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RESEARCH PAPERS

# Mixture of calcium, magnesium and seaweed affects leaf phytoalexin contents and grape ripening on vines with grapevine leaf stripe disease

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**Summary.** Grapevine leaf stripe disease (GLSD) is a tracheomycosis caused by *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum*. Impacts on yields of grapes were correlated with the incidence and severity of GLSD symptoms on vine canopies. In 2012 and 2013, vines in two vineyards were treated with nine applications of a mixture of leaf fertilizers containing calcium, magnesium and seaweed extracts. At different growth stages, leaves were sampled from treated and control plots of healthy, GLSD-asymptomatic, or GLSD-symptomatic vines and contents were measured of the phytoalexins *trans*-resveratrol, *trans*-ε-viniferin, *trans*-δ-viniferin and *trans*-pterostilbene. Grape ripening was also monitored from veraison to harvest during both vintages. The treatments caused significant reductions in canopy symptom expression. Increased phytoalexin contents were measured from 'fruit set' to 'berries developing colour' stages. *Trans*-resveratrol peak was recorded in asymptomatic diseased vines at the 'berries pea-sized' stage, and *trans*-ε-viniferin and *trans*-δ-viniferin increased at the 'berries beginning to touch' stage, compared to the contents recorded in untreated asymptomatic vines. From 'berries developing colour' to harvest, all treated vines had lower amounts of phytoalexins than the control ones. At harvest, treated healthy and symptomatic vines produced berries with similar amounts of total sugars compared to untreated vines. Treated asymptomatic vines produced berries with greater amounts of total sugars compared to the untreated vines. These results indicate that increased phytoalexin content recorded from 'fruit set' to 'berries beginning to touch' in asymptomatic vines treated with the mineral/seaweed mixture may reduce symptoms of GLSD.

**Key words:** leaf fertilizers, GLSD, leaf symptoms, maturation.

## Introduction

Grapevine trunk diseases are considered the most destructive diseases in grape growing areas worldwide. The most important and common trunk disease in European vineyards is grapevine leaf stripe disease (GLSD) of the esca complex (Surico *et al*., 2008; 2009). GLSD is caused mainly by *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp., of which *P. minimum* is the most frequently isolated. These tracheomycotic fungi colonize the woody tissues inside grapevines, producing black streaking and brown or red-brown necrosis (Marchi *et al*., 2001; Calzarano and Di Marco, 2007; Surico, 2009). The most common hypothesis for the formation of the characteristic symptoms of the disease is based on phytotoxic substances produced by the pathogens, that are transported through the host vascular tissues into the leaves (Sparapano *et al*., 1998; Evidente *et al*., 2000; Tabacchi *et al*., 2000). The leaves develop interveinal chlorosis, which then becomes necrotic resulting in the leaf symptom known as "tiger stripes". GLSD causes qualitative damage in vineyards when symptoms appear on

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the canopies. The disease also causes shoot wilting and, in some cases, black measles in the fruits (Chiarappa, 2000), with direct yield losses. Some of the plants affected by GLSD do not show symptoms every year, making them indistinguishable from healthy plants. These asymptomatic plants are able to grow and produce quality and quantity of yield similar to that of healthy plants (Calzarano *et al*., 2001; 2004; Bertsch *et al*., 2013). Since manifestation of GLSD symptoms on vine canopies is correlated with quantitative and qualitative impacts on grape yields (Calzarano *et al*., 2001; 2004; Bertsch *et al*., 2013), and given the impossibility of eradicating the disease once established, studies are aimed at reducing symptom incidence and severity, and increasing the ratio of asymptomatic vines over these affected by GLSD in vineyards.

Many control methods against GLSD have been attempted, but no effective control strategy has yet been developed (Di Marco *et al*., 2011a; 2011b). Recent studies show promising results with *Trichoderma* applications to pruning wounds (Di Marco *et al*., 2004), or treating plants with nutrients and seaweed mixture that reduced the canopy symptoms, associated with significant increases in *trans*-resveratrol levels in diseased treated vines (Calzarano *et al*., 2014). In both cases, the treatments reduced symptom expression of vine canopies, with consistent reductions of yield losses. Further studies have recently focused on the role of the main vine phytoalexins during vegetative growth, before and during the formation of leaf symptoms (Calzarano *et al*., 2008, 2016). In particular, amounts of these phytoalexins increase in the leaves of symptomatic plants, after symptom formation, especially at the pre-bunch closure growth stage, but this increase was not associated with reduced foliar symptom expression (Calzarano *et al*., 2016).

The present research studied the possible role of different phytoalexins from leaves of infected plants (Calzarano *et al*., 2016) in the mechanism of action of a leaf fertilizer mixture based on nutrients and seaweed extract. Investigations were carried out at different vine growth stages, measuring phytoalexin contents in leaves of healthy, GLSD-symptomatic and GLSD-asymptomatic vines, either untreated or treated with the leaf fertilizer mixture. Impacts of nutrient applications on the grape ripening and composition were also evaluated.

## Materials and methods

### Leaf nutrient applications

A field trial was carried out on a 39-year-old vineyard, of grape cv. Trebbiano d'Abruzzo, located in Piane Tronto, Controguerra (Teramo, Central Italy) and GDC trained, with a planting arrangement of 2 m (within rows) × 4 m (between rows), and with an average yield of up to 16.5 kg per vine in years of optimum yield. The vineyard had a clay-calcareous soil with a pH of 8.45, an active limestone content of 16.5% and a low soil organic matter content of 1.25% (Calzarano *et al*., 2009). The previous vineyard soil fertilizations were carried out in 2005 and in 2010 using triple N-$P_2O_5$-$K_2O$ fertiliser, with titres of 16-10-16, at a rate of 300 kg $ha^{-1}$. Since 1994, incidence and severity of foliar symptoms of GLSD were assessed on approx. 740 plants. The experiment considered two treatments (leaf fertilizer application and control) with two repetitions per treatment, each consisting of 70 vines. As a result of a long-term survey, it was possible to discriminate three groups of vines for the present study (carried out in 2012 and 2013): GLSD symptomatic vines, healthy vines (that had never shown any symptoms since 1994), and GLSD asymptomatic vines (plants without symptoms in 2012 and 2013 but had shown symptoms in one of the previous 18 years). The data obtained in the present study are related to those three statuses of vines. In 2012 and 2013 nine applications of a leaf fertilizer mixture containing 466 g of $CaCl_2$, 403 g of $Mg(NO_3)_2$, 75 mL of *Fucales* seaweed extract and 466 mL distilled $H_2O$ per liter of solution were carried out in the vineyard. The mixture was applied at each application at 4 L $ha^{-1}$. Applications were carried out at 10 d intervals each year, commencing from the "three leaves unfolded" BBCH 13 stage (Lorenz *et al*., 1995) at the beginning of May, up to pre-bunch closure ('berries beginning to touch' BBCH 77) at the end of July.

