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RESEARCH PAPERS - 10TH SPECIAL ISSUE ON GRAPEVINE TRUNK DISEASES

Further evidence that calcium, magnesium and seaweed mixtures reduce grapevine leaf stripe symptoms and increase grape yields

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Summary. Grapevine leaf stripe disease (GLSD) is the most important syndrome of the esca disease complex. GLSD foliar symptoms are associated with grapevine yield losses and decline in vigour of host plants. Relationship between incidence and severity of GLSD symptoms and reductions grape quantity and quality has been demonstrated. In 2010–2012, foliar applications of mixtures of fertilizers based on calcium, magnesium and seaweed reduced symptom expression and increased yield quantity and quality. In the present study, mixture applications were carried out in 2013-2015 in different vineyards located in Abruzzo and Emilia-Romagna Regions (north-central Italy). These treatments reduced GLSD symptom incidence and severity, and applications of seaweed without calcium and magnesium was not effective. This confirmed involvement of the nutrients in the reduction of leaf symptom expression. Mechanical properties and absorbance difference measurements demonstrated increased berry quality from vines treated with the mixture. In trials carried out in 2015, the efficacy of the mixture applied in tank mix with other pest and disease control products was not reduced. We conclude that applications of calcium, magnesium and seaweed mixtures are effective for reducing the impacts of GLSD in vineyards.

Key words: foliar fertilizer, ripening, yield quality, mechanical properties, absorbance difference.

Introduction

The esca disease complex affects almost all grape growing areas (Gramaje *et al*., 2018). Grapevine leaf stripe disease (GLSD) is the most important and widespread component of the esca complex. Although recent studies have clarified GLSD etiology and epidemiology, effective control strategies are still lacking (Di Marco *et al*., 2011a; 2011b).

GLSD foliar symptoms normally increase during the grapevine growth stages, from the pre-bunch closure to harvest (Calzarano *et al*., 2016). Symptoms affecting leaves consist of typical chloro-necrosis of laminae (tiger-stripe), and those on bunches are purple spots on the berry skins. Symptomatic vines can also show wilting of clusters or canes (Mugnai *et al*., 1999).

GLSD is characterized by discontinuity of foliar symptom expression from year to year, depending on climatic factors and other mechanism yet to be defined (Marchi *et al*., 2006; Calzarano *et al*., 2018). Diseased vines may not show external or yield symptoms, but appear similar to healthy vines (i.e. vines that do not show external symptoms during more than 20 years of inspection) (Calzarano and Di Marco, 2007). Clear correlation between symptom severity and decreased yields has been demonstrated (Calzarano *et al*., 2001; 2004; Bertsch *et al*., 2013). Furthermore, studies have not showed significant decreases in fruit quality of asymptomatic diseased vines compared to that from healthy vines (Calzarano *et al*., 2001; 2004). Therefore, efforts have been directed to developing strategies for reducing incidence and severity of foliar symptom expression in vineyards (Calzarano *et al*., 2007).

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Recent studies have showed the presence of high levels of calcium in asymptomatic leaves from GLSD-affected vines compared to healthy leaves (Calzarano *et al*., 2009), and reductions of foliar symptom expression after treatments with mixtures of foliar fertilizers containing calcium, magnesium and seaweed (Calzarano *et al*., 2014). The effects of the mixtures on vegetative growth and the mechanisms involved in activity of the mixture or its components have been investigated. Histological observations and determination of contents of *trans*-resveratrol, *trans*-viniferin, *trans*-δ-viniferin and *trans*-pterostilbene in leaves were carried out (Calzarano *et al*., 2014; 2017a; 2017b). These studies highlighted capability of the mixture for reducing GLSD symptoms and increasing yield quantity and quality from mixture-treated vines[1].

The results obtained from a further 3 years of trial applications of the mixture, and of the seaweed component alone, that were carried out in vineyards located in Abruzzo and in Emilia-Romagna Regions, are reported here. To expand information from data reported in our previous study (Calzarano *et al*., 2014), additional analyses of yield quality have been performed.

Materials and methods

Foliar applications

Trials were carried out in 2013-2015 in five vineyards located in the provinces of Teramo and Ravenna, respectively in the Abruzzo and Emilia-Romagna regions of Italy (Table 1). The treatments were a foliar fertilizer based on a mixture of $CaCl_2$, $Mg(NO_3)_2$, and *Fucales* seaweed extract, termed the "full mixture" (Calzarano *et al*., 2014).

The full mixture (4 L ha^{-1}) was applied with a pneumatic air sprayer in a water volume of 400 L ha^{-1} in the vineyards containing Montepulciano d'Abruzzo, Sangiovese and Cabernet Sauvignon grapevine varieties. In the vineyard of Trebbiano d'Abruzzo vines, the same quantity of full mixture was diluted in 800 L ha^{-1} and applied with an air blast sprayer.

