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RESEARCH PAPERS

Grapevine leaf stripe disease symptoms (esca complex) are reduced by a nutrients and seaweed mixture

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Summary. Grapevine leaf stripe disease (GLSD) seriously reduces the quality and quantity of grape production, and results in a shorter lifespan of vineyards. Recent research has shown that foliar applications of nutrients influence the development of GLSD foliar symptoms. Based on this knowledge the effect of foliar applications of a mixture of calcium chloride, magnesium nitrate and *Fucales* seaweed extract on the development of leaf symptoms was evaluated over a 3-year period from 2010 to 2012. Nine foliar applications of the full mixture and its individual mineral components, also in different combinations, were tested in three different vineyards, one of cv. Trebbiano d'Abruzzo and two of cv. Montepulciano d'Abruzzo in the Teramo province (Abruzzo, Italy). Treatments were applied every 10 days from the beginning of vegetative growth to pre-bunch closure. The final results were similar in all the three vineyards and in the three years leading to a significant reduction of symptom development in the vines treated with the full mixture, while lower effects were obtained by applying partial combinations or single components. Both quantity and quality of grapes from the treated vines increased, while no phytotoxic or other unwanted effects on grape growth were detected. Vines treated with the full mixture showed an increase in *trans*-resveratrol and flavonoids content, and a higher accumulation of calcium oxalate in crystal druses in the leaf mesophyll. These data can be a useful base to set up a control strategy against GLSD and give some input for better understanding the mechanisms involved in foliar symptom expression in GLSD.

Key words: foliar fertilisation, *trans*-resveratrol, vegetative growth, defence response, Ca^{2+}.

Introduction[1]

The diseases included in the esca complex (Surico *et al*., 2008) are the most common and widespread of the fungal wood diseases of grapevine in Europe. Etiological and epidemiological studies have increased the knowledge on the fungal agents involved and their interaction with the plant and environment. However, despite many efforts efficient control methods are still lacking (Di Marco *et al*., 2011a; 2011b).

Wood decay agents (in Europe mainly *Fomitiporia mediterranea*), wood canker agents (mostly species of *Botryosphaeriaceae*, *Diatrypaceae* and *Diaporthaceae*) and vascular pathogens, such as species of *Phaeoacremonium*, mainly *P. aleophilum* (Pal), and *Phaeomoniella chlamydospora* (Pch) can reduce the efficiency of wa-

[1] The significant reduction of foliar symptoms and the values obtained in associated physiological/histological parameters lead to deposit a patent application (Italian patent application No. RM2014A000097 of March 3, 2014) on the use of mixtures of calcium chloride and/or magnesium nitrate with Fucales seaweed extract to reduce the foliar stripe symptoms in grapevines affected by esca complex diseases.

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ter and nutrients movement as they cause necrosis and disruption of the wood. Therefore considerable attention has been devoted to preventing wood infections by different approaches in the various life stages of the vine (Rolshausen *et al*., 2010; Gramaje *et al*., 2011; Kotze *et al*., 2011). Furthermore, many of these pathogens produce phytotoxic metabolites (Andolfi *et al*., 2011) that interact with normal growth activity causing specific disruption of plant metabolism. This results in a reduction in the quality and quantity of grapes at harvest and contribute to a shorter lifespan of the vines. This is the case with grapevine leaf stripe disease (GLSD) (Surico, 2009), one of the diseases within the esca complex (Surico *et al*., 2008). The wood symptoms most commonly associated with this disease are brown streaking and brown red/brown necrosis caused by Pch and *Phaeoacremonium* species (see "young esca", Marchi *et al*., 2001; Surico, 2009). Foliar symptoms of GLSD are characterized by typical interveinal chlorosis and/or reddening of the leaves that soon become necrotic (Figure 1). This results in a reduction of quality

Figure 1. Grapevine leaf stripe disease foliar symptoms in the Trebbiano d'Abruzzo vineyard (a) Foliar symptoms evolve from yellow spots in the interveinal area to necrotic areas surrounded, in this cultivar, by a yellow border (b).

(Calzarano *et al*., 2004a) and quantity of grape yield (Bertsch *et al*., 2013). This leaf symptom can be associated with a wilting of clusters or canes, or black measles on the berries.

The damage to yield that the disease causes is not directly related to the presence of fungal pathogens in the wood (infected vines can remain asymptomatic), but to the response that the pathogens activate in the plant foliage by metabolite production (Andolfi *et al*., 2011; Bertsch *et al*., 2013; Calzarano *et al*., 2013). This activity is strongly influenced by external or environmental factors that have still not been clearly defined (Surico *et al*., 2006), and this, most probably, is the main reason why full and consistent reproduction of the development of the leaf stripe symptoms by artificial inoculation has not yet been achieved. Excessive watering induced a significant increase in foliar symptoms in 20 year-old potted vines (Surico *et al*., 2010), confirming the role of rainy seasons and water in the soil in symptom development (Marchi *et al*., 2006).

The fluctuation of symptoms from one year to another on the same vines, which can appear symptomatic or asymptomatic in subsequent years, remains a particular characteristic of GLSD. It is noteworthy that the yield quality parameters in asymptomatic infected vines (symptomatic in previous years but not in that specific year) and in apparently healthy vines (never symptomatic) were actually comparable (Calzarano *et al*., 2001; 2004a). Furthermore, grapevine lifespan even when affected by GLSD can extend over many years in some vineyards (Calzarano *et al*., 2007; 2010). All this underlines the importance of a different approach to control and reduce the number of vines showing GLSD foliar symptoms in any given year, as well as protecting the wounds from fungal infections (Eskalen *et al*., 2007; Rolshausen *et al*., 2010; Kotze *et al*., 2011; Di Marco *et al*., 1999; 2004; 2011a; 2011b).

Nutrients are reported to affect the disease either by directly influencing the fungal infection processes (Osti and Di Marco, 2010; Oliveira and Santos, 2011; Whiting *et al*., 2001) or by influencing the physiology of the plant (Calzarano *et al*., 2009; Di Marco *et al*., 2001). Studies on the nutritional status of "esca proper" (*sensu* Surico, 2006) affected vines and the influence of leaf fertilization and biostimulants on foliar symptoms suggested that elements such as calcium reach a higher concentration in asymptomatic infected vines, thus suggesting they have a role in symptom development (Calzarano *et al*., 2009). On the other hand, leaf symptoms can increase following applications of some biostimulants and nutrients (including the main macro and micronutrients) (Calzarano *et al*., 2007; Di Marco and Osti, 2009).

On the basis of these observations the role of a specific foliar nutrition protocol, to be applied from the time of the initial stages of symptom formation, was tested in the field, selecting the nutrients that were shown to reach higher concentrations in the leaves of infected but asymptomatic vines, such as calcium (Calzarano *et al*., 2009). Side effects of the applied treatments on vegetative growth and on quality and quantity parameters of yield were also recorded. Moreover, the treated tissues were evaluated histologically for changes induced by the treatment in the leaf blade tissue and in aspects of the defence related response, in particular evaluating the *trans*-resveratrol (the main grapevine phytoalexin) content in the treated foliage.

Materials and methods

Vineyards

Vineyard cv. Trebbiano d'Abruzzo. This 37-year old vineyard in Piane Tronto, Controguerra (Teramo), Geneva Double Courtain (GDC) trained, had been monitored for GLSD foliar symptoms since 1994. After 19 years of survey, an adequate number of vines could be assessed in the following categories: i) healthy vines that had never shown symptoms; ii) asymptomatic vines that had previously shown GLSD symptoms at least once but were asymptomatic in the season of the experiment; and iii) diseased, symptomatic vines.

Vineyard cv. Montepulciano d'Abruzzo-1 and 2. Two vineyards were chosen in a different area of the Teramo province; S. Maria Assunta, Mosciano S.A. (Teramo). Both vineyards, monitored for GLSD foliar symptoms since 2010, were 34-years old and grown with the Tendone trellising system.

Treatments

In the 2010–2012 period, in each of the three vineyards, nine foliar spray applications per treatment were applied, each containing i) a mixture of nutrients commonly used as leaf fertilizers and amended

with brown seaweed extract (*Fucales*, Phaeophyceae), ii) a mixture of nutrients without seaweed extract, iii) individual nutrients without seaweed extract (Table 1). Treatments were applied at 10 day intervals starting from the "three leaves unfolded" phase (Lorenz *et al*., 1995), up to pre-bunch closure (beginning of May up to the end of July). The full mixture contained 466 g of $CaCl_2$, 403 g of $Mg(NO_3)_2$, 75 mL of *Fucales* seaweed extract and 466 mL distilled H_2O for 1 L of solution, and applied at 4 L ha^{-1}. All different combinations or single components were applied at the same dosage as the full mixture. The volume of water used for the field treatment varied from 8 hL ha^{-1} to 4 hL ha^{-1}, depending on the spray machine that was used. Thus, the product concentration varied from 500 mL ha^{-1}, in the Trebbiano d'Abruzzo vineyard (by Air blast sprayer), to 1000 mL ha^{-1} in the two Montepulciano d'Abruzzo vineyards (by Pneumatic air sprayer).

