluated for each leaf and the percentage of sporulating leaf surface determined. Results were analyzed using the ANOVA test.

Epifluorescence microscopy: For microscopic analysis, three leaves from the 4th to 6th nodes from the apex of potted vines were detached, rinsed with distilled water, and soft dried. Inoculation was performed by putting single drops of a suspension of 5×10^5 zoosporangia on the abaxial leaf surface. At 24, 48, and 72 h post inoculation (hpi), four sections, corresponding to different infected areas, were sampled per inoculated leaf. Excised sections were dipped in a 1 M solution of KOH for 15 min at 60 °C, and then washed and dipped in a 0.02 % solution of aniline blue in a 5 % sodium bicarbonate buffer (KORTEKAMP *et al.* 1997, GINDRO *et al.* 2006, DIEZ-NAVAJAS *et al.* 2007). For each section, two areas of 0.16 mm² were observed under epifluorescence microscopy using a Leica DLMB (Leica Inc., Heidelberg, Germany) (UV filter Leitz, excitation at 340 nm, emission at 380 nm, stop filter LP 430 nm). Epifluorescence microscopy was used to determine the average number of total stomata per leaf surface unit (BOSO *et al.* 2010), the average number of infected stomata per leaf surface unit, the percentage of infected stomata (ALONSO-VILLAVARDE *et al.* 2011a, GINDRO *et al.* 2006), the average number of zoospores successfully penetrated per stomata and average number of zoospores penetrated per leaf surface unit. Furthermore pathogen colonization of host mesophyll was assessed at 24, 48, and 72 hpi and expressed as frequency of the developmental phases of *P. viticola* from S1 to S6 (UNGER *et al.* 2007, GODARD *et al.* 2009).

Stilbene analysis: Stilbene production at 72 hpi was detected according to PEZET *et al.* 2004b on three leaves inoculated as previously described. Three sections of leaf corresponding to the droplet surface were cut from each inoculated leaf and subsequently tissue extract (30 μ L) was

analyzed by HPLC (PEZET *et al.* 2003, JEAN-DENIS *et al.* 2006, GINDRO *et al.* 2006). Results are expressed as average values of $\mu\text{mol}\cdot\text{mg}^{-1}$ of fresh weight.

Results

Resistance to downy mildew evaluated under natural conditions showed differences among varieties in the response to pathogen infection. Disease incidence was significantly low in ‘Trebiano g.’ and disease severity ranged from 25 % in ‘Romanesco’ to 45 % in ‘Canaiole n.’ although without significant differences among grapevines (Fig. 1).

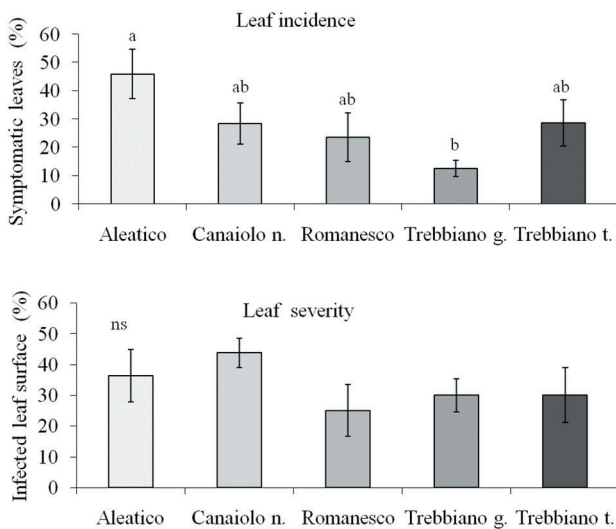


Fig. 1: Values of disease incidence and of disease severity. Results are expressed as average values of three years. Bars indicate standard errors of means. Values with different letters show significant differences at Tukey's test ($p \leq 0.05$); ns = not significant differences.