Nine foliar applications were carried out, commencing from the "three leaves unfolded" stage (12 BBCH) (Lorenz *et al*., 1995), through to the end of pre-bunch closure (79 BBCH) (beginning of May through to the end of July). Six foliar applications were also considered; in this case the applications commenced at the "nine leaves unfolded" stage (19 BBCH), through to the beginning of pre-bunch closure (77 BBCH) (end of May through to the beginning of July). In both of these cases, the treatments were applied at 10-d intervals (Table 2).

In our previous study, all the components of the full mixture were examined for their activity in reducing expression of foliar symptoms, except for the *Fucales* seaweed extract (Calzarano *et al*., 2014). In 2015, in the Trebbiano d'Abruzzo vineyard, seaweed applications were performed. Furthermore, the full mixture was also applied with the fungicides or insecticides normally used against other diseases and pests (Tables 2 and 3).

For each treatment and year of trials, replicates were distributed taking care to avoid replicates of the same treatments being adjacent to each other.

Evaluation of foliar symptoms

Incidence and severity of foliar symptoms were assessed in the second half of September each year, at the time of maximum GLSD symptom expression in the investigated vineyards, and close to technological berry ripeness. Data of surveys are reported in Table 2. Incidence and severity of GLSD for each treatment were recorded, and were statistically analyzed using the method described by Calzarano *et al*. (2014).

Mechanical properties of berries

In the Trebbiano d'Abruzzo vineyard, samples of healthy berries were collected from veraison to harvest, on 26 July, 9, 16, 30 August and 10 September, 2015. Samples were collected from healthy vines (those that did not show any external symptoms during more than 20 years of inspection) and from GLSD symptomatic or asymptomatic plants (vines that showed symptoms in a one or more previous inspection years but not in the year of assessment), and from vines that had been either treated and not treated with the full mixture. Visually healthy berries

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[1] The significant reduction of foliar symptoms and the values obtained in associated physiological/histological parameters (Calzarano et al., 2014) lead to deposit a patent application at the Italian Patent and Trade Mark Office (UIBM) 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. The Italian patent (No. 102014902238891) was released on 21 June 2016. The patent application has also been deposited at the European Patent Office (EPO), (Patent application no. EP15157207.0; 2 March 2015).

Table 1. Main characteristics of the vineyards treated with mixtures of $CaCl_2$, $Mg(NO_3)_2$ and seaweed extract in 2013–2015.

Vineyard	Location	Region	Age	Vine planting pattern (m x m)	Surface (m^2)	No.of vines
Trebbiano d'Abruzzo	Controguerra (TE)	Abruzzo	40	3x3	7000	700
Montepulciano d'Abruzzo-1	Mosciano S.A. (TE)	Abruzzo	37	2.5x2.5	8000	1280
Montepulciano d'Abruzzo-2	Mosciano S.A. (TE)	Abruzzo	34	2.5x2.5	8000	1280
Cabernet Sauvignon	Faenza (RA)	Emilia-Romagna	20	3x1.1	10.000	3030
Sangiovese	Faenza (RA)	Emilia-Romagna	20	3x1.1	10.000	3030

Table 2. Treatment plan applied in five GLSD affected vineyards in 2013-2015.

Year	Vineyard	Treatment	No. of applications	Plots	No. of vines	Survey date
2013	Trebbiano d'Abruzzo	Full mixture[a]	9	2	140	20 September
		Full mixture	6	2	140	
		Untreated control	-	2	140	
	Montepulciano d'Abruzzo-1	Full mixture	9	2	140	26 September
		Full mixture	6	2	140	
		Untreated control	-	2	140	
	Montepulciano d'Abruzzo-2	Full mixture	9	3	150	27 September
		Untreated control	-	3	150	
2014	Trebbiano d'Abruzzo	Full mixture	9	2	140	19 September
		Full mixture	6	2	140	
		Untreated control	-	2	140	
	Sangiovese	Full mixture	9	4	360	23 September
		Untreated control	-	4	360	
	Cabernet Sauvignon	Full mixture	9	4	360	24 September
		Untreated control	-	4	360	
2015	Trebbiano d'Abruzzo	Full mixture	9	2	100	14 September
		Full mixture + other a.i.s[b]	9	2	100	
		Seaweed	9	2	100	
		Untreated control	9	2	100	
	Sangiovese	Full mixture	9	4	360	22 September
		Untreated control	-	4	360	
	Cabernet Sauvignon	Full mixture	9	4	360	23 September
		Untreated control	-	4	360	

[a] Full mixture = $CaCl_2 + Mg(NO_3)_2$ + seaweed extract.

[b] All the applications were carried out with the full mixture, except in 2015 in the Trebbiano d'Abruzzo vineyard, where the full mixture in tank mix with the active ingredients (a.i.s) used for control of other diseases or pests, and the seaweed extract alone, were applied.