Treatments were carried out during the three years following the scheme in Table 1. Initially, in 2010, in Trebbiano d'Abruzzo vineyard, only 1 treated and 1 untreated plots, with four replicates of 90 vines each, were set up. In 2011 and in 2012 in Trebbiano d'Abruzzo and Montepulciano d'Abruzzo-1 vineyards, 4 treatments and one untreated control were set up. Each treatment included two replicates of 70 vines (Trebbiano d'Abruzzo) or 50 vines (Montepulciano d'Abruzzo 1). In the Montepulciano d'Abruzzo-2 vineyard only the full mixture was compared with the untreated control, with three replicates of 50 vines each (Table 1). Replicates in both vineyards were distributed randomly, taking care that replicates of the same treatment were not close to each other.

Leaf symptoms evaluation

Field surveys were carried out each year in September, i.e. just before harvest, because at that time the number of plants showing foliar symptom is at its highest. In the Trebbiano d'Abruzzo vineyard, where the history of each vines had been recorded for 19 years, the incidence of esca was calculated, every year, by dividing the number of vines with visible symptoms by the total number of diseased vines (symptomatic for at least one of the years of

Table 1. Treatment plan applied in 3 vineyards showing GLSD symptoms in the years prior to treatment application.

Year of treatment	Vineyard Trebbiano d'Abruzzo		Vineyard Montepulciano d'Abruzzo 1		Vineyard Montepulciano d'Abruzzo 2	
	Treatment	No. of vines	Treatment	No. of vines	Treatment	No. of vines
2010	$CaCl_2$ + $Mg(NO_3)_2$ + seaweed extract	360	-	-	-	-
	Untreated	360	-	-	-	-
2011	$CaCl_2$ + $Mg(NO_3)_2$ + seaweed extract	140	$CaCl_2$ + $Mg(NO_3)_2$ + seaweed extract	100	$CaCl_2$ + $Mg(NO_3)_2$ + seaweed extract	150
	$CaCl_2$	140	$CaCl_2$	100	Untreated	150
	$Mg(NO_3)_2$	140	$Mg(NO_3)_2$	100	-	-
	$CaCl_2$ + $Mg(NO_3)_2$	140	$CaCl_2$ + $Mg(NO_3)_2$	100	-	-
	Untreated	140	Untreated	100	-	-
2012	$CaCl_2$ + $Mg(NO3)_2$ + seaweed extract	140	$CaCl_2$ + $Mg(NO3)_2$ + seaweed extract	100	$CaCl_2$ + $Mg(NO3)_2$ + seaweed extract	150
	$CaCl_2$	140	$CaCl_2$	100	Untreated	150
	$Mg(NO3)_2$	140	$Mg(NO3)_2$	100	-	-
	$CaCl_2$ + $Mg(NO3)_2$	140	$CaCl_2$ + $Mg(NO3)_2$	100	-	-
	Untreated	140	Untreated	100	-	-

survey) (Calzarano *et al*., 2004a; 2007; 2009). In the two Montepulciano d'Abruzzo vineyards, where surveys were started in 2010, disease incidence was calculated, every year, by dividing the number of symptomatic vines by the number of standing vines.

Leaf symptom severity (as portion of symptomatic crown) was recorded on a disease rating scale of 0 to 5, where 0=no leaf symptom; 1=1–10%; 2=11–30%; 3=31–50%; 4=51–70%; 5=71–100%.

Percent disease severity was calculated from the McKinney index:

$$\sum N \times 100 \; / \; (Y \times Z)$$

where $\sum N$ = sum of severity rating in each plant; Y = number of vines surveyed (in the Trebbiano d'Abruzzo vineyard this was the number of diseased vines, both symptomatic and asymptomatic; in the Montepulciano d'Abruzzo-1 and -2 vineyards it was the number of standing vines); Z = 5, which is the maximum rating in the disease assessment scale (McKinney, 1923). The number of dead vines during the three years trials was not analysed as, being always lower than 1%, could not be considered informative in differences among treatments.

Leaf area measurement

Side effects of the treatments on vegetative growth were evaluated in 2012 in the Trebbiano d'Abruzzo vineyard. Leaf area of healthy and asymptomatic vines treated with the full mixture was compared with leaf area of the healthy and asymptomatic vines in the untreated plots. In each treatment 24 canes were selected, 12 exposed to east and 12 to west. All canes were collected at the end of the vegetative growth phase (before veraison), all leaves were excised and photographed keeping separate the primary and secondary shoot leaves. Each leaf area was recorded and processed by "Image-pro plus" version 7.0, Media Cybernetic Inc., Silver Spring, MD, USA. Single leaf measurements were taken in each of the four groups (healthy and asymptomatic, treated and non treated) to be evaluated statistically.

Quantitative and qualitative yield parameters and *trans*-resveratrol content

Grape yield was recorded in 2012 in the Trebbiano d'Abruzzo vineyard at harvest in healthy and asymptomatic vines treated with the full mixture and in the untreated ones. Six vines were harvested in each of the two treatments. The number of clusters and their weight were recorded. In the same vineyard, in the same year and for each vine group (healthy and asymptomatic vines, treated and untreated), reducing sugars and total acidity were measured on 3 berry-samples, 500 g each, taking the berries from the cluster wings, central and apical position of 12 vines. The analyses were carried out following the official protocol of the Regulation No. 2676/90/EEC annex 13, 24 and 5 (AA.VV., 1990). The *trans*-resveratrol content of the leaves of the different vine groups treated with the full mixture and in the untreated controls were measured at the end of the vegetative growth phase in 2010 and 2011 by the method of Calzarano *et al*. (2008).

Histological analyses

Micromorphology of leaves collected from untreated healthy and asymptomatic vines and from asymptomatic vines treated with the complete mixture, was determined in 2010 and 2011 in the Trebbiano d'Abruzzo vineyard. For each year and treatment two mature leaves per plant, for the observation of crystal druses, and one mature leaf per plant for the determination of flavonoids, were collected from three different vines, for a total of six leaves in the case of crystal druses and three leaves in the case of flavonoids. Each leaf was taken from the midpoint along a different primary vine shoot. Leaves were sampled before veraison at the end of treatment regime.

For detection of druse crystals by light microscopy, leaves were fixed in formaldehyde/acetic acid/alcohol [10% (v/v) formaldehyde, 5% (v/v) acetic acid and 70% (v/v) ethanol]. Samples were cut from the middle area of the leaf blade near one of the main veins, dehydrated in ethanol and embedded in Technovit®7100 resin. Sections, 5 μm thick, were cut with a manual microtome and examined without any additional staining. From each of the 6 leaves of each group nine sections were obtained for a total of 54 sections per vine group (untreated healthy and asymptomatic vines, and asymptomatic vines treated with the complete mixture). Slides were examined with a Nikon Eclipse 400 microscope equipped with crossed polarized light and an ocular micrometer. The number of druses was counted along 1 mm

of leaf. Images were recorded with a Nikon digital camera DN 100.

For observations of phenolic compounds, fresh leaves were selected and immediately taken to the laboratory. Sections, approx. 40–50 µm thick, were cut with a Vibratome® 1000 Plus and stained with Naturstoffreagenz-A (diphenylboric acid 2-aminoethyl ester), Sigma-Aldrich. From each of the 3 leaves of each group of vines 3 sections were obtained for a total of nine sections per group. The stained sections were examined and photographed under fluorescence light with a Zeiss Axioplan Microscope (excitation filter: 365-395 nm) equipped with a Nikon digital sight DS-MS.

Statistical analysis

Significance of the treatment effects on recurrence of symptoms in vines that had proved to be diseased were determined by statistical analyses. In the Trebbiano d'Abruzzo vineyard all the vines that had been healthy for 19 years were excluded from the counting. In the other vineyards all calculations were done on the standing vines.

For the incidence of vine plants with esca symptoms, frequency analysis on two-way contingency table was performed using treatment as a group variable and the presence of symptoms in a binomial scale (yes/not). Association between variables were analysed using χ^2 (Chi-square) test and Fisher's exact test. For the severity of foliar symptoms, analysis of variance of categorical data (CATMOD procedure) was performed using treatment as the independent variable and the occurrence of symptoms scored on a categorical scale using the same scale that was used to score the symptoms as dependent variable. Differences were again compared by χ^2 (Chi-square) test along with the associated P value. Data were processed using SAS 9.3 (SAS Institute Inc., Cary, NC, USA).