### Disease incidence and severity

Foliar symptoms were assessed near technological ripeness on 18$^{th}$ September 2012 and 20$^{th}$ September 2013, which corresponded to the greatest expression of GLSD symptoms in each growth season. Incidence and severity of the disease in each treatment were recorded and analyzed using the methods described by Calzarano *et al*. (2014).

### Leaf sampling and phytoalexin determinations

For each vine status (healthy, GLSD asymptomatic or GLSD symptomatic) the contents of phytoalexins were measured in the leaves of six individual vines per treatment. From each vine, 12 leaves were collected from the middle part of grape-bearing shoots, opposite to respective bunches. For each of the six plant groups (three treated with the mixture and three untreated), six samples of 12 leaves were obtained, with each sample consisting of leaves taken from a single plant. Sampling of leaves was carried out at six different vine growth stages, described by the BBCH classification scheme (Lorenz *et al*., 1995), including: 'Fruit set' (BBCH 71); 'Berries pea-sized' (BBCH 75); 'Berries beginning to touch' (BBCH 77); 'Berries developing colour' (BBCH 83); 'Softening of berries' (BBCH 85); and 'Berries ripe for harvest' (BBCH 89). First seasonal appearance of foliar GLSD symptoms in the vineyard usually occurred no earlier than July. For this reason, it was possible to collect the first symptomatic leaves starting from the BBCH 77 stage. Leaf contents of extracted phytoalexins were measured according to the methods reported by Calzarano *et al*. (2016) and expressed on a leaf dry weight basis.

### Total sugars and total acids contents of grape berries

In each of 2012 and 2013, three samples of berries of 500 g each from each different group of vines were harvested at four different phenological phases: 'Beginning of ripening: berries start to develop variety-specific colour' (BBCH 81); 'Berries developing colour' (BBCH 83); 'Softening of berries' (BBCH 85); and 'Berries ripe for harvest' (BBCH 89). In the GLSD symptomatic vines, two subgroups were created by collecting the three samples of berries both from symptomatic and asymptomatic shoots. In all cases, berries were visually healthy and suitable for vinification. In each group of vines, berry samples were taken similarly by the margins, apical and central parts of the bunches of 12 vines. At each of the growth stages, total sugars and total acidity were measured in each group and subgroup. Each 500 g berry sample was separately crushed with a manual press. Must yield was standardized to 70 % (w:v). The obtained musts were analysed for total sugars using Feheling analysis, and total acids using Acid/Base Titration. The analyses were carried out following the official protocols of the Regulation No.2676/90/EEC annex 13, 24 and 5 (AA.VV., 1990).

### Statistical analyses

Differences of the incidence and severity of foliar symptoms recorded in control plots and those treated with foliar nutrient mixture were compared by (Chi-square) ($\chi^2$) tests, according to the methods reported by Calzarano *et al*. (2014). For each vine status (healthy, GLSD-asymptomatic, and GLSD-symptomatic), phenological growth stage and year of study, measurements of the leaf phytoalexin contents from treated and untreated vines were compared by Student's *t*-test. For each vine status, data of total sugars and total acidity in berry samples collected at harvest from treated and untreated vines were compared by Student's *t*-test. Data were processed with the SAS 9.3 statistical program (SAS Institute Inc., Cary, NC, USA).

## Results

### Disease incidence and severity

The treatments with the mixture of foliar nutrients containing calcium, magnesium and seaweed extracts, carried out during the vine growing season, significantly reduced the expression of foliar symptoms in the vine canopies of treated plots in both years of the trial. Mean disease incidence in 2012 was 4.2% in treated plots, and 26.2% in untreated plots ($\chi^2$ = 35.31, d.f. = 1, $P$<0.0001), and in 2013 was 8.02% in treated plots and 24.5% in untreated plots ($\chi^2$ = 69.77, d.f. = 1, $P$<0.0001). Mean disease severity (McKinney index) in 2012 was 1.32% in treated plots and 9.17% in untreated plots ($\chi^2$ = 22.74, d.f. = 1, $P$<0.0001), and in 2013 was 3.62% in treated plots and 10.62% in untreated plots ($\chi^2$ = 44.74, d.f. = 1, $P$<0.0001).

### Phytoalexin measurements

Four different stilbenes, *trans*-resveratrol, its *trans*-ε-viniferin and *trans*-δ-viniferin dimers, and *trans*-pterostilbene were extracted from sampled grapevine leaves. The *trans*-resveratrol content was always greater than that of the other compounds. For each of the three different vine statuses, application of the mixture of foliar nutrients induced greater contents of phytoalexins, from the BBCH 71 to the BBCH 83 growth stages, compared to the contents recorded in the corresponding untreated control vines (Figures 1 and 2, Table 1). The contents of phytoalexins recorded after the applications of the mixture, showed

**Table 1.** Mean amounts of *trans*-ε-viniferin (a, b), *trans*-δ-viniferin (c, d) and *trans*-pterostilbene (e, f) in leaves of healthy grapevines, and of asymptomatic and symptomatic vines affected by GLSD, treated or untreated with foliar nutrient mixture in 2012 and 2013. Each value is the mean of six replications of the leaf data individually obtained from six vines for each grapevine status. The statistical analysis separately compared leaf data from each grapevine status (healthy, asymptomatic and symptomatic; data comparison of treated-untreated pairs) for each phenological growth stage in each year (following Lorenz *et al*., 1995). Mean ± standard deviations are indicated. Values for each pair of means followed by symbols are statistically different (* for $P = 0.05$; ** for $P = 0.01$), as indicated by Student's $t$ –test.