Differences in the degree of resistance to *P. viticola* among varieties were detected in infections performed in controlled conditions. Among *V. vinifera* varieties, ‘Trebiano g.’ showed significant higher resistance to downy mildew compared to black-berried ‘Aleatico’ and ‘Canaiole n.’ (Fig. 2). The assessment of hair density of the abaxial leaf surface also highlighted differences among varieties. Young and mature leaves of ‘Canaiole n.’ and ‘Romanesco’ were

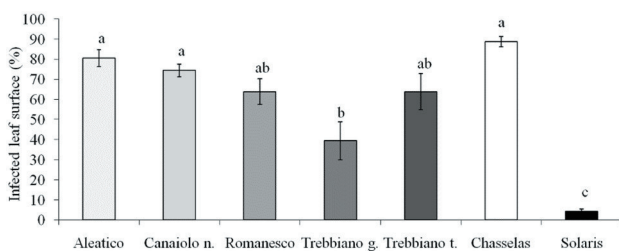


Fig. 2: Disease severity observed in controlled infection on detached leaves. Values are expressed as percentage of symptomatic leaf surface. Bars represents standard errors of means. Values with different letters show significant differences at Tukey's test ($p \leq 0.05$).

characterized by a dense white felt of leaf hairs determined by the presence of reclining trichomes. The leaves of ‘Trebiano t.’ showed a low density of hairs. ‘Trebiano g.’ had a very low hair density, with rare reclining trichomes, and finally, in the ‘Aleatico’ variety, trichomes were not found on the abaxial surface (data not shown). The average number of total stomata determined by epifluorescence microscopy varied according to genotype. ‘Trebiano g.’ showed a lower average number of leaf stomata per surface unit compared to ‘Aleatico’, ‘Canaiole n.’, ‘Romanesco’, and ‘Trebiano t.’ and to the control variety ‘Chasselas’, and ‘Solaris’ showed the highest value of stomatal density (Table).

The percentage of infected stomata differed among varieties and the lowest values was detected in ‘Trebiano g.’ and in the resistant control variety ‘Solaris’ (Fig. 3). The average number of infected stomata per leaf surface unit was significantly lower in ‘Trebiano g.’ compared to other varieties (Fig. 4). The regression analysis showed the positive correlation between the number of stomata per leaf surface unit and the number of infected stomata per leaf surface unit, with a low value of r^2 ($r^2 = 0.124$; $p < 0.0001$).

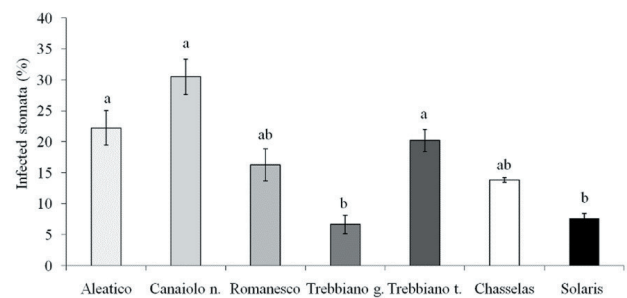


Fig. 3: Percentage of infected stomata observed in the different varieties. Bars represent standard errors of means. Values with different letters are significantly different according to Tukey's test ($p \leq 0.05$).

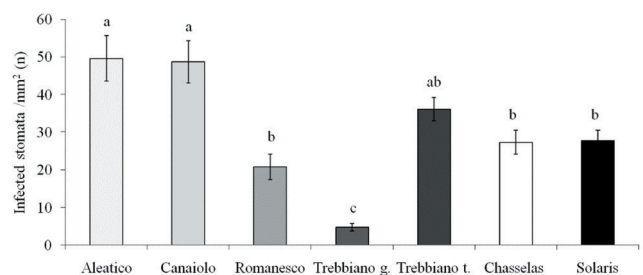


Fig. 4: Average number of infected stomata per leaf surface unit in the different varieties. Bars represent standard errors of means. Values with different letters are significantly different according to Tukey's test ($p \leq 0.05$).

Also the average number of successfully penetrated zoospores significantly differed among varieties: ‘Trebiano g.’ proved to have the highest average value of penetrated zoospores per stomata and the lowest number of zoospores penetrated per surface unit (Figs 5 and 6).