Two samples Student's t-test (treated-untreated) were appllied to compare: reducing sugars and total acidity values in the berries of healthy and asymptomatic treated and untreated vines at harvest maturity; *trans*-resveratrol levels of leaves of healthy, asymptomatic and symptomatic treated and untreated vines, leaf area and the number and weight of grape clusters from healthy and asymptomatic treated and untreated vines.

Chi-square test and Fisher's exact test and Student *t*-test were performed for $P \leq 0.05$, while a probability level between 0.05 and 0.1 indicated the minimum significant level. Statistical analyses were carried out using SAS version 9.3 (SAS Institute Inc., Cary, North Carolina, USA).

Data on druse number in the different vine groups were analyzed by one way analysis of variance followed by LSD tests ($P \leq 0.05$) (Statistica, version 7.1, Statsoft, Tulsa, USA) for comparisons of pairs between leaves of untreated healthy vines and leaves of treated asymptomatic diseased vines and between leaves of untreated asymptomatic diseased vines and leaves of treated asymptomatic diseased vines.

Results

Effects on GLSD foliar symptoms

In 2010, both incidence and severity of GLSD symptoms surveyed just before harvest time (September) were strongly reduced by the complete mixture of calcium chloride, magnesium nitrate and seaweed extract (Figure 2a and Table 2). In 2011 and 2012, the efficacy of the full mixture was confirmed in all vineyards (Table 2 and 3a-h; Figures 2b, 2c, 3a, 3b, 3c, 3d). A reduction in incidence and severity was obtained also by the application of the mixture calcium chloride + magnesium nitrate without the seaweed extract (Figure 2b, 2c; Figure 3a, 3b), but the reduction in disease incidence and severity was lower while the single separate components application, calcium chloride and magnesium nitrate, showed even lower effects (Table 3). Only the full mixture gave significant *P*-values in all vineyards and years, except showing only a clear tendency in 2012 in the Montepulciano d'Abruzzo 1 vineyard symptom severity (Table 3h). This appeared to be linked to the lower disease incidence recorded, also in the control untreated plots, in that year (Figure 3b).

Effects on vegetative growth (leaf area)

No phytotoxicity or any growth inhibition was recorded in the treated vines. The leaf area measurements carried out in the Trebbiano d'Abruzzo vineyard in 2012 showed no difference between treated and untreated vines both on primary and secondary shoot leaves. A significant increase in primary leaf area was observed only in the asymptomatic diseased vines treated with the full mixture (Table 4).

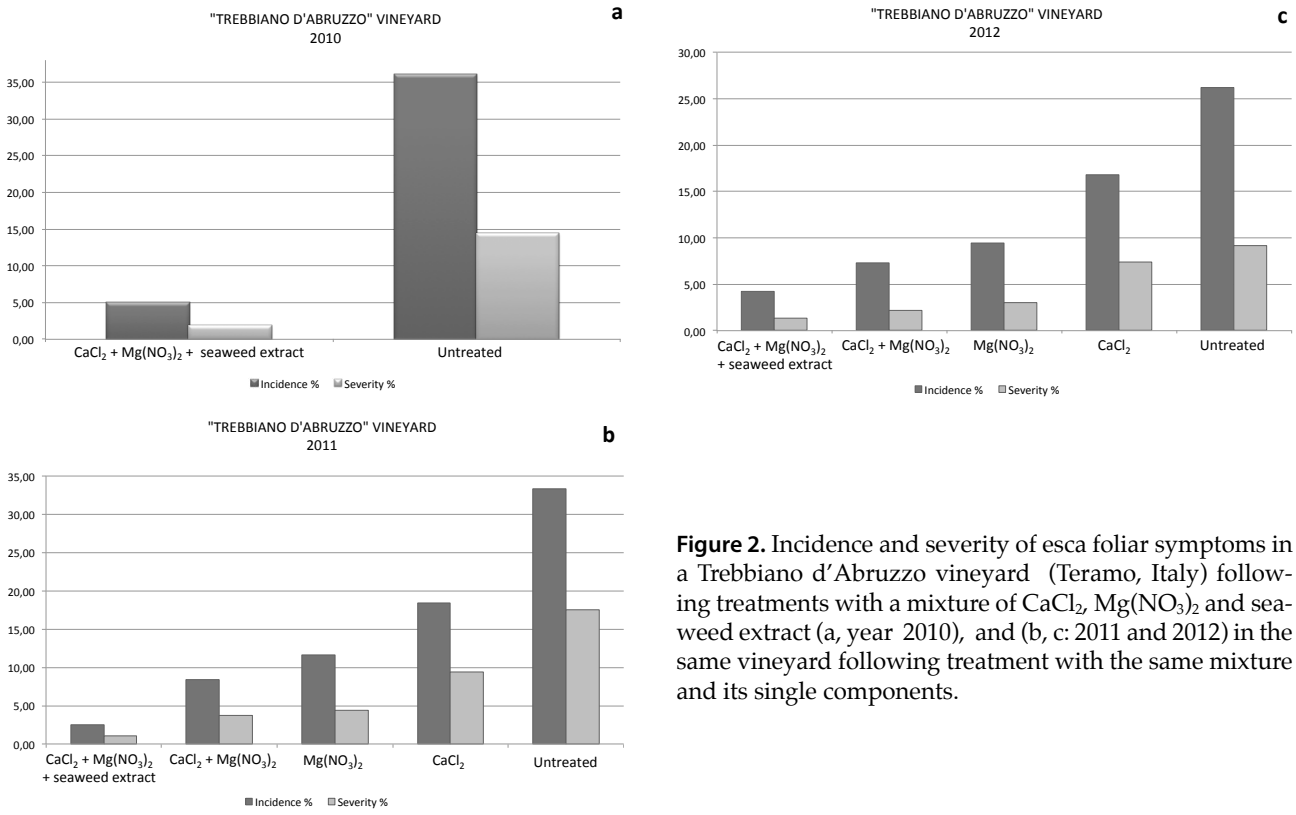

Figure 2. Incidence and severity of esca foliar symptoms in a Trebbiano d'Abruzzo vineyard (Teramo, Italy) following treatments with a mixture of $CaCl_2$, $Mg(NO_3)_2$ and seaweed extract (a, year 2010), and (b, c: 2011 and 2012) in the same vineyard following treatment with the same mixture and its single components.

Table 2. Chi-square statistics comparing the full mixture treatment to the untreated control in three trials, showing the highly significant (in bold-italics, $P<0.001$) differences, and the moderately significant (in italic, $P<0.1$) differences, both in symptoms incidence and severity.

Untreated control	$CaCl_2$ + $Mg(NO3)_2$ + seaweed extract	
	Incidence	**Severity**
2010 - Vineyard Trebbiano d'Abruzzo	***<0.0001***	***<0.0001***
2011 - Vineyard Montepulciano d'Abruzzo 2	***<0.0001***	***0.0004***
2012 - Vineyard Montepulciano d'Abruzzo 2	*0.0650*	*0.0581*

Effects on quantitative and qualitative yield parameters and on defence related compounds (*trans*-resveratrol content)

A statistically significant increase in yield was recorded in the Trebbiano d'Abruzzo vineyard treated in 2012 with the full mixture both for weight (P=0.035) and number of clusters (P=0.036) with values of 136.1 Kg and 337 clusters in healthy treated vines, and 88.8 Kg and 241 clusters in healthy non treated vines. The yield of the asymptomatic treated vines was moderately statistically significant: 180.1 Kg with 409 clusters, and 120.1 Kg with 310 clusters in asymptomatic non treated vines (yield: P=0.073; number of clusters: P=0.093) (Table 5).

No decrease in the quality of the yield was recorded following the full mixture treatment. In fact, on several occasions the quality parameters were improved. In the Trebbiano d'Abruzzo, asymptomatic

Table 3. Significance of the differences in incidence and severity of GLSD symptoms after treatment with the mixture $CaCl_2$, $Mg(NO_3)_2$ and seaweed extract or with the separate mixture components surveyed in the Trebbiano d'Abruzzo and in the Montepulciano d'Abruzzo 1 vineyards during 2010–2012 trials (χ^2 test). Moderately significant ($P<0.1$) values are in italics, significant ($P<0.05$) values are in bold, highly significant ($P<0.001$) are in bold-italics.