**a**

| Vine status | ***Trans*-ε-viniferin - 2012 (mg kg$^{-1}$ d wt)** | | | | | |
|---|---|---|---|---|---|---|
| | **BBCH 71 20/06/2012** | **BBCH 75 13/07/2012** | **BBCH 77 30/07/2012** | **BBCH 83 29/08/2012** | **BBCH 85 06/09/2012** | **BBCH 89 24/09/2012** |
| Untreated healthy vines | 0.03±0.05 | 0.07±0.09 | 0.05±0.06 | 1.29±0.23 ** | 2.65±0.20 ** | 1.64±0.22 |
| Treated healthy vines | 0.26±0.45 | 1.61±0.16 | 1.05±0.91 | 0.00±0.00 | 1.18±0.36 | 1.31±1.16 |
| Untreated asymptomatic vines | 0.51±0.89 | 0.87±0.75 | 1.35±0.16 | 0.45±0.78 * | 4,11±0.53 ** | 4.14±0.81 * |
| Treated asymptomatic vines | 0.75±0.73 | 1.35±1.38 | 2.61±0.25 ** | 0.00±0.00 | 2.14±0.22 | 2.22±0.10 |
| Untreated symptomatic vines | n.p. | n.p. | 2.35±0.90 | 3.11±0.28 | 13.10±1.89 | 8.04±4.27 |
| Treated symptomatic vines | n.p. | n.p. | 5.43±0.78 * | 5.81±1.35 * | 9.70±1.02 | 14.99±0.87 |

**b**

| Vine status | ***Trans*-ε-viniferin - 2013 (mg kg$^{-1}$ d wt)** | | | | | |
|---|---|---|---|---|---|---|
| | **BBCH 71 21/06/2013** | **BBCH 75 12/07/2013** | **BBCH 77 26/07/2013** | **BBCH 83 27/08/2013** | **BBCH 85 07/09/2013** | **BBCH 89 21/09/2013** |
| Untreated healthy vines | 1.15±1.01 | 1.79±0.43 | 1.48±1.35 | 1.66±0.10 ** | 2.67±0.41 | 1.85±0.17 |
| Treated healthy vines | 0.71±1.22 | 1.38±1.24 | 0.98±0.85 | 0.00±0.00 | 1.25+1.14 | 1.27±1.11 |
| Untreated asymptomatic vines | 0.78±1.35 | 1.11±0.99 | 0.87±0.82 | 0.79±1.36 | 2.93±0.98 | 3.26±1.03 |
| Treated asymptomatic vines | 1.08±0.94 | 0.99±0.97 | 2.33±0.28 * | 0.00±0.00 | 1.47±0.20 | 2.27+0.06 |
| Untreated symptomatic vines | n.p. | n.p. | 2.72±0.10 | 3.24+0.47 | 7.80±3.31 | 7.75±3.95 |
| Treated symptomatic vines | n.p. | n.p. | 4.49±1.95 | 7.74±5.03 | 9.70±1.02 | 9.42±4.80 |

**c**

| Vine status | ***Trans*-δ-viniferin - 2012 (mg kg$^{-1}$ d wt)** | | | | | |
|---|---|---|---|---|---|---|
| | **BBCH 71 20/06/2012** | **BBCH 75 13/07/2012** | **BBCH 77 30/07/2012** | **BBCH 83 29/08/2012** | **BBCH 85 06/09/2012** | **BBCH 89 24/09/2012** |
| Untreated healthy vines | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 1.19±0.09 ** | 0.90±0.80 |
| Treated healthy vines | 0.80±0.69 | 0.00±0.00 | 0.36±0.34 | 0.00±0.00 | 0.20±0.35 | 0.00±0.00 |
| Untreated asymptomatic vines | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.63±1.09 | 1.41±0.33 |
| Treated asymptomatic vines | 0.00±0.00 | 0.00±0.00 | 1.01±0.18 ** | 0.89±0.19 ** | 0.14±0.25 | 1.26±0.18 |
| Untreated symptomatic vines | n.p. | n.p. | 1.70±0.26 | 1.79±0.19 | 3.23±0.07 | 2.51±0.52 |
| Treated symptomatic vines | n.p. | n.p. | 3.01±0.25 ** | 1.89±0.11 | 3.37±0.19 | 2.25±0.53 |

(Continued)

**Table 1.** (Continued).

**d**

| **Vine status** | ***Trans*-δ-viniferin - 2013 (mg kg$^{-1}$ d wt)** | | | | | |
|---|---|---|---|---|---|---|
| | **BBCH 71 21/06/2013** | **BBCH 75 12/07/2013** | **BBCH 77 26/07/2013** | **BBCH 83 27/08/2013** | **BBCH 85 07/09/2013** | **BBCH 89 21/09/2013** |
| Untreated healthy vines | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 1.92±0.36 | 0.51±0.88 |
| Treated healthy vines | 0.80±1.39 | 0.00±0.00 | 0.66±0.51 | 0.00±0.00 | 0.56±0.98 | 0.00±0.00 |
| Untreated asymptomatic vines | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.74±1.29 | 1.63+0.02 |
| Treated asymptomatic vines | 0.00±0.00 | 0.00±0.00 | 1.31±0.04 ** | 0.00±0.00 | 0.00±0.00 | 1.72±0.16 |
| Untreated symptomatic vines | n.p. | n.p. | 1.55±0.11 | 1.70±0.30 | 2.72±0.45 | 2.19+0.01 |
| Treated symptomatic vines | n.p. | n.p. | 3.44±1.66 | 1.78±0.32 | 2.98±0.54 | 2.76+0.91 |

**e**

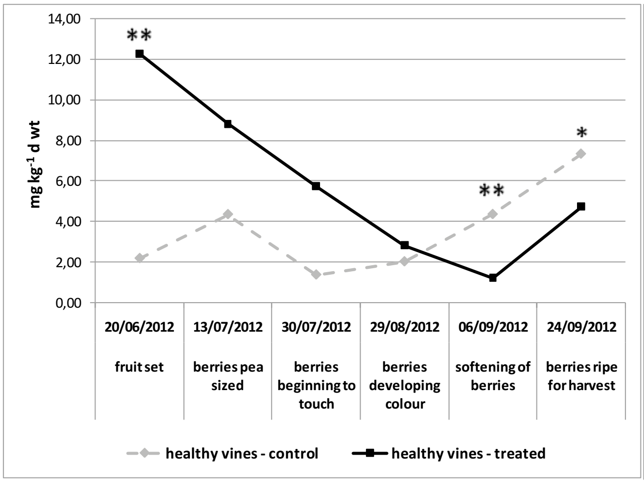
| **Vine status** | ***Trans*-pterostilbene - 2012 (mg kg$^{-1}$ d wt)** | | | | | |
|---|---|---|---|---|---|---|
| | **BBCH 71 20/06/2012** | **BBCH 75 13/07/2012** | **BBCH 77 30/07/2012** | **BBCH 83 29/08/2012** | **BBCH 85 06/09/2012** | **BBCH 89 24/09/2012** |
| Untreated healthy vines | 0.00±0.00 | 0.00±0.00 | 0.08+0.14 | 0.00±0.00 | 1.38±0.54 * | 2.41±0.31 |
| Treated healthy vines | 0.00±0.00 | 0.00±0.00 | 0.39±0.40 | 0.00±0.00 | 0.00±0.00 | 1.40±1.22 |
| Untreated asymptomatic vines | 0.00±0.00 | 0.00±0.00 | 0.11±0.19 | 0.00±0.00 | 3.00+0.37 * | 2.87+0.56 |
| Treated asymptomatic vines | 0.00±0.00 | 0.00±0.00 | 0.42±0.41 | 0.00±0.00 | 0.89±0.79 | 2.18±0.38 |
| Untreated symptomatic vines | n.p. | n.p. | 4.91±0.63 | 3.05+1.23 | 4.35±1.11 | 4.16±0.77 |
| Treated symptomatic vines | n.p. | n.p. | 13.91±2.84 ** | 3.72±2.16 | 4.36±0.69 | 3.37±0.07 |

