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SHORT NOTES

Levels of phytoalexins in vine leaves with different degrees of grapevine leaf stripe disease symptoms (Esca complex of diseases)

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Summary. Grapevine leaf stripe disease (GLSD) is one of the most common diseases in the esca complex. Losses in grape yields and quality caused by GLSD are correlated with the severity of the leaf symptoms. The time course of phytoalexin levels was examined in vine leaves of two vineyards, in leaves of healthy, asymptomatic/diseased and symptomatic vines. Symptomatic leaves were further divided into four categories according to symptom severity: 1, 5% chlorosis; 2, 20% chlorosis; 3, 40% leaf surface covered with tiger stripes; or 4, 65% leaf surface covered with tiger stripes. Leaves were sampled at three growth stages: 'berries beginning to touch'; 'berries developing colour'; and 'berries ripe for harvesting'. The phytoalexins *trans*-resveratrol, *trans*-ε-viniferin; *trans*-δ-viniferin; and *trans*-pterostilbene were detected and assayed. Patterns of each of the phytoalexins were similar at each growth stage to those found earlier for *trans*-resveratrol, with an increased phytoalexin levels with increasing leaf symptom severity on the leaf blade, especially at the stages 'berries beginning to touch' and 'berries ripe for harvest'. A laboratory test was also carried out. Petioles of healthy grapevine leaves were immersed in culture filtrates of *Phaeomoniella chlamydospora* and then dipped in solutions of *trans*-resveratrol or *trans*-pterostilbene to assess the effects of these substances on the leaf blades. Adding these phytoalexins did not influence the effects of *P. chlamydospora*. These results indicated that phytoalexins increased in the leaves after GLSD symptoms appeared, and not before host response (phytoalexin synthesis) to control symptom development.

Key words: phytoalexins, GLSD, leaf symptoms.

Introduction

Grapevine leaf stripe disease (GLSD) is a vine wood disease, and one of the Esca complex of diseases. GLSD is widespread in all vine-growing areas, but despite considerable experimental advances made in recent years (Di Marco *et al.*, 2011a; 2011b), it is still difficult to control.

Initially, GLSD was associated with white rot caused by the basidiomycete *Fomitiporia mediterranea* (Fischer, 2002; Surico *et al.*, 2006) and was named 'esca proper', but experimental findings have made it clear that the three fungi mainly involved in esca proper, *Phaeomoniella chlamydospora* (Pch) (Crous and Gams, 2000), *Phaeoacremonium minimum* (syn. *P. aleophilum*, Gramaje *et al.*, 2015) and *F. mediterranea*, can each colonise vines without any of the others, and in no particular chronological order (Sparapano *et al.* 2000). Esca proper has now been confirmed to derive from at least two distinct diseases; a trachaeomycosis, caused by *P. chlamydospora, P. minimum* and probably some other species of *Phaeoacremonium*; and a white wood rot, caused by *F. mediterranea*. It was subsequently found that white rot caused by *F. mediterranea* was not necessary for the expression of leaf symptoms attributed to *P. chlamydospora* and

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P. minimum (Calzarano and Di Marco, 2007; 2008). Consequently, the term GLSD was proposed to designate specifically the disease that causes the typical leaf symptoms on infected grapevines (Surico, 2009).

The fungi mainly associated with GLSD are *P. chlamydospora* and *P. minimum*. These fungi colonise the grapevine wood and produce brown necrosis and dark streaking of the vessels (Marchi *et al.*, 2001; Calzarano and Di Marco, 2007; Surico, 2009). The toxic metabolites produced by these fungi in colonised portions of vines are translocated to the crowns by the sap stream, and the GLSD symptoms develop on leaves (Sparapano *et al.*, 1998; Mugnai *et al.*, 1999; Evidente *et al*., 2000; Tabacchi *et al.*, 2000; Surico, 2009). These symptoms are: leaf chlorosis and necrosis (tiger stripes), purple spots, and more or less extensive withering of the grape bunches that result in losses in yield and quality proportional to the severity of the leaf symptoms (Calzarano *et al.*, 2001; 2004; Bertsch *et al.*, 2013).