Treatment	$CaCl_2 + Mg(NO_3)_2$ + seaweed extract	$CaCl_2$	$Mg(NO_3)_2$	$CaCl_2 + Mg(NO_3)_2$	Untreated
a) GLSD incidence 2011 - Trebbiano d'Abruzzo					
$CaCl_2 + Mg(NO_3)_2$ + seaweed extract	1	***<.0001***	**0.0087**	*0.0540*	***<.0001***
$CaCl_2$	***<.0001***	1	0.1785	**0.0333**	**0.0135**
$Mg(NO_3)_2$	**0.0087**	0.1785	1	0.4716	***0.0008***
$CaCl_2 + Mg(NO_3)_2$	*0.0540*	**0.0333**	0.4716	1	***<.0001***
Untreated	***<.0001***	**0.0135**	***0.0008***	***<.0001***	1
b) GLSD incidence 2011 - Montepulciano d'Abruzzo 1					
$CaCl_2 + Mg(NO_3)_2$ + seaweed extract	1	*0.0693*	0.3043	0.5694	**0.0039**
$CaCl_2$	*0.0693*	1	0.5269	0.3219	0.4252
$Mg(NO_3)_2$	0.3043	0.5269	1	0.7119	0.1516
$CaCl_2 + Mg(NO_3)_2$	0.5694	0.3219	0.7119	1	*0.0765*
Untreated	**0.0039**	0.4252	0.1516	*0.0765*	1
c) GLSD incidence 2012 - Trebbiano d'Abruzzo					
$CaCl_2 + Mg(NO_3)_2$ + seaweed extract	1	**0.0014**	0.1374	0.3341	***<.0001***
$CaCl_2$	**0.0014**	1	0.1232	**0.0331**	*0.0944*
$Mg(NO_3)_2$	0.1374	0.1232	1	0.6056	**0.0045**
$CaCl_2 + Mg(NO_3)_2$	0.3341	**0.0331**	0.6056	1	***0.0006***
Untreated	***<.0001***	*0.0944*	**0.0045**	***0.0006***	1
d) GLSD incidence 2012 - Montepulciano d'Abruzzo 1					
$CaCl_2 + Mg(NO_3)_2$ + seaweed extract	1	*0.0815*	0.2306	0.2044	**0.0022**
$CaCl_2$	*0.0815*	1	0.6493	0.7055	0.2541
$Mg(NO_3)_2$	0.2306	0.6493	1	0.9450	0.2539
$CaCl_2 + Mg(NO_3)_2$	0.2044	0.7055	0.9450	1	0.1460
Untreated	**0.0022**	0.2541	0.2539	0.1460	1

Treatment	$CaCl_2 + Mg(NO_3)_2$ + seaweed extract	$CaCl_2$	$Mg(NO_3)_2$	$CaCl_2 + Mg(NO_3)_2$	Untreated
e) GLSD severity 2011 - Trebbianod'Abruzzo					
$CaCl_2 + Mg(NO_3)_2$ + seaweed extract	1	***<.0001***	**0.0345**	*0.0894*	***<.0001***
$CaCl_2$	***<.0001***	1	**0.0326**	**0.0159**	**0.0353**
$Mg(NO_3)_2$	**0.0345**	**0.0326**	1	0.7504	***0.0004***
$CaCl_2 + Mg(NO_3)_2$	*0.0894*	**0.0159**	0.7504	1	***0.0002***
Untreated	***<.0001***	**0.0353**	***0.0004***	***0.0002***	1
f) GLSD severity 2011 – Montepulcianod'Abruzzo 1					
$CaCl_2 + Mg(NO_3)_2$ + seaweed extract	1	0.1080	0.7629	0.5093	**0.0243**
$CaCl_2$	0.1080	1	0.1683	0.3484	0.8354
$Mg(NO_3)_2$	0.7629	0.1683	1	0.6807	*0.0545*
$CaCl_2 + Mg(NO_3)_2$	0.5093	0.3484	0.6807	1	0.1888
Untreated	**0.0243**	0.8354	*0.0545*	0.1888	1
g) GLSD severity 2012 - Trebbianod'Abruzzo					
$CaCl_2 + Mg(NO_3)_2$ + seaweed extract	1	**0.0015**	0.2137	0.4042	***0.0006***
$CaCl_2$	**0.0015**	1	**0.0420**	**0.0087**	0.5274
$Mg(NO_3)_2$	0.2137	**0.0420**	1	0.5775	**0.0133**
$CaCl_2 + Mg(NO_3)_2$	0.4042	**0.0087**	0.5775	1	**0.0029**
Untreated	***0.0006***	0.5274	**0.0133**	**0.0029**	1
h) GLSD severity 2012 – Montepulcianod'Abruzzo 1					
$CaCl_2 + Mg(NO_3)_2$ + seaweed extract	1	0.3918	0.3670	0.3901	0.1037
$CaCl_2$	0.3918	1	0.8137	0.8744	0.4487
$Mg(NO_3)_2$	0.3670	0.8137	1	0.9396	0.9737
$CaCl_2 + Mg(NO_3)_2$	0.3901	0.8744	0.9396	1	0.6110
Untreated	0.1037	0.4487	0.9737	0.6110	1

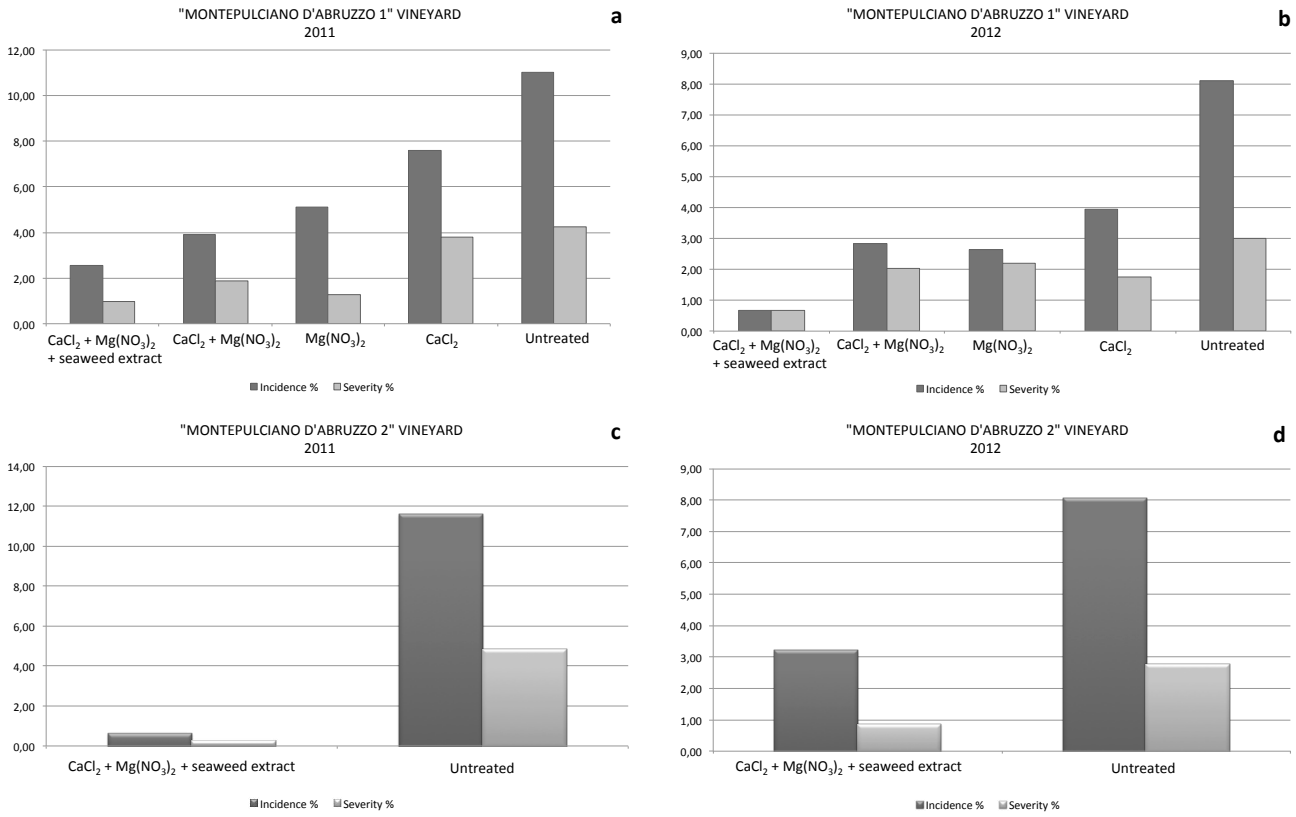

Figure 3. Incidence and severity of esca foliar symptoms in two vineyards cv. Montepulciano d'Abruzzo (Teramo, Italy) after treatments with a mixture of $CaCl_2$, $Mg(NO_3)_2$ and seaweed extract or its single components (a, b: 2011 and 2012, Montepulciano d'Abruzzo 1) and with the full mixture only (c, d: 2011 and 2012, Montepulciano d'Abruzzo 2).