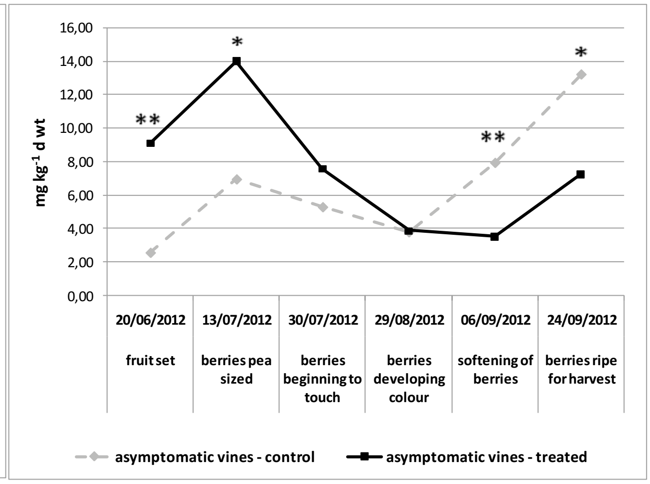
**f**

| **Vine status** | ***Trans*-pterostilbene - 2013 (mg kg$^{-1}$ d wt)** | | | | | |
|---|---|---|---|---|---|---|
| | **BBCH 71 21/06/2013** | **BBCH 75 12/07/2013** | **BBCH 77 26/07/2013** | **BBCH 83 27/08/2013** | **BBCH 85 07/09/2013** | **BBCH 89 21/09/2013** |
| Untreated healthy vines | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 1.68±1.45 | 1.66+0.17 |
| Treated healthy vines | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.88±0.77 |
| Untreated asymptomatic vines | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 2.66±0.65 * | 1.89±0.49 |
| Treated asymptomatic vines | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.52±0.90 | 1.42±0.08 |
| Untreated symptomatic vines | n.p. | n.p. | 5.41±1.00 | 2.80±1.24 | 3.25±0.15 | 2.87±0.78 |
| Treated symptomatic vines | n.p. | n.p. | 10.62±4.04 | 3.04±1.06 | 3.23±0.65 | 2.75±0.64 |

n.p. No symptomatic vine was present.

different dynamics among the leaves of treated vines of the three vine statuses. After fifth application of the mixture, healthy treated leaves showed significant increases in mean *trans*-resveratrol content at an early growth stage (BBCH 71), at 12.30 mg kg$^{-1}$ d wt, compared to 2.19 mg kg$^{-1}$ d wt of untreated healthy vines in 2012 ($t$ = 116.5561, d.f. = 1, $P$=0.0002), and 9.64 mg kg$^{-1}$ d wt compared to 2.47 mg kg$^{-1}$ d



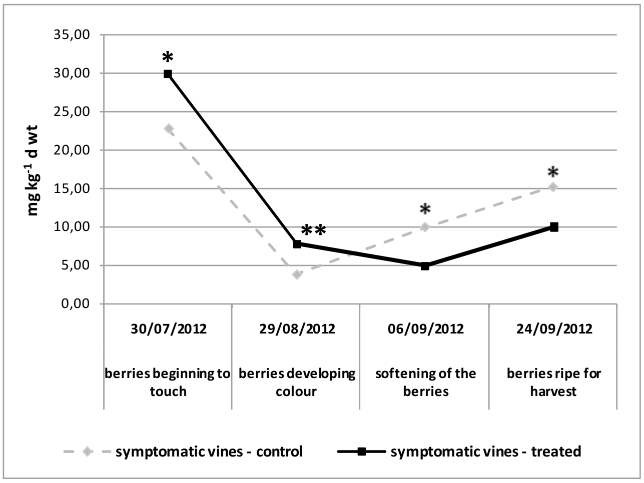
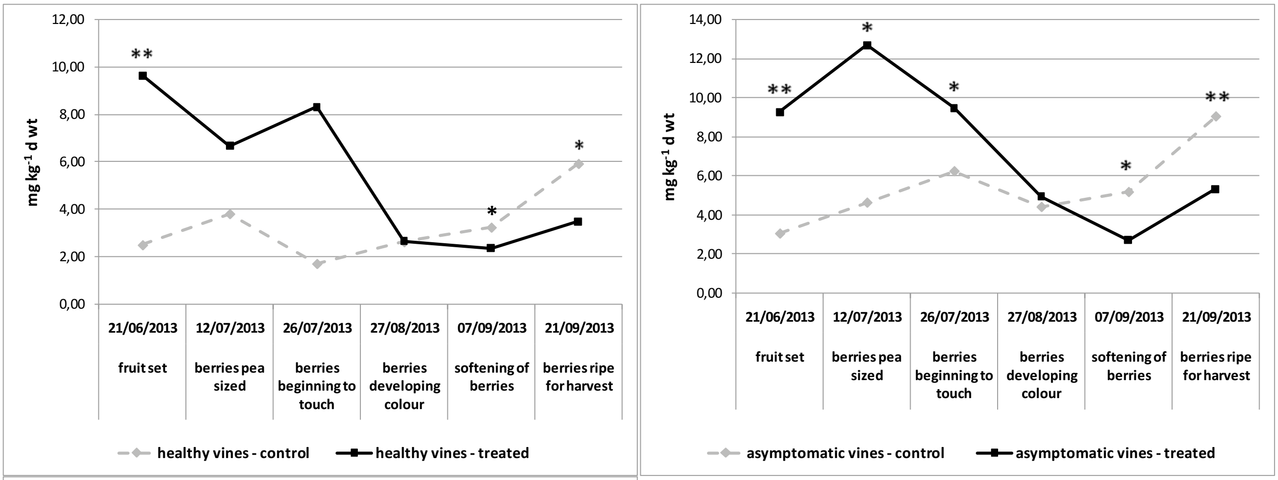




**Figure 1.** Mean amounts, in 2012, of *trans*-resveratrol in leaves from healthy grapevines, and from asymptomatic and symptomatic vines affected by GLSD, that were treated or untreated with foliar fertilizer. Each value is the mean of six replications of the leaf data individually obtained from six vines for each grapevine status. The statistical analysis separately compared leaf data from each grapevine status (healthy, asymptomatic and symptomatic; data comparison of treated and untreated pairs) in each phenological growth stage (following Lorenz *et al*., 1995). Values for each pair of means followed by symbols are statistically different (* for $P$=0.05; ** for $P$=0.01), as indicated by Student's $t$-test.