Despite the advances of knowledge on these diseases, the idea that the leaf symptoms of GLSD are due to translocated toxic metabolites is still only a hypothesis. Tests undertaken with culture filtrates or with the constitutent fungal metabolites involved have produced uncertain results (Sparapano *et al*., 1998, 2000; Abou-Mansour *et al*., 2004). Only Sparapano *et al*. (2001) and Feliciano *et al.* (2004) were able to induce leaf symptoms, but these were different from typical GLSD symptoms. The well-known phenomenon of the partial remission or total disappearance of the foliar symptoms (but not of the disease itself) on certain plants in certain years suggests that other physiological or environmental factors may also be involved in symptom expression. These may include rainfall early in the growing season (Marchi *et al*., 2006; Calzarano and Di Marco, 2007; 2008).

More recently, variations in the levels of the phytoalexin *trans*-resveratrol, the main phytoalexin in grapevine (Jeandet *et al.*, 2002), have been examined in leaves of vines infected by GLSD in some vineyards at different growth stages (Calzarano *et al.*, 2008). In a following study, *trans*-resveratrol levels were measured in leaves differing for GLSD symptoms severity (Calzarano *et al.*, 2013). Furthermore, laboratory tests with healthy vine leaves dipped by petiole, first in *P. chlamydospora* culture filtrate and then in a *trans*-resveratrol suspension, showed that *trans*-resveratrol did not influence the effects of Pch filtrates on the withering of the leaves (Calzarano *et al*., 2013). The availability of a new analytical technique for the extraction and identification of *trans*-resveratrol and other derived phytoalexins, namely viniferins and *trans*-pterostilbene, has made it possible to better assess the levels of *trans*-resveratrol, and to measure the amounts of the other stilbene phytoalexins in healthy and diseased leaves (Calzarano *et al*. 2016).

The aim of the present study was to advance understanding of the roles of these phytoalexins in the formation of leaf symptoms in the crowns of grapevines infected with GLSD. In particular, we determined the levels of various phytoalexins in leaves with different GLSD symptom severity at different vine growth stages. The laboratory test with the Pch culture filtrates was repeated measuring the effects of *trans*-resveratrol and *trans*-pterostilbene.

Materials and methods

Foliar symptom survey, sampling of leaves and phytoalexin extraction

The study was carried out in 2014 in two 36-year-old vineyards, planted with the cv. Trebbiano d'Abruzzo on 420A rootstock, managed with IPM strategies, and located at Controguerra (Geneva Double Curtain system) and Giulianova (Tendone system) in the province of Teramo, Abruzzo, Italy. Both vineyards were on clay-calcareous soils. The cv. Trebbiano d'Abruzzo is usually severely affected by GLSD.

The sanitary status of each vine (healthy or GLSD-affected) in both vineyards was known because the vines had been monitored for GLSD symptoms since 1994. In the 2014 test year, the vines could be positively identified as either healthy, diseased/asymptomatic or diseased/symptomatic.

Incidence and severity of GLSD foliar symptoms were evaluated in 2014 on 20 September in Controguerra and 22 September in Giulianova, during the maximum of symptom expression, recorded and analyzed using the methods described in Calzarano *et al*. (2014).

Leaf samples were collected from each of the three groups of vines, designated: H (healthy), AS (diseased/asymptomatic) or T (diseased/symptomatic). Leaves from T vines were arbitrarily divided into four subgroups: T1, exhibiting 5% chlorosis; T2, 20% chlorosis; T3, 40% leaf surface covered with ti-

ger-stripes (chlorosis + necrosis); or T4, 65% leaf surface covered with tiger-stripes (chlorosis + necrosis). This gave a total of six categories of leaves. Leaves were sampled at three growth stages, according to BBCH-scale for grapevine (Lorenz *et al*., 1995): i, 'berries beginning to touch' (BBCH 77); ii, 'berries developing colour' (BBCH 83); or iii, 'berries ripe for harvest' (BBCH 89). From each of the three groups of vines, six vines were selected, and from each of H and AS vines eight leaves, and from each of T vines 32 leaves (eight leaves for each symptom subgroup), at each growth stage, were sampled from the central portion of a vine shoot, located in a position opposite a grape bunch, to minimise natural variations between leaves due to leaf position along each shoot. The leaves sampled at the three growth stages were always taken from the same vines in each groups. Each sample then consisted of eight leaves. The 8 leaves of each sample were combined and treated as one replicate, from which phytoalexins were extracted and identified according to the procedure described in Calzarano *et al*. (2016).