Table 4. Average leaf area (cm^2) in treated and untreated healthy and infected asymptomatic vines of the cv. Trebbiano d'Abruzzo in 2012 (24 vineshoots per treatment).

Leaf type	Treatment	Healthy vines	Asymptomatic vines
Primary shoot leaves	Treated	29293	37656
	Untreated	27036	27829
	P value	0.41	**0.0005**[a]
Secondary shoot leaves	Treated	35312	46227
	Untreated	41702	53958
	P value	0.42	0.62

[a] Values in bold refer to significant differences following Student's *t*-test ($P \leq 0.05$)

treated vines showed a significant increase in levels of reducing sugars (10.12 g L^{-1} increase) and a significant decrease in total acidity (1.32 g L^{-1}) with respect to asymptomatic untreated vines (Table 6).

In the Montepulciano d'Abruzzo 1 vineyard, reducing sugar levels were significantly higher in grapes from treated vines compared to untreated of both healthy (15.82 g L^{-1} increase) and asymptomatic

Table 5. Grape yield in the cv. Trebbiano d'Abruzzo vineyard in 2012 following treatment in healthy and asymptomatic vines.

Yield parameters	Treatment	Healthy vines	Asymptomatic vines
Yield mass	Treated	136.1	180.1
	Untreated	88.8	120.1
	P value	**0.035** [a]	**0.073**
No. of clusters	Treated	337	409
	Untreated	241	310
	P value	**0.036**	**0.093**

[a] Values in bold refer to significant differences following Student's t-test ($P \leq 0.05$) and ($P \leq 0.1$).

Table 6. Reducing sugar and total acidity in berries of vines treated with the full mixture $CaCl_2$, $Mg(NO_3)_2$ and seaweed extract and untreated vines in the Trebbiano d'Abruzzo and Montepulciano d'Abruzzo 1 vineyards in 2012. Data were compared by the Student *t*-test ($P \leq 0.05$).

Treatment	Trebbiano d'Abruzzo		Montepulciano d'Abruzzo 1	
	Reducing sugar (g L^{-1})	Total acidity (g L^{-1})	Reducing sugar (g L^{-1})	Total acidity (g L^{-1})
Untreated healthy	198.33	5.30	220.00	8.87
Treated healthy	193.82	5.57	235.82	8.29
Student's *t*-test	0.37	0.27	0.00004	0.047
Untreated asymptomatic	188.30	7.13	220.11	8.43
Treated asymptomatic	198.42	5.81	238.36	8.30
Student's *t*-test	0.02	0.007	0.00002	0.71

Table 7. Mean *Trans*-resveratrol level in leaves of treated and untreated vines in the Trebbiano d'Abruzzo vineyard in 2010 and 2011 following treatement with the full mixture $CaCl_2$, $Mg(NO_3)_2$ and sea weed extract. Data were compared by the Student *t*-test ($P \leq 0.05$).

Treatment	Resveratrol content (ppm d.w.)		
	08/06/2010	26/07/2010	29/07/2011
Untreated healthy	5.87	6.95	6.94
Treated healthy	9.35	11.17	10.03
Student's *t*-test	0.0104	0.0067	0.0001
Untreated asymptomatic	8.15	8.53	8.53
Treated asymptomatic	8.98	10.43	12.87
Student's *t*-test	0.1487	0.0040	0.0002
Untreated symptomatic	/	17.83	19.44
Treated symptomatic	/	25.46	27.43
Student's *t*-test	/	0.0181	0.0062

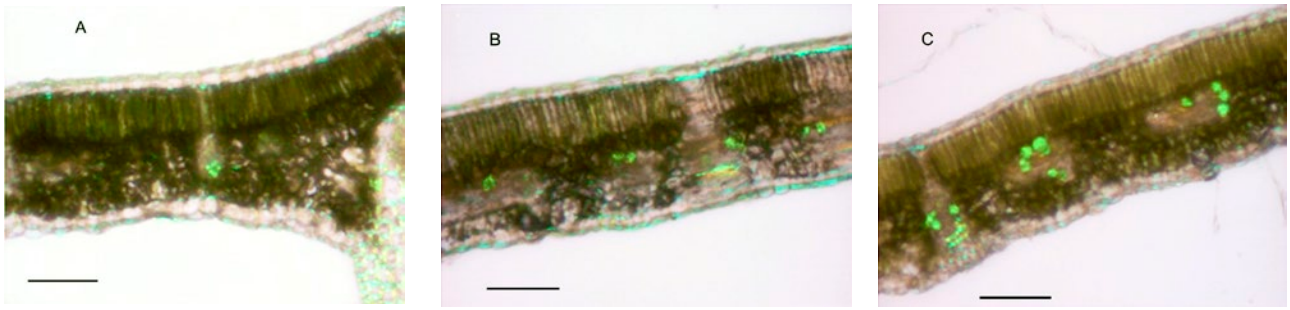

Figure 4. Polarized light micrographs of cross section of leaf of untreated healthy vines (A) and leaf of untreated asymptomatic vines (B) showing a druse population at minimum density and leaf of treated asymptomatic vines (C) showing a higher druse number, some of which are near the vascular bundle (arrows). Scale bar: 100 μm.

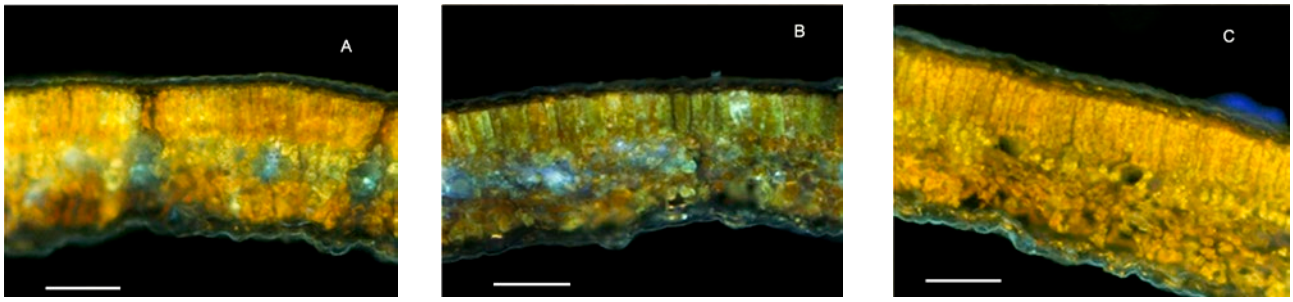

Figure 5. Fluorescence micrographs of cross section of leaf of untreated healthy vines (A) and leaf of untreated asymptomatic vines (B) showing a light yellow fluorescence not evenly distributed in the tissues, and leaf of treated asymptomatic vines (C) with bright and uniform yellow fluorescence. Scale bar: 100 μm.

vines (18.36 g L^{-1} increase). Total acidity was significantly lower in grapes from healthy treated vines versus non treated ones (8.29 versus 8.87 g L^{-1}, P=0.047), and similar between treated and non treated asymptomatic ones (8.30 versus 8.43 g L^{-1}, P=0.71) (Table 6).

A clear increase in *trans*-resveratrol content was recorded following the full mixture treatment, which was evaluated in Trebbiano d'Abruzzo leaves in 2010 and 2011. In all groups of treated vines ("healthy", asymptomatic and diseased), the *trans*-resveratrol content was significantly higher at the end of the entire treatment program (Table 7). In 2010, an intermediate sampling showed a statistically significant increase in *trans*-resveratrol content of treated healthy vines on June 8 after only four treatments.

Histological changes

Polarizing optics allowed easy visualization and observation of the druse crystals in the parenchymatous spongy tissue of untreated and treated leaves. In the leaves of untreated vines, both healthy and asymptomatic, relatively few druse crystals could be seen and these were located far from the midrib and vascular bundles (Figure 4a, 4b). In the leaves from treated asymptomatic vines druse crystals were larger and more abundant and were localized near the vascular bundles (Figure 4c). In both years of treatment, druse crystals were consistently found in higher numbers per unit area in treated leaves compared to untreated leaves. In 2010, the average number of druses in untreated healthy vines was similar to the numbers found in untreated asymptomatic vines (7.99 and 8.05 druses mm^{-1} respectively), while a high number of druses (11.83 mm^{-1}) was observed only in treated asymptomatic vines. In 2011, again a higher number of druses (15.4 druses mm^{-1}) was observed in treated leaves of asymptomatic vines, while both untreated healthy vines and asymptomatic vines showed an average number of 11.1 and

11.05 druses mm^{-1}. The pairwise comparison between leaves of untreated healthy vines and leaves of treated asymptomatic vines revealed significant differences at P=0.003 in 2010, and at P=0.010 in 2011, as well as between leaves of untreated asymptomatic vines and treated asymptomatic vines (P=0.016 in 2010, and P=0.028 in 2011). The pairwise comparison of leaves of untreated healthy vines and leaves of untreated asymptomatic vines showed that the differences were not significant (P=0.22 in 2010, and P=0.18 in 2011).