wt of untreated healthy vines in 2013 ($t$ = 46.4654, d.f. = 1, $P$=0.0023) (Figures 1 and 2). After BBCH 71 stage, the contents of *trans*-resveratrol gradually decreased, becoming similar to those of the untreated healthy leaves at BBCH 83 growth stage (Figures 1 and 2). In the leaves of treated asymptomatic vines, the greatest mean amount peak of *trans*-resveratrol in 2012 was significantly more at 14.02 mg $kg^{-1}$ d wt than the content in untreated asymptomatic leaves, at 6.95 mg $kg^{-1}$ d wt ($t$ = 10.8558, d.f. = 1, $P$=0.0301). A similar result was recorded in 2013, with 12.71 mg $kg^{-1}$ d wt in treated asymptomatic leaves and 4.62 mg $kg^{-1}$ d wt in untreated asymptomatic leaves ($t$ = 10.8492, d.f. = 1, $P$=0.0301). In both years, the peak of *trans*-resveratrol in treated asymptomatic leaves occurred later, after seven applications, at the BBCH 75 growth stage (Figures 1 and 2). In treated asymptomatic vines the contents of *trans*-resveratrol progressively decreased, becoming similar to the untreated asymptomatic vines at the BBCH 83 growth stage (Figures 1 and 2). In both years, after this stage, the contents of *trans*-resveratrol in leaves from treated healthy and asymptomatic vines decreased significantly from BBCH 85, compared to the contents of the corresponding untreated vines (Figures 1 and 2). Thus, there was an inversion in the dynamic of *trans*-resveratrol content between treated and untreated vines. In 2012, at BBCH 85 stage, the mean contents of *trans*-resveratrol were 1.22 mg $kg^{-1}$ d wt in treated healthy vine leaves and 4.36 mg $kg^{-1}$ d wt in untreated healthy leaves ($t$ = 36.5112, d.f. = 1, $P$=0.0037). These values were 3.55 mg $kg^{-1}$ d wt in treated asymptomatic vine leaves, and 7.93 mg $kg^{-1}$ d wt in untreated asymptomatic leaves ($t$ = 26.8741,



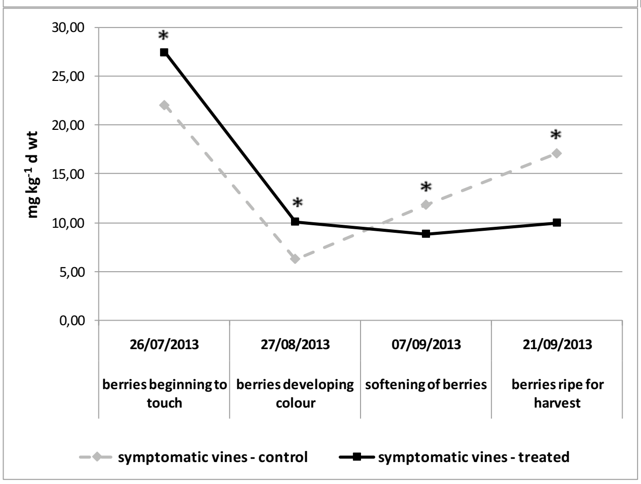




**Figure 2.** Mean amounts, in 2013, of *trans*-resveratrol in leaves from healthy grapevines, and from asymptomatic and symptomatic vines affected by GLSD, that were either treated or untreated with foliar fertilizer. Each value is the mean of six replications of the leaf data individually obtained from six vines for each grapevine status. The statistical analysis separately compared leaf data from each grapevine status (healthy, asymptomatic and symptomatic; data comparison of treated and untreated pairs) in each phenological growth stage (following Lorenz *et al*., 1995). Values for each pair of means followed by symbols are statistically different (* for $P$=0.05; ** for $P$=0.01) as indicated by Student's $t$-test.

d.f. = 1, $P$=0.0065). In 2013 at the same growth stage, and at BBCH 89 stage in both years, treated healthy and asymptomatic vine leaves equally showed significantly lower contents of *trans*-resveratrol compared to the corresponding untreated plants (Figures 1 and 2).

The leaves from treated asymptomatic vines, which showed maximum mean *trans*-resveratrol content at BBCH 75 stage, had significantly greater amounts of *trans*-ε-viniferin at 2.61 mg kg$^{-1}$ d wt, compared to 1.35 mg kg$^{-1}$ d wt in leaves from untreated asymptomatic vines in 2012 ($t$ = 54.0192, d.f. = 1, $P$=0.0017) at the BBCH 77 stage. In 2013, these values were 2.33 mg kg$^{-1}$ d wt in leaves from treated asymptomatic vines compared to 0.87 mg kg$^{-1}$ d wt for untreated asymptomatic vines ($t$ = 8.5297, d.f. = 1, $P$=0.0432), at the same growth stage. At BBCH 77 growth stage, treated asymptomatic vines also had increased contents of *trans*-δ-viniferin (1.01 mg kg$^{-1}$ d wt in 2012, and 1.31 mg kg$^{-1}$ d wt in 2013), while this phytoalexin was absent at BBCH 77 stage in the asymptomatic untreated vines in both years (Table 1). From the BBCH 83 stage, and during maturation, a reverse of the data for the other phytoalexins was observed, as noticed for *trans*-resveratrol, with increased contents of *trans*-ε-viniferin and *trans*-δ-viniferin in both untreated healthy and asymptomatic vines, compared to the corresponding treated vines (Table 1). Contrary to asymptomatic vines, untreated and treated healthy vines showed similar contents of *trans*-ε-viniferin and *trans*-δ-viniferin, in the growth stages preceding the BBCH 83 stage (Table 1). In both healthy and asymptomatic vines, *trans*-pterostilbene was never detected or was detected only at trace contents until the BBCH 83 stage (Table 1). During the grape maturation stages, greater contents of *trans*-pterostilbene were observed in untreated vines that were both healthy and asymptomatic (Table 1).