Laboratory assay

A laboratory assay was carried out to determine the effect of the interactions among phytoalexins - grapevine leaf - Pch filtrate. To obtain Pch filtrates, an adequate volume of Czapeck–Dox medium, modified by adding 0.5% yeast and 0.5% malt extract (pH 6.8), was autoclaved, and 150 mL was added to each of 1 L capacity Roux flasks. Half of the prepared flasks were inoculated with of agar/mycelium obtained from the edges of 2-week-old Pch colonies grown on potato dextrose agar. The resulting liquid cultures were incubated in stationary phase at 25°C in darkness. After 28 d, mycelium was removed by filtration through Whatman No. 4 filter paper (Martos *et al*., 2008; Osti and Di Marco, 2010). Filtrates form the liquid cultures were pooled, mixed and observed with a light microscope to ensure that fungal structures had been excluded by the filtration. The remaining half of the flasks were subjected to the same procedure except that they were not inoculated, to provide sterile liquid growth medium.

The petioles of healthy grapevine leaves were immersed in Pch culture filtrate, and then in solutions of *trans*-resveratrol or *trans*-pterostilbene. The leaves were taken from the middle portions of the primary grapevine shoots at the BBCH 77 growth stage, from positions opposite to grape bunches to minimise natural variations along the shoots. Petioles of leaves from healthy vines were individually immersed in 50 mL of Pch culture filtrate (for a total of 18 leaves) or in sterile liquid growth medium as controls (additional 18 leaves) for 3 h, and were then evaluated using an arbitrary leaf withering severity scale; where 1 was maximum withering and 5 was absence of withering (fresh green leaf). Leaves from Pch culture filtrate treatment were then randomly subdivided into three groups of six leaves each. Leaves in these groups were individually immersed in 50 mL of distilled water or in distilled water containing 80 mg L^{-1} of *trans*-resveratrol, or 15 mg L^{-1} of *trans*-pterostilbene, corresponding to the maximum concentrations found previously in GLSD-affected Trebbiano d'Abruzzo grapevine leaves in both vineyards (Calzarano *et al*., 2001; 2004; 2008; 2013; 2014). The control was a group of six leaves immersed in sterile growth medium and in distilled water. All leaves were evaluated for leaf withering at 15 h, 58 h, or 106 h of immersion.

Statistical analyses

Data of the concentrations of each phytoalexin in the different GLSD categories of leaves were compared separately at each growth stage using Tukey's honest significant difference (HSD) test at $P = 0.05$. In the laboratory test for each time of immersion, treatments were compared and data were statistically analyzed using Tukey's honest significant difference (HSD). Statistical analyses were carried out using SAS version 9.3 (SAS Institute Inc.).

Results

Phytoalexins determinations

In 2014, the mean amounts of *trans*-resveratrol levels in T4 leaves, recorded at the BBCH 77 vine growth stage, were 34.67 mg kg^{-1} d wt in Controguerra and 47.99 mg kg^{-1} d wt in Giulianova. These levels were among the greatest amounts of *trans*-resveratrol recorded in those vineyards (Calzarano *et al*., 2016). These high levels were consistent with the high incidence (30.4%) and severity (11.8%) of GLSD leaf symptoms recorded in the Controguerra vineyard, and 9.2% incidence and 5.8 % severity recorded in the Giulianova vineyard.