At 24 hpi, the analysis of the colonization of *P. viticola* in the mesophyll (S1, S2, and S3 developmental phases) showed high pathogen growth in ‘Aleatico’, ‘Canaiole n.’ and ‘Trebiano t.’ leaf tissues, similar to the growth ob-

Table

Stomata number per surface unit assessed in the different grapevines by epifluorescence microscopy. Results are expressed as the average of 24 repetitions (\pm standard deviation)

Variety	Aleatico	Canaio n.	Romanesco	Trebbiano g.	Trebbiano t.	Chasselas	Solaris
stomata/mm ²	205.4 \pm 19.1	132.7 \pm 19.6	116.2 \pm 12.1	72.7 \pm 15.7	167.2 \pm 27.9	195.2 \pm 23.0	345.1 \pm 38.9

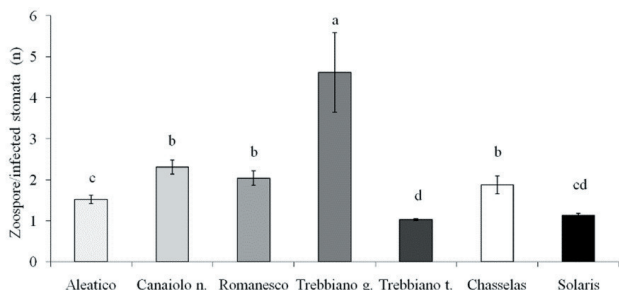


Fig. 5: Average number of zoospores detected per infected stomata in the different varieties. Bars represent standard errors of means. Values with different letters are significantly different according to Tukey's test ($p \leq 0.05$).

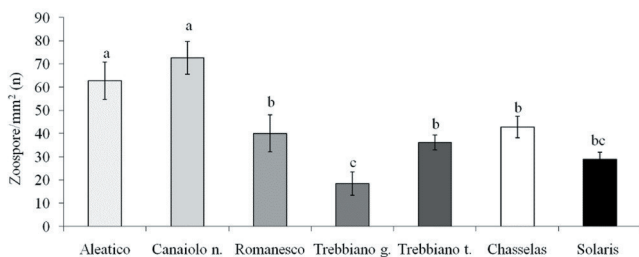


Fig. 6: Average number of zoospores penetrated per leaf surface unit. Bars represent standard errors of means. Values with different letters are significantly different according to Tukey's test ($p \leq 0.05$).

served in the susceptible variety 'Chasselas' (Fig. 7). At 48 hpi, the pathogen growth was similar in all varieties with the exception of 'Trebbiano g.', which showed the slowest hyphal growth (Fig. 8). At 72 hpi, little difference among selected varieties was observed in the development of mycelium (Figs 9 and 10). In the resistant control variety 'Solaris', pathogen growth was interrupted from 48 to 72 hpi. Stilbene production, analyzed at 72 hpi, revealed differences in the amount of detected compounds among the varieties. The major synthesized stilbenic phytoalexins were resveratrol and piceid, and the resistant variety 'Solaris' showed the highest production of δ - and ϵ -viniferins. 'Romanesco' showed the highest production of pterostilbene and, among *V. vinifera* varieties, of δ - and ϵ -viniferins (Fig. 11).

Discussion

Recently, different researches confirmed the heterogeneous response of *V. vinifera* varieties to downy mildew challenge (BOSO *et al.* 2006, CADLE-DAVIDSON 2008, BOSO *et al.* 2011). In the present study *V. vinifera* varieties belonging to the oenological tradition of Central Italy showed

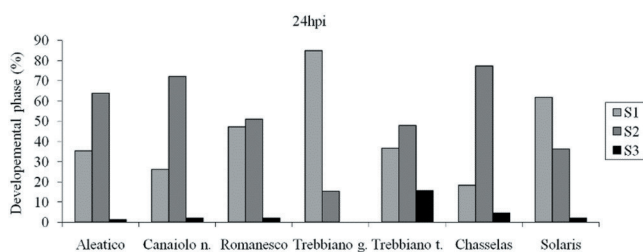


Fig. 7: Percentage for each developmental stage of *P. viticola* detected in the different varieties at 24 h post inoculation.