Mean *trans*-resveratrol contents in tiger stripe (symptomatic) leaves were greater than those recorded in the leaves of the other two groups of vines. In particular, at the BBCH 77 stage, the greatest seasonal contents in untreated tiger stripe leaves were measured, with values of 22.70 mg $kg^{-1}$ d wt in 2012 and 22.10 mg $kg^{-1}$ d wt in 2013 (Figure 1 and 2). In these symptomatic leaves at the BBCH 83 stage the contents of *trans*-resveratrol decreased, but increased in the following stages, particularly at the BBCH 89 stage. As observed for healthy and asymptomatic vines, the treatments caused significantly greater amounts of *trans*-resveratrol, recorded in symptomatic leaves, particularly at the BBCH 77 stage, with 29.96 mg $kg^{-1}$ d wt compared to 22.70 mg $kg^{-1}$ d wt for untreated symptomatic leaves in 2012 ($t$ = 20.0120, d.f. = 1, $P$=0.011). In 2013 the equivalent values were 27.51 mg $kg^{-1}$ d wt for treated vines and 22.10 mg $kg^{-1}$ d wt for untreated symptomatic leaves ($t$ = 10.0987, d.f. = 1, $P$=0.0336). This was unlike the other two groups of vines, even at the BBCH 83 stage (Figures 1 and 2). At both growth stages, increases of *trans*-ε-viniferin and *trans*-δ-viniferin were also observed in the treated symptomatic leaves, compared to untreated ones (Table 1). From the BBCH 83 stage and at BBCH 89 stage, treated symptomatic vines produced significantly less *trans*-resveratrol, but similar amounts the other analyzed phytoalexins compared to leaves from the untreated symptomatic vines (Figures 1 and 2; Table 1).

### Total sugars and total acids in grape berries

Grape berries from treated healthy vines showed initial decreases in sugar accumulation and increases of total acidity at the growth stages before harvest, compared to those from untreated healthy vines (Figures 3 and 4). However, these differences were never found at harvest, and these two parameters were not significantly different for both groups of vines, and in the 2 years of study. At harvest, the mean contents of total sugars were, in 2012, 193.82 g $L^{-1}$ in berries from treated healthy vines and 198.30 g $L^{-1}$ from untreated healthy vines, and, in 2013, 200.82 g $L^{-1}$ for treated healthy vines and 200.30 g $L^{-1}$ for un-

**Table 2.** Mean amounts of total sugars and total acids in berries from vines of different disease status (healthy, asymptomatic, symptomatic (asymptomatic and symptomatic shoots) at harvest in 2012 and 2013 growing seasons. In each status, the contents of total sugars and total acids of treated and untreated vines were compared by Student's *t*-test.

| Vintage and vine status | Total sugars (g $L^{-1}$) | | Student's *t*-test | | | Total acidity (g $L^{-1}$) | | Student's *t*-test | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | treated | untreated | *t*-value | df | P | treated | untreated | *t*-value | df | P |
| **2012** | | | | | | | | | | |
| Healthy vines | 193.82 | 198.30 | 0.9874 | 1 | 0.3765 | 5.63 | 5.30 | 4.5455 | 1 | 0.998 |
| Asymptomatic vines | 198.33 | 188.30 | 71.0039 | 1 | 0.001 | 5.80 | 7.13 | 84.2105 | 1 | 0.006 |
| Symptomatic vines (asymptomatic shoots) | 172.50 | 175.00 | 0.6369 | 1 | 0.4694 | 8.87 | 8.57 | 4.7647 | 1 | 0.0943 |
| Symptomatic vines (symptomatic shoots) | 155.83 | 159.17 | 4.0032 | 1 | 0.1159 | 9.83 | 9.23 | 3.1154 | 1 | 0.1522 |
| **2013** | | | | | | | | | | |
| Healthy vines | 200.82 | 200.30 | 2.7982 | 1 | 0.1695 | 4.67 | 4.87 | 2.5857 | 1 | 0.183 |
| Asymptomatic vines | 199.80 | 187.80 | 29.7826 | 1 | 0.0054 | 5.33 | 6.87 | 471.2695 | 1 | <0.0001 |
| Symptomatic vines (asymptomatic shoots) | 169.69 | 171.20 | 1.2596 | 1 | 0.3244 | 8.86 | 8.83 | 0.2477 | 1 | 0.6447 |
| Symptomatic vines (symptomatic shoots) | 139.17 | 140.28 | 0.2606 | 1 | 0.6365 | 9.10 | 8.23 | 3.9334 | 1 | 0.1182 |