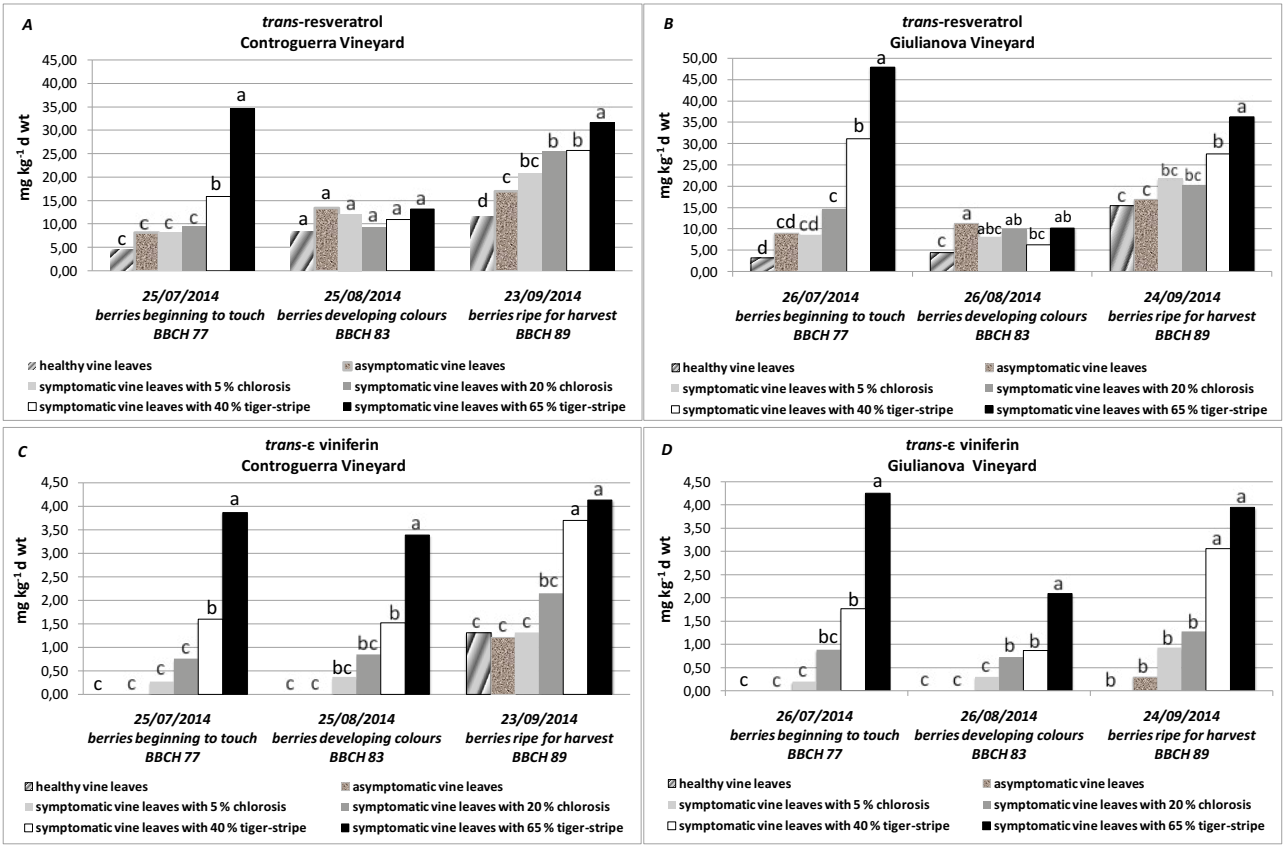

Figure 1. Levels of trans-resveratrol (A, B) and *trans*-ε-viniferin (C, D) in the leaves of vines infected with GLSD with varying degrees of chlorosis or tiger-stripes and in the leaves of diseased/asymptomatic and healthy vines at various growth stages in the 2014 growing season in the Controguerra and Giulianova vineyards. Statistical analysis was performed according to Tukey's honest significant differences (HSD) test. Each of the six grapevine category (4 subgroups of diseased/symptomatic, 1 group of diseased/asymptomatic and 1 group of healthy) had 6 replications for each category of leaves in each growth stage and in each vineyard. Different letters represent significant differences at P=0.05.

In both vineyards, *trans*-resveratrol levels were particularly high in symptomatic leaves at the BBCH 77 and BCCH 89 growth stages, compared with healthy and asymptomatic/diseased leaves. At the BBCH 77 stage, *trans*-resveratrol levels declined showing similar values for the three groups of vines (Figure 1A–1B). Levels of ε-viniferin and δ-viniferin in symptomatic leaves exhibited similar patterns over the growth stages, as compared with healthy and diseased/asymptomatic levels, except that they were higher than in healthy and diseased/asymptomatic leaves, also at the 'berries developing colour' stage. This was despite the generally lower amounts of the phytoalexins at this stage (Figures 1C–1D and 2A–2B).

Concentrations of *trans*-resveratrol, ε-viniferin and δ-viniferin, increased in proportion to the chlorosis and necrosis severity on symptomatic leaves (Figures 1A–1D and 2A–2B), as observed for *trans*-resveratrol at BBCH 77 and BCCH 89 stages, and for ε-viniferin and δ-viniferin at all of the growth stages (Figures 1 and 2). These findings agree with those for *trans*-resveratrol reported by Calzarano *et al.* (2013). *Trans*-pterostilbene levels were always very low and similar in all types of leaves and at all growth stages (Figure 2C–2D).