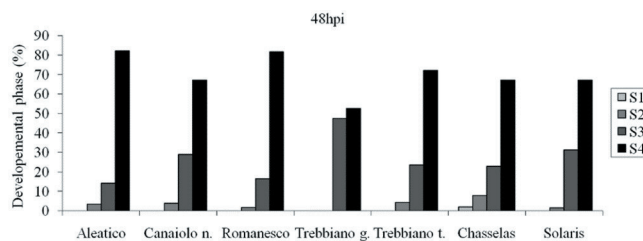


Fig. 8: Percentage for each developmental stage of *P. viticola* detected in the different varieties at 48 h post inoculation.

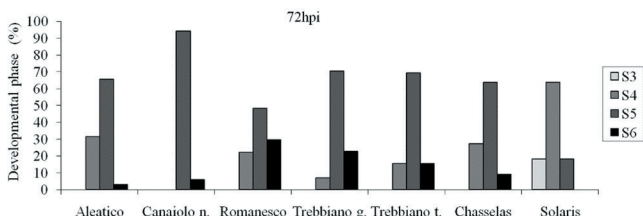


Fig. 9: Percentage for each developmental stage of *P. viticola* detected in the different varieties at 72 h post inoculation.

significant differences in the resistance to *P. viticola*, with a comparable behaviour both in field conditions and in controlled environment. Considering morphological characteristics, leaf trichomes did not influence plant infection by downy mildew, as the pathogen resistance of the hairiest variety 'Canaiolo n.' was similar to hairless 'Aleatico'. Previous studies analyzed the influence of stomatal density on grapevine susceptibility to downy mildew (Boso *et al.* 2010): in this study observations carried out by epifluorescence microscopy led to the identification, among *V. vinifera* varieties, of a relationship between the number of infected stomata per leaf surface unit and stomatal density. The low r^2 value from the regression analysis can testify the possible involvement of further mechanisms which could influence the infection process, in the light of the different resistance to the disease shown by selected varieties. At 24 hpi, 'Trebbiano g.', which proved to have the lowest number of stomata per surface unit, showed the lowest number of infected stomata per surface unit and the lowest

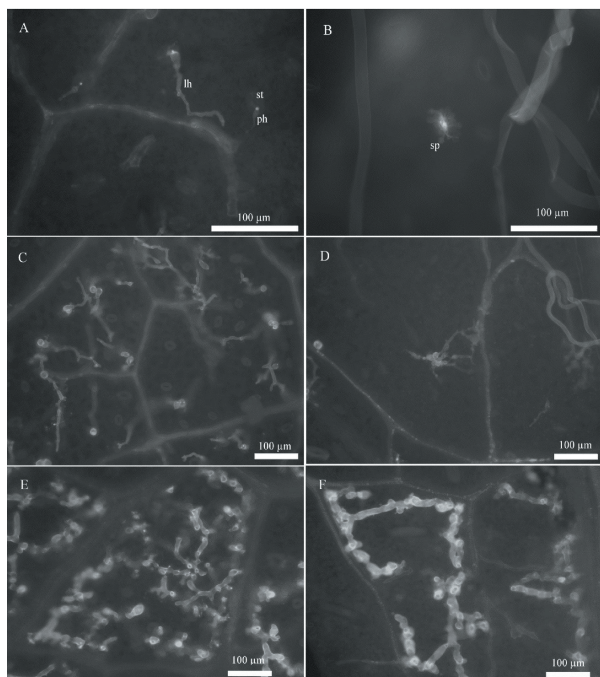


Fig. 10: Time course of infection of *P. viticola* observed by epifluorescence microscopy at 24, 48, and 72 hours post inoculation in 'Aleatico' (A-C-E) and in 'Trebiano g.' (B-D-F). (st) stomata; (ph) primary hypha, corresponding to the S2 developmental phase of *P. viticola*; (lh) long hypha, corresponding to the S3 developmental phase of *P. viticola*; (sp) empty zoospore.