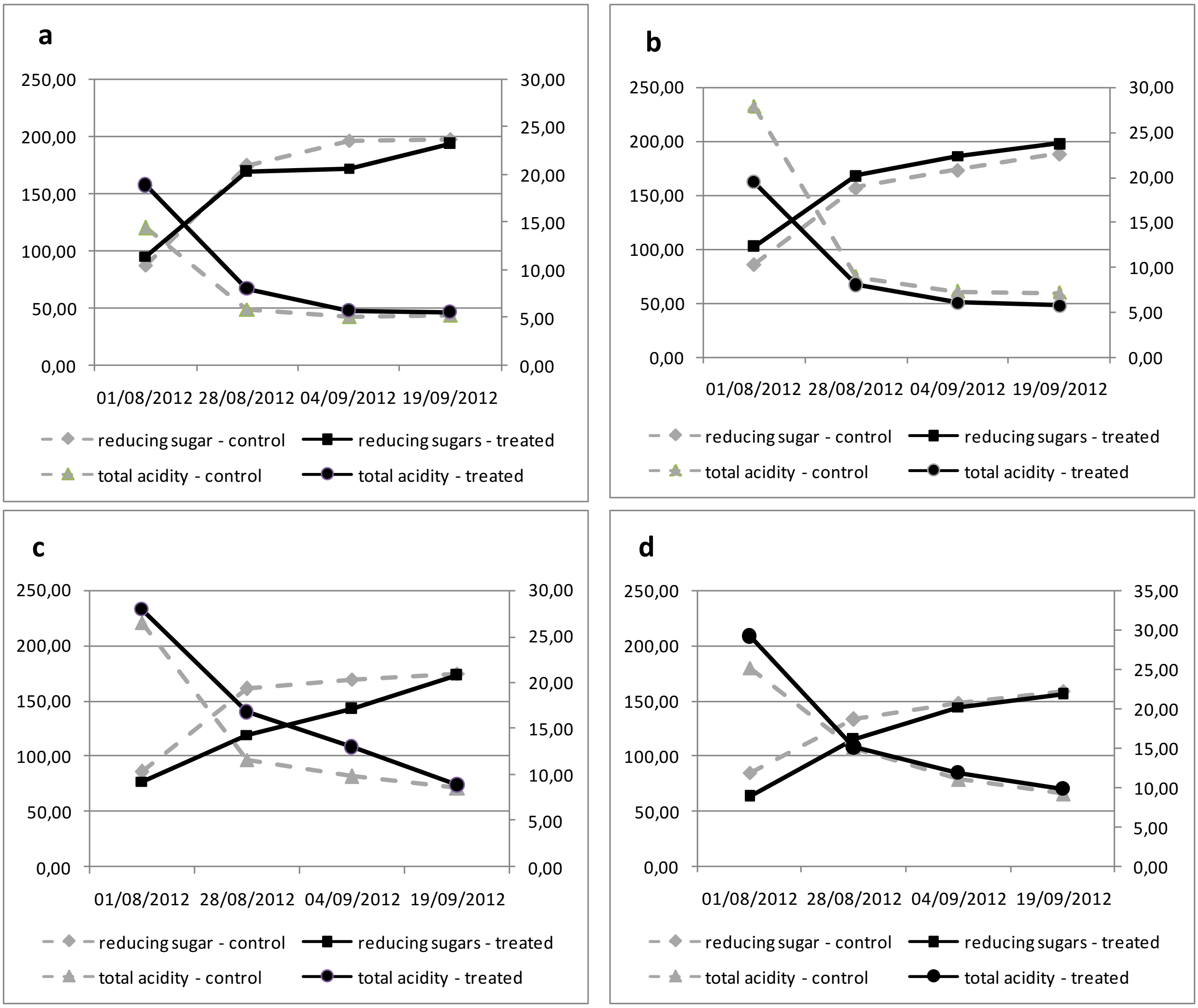



**Figure 3.** Maturation curves, in 2012, of total berry sugars and total berry acids from healthy (a), asymptomatic (b) and symptomatic [asymptomatic (c) and symptomatic (d) shoots] grapevines affected by GLSD, at four growth stages: 'Beginning of ripening: berries start to develop variety-specific colour' (81); 'Berries developing colour' (83); 'Softening of berries' (85); and 'Berries ripe for harvest' (89).

treated healthy vines (Table 2; Figures 3 and 4). In 2012, at harvest, the total acidity was 5.63 g $L^{-1}$ and 5.30 g $L^{-1}$ and in 2013 was 4.67 g $L^{-1}$ and 4.87 g $L^{-1}$ in treated and untreated healthy vines, respectively (Table 2; Figure 3 and 4).

Berries from treated asymptomatic vines had greater mean amounts of total sugars from BBCH 83 stage until harvest, compared to the untreated asymptomatic vines (Figures 3 and 4). At harvest, significantly greater amounts of total sugars were recorded for treated asymptomatic vines, 198.33 g $L^{-1}$, compared to 188.30 g $L^{-1}$ for untreated asymptomatic vines, in 2012, and 199.80 g $L^{-1}$ for treated asymptomatic vines and 187.80 g $L^{-1}$ for untreated asymptomatic vines, in 2013 (Table 2; Figures 3 and 4). Total acidity at harvest was significantly less in berries from treated vines; in 2012 at 5.80 g $L^{-1}$ and 7.13 g $L^{-1}$ for, respectively, treated and untreated asymptomatic vines, and 5.33 g $L^{-1}$ and 6.87 g $L^{-1}$ in 2013 (Table 2; Figure 3 and 4). The total sugar con-

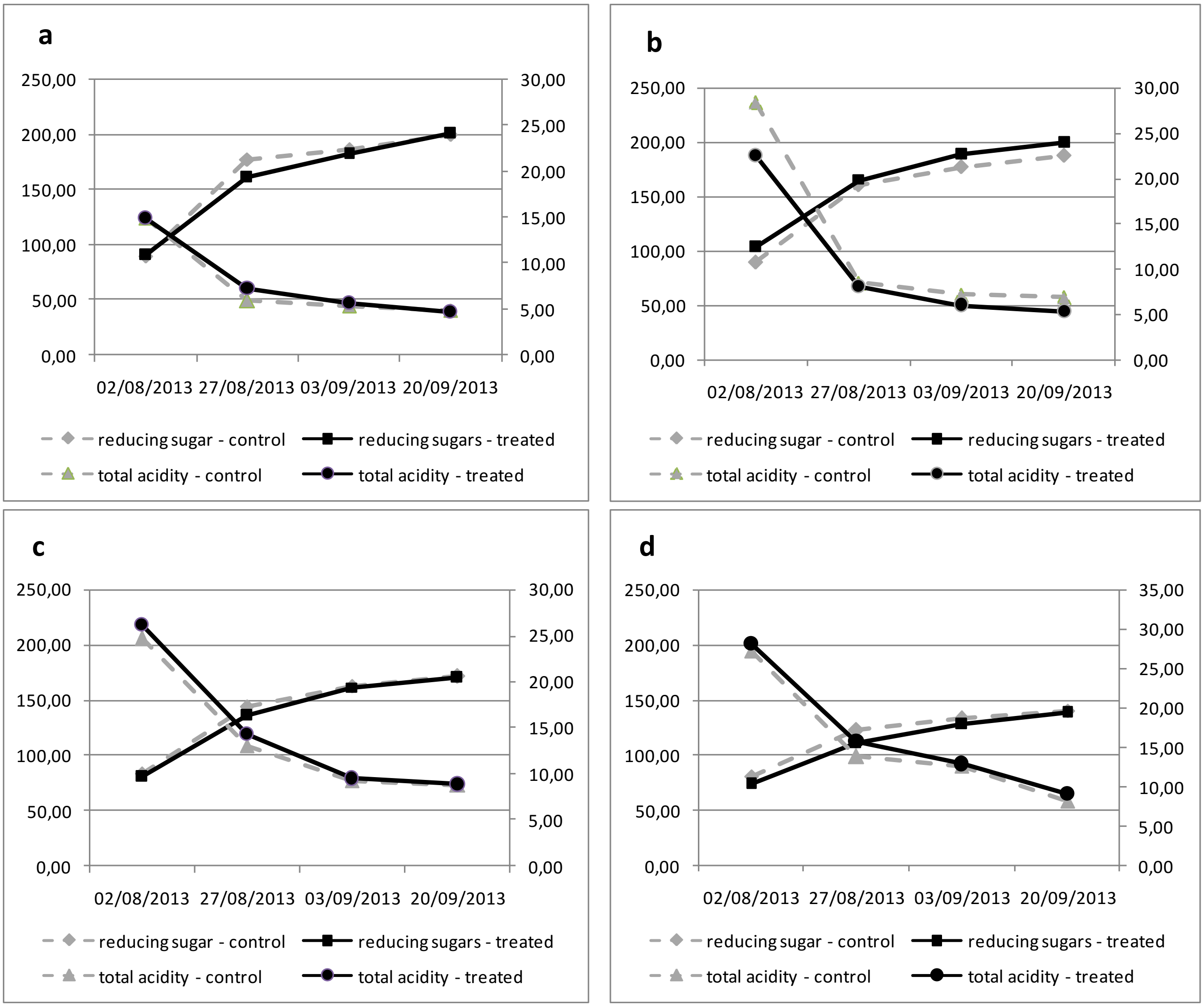



**Figure 4.** Maturation curves, in 2013, of total berry sugars and total berry acids from healthy (a), asymptomatic (b) and symptomatic [asymptomatic (c) and symptomatic (d) shoots] grapevines affected by GLSD, at four growth stages: 'Beginning of ripening: berries to develop variety-specific colour' (81); 'Berries developing colour' (83); 'Softening of berries' (85); and 'Berries ripe for harvest' (89).

tents recorded in berries from treated asymptomatic vines were similar to those found in both treated and untreated healthy plants.