Laboratory assay

Trans-resveratrol or *trans*-pterostilbene failed to improve the turgor of the healthy grapevine leaves

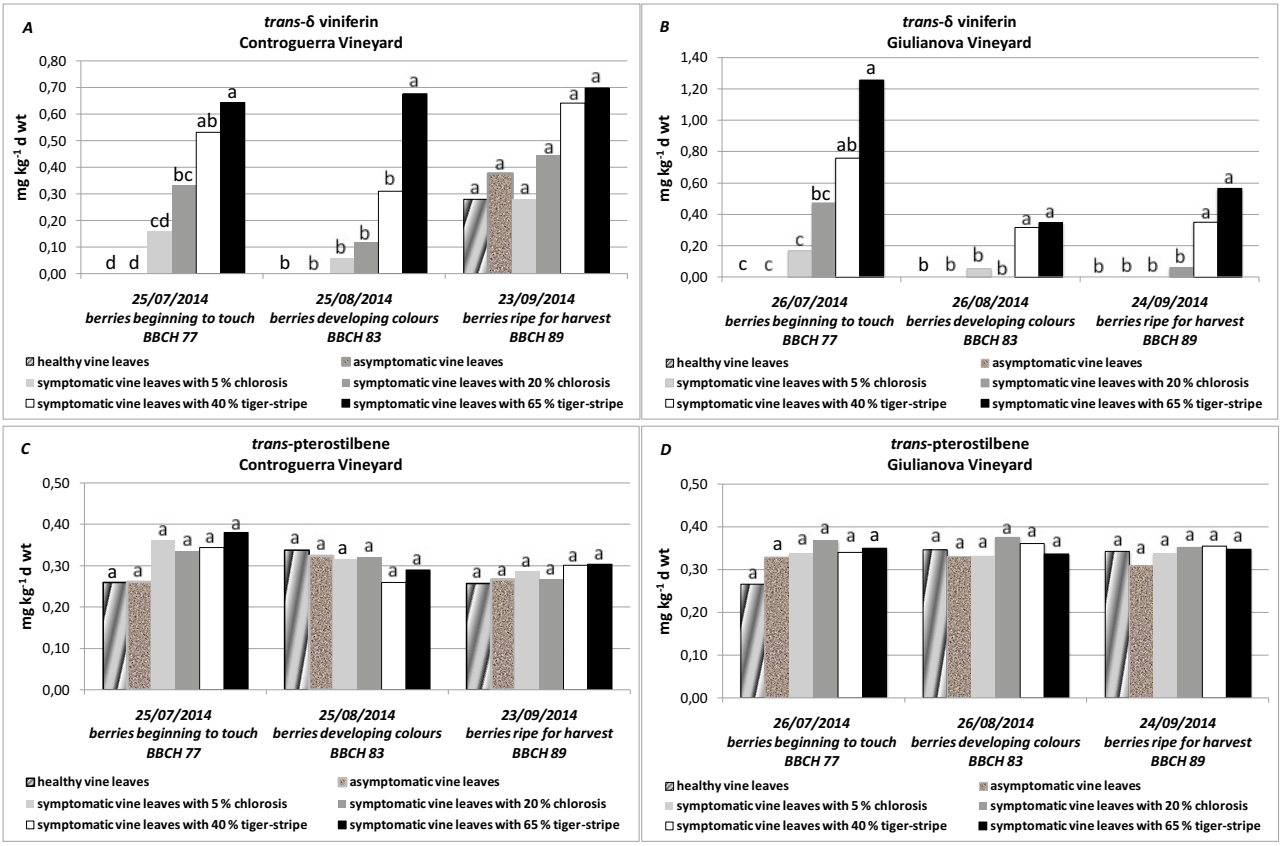

Figure 2. Levels of trans-δ-viniferin (A, B) and *trans*-pterostilbene (C, D) in the leaves of vines infected with GLSD with varying degrees of chlorosis or tiger-stripes and in the leaves of diseased/asymptomatic and healthy vines at various growth stages in the 2014 growing season in the Controguerra and Giulianova vineyards. Statistical analysis was performed according to Tukey's honest significant differences (HSD) test. Each of the six grapevine category (4 subgroups of diseased/symptomatic, 1 group of diseased/asymptomatic and 1 group of healthy) had 6 replications for each category of leaves in each growth stage and in each vineyard. Different letters represent significant differences at P=0.05.

collected at BBCH 77 growth stage and immersed in Pch culture filtrate and then in phytoalexin suspensions (Figure 3). In the first part of the laboratory assay, results showed statistically significant differences comparing the 3 hours Pch filtrate treatment (mean leaf withering score = 1.39) with the 3 h sterile growth medium treatment (mean leaf withering score = 5) (Q = 229.8043, d.f. = 1, P<0.0001).