In all collected samples flavonoid content was estimated by fluorescence in fresh leaf sections. In leaves from untreated healthy vines and from untreated asymptomatic vines a light yellow fluorescence unevenly distributed in the tissues was detected (Figure 5a, 5b). In all leaves of treated asymptomatic vines, the bright yellow fluorescence was intense throughout the entire cross section demonstrating the accumulation of flavonoids in those tissues (Figure 5c).

Discussion

Foliar applications of a calcium chloride and magnesium nitrate mixture containing brown seaweed extract were shown to efficiently reduce the incidence and severity of GLSD foliar symptoms. The treatment does not act on the fungal pathogens colonizing the wood of symptomatic vines as it is a foliar treatment applied during the growth season, but it appears to interfere with the mechanisms involved in the development of chlorosis and necrosis in the interveinal leaf blade. GLSD is typically characterised by a fluctuation in the appearance of foliar symptoms from one year to another. In this way only a portion of the infected vines in year *n* will show symptoms in year *n+1* or the following years. The proportion of diseased and asymptomatic vines in a given year has been named "hidden esca" (Marchi *et al*., 2006; Surico *et al*., 2006).

The spray programmes described in this paper included nine treatments at 10-day intervals and was shown to have a direct effect on the ability of diseased vines to counteract the (still unknown) symptom inducing process, leading to a significant reduction of symptoms compared to the untreated diseased vines. Therefore, it would appear that the natural process whereby a diseased vine may remain asymptomatic (Surico *et al*., 2006) was enhanced.

The greatest reduction of foliar symptoms was obtained by applications of the full mixture of calcium chloride, magnesium nitrate and seaweed extract. The seaweed extract enhances the activity of the nutrients, possibly by acting as a carrier of the nutrients present in the mixture. A direct action of the seaweed extract on symptom development seems less feasible. For example, applications of a seaweed extract (*Ascophyllum nodosum*) in previous trials showed only a slight, and non-significant, decrease in foliar symptom expression in treated vines affected by GLSD (Di Marco and Osti, 2009).

Induction of a disease response mechanism is suggested by the increase in *trans*-resveratrol levels recorded both in 2010 and 2011 in each group of treated leaves. Also the higher content of flavonoids revealed by the histological studies confirms that the full mixture interacts with the defence response of the plant since it is widely accepted that secondary metabolites, such as flavonoids, are involved in plant defence against biotic and abiotic stress (Buschmann *et al*., 2000; Braidot *et al*., 2008; Lima *et al*., 2012; Crupi *et al*., 2013).

The application of the treatment starting at the very beginning of the growth season appears to cause an early activation of this defence response, thus interfering with the oxidative burst leading to leaf damage which is supposed to be incited by the virulence factors brought by the sap (Andolfi *et al*., 2009). Valtaud *et al*. (2011) suggested a relation between the increase in reactive oxygen species (ROS) with a decrease of carotenoids and their associated "protective" action from the oxidative damage in leaves of "esca" affected vines that are beginning to show symptoms. In this paper histological observations revealed an increase in flavonoids in the treated diseased vines that remained asymptomatic, thus suggesting the activation of a defence response (Friend, 1981; Ojeda *et al*., 2002; Treutter, 2006).

The most commonly accepted hypothesis on the factors that trigger symptom development in GLSD is that phytotoxic metabolites produced by the fungal pathogens in the infected wood tissues are transported by the sap stream to the leaves (Evidente *et al*., 2000; Tabacchi *et al*., 2000; Andolfi *et al*., 2009). Those phytotoxic metabolites or their derivatives, following this hypothesis, are the ones that activate the above cited reaction of the tissue with interveinal chlorosis or pigments accumulation, and later the interveinal necrosis (the typical leaf stripes), i.e. chlorosis/reddening

and necrotic stripes (Andolfi *et al*., 2009; Calzarano *et al*., 2009; Magnin-Robert *et al*., 2011). In this process the minerals applied in the trials reported here may have a specific role in reducing an intensive oxidative response, as in the case of Ca^{2+} (Lima *et al*., 2012). Ca^{2+} have been shown to have beneficial effects on the host in many pathosystems and different host plants (Biggs *et al*., 1994; Gadoury *et al*., 1994; Campanella *et al*., 2002). Those effects may be related to calmodulin accumulation that regulates salicylic acid and thereby triggers the disease defence reaction and reduces the hypersensitive reaction (Lecourieux *et al*., 2006; Du *et al*., 2009). Furthermore, the high calcium content in the extracellular space can also be linked to increased phytoalexin synthesis (Kurosaki *et al*., 1987; Stäb and Ebel, 1987; Ebel, 1995; Tavernier *et al*., 1995). Calcium is known to bind to pectins (Conway *et al*., 1991) giving greater mechanical resistance to cell walls (Kratzke, 1988). The ability of calcium to strengthen cell walls, is also suggested by the higher calcium oxalate druse populations in the leaves of treated diseased vines that remained asymptomatic. Druse crystals are just one of the possible storage forms of Ca^{2+} in leaf tissue (Lecouriex *et al*., 2006). Other accumulation forms could be further investigated.

A very relevant aspect of calcium in a disease like GLSD could be its role in delaying leaf senescence as demonstrated in *Arabidopsis* and other plants (Poovaiah and Leopold, 1973; Ma *et al*., 2010). As a matter of fact leaf stripe symptoms have been described as an early leaf senescence in the interveinal mesophyll areas (Andolfi *et al*., 2011) and the role of calcium on delaying the process can also be relevant.

The role of magnesium in disease control is less clear and less studied than that of calcium, and the results are sometimes contradictory (Jones *et al*., 1983; Jones and Huber, 2007), even though its many functions in plant physiology are widely described (Schaul, 2002; Marschner, 2012). Magnesium is an essential constituent of chlorophyll and deficiency generally leads to chlorosis. In grapevine leaves magnesium deficiency results in symptoms similar to GLSD, namely an interveinal discoloration and subsequently necrosis (Figure 6).

Magnesium could also have a more direct role in the interaction between pathogens and grapevines. Thus, Colrat (1999) reported the activation of a phytotoxin detoxification process by Mn^{2+} and Mg^{2+} in *Eutypa lata* infections. Eutypin is actively detoxified to eutypinol, a non toxic compound, by grapevine cells and it is hypothesized that the differences in susceptibility to this wood disease is strongly linked to the ability of grapevine cells to detoxify eutypin. A similar mode of action for toxins produced by esca complex pathogens is possible. It should also be clarified if the seaweed extract has mainly a carrier role, improving the distribution of the macro-elements, or if (in this case and with this type of seaweed extract containing isopentenyl adenine and glycine-betaine) it also enhances the defence reaction of the host, since in all cases the full mixture gave the greatest control effect. Nevertheless, the synergistic effect of the seaweed extract on the activity of the nutrients is clear.

Figure 6. Magnesium deficiency is associated with a typical chlorosis or, in red cultivars, anthocyanins accumulation, that resemble GLSD symptoms. The chlorosis develops in the interveinal areas of the leaf blade with a more regular pattern than GLSD, and typical pointed ends shape. A necrosis can also follow.

The treatment described here significantly reduced the severity of the disease symptoms, stimulated vegetative activity, as shown by significantly greater surface area of primary leaves in the treated, asymptomatic vines. Moreover the positive effects on quality and quantity of the berries can only encourage a profitable application of the full mixture in GLSD control. Furthermore, phytotoxicity was never recorded, neither in the current season nor in following years of repeated treatment. These results, which represent the first efficient leaf treatment to reduce GLSD after the moderate reduction obtained with Fosetyl Al (Di Marco *et al*., 2011a), may improve a new approach for the development of control strategies against this complex disease.

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Literature cited

AA.VV., 1990. Methods of community analysis to use in wine sector. *Official Gazette of the European Communities*, 3 October 1990. Commission Regulation (EEC). No. 2676/90.