Strong reductions in the mean amounts of total berry sugars were measured for the untreated symptomatic vines, compared to treated and untreated healthy and asymptomatic vines. This was mainly in berries sampled from symptomatic shoots, at means of 159.2 g $L^{-1}$, in 2012, and 140.3 g $L^{-1}$, in 2013. Mean total acid contents were 9.23 g $L^{-1}$ in 2012 and 8.23 g $L^{-1}$, in 2013 (Figures 3 and 4). The decreases of total sugar content were also recorded in berries from asymptomatic shoots of untreated symptomatic vines, at 175.0 g $L^{-1}$ in 2012, and 171.2 g $L^{-1}$ in 2013. Total acids were 8.57 g $L^{-1}$ in 2012 and 8.83 g $L^{-1}$ in 2013. These amounts were not significantly different from those found in the treated symptomatic vines (Table 2; Figures 3 and 4).

## Discussion

The results obtained in this study show that induction of phytoalexin synthesis assessed at 'fruit set', 'pea-sized berries' and 'berries beginning to touch' growth stages, caused by applications of nutrient mixture, can be linked to reductions of GLSD symptoms on treated vines. All plants treated with the nutrient mixture initially showed higher contents of phytoalexins in their leaves than the corresponding untreated plants. From the "berries developing colour" growth stage, phytoalexin contents then decreased, which, in most cases, became significantly less than those for the untreated plants. This impact probably contributed to grape ripening. At harvest, berries from treated healthy vines had total sugar contents similar to those from untreated healthy vines, and berries from treated asymptomatic vines had total sugar contents significantly greater than those from untreated asymptomatic vines.

In all the three statuses of vines, in the absence of the pathogens in the leaf tissue, *trans*-resveratrol levels were greater than the other phytoalexins, in accordance with the results of Pezet *et al*. (2004). Viniferins and *trans*-pterostilbene, because of their high levels of antimicrobial activity, are mainly produced in tissues directly infected by pathogens (Langcake and McCarthy, 1979; Jeandet *et al*., 2002; Pezet *et al*., 2004).

It is possible that in treated vines (healthy and asymptomatic), the increase of phytoalexin synthesis at the early growth stages could be compensated by synthesis of smaller phytoalexin quantities observed from veraison to the harvest. This could lead to the reverse of phytoalexin synthesis between treated and untreated vines, probably favouring grape ripening. The initial decrease of sugar accumulation and increase of total acids in berries from treated healthy vines compared to the untreated healthy vines, were never found at harvest, so that the levels of the two parameters were similar for both groups of vines and in both years of this study. Most likely, from veraison the magnesium contained in the nutrient mixture may have been an essential constituent of chlorophyll, the deficiency of which in grapevine leaves generally leads to chlorosis symptoms similar to GLSD, and to decreased sugars in berries (Schaul, 2002; Marschner, 2012).

Although GLSD asymptomatic vines are visually similar in growth to healthy vines, they differ in vegetative characteristics (Di Gennaro *et al*., 2016). For example, Calzarano *et al*. (2001, 2004, 2009) showed that asymptomatic vines produced berries with lower total sugar contents than healthy vines. For that reason, improvement of physiological processes in asymptomatic treated vines was greater than application of the same treatments was for healthy vines. Treatments causing an increases of leaf area can positively affect grape ripening in asymptomatic vines after the 'berries developing colour' stage (Calzarano *et al*., 2014). The content of total sugars in berries from treated asymptomatic vines, similar to the content for healthy vines assessed in this study, corresponded to the greatest content commonly recorded for the studied cultivar, also reported by Calzarano *et al*. (2001, 2004, 2009).

In treated symptomatic vines, as well as in healthy and asymptomatic treated vines, phytoalexin contents in leaves decreased from veraison to harvest. Contrary to healthy and asymptomatic vines, in symptomatic vines imbalances caused by the appearance of symptoms resulted in statistically significant reductions in the contents of total sugars, particularly in berries from symptomatic shoots, with contents similar between treated and untreated symptomatic vines.

The results obtained in the present study suggest that the significant reductions in the manifestation of symptoms caused by applications of the mixture nutrients can be linked to increases in *trans*-resveratrol content in leaves at 'fruit set' and 'pea-sized berries' stages, and in *trans*-ε-viniferin and *trans*-δ-viniferin at the bunch closure stage. This increase was verified in treated infected plants that remained asymptomatic throughout the vine growth seasons. It can be assumed that the increases of phytoalexins in leaves can be one of the impacts induced by the nutrient mixture, in a complex mechanism of plant defense responses. In particular, the minerals in the mixture, especially calcium, may play a role in reducing the plant oxidative response (Lima *et al*., 2012). Calcium penetrates the foliar tissues in association with the seaweed, which acts as a carrier, and can cause accumulations of calmodulin that regulates salicylic acid and triggers plant defense reactions and reductions of hypersensitive responses (Lecourieux *et al*., 2006; Du *et al*., 2009). The increase of *trans*-resveratrol found in the leaves of treated vines may also be due to increased extracellular $Ca^{2+}$ contents caused by the nutrient mixture, and increases in phytoalexins have been similarly reported from other studies

(Kurosaki *et al*., 1987; Stäb and Ebel, 1987; Ebel, 1995; Tavernier *et al*., 1995). Moreover, calcium treatment can also induce the accumulation of calcium oxalate in crystal druses in the leaves of treated diseased vines that remained asymptomatic (Calzarano *et al*., 2014). Magnesium may have a role in detoxifying the toxic metabolites produced by pathogens in the wood and translocated to the foliage through the vascular tissues, as occurs for eutipine in infections by *Eutypa lata* (Colrat, 1999).

In conclusion, this study has confirmed that treatments with the foliar nutrient and seaweed mixture substantially reduced the foliar symptoms on vines affected by GLSD. The results also indicate the possible role of the induction of phytoalexin synthesis at early growth stages, before symptom appearance in the season, as the mechanism of action of the nutrient mixture. In addition to the increase in yields from healthy and GLSD asymptomatic plants verified in a previous study (Calzarano *et al*., 2014), and an improvement of the quality of the grape berries from asymptomatic diseased vines, these results indicate that the mixture of calcium, magnesium and seaweed extracts could be an effective treatment in the management of GLSD in commercial vineyards.

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