The three groups of six leaves each treated with Pch culture filtrate did not differ significantly for leaf turgor, with mean leaf withering scores ranging from 1.3 to 1.5 (Q = 0.2, d.f: = 2, P=0.8209). These leaves immersed for 15 or 58 h in suspensions of *trans*-resveratrol, *trans*-pterostilbene or distilled water after immersion in Pch filtrates, showed similar levels of withering after 15 or 58 h, but these were significantly different from the leaves immersed in distilled water after immersion in sterile growth medium as controls (Q =44.6825, d.f: = 3, P<0.0001 after 15 h; and Q = 40.8333 d.f: = 3, P<0.0001, after 58 h) (Figure 3). At these times, control leaves showed no withering. At 106 h, all leaves dried up and no significant differences in withering scores could be detected among treatments, according to Tukey's HSD test (Q = 0.1724, d.f. = 3; P=0.9138) (Figure 3).

Discussion

Results of this study highlighted the differences in phytoalexin levels in GLSD-symptomatic grape-

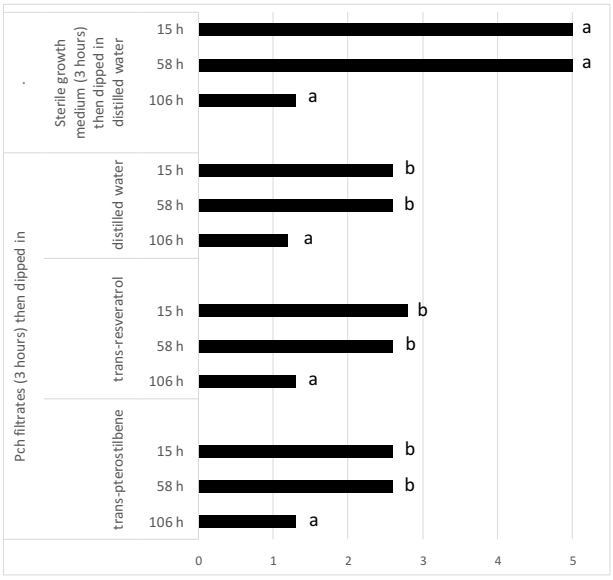

Figure 3. Effect on leaves of the immersion of petioles in Pch filtrates or sterile growth medium then dipped in suspensions of *trans*-resveratrol or *trans*-pterostilbene in distilled water, or in distilled water alone. Results were expressed using an arbitrary 1-5 scale of leaf withering severity where 1 is the maximum withering and 5 is the absence of withering (fresh green leaf). The statistical analysis was carried out at each time of immersion using Tukey's honest significant difference (HSD).

vine leaves, depending on different symptom severities, at different plant growth stages. This study has also identified *in vitro* activity of *trans*-resveratrol and *trans*-pterostilbene towards the phytotoxic effects of Pch culture filtrates on grapevine leaves.

In the Controguerra vineyard, the high *trans*-resveratrol levels in leaves corresponding to the maximum symptom severity were consistent with the high incidence and severity of GLSD leaf symptoms recorded in 2014. In Giulianova, the incidence and severity of leaf symptoms were less than in Controguerra, but not so low as to lead to decreases in phytoalexin levels, even though lower severity of leaf symptoms are always associated with lower levels of phytoalexins (Calzarano *et al.*, 2016). A substantial difference between the levels of *trans*-resveratrol, *trans*-ε-viniferin, *trans*-δ-viniferin and *trans*-pterostilbene recorded in the present study and those previously noted in the same vineyards (Calzarano *et al.*, 2016) was observed. Thus, in the grapevines analysed in this study, the dimerisation and demethylation of *trans*-resveratrol was much less; consequently, amounts of viniferin, and especially *trans*-pterostilbene, were much less in 2014 than in previous growing seasons (Calzarano *et al.*, 2016).

Amounts of phytoalexins in leaves at the 2014 growth stages exhibited the same fluctuations as those seen in previous years (Calzarano *et al.*, 2008; 2013; 2016). These findings suggest that the time-course of phytoalexin amounts may also depend on variations in vine physiology during the growing season. The renewal of carbohydrate reserves in the vine wood at the 'berries developing colour' stage (Lebon *et al.*, 2008), and the need to translocate energy for berry ripening, could explain the reduced synthesis of the phytoalexins at that stage.