Andolfi A., A. Cimmino, A. Evidente, M. Iannaccone, R. Capparelli, L. Mugnai and G. Surico, 2009. A new flow cytometry technique to identify *Phaeomoniella chlamydospora* exopolysaccharides and study mechanisms of esca grapevine foliar symptoms. *Plant Disease* 93 (7), 680–684.

Andolfi A., L. Mugnai, J. Luque, G. Surico, A. Cimmino and A. Evidente, 2011. Phytotoxins produced by fungi associated with grapevine trunk diseases. *Toxins* 3 (12), 1569–1605.

Bertsch C., M. Ramírez-Suero, M. Magnin-Robert, P. Larignon, J. Chong, E. Abou-Mansour, A. Spagnolo, C. Clèment and F. Fontaine, 2013. Grapevine trunk diseases: complex and still poorly understood. *Plant Pathology* 62 (2), 243–265.

Biggs A.R., M.M. El-Kholi and S.M. El-Neshawy, 1994. Effect of calcium salts on growth, pectic enzyme activity, and colonization of peach twigs by *Leucostoma persoonii*. *Plant Disease* 78, 886–890.

Braidot E., M. Zancani, E. Petrussa, C. Peresson, A. Bertolini, S. Patui, F. Macrì, and A. Vianello, 2008. Transport and accumulation of flavonoids in grapevine (*Vitis vinifera* L.). *Plant Signaling & Behaviour* 3 (9), 626–632.

Buschmann H., K. Reilly, M.X. Rodriguez, J. Thome and J.R. Beeching, 2000. Hydrogen peroxide and flavan-3-ols in storage roots of Cassava (*Manihot esculenta* Crantz) during postharvest deterioration. *Journal of Agricultural and Food Chemistry* 48, 5522–5529.

Calzarano F. and S. Di Marco, 1997. Il "mal dell'esca" della vite: stato della ricerca. *Informatore Fitopatologico* 47 (9), 9–20.

Calzarano F., A. Cichelli and M. Odoardi, 2001. Preliminary evaluation of variations in composition induced by esca on cv. Trebbiano D'Abruzzo grapes and wines. *Phytopathologia Mediterranea* 40, S443–S448.

Calzarano F., L. Seghetti, M. Del Carlo and A. Cichelli, 2004a. Effect of esca on the quality of berries, musts and wines. *Phytopathologia Mediterranea* 43, 125–135.

Calzarano F., S. Di Marco and A. Cesari, 2004b. Benefit of fungicide treatment after trunk renewal of vines with different types of esca necrosis. *Phytopathologia Mediterranea* 43, 116–124.

Calzarano F., C. Amalfitano, L. Seghetti, and V. D'Agostino. 2007. Foliar treatment of esca proper affected vines with nutrients and bio activators. *Phytopathologia Mediterranea* 46, 207–217.

Calzarano F., V. D'Agostino and M. Del Carlo, 2008. Trans-resveratrol extraction from grapevine: application to berries and leaves from vines affected by esca proper. *Analytical Letters* 41, 1–13.

Calzarano F., C. Amalfitano, L. Seghetti, and V. Cozzolino, 2009. Nutritional status of vines affected with esca proper. *Phytopathologia Mediterranea* 48, 20–31.

Calzarano F. and V. D'Agostino, 2010. Il mal dell'esca della vite in Abruzzo. In: *Il Mal dell'Esca della Vite: Interventi di ricerca e Sperimentazione per il Contenimento Della Malattia. Progetto MesVit*. (Surico G., Mugnai L., ed.), Arsia Regione Toscana, Firenze, Italy, 132–135.

Calzarano F., V. D'Agostino, F. Osti and S. Di Marco, 2013. Levels of *trans*-resveratrol in leaves of vines affected with "esca". In: Protection and Production in Viticulture, IOBC/WPRS Bulletin Vol. 85, 129–136.

Campanella V., A. Ippolito and F. Nigro, 2002. Activity of calcium salts in controlling *Phytophthora* root rot of citrus. *Crop Protection* 21, 751–756.

Cesari A., S. Di Marco and F. Calzarano, 2005. *Presenza del Mal dell'esca nell'area Viticola della Provincia di Teramo. Strategie Sperimentali di Difesa a Basso Impatto Ambientale*. Monografia, Media Edizioni, Teramo, Italy, 59 pp.

Chung M.I., 1992. An antiplatelet principle of *Veratrum formosanum*. *Planta Medica* 58, 274–276.

Colrat S., C. Deswarte, A. Latché, K. Klaébe, M. Bouzayen, J. Fallot and J.P. Roustan, 1999. Enzymatic detoxification of eutypine, a toxin from *Eutypa lata*, by *Vitis vinifera* cells: partial purification of an NADPH-dependent aldehyde reductase. *Planta* 207, 544–550.

Conway W.S., C.E. Sams, J.A. Abbott, and B.D. Bruton, 1991. Postharvest calcium treatment of apple fruit to provide broad-spectrum protection against postharvest pathogens. *Plant Disease* 75, 620–622.

Crupi P., A. Picchierri, T. Basile and D. Antonacci, 2013. Postharvest stilbenes and flavonoids enrichment of table grape cv Redglobe (*Vitis vinifera* L.) as affected by interactive UV-C exposure. *Food Chemistry* 141, 802–808.

Datnoff L.E., W.H. Elmer and D.M. Huber, 2007. *Mineral Nutrition and Plant Disease*. The American Phytopathological Society. St. Paul, MN, USA, 278 pp.

Di Marco S., A. Mazzullo, F. Calzarano and A. Cesari, 1999. *In*

vitro studies on the phosphorous acid and vitis stilbenes interaction, and *in vivo* Fosetyl Al activity towards *Phaeoacremonium* spp. grapevine wood decay agents. In: *Modern Fungicides and Antifungal Compounds* II, BCPC monograph (British Crop Protection Council) 171–177.

Di Marco S., Osti F., Mazzullo A. and A. Cesari, 2001. How iron could be involved in esca fungi development. *Phytopathologia. Mediterranea* 40, S449–S452.

Di Marco S., F. Osti and A. Cesari, 2004. Experiments on the Control of Esca by *Trichoderma*. *Phytopathologia Mediterranea* 43 (1), 108–115.

Di Marco S. and F. Osti, 2009. Effect of biostimulant sprays on *Phaeomoniella chlamydospora* and esca proper infected vines under greenhouse and field conditions. *Phytopathologia Mediterranea* 48, 47–58.

Di Marco S., F. Osti, F. Calzarano, R. Roberti, A. Veronesi, and C. Amalfitano, 2011a. Effects of grapevine applications of fosetyl-aluminium formulations for downy mildew control on "esca" and associated fungi. *Phytopathologia Mediterranea* 50, S285–S299.

Di Marco S., F. Osti, and L. Mugnai, 2011b. First studies on the potential of a copper formulation for the control of leaf stripe disease within esca complex in grapevine. *Phytopathologia Mediterranea* 50, S300–309.

Du L., G.S. Ali, K.A. Simons, J. Hou, T. Yang, A.S.N. Reddy and B.W. Poovaiah, 2009. Ca^{2+}/ calmodulin regulate salicylic-acid-mediated plant immunity. *Nature* 457, 1154–1158.

Ebel J., 1995. Oligoglicoside elicitor-mediated activation of plant defence. *Bioessays*, 20, 569-576.

Evidente A., L. Sparapano, A. Andolfi and G. Bruno, 2000. Two naphthalenone pentaketides from liquid cultures of *Phaeoacremonium aleophilum*, a fungus associated with esca of grapevine. *Phytopathologia Mediterranea* 39, 162–168.

Friend J., 1981. Plant phenolics, lignification and plant disease. *Progress in Phytochemistry* 7, 197–261.

Gadoury D.M., R.C. Pearson, D.G. Riegel, R.C. Seem, C.M. Becker and J.W. Pscheidt, 1994. Reduction of powdery mildew and other diseases by over-the-trellis applications of lime sulfur to dormant grapevines. *Plant Disease* 78, 83–87.

Gramaje D., L. Mostert and J. Armengol, 2011. Characterization of *Cadophora luteo-olivacea* and *C. melinii* isolates obtained from grapevines and environmental samples from grapevine nurseries in Spain. *Phytopathologia Mediterranea* 50, S112–S126.

Hart J.H., 1981. Role of phytostilbenes in decay and disease resistance. *Annual Review of Phytopathology* 19, 437–458.

Heath M.C., 2000. Hypersensitive response-related death. *Plant Molecular Biology* 44, 321–334.

Jones J.B., S.S. Woltz and J.P. Jones, 1983. Effect of foliar and soil magnesium application on bacterial leaf spot of peppers. *Plant Disease* 67, 623–624.