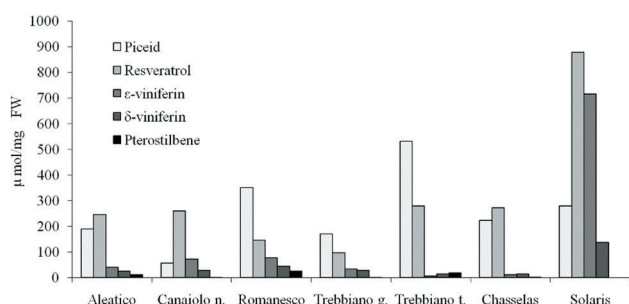


Fig. 11: Average values of stilbenic phytoalexins (µmol/mg of fresh weight) detected at 72 h post inoculation in infected leaf tissues.

number of successfully penetrated zoospores per surface unit. Otherwise in 'Trebiano g.' the percentage of infected stomata was unexpectedly lower compared to other varieties and without significant differences with respect to resistant 'Solaris', which showed the highest number of stomata per mm². Furthermore 'Trebiano g.' showed the highest number of successfully penetrated zoospores per each infected stomata (about five zoospores per infected stomata). The accumulation of pathogen propagules in the same stomata could have influenced the low pathogen growth observed in 'Trebiano g.' both at 24 and 48 hpi, possibly because of trophic competition among zoospores. This accumulation of zoospores might be explained by an adelphotactic process, a functional relationship between zoospores and infected stomata (LANGCAKE and LOVELL 1980, LALANCETTE *et al.* 1987, THOMAS and PETERSON 1990, KIEFER *et al.* 2002, KORTEKAMP 2003). Later, at 72 hpi, in

'Trebiano g.' the colonization of intercellular spaces by the pathogen was similar to susceptible control variety 'Chasselas' and no significant difference in mycelium growth was observed among selected varieties. At 72 hpi the control variety 'Chasselas' showed the lowest values of stilbenes and 'Solaris' the highest content of viniferins in infected leaves. In our study, stilbene production in infected tissues of the two control varieties 'Solaris' and 'Chasselas' correlated well with respective levels of resistance to downy mildew previously characterized (PEZET *et al.* 2004b, GINDRO *et al.* 2006), which showed that defence mechanisms in the resistant variety 'Solaris' rely on the synthesis and accumulation of stilbenic phytoalexins, particularly viniferins. At 24 and 48 hpi, the pathogen growth in resistant control variety 'Solaris' was similar to that in susceptible varieties, but from 48 to 72 hpi it was stopped with values of toxic stilbenes usually associated with resistant varieties (GODARD *et al.* 2009, ALONSO-VILLAVARDE *et al.* 2011b). The synthesis of stilbenes detected at 72 hpi in selected *V. vinifera* varieties proved to be weak for intensity and produced the accumulation of resveratrol and piceid and a low level of toxic viniferins that did not limit pathogen growth. This outcome was confirmed both by disease progression observed at 72 hpi in 'Trebiano g.' and by production of toxic pterostilbene in 'Romanesco' which content appeared to be ineffective to block the disease.

Conclusions

A wide ampelographic platform could respond to growers' needs to face market competition by diversifying their products and to improve sustainable production, also in the light of climatic changes which can cause the increase of grapevine diseases. The results of the present study show differences in the resistance to *P. viticola* among *V. vinifera* varieties and provide information about the defence mechanisms involved in the plant response. We observed that leaf morphological characteristics play a role in constitutive defence among *V. vinifera* varieties and that they seem to act in the initial phases of pathogen infection. The different number of successfully penetrated zoospores per stomata shows a possible functional relationship between stomata and zoospores of *P. viticola* that, in addition to depending on stomatal density, might be related to specific characteristics of the stomatal openings, stomata distribution on the leaf surface, or chemotactic processes that could be targets for future studies. Furthermore, although stilbenic phytoalexin synthesis resulted mainly in non-toxic compounds against downy mildew, it might be useful to evaluate the response of studied grapevines to exogenous application of stilbene synthesis promoters.

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