Leaves showing the same extension of tiger-stripe symptoms synthesised different amounts of phytoalexins in different years. The amounts of the phytoalexins in leaves with the greatest severity of tiger-stripe symptoms varied considerably between growing seasons (Calzarano *et al.*, 2016). For example, amounts of *trans*-pterostilbene in 2014 (0.25–0.35 mg kg^{-1} d wt) were considerably less than those recorded in previous years (2.0–5.5 mg kg^{-1} d wt; Calzarano *et al.*, 2016). Similarly, *trans*-resveratrol amounts fluctuated from 20 to 50 mg kg^{-1} d wt in the years examined (Calzarano *et al.*, 2016). These fluctuations probably indicate that environmental factors also affect the amounts of phytoalexins produced in symptomatic leaves, i.e., the rainfall amount during the growing season. These factors could increase the incidence of GLSD symptoms (Marchi *et al.*, 2006), and could also determine the amounts of phytoalexins produced in grapevine leaves.

The fact that levels of *trans*-resveratrol, ε-viniferin and δ-viniferin increased with increasing severity of GLSD symptoms on the leaf, confirmed that all these substances are largely synthesised as a reaction to lesions suffered by leaves, similarly to any biotic or abiotic events that damage the leaves (Kuć, 1995; Smith, 1996; Bavaresco and Fregoni, 2001). Toxic metabolites produced by fungi in wood (Andolfi *et al.*, 2011) and translocated to the leaves may trigger plant responses that include the formation of necrotic lesions on leaves, as a hypersensitive reaction. These reactions are usually followed by formation of antimicrobial compounds such as stilbene derivatives (Heath, 2000). In the green portions of symptomatic leaves, gene expression of phenylalanine ammonia lyase and stilbene synthase, involved in the

trans-resveratrol synthesis pathway, did not occur. These enzymes are found only in the chlorotic areas of leaves where symptoms have already formed (Magnin-Robert *et al*., 2011). Moreover, in asymptomatic grapevine leaves before the 'berries beginning to touch' growth stage (when any GLSD symptoms that may appear later are not yet visible), levels of *trans*-resveratrol and ε-viniferin were low and levels of δ-viniferin and *trans*-pterostilbene were undetectable (Calzarano *et al*., 2016). The amounts of all these substances in asymptomatic/diseased leaves then increased at the 'berries beginning to touch' stage, when GLSD symptoms begin to appear, as reported in other studies (Magnin-Robert *et al*., 2011; Valtaud *et al*., 2011; Lambert *et al*., 2013), and levels of phytoalexins were especially high in symptomatic leaves. All these results indicate that the phytoalexins do not prevent leaf symptoms to form until the 'berries beginning to touch' growth stage (Calzarano *et al*., 2016). From that stage onward, the differences in the levels of the phytoalexins between healthy and asymptomatic/diseased leaves were so small that it is unlikely that these phytoalexins have any roles in the mechanisms that inhibit symptom formation. An alternative hypothesis could be that symptoms sometimes do not appear in diseased vines during an entire growing season is due to environmental factors, or to the particular conditions of individual vines, or to interactions between environmental factors and the conditions of vines. That phytoalexins have no role in prevention of leaf symptom development seems more likely since both *trans*-resveratrol and *trans*-pterostilbene failed to improve the turgor of the leaves, when these leaves had previously been immersed in Pch culture filtrates, any more than did immersion in distilled water. Nevertheless, it is yet to be determined if increased amounts of the phytoalexins induced in asymptomatic/diseased vine leaves before leaf symptom appearance (i.e. prior to the 'berries beginning to touch' growth stage) could contribute vines remaining asymptomatic during particular growing seasons. Repeated applications of a leaf fertilising mix containing calcium, magnesium and extracts of algae during vegetative growth strongly reduced the incidence of GLSD symptoms, and caused high phytoalexins levels at the 'fruit-set' and the 'pea-sized berries' stages (Calzarano *et al*., 2014; 2017).

In conclusion, this study has confirmed that the growth stage of grapevines plays an important role in determining the amount of phytoalexins occurring in leaves. The study also revealed that these phytoalexins are not involved, under natural conditions, in the mechanisms that inhibit development of GLSD leaf symptoms.

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