Jones J.B. and D.M. Huber, 2007. Magnesium and plant disease. In: *Mineral Nutrition and Plant Disease*, (Datnoff L.E., ed.), American Phytopathological Society, St. Paul, MN, USA, 95–100,

Klein L.B., S. Chandra, N.I. Mondy, 1981. Effect of magnesium fertilization on the quality of potatoes: yield, discolouration, phenols, and lipids. *Journal of Agricultural and Food Chemistry* 29, 384–387.

Kotze C., J. Van Niekerk, F. Halleen, L. Mostert and P. Fourie, 2011. Evaluation of biocontrol agents for grapevine pruning wound protection against trunk pathogen infection. *Phytopathologia Mediterranea* 50 (4), 247–263.

Kratzke M.G., 1988. Study of mechanism of calcium uptake by potato tubers and of cellular properties affecting soft rot. Ph. D. Thesis, University of Wisconsin, MD, USA.

Kurosaki F., Y. Tsurusawa and A. Nishi, 1987. The elicitation of phytoalexins by Ca^{2+} and cyclic AMP in carrot cells. *Phytochemistry* 26, 1919–1923.

Lecomte P., G. Darrieutort, J.M. Liminana, G. Comont, A. Muruamendiaraz, F.J. Legorburu, E. Choueiri, F. Jreijiri, Roula El Amil and M. Fermaud, 2012. New insights into esca of grapevine: the development of foliar symptoms and their association with xylem discoloration. *Plant Disease* 96, 924–934.

Lecourieux D., R. Ranjeva and A. Pugin, 2006. Calcium in plant defence-signalling pathways. *New Phytologist* 171, 249–269.

Lima M.R.M., F. Ferreres and A.C.P. Dias, 2012. Response of *Vitis vinifera* cell cultures to *Phaeomoniella chlamydospora*: changes in phenolic production, oxidative state and expression of defence-related genes. *European Journal of Plant Pathology* 132, 133–146.

Lorenz D.H., K.W. Eichhorn, H. Bleiholder, R. Close, U. Meier and E. Weber, 1995. Phenological growth stages of the grapevine (*Vitis vinifera* L. ssp. *vinifera*). Encoding and description of the phenological stages of the grapevine according to the extended BBCH scheme. *Australian Journal of Grape and Wine Research* 1 (2), 100–103.

Ma W., Smigel, A., Walker, R. K., Moeder, W., Yoshioka, K., and Berkowitz, G. A., 2010. Leaf senescence signaling: The Ca^{2+}-conducting Arabidopsis cyclic nucleotide gated channel2 acts through nitric oxide to repress senescence programming. *Plant Physiology 154*, 733–743.

Magnin-Robert M., Letousey, P., Spagnolo, A., Rabenoelina, F., Jacquens, L., Mercier, L., Christophe Clément and Florence Fontaine, 2011. Leaf stripe form of esca induces alteration of photosynthesis and defence reactions in presymptomatic leaves. *Functional Plant Biology* 38, 856–866.

Marchi G., I.G. Pascoe and J. Edwards, 2001. Young esca in Australia. *Phytopathologia Mediterranea* 40 (3), 303–310.

Marschner P. (ed), 2012. *Marschner's Mineral Nutrition of Higher Plants* (Third Edition). Elsevier Ltd.

McKinney H.H., 1923. Influence of soil temperature and moisture on infection of wheat seedlings by *Helminthosporium sativum*. *Journal of Agricultural Research* 26, 195–218.

Ojeda H., C. Andary, E. Kraeva, A. Carbonneau, A. Deloire, 2002. Influence of pre- and post-veraison water deficit on synthesis and concentration of skin phenolic compounds during berry growth of *Vitis vinifera* cv. Shiraz. *American Journal of Enology and Viticulture* 53, 261–267.

Oliveira H. and C. Santos, 2011. An integrative view of sodium chloride stress and *Phaeomoniella* sp. inoculation on growth and nutrient accumulation and patterning in *in vitro* grapevine plants. *Journal of Plant Nutrition* 34, 557–572.

Osti F. and S. Di Marco, 2011. Iron-dependent, non-enzymatic processes promoted by *Phaeomoniella chlamydospora* and

Phaeoacremonium aleophilum, agents of esca in grapevine. *Physiological and Molecular Plant Pathology* 74, 309–316.
Poovaiah B.W. and A.C. Leopold, 1973. Deferral of leaf senescence with calcium. *Plant Physiology* 52 (3), 236–239.
Reddy A.S.N., 2001. Calcium: silver bullet in signalling. *Plant Science* 160, 381–404.
Rolshausen P.E., J.R. Úrbez-Torres, S. Rooney-Latham, A. Eskalen, R.J. Smith and W.D Gubler, 2010. Evaluation of pruning wound susceptibility and protection against fungi associated with grapevine trunk diseases. *American Journal of Enology and Viticulture* 61 (1), 113–119.
Rudd J.J. and V.E. Franklin-Tong, 2001. Unravelling response-specificity in Ca^{2+} signalling pathways in plant cells. *New Phytologist* 151, 733–749.
Sanders D., C. Brownlee and J.F. Harper, 1999. Communicating with Calcium. *Plant Cell* 11, 691–706.
Santos C., S. Fragoeiro and A. Phillips, 2005. Physiological response of grapevine cultivars and a rootstock to infection with *Phaeoacremonium* and *Phaeomoniella* isolates: an in vitro approach using plants and calluses. *Scientia Horticulturae* 103 (2), 187–198.
Shaul O., 2002. Magnesium transport and function in plants: the tip of the iceberg. *BioMetals* 15, 309–323.
Spagnolo A., M. Magnin-Robert, T.D. Alayi, C. Cilindre, L. Mercier, C. Schaeffer-Reiss, A. Van Dorsselaer, C. Clément and F. Fontaine, 2011. Physiological changes in green stems of *Vitis vinifera* L. cv. Chardonnay in response to esca proper and apoplexy revealed by proteomic and transcriptomic analyses. *Journal of Proteome Research* 11 (1), 461–475.
Stäb M.R. and J. Ebel, 1987. Effects of Ca^{2+} on phytoalexin induction by fungal elicitor in soybean cells. *Archives of Biochemistry and Biophysics* 257 (2), 416–423.
Surico G., 2009. Towards a redefinition of the diseases within the esca complex. *Phytopathologia Mediterranea* 48, 5–10.
Surico G., L. Mugnai and G. Marchi, 2006. Older and more recent observations on esca, a critical overview. *Phytopathologia Mediterranea* 45, S68–S86.
Surico G., L. Mugnai and G. Marchi, 2008. The esca disease complex. In: *Integrated Management of Diseases Caused by Fungi, Phytoplasma and Bacteria.* (Ciancio A., Mukerji K.G., ed.) Springer, Heidelberg, Germany, 119–136.
Surico G., G. Marchi, L. Mugnai and F. Peduto, 2010. Espressione dei sintomi fogliari dell'esca in funzione della disponibilità di acqua nel suolo e germogliamento delle gemme. In: *Il Mal dell'Esca della Vite: Interventi di ricerca e Sperimentazione per il Contenimento della Malattia. Progetto MesVit*. (Surico G., Mugnai L., ed.), Arsia Regione Toscana, Firenze, Italy, 276–291.
Tabacchi R., A. Fkyerat, C. Poliart and G. Dubin, 2000. Phytotoxins from fungi of esca of grapevine. *Phytopathologia Mediterranea* 39, 156–161.
Tavernier E., D. Wendehenne, J.P. Blein and A. Pugin, 1995. Involvement of free calcium in action of cryptogein, a proteinaceous elicitor of hypersensitive reaction in tobacco cells. *Plant Physiology* 109, 3, 1025–1031.
Treutter D., 2006. Significance of flavonoids in plant resistance: a review. *Environmental Chemistry Letters* 4, 147–157.
Valtaud C., P. Larignon, G. Roblin and P. Fleurat-Lessard, 2009. Developmental and ultrastructural features of *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* in relation to xylem degradation in esca disease of the grapevine. *Journal of Plant Pathology* 91 (1), 37–51.
Valtaud C., F. Thibault, P. Larignon, C. Bertsch, P. Fleurat-Lessard and A. Bourbouloux, 2011. Systemic damage in leaf metabolism caused by esca infection in grapevines. *Australian Journal of Grape and Wine Research* 17 (1), 101–110.
White P.J. and M.R. Broadley, 2003. Calcium in plants. *Annals of Botany* 92, 487–-511.
Whiting E.C., A. Khan and W. D. Gubler, 2001. Effect of temperature and water potential on survival and mycelial growth of *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. *Plant Disease* 85, 195–201.

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