Table 3. Active ingredients distributed in tank mixes with the mixture of $CaCl_2$, $Mg(NO_3)_2$ and seaweed extract in the Trebbiano d'Abruzzo vineyard in 2015.

Application number	Application date	Active Ingredients	Concentration (%)	Dose (Kg/L ha^{-1})
1	04/05/2015	Cymoxanil + Mancozeb	4+40	3
		Spiroxamine	50	0.4
		Sulphur	80	2
2	14/05/2015	Cymoxanil + Mancozeb	4+40	3
		Spiroxamine	50	0.4
		Sulphur	80	2
3	25/05/2015	Propineb + Fluoplicolide	65+5	2
		Meptyldinocap	35.71	0.4
		Sulphur	57.3	1.5
4	04/06/2015	Fenamidone + Fosetyl Al + Iprovalicarb	4+52+4.8	2.5
		Quinoxyfen + Myclobutanil	4+3.81	1.25
		Sulphur	57.3	1
5	15/06/2015	Fenamidone + Fosetyl Al + Iprovalicarb	4+52+4.8	2.5
		Quinoxyfen + Myclobutanil	4+3.81	1.25
		Sulphur	57.3	1
6	25/06/2015	Ametoctradin + Metiram	12+44	2.5
		Fluopyram + Tebuconazole	17.7+17.7	0.35
		Emamectin benzoate	0.95	1.5
		Sulphur	57.3	1
7	06/07/2015	Cymoxanil + Zoxamide + Fosetyl Al	2.5+4+32.5	4
		Cyflufenamid	5.1	0.5
		Sulphur	80	2
8	16/07/2015	Cymoxanil + Zoxamide + Fosetyl Al	2.5+4+32.5	4
		Meptyldinocap	35.71	0.4
		Sulphur	80	2
9	27/07/2015	Copper oxychoride + Zoxamide	25+5.88	3
		Sulphur	80	2
		Emamectin benzoate	0.95	1.5

collected from GLSD-symptomatic vines came both from symptomatic and asymptomatic shoots.

For each group of plants (healthy, diseased asymptomatic and diseased symptomatic), treated and untreated with mixture, three vines were selected; in each healthy and diseased asymptomatic vine three clusters were chosen. In each diseased symptomatic vine, six clusters were chosen, three from the symptomatic shoots and three from asymptomatic shoots. Each cluster was at the mid-point along the vineshoot. From each cluster nine berries were collected (three each from the cluster wings, center or tip).

Berry samples were subjected to compression-relaxation tests, using the Instron U.T.M. mod. 5542 (Wycombe, UK) equipped with a 500 N load cell and a piston of 35 mm diam., with a speed of 20 mm min^{-1} in the compression phase. Relaxation time was 70 s. The following parameters were registered: maximum load (N), which expresses the force needed to deform each berry by 20%; displacement (mm), a measure of 1/5 of the berry radius and define the berry size; and relative relaxation load (%), which expresses the force dissipated in viscous flow.

Measurements of berry absorbance difference

In the Trebbiano d'Abruzzo vineyard, at harvest ripening (September 2015), a non-destructive measurement of the absorbance difference (IAD) between treated and untreated visually healthy berries of healthy, asymptomatic or symptomatic vines was carried out using a DA-Meter hand-held device, Mod. 53500, T.R. Turoni srl (Noferini *et al*. 2006). This vis/NIR portable spectrometer measured the chlorophyll content (Chl-a) using absorbance properties (difference between 670 nm and 720 nm wavelengths), as an index of berry ripening. IAD is directly correlated with Chl-a content in fruit mesocarps ($R^2 = 0.979$) during ripening (Ziosi *et al.*, 2008).

For each group of plants, healthy, diseased asymptomatic and diseased symptomatic, treated or untreated, six plants were chosen; in each vine, *in planta* measurements were carried out on 16 clusters; in each cluster three berries were measured in the central portion only, to avoid differences in the ripening of the different berries of the cluster. In the symptomatic vines, the measurements were carried out in 16 clusters each from symptomatic and asymptomatic shoots.

Statistical analyses

Differences of incidence and severity of foliar symptoms recorded in control plots, and those treated with the foliar nutrient mixture, were compared by (Chi-square) tests at $P \leq 0.05$, according to the methods reported by Calzarano *et al*. (2014).

For each sampling date, data of mechanical measurements (maximum load, relative relaxation load and displacement) on the berries of the different vines and shoots were subjected to analysis of variance (ANOVA). Mean values were compared by Tukey's honest significant difference (HSD) test at $P \leq 0.05$.

Absorbance differences of the berries were measured at harvest. Data of each treated group of vines and shoots were compared with the correspondent untreated group by analysis of variance (ANOVA) at $P \leq 0.05$.

Statistical analyses were carried out using SAS version 9.3 (SAS Institute Inc.).

Results

Activity of foliar applications on GLSD symptom expression

In all trials, the full mixture significantly reduced the incidence and severity of GLSD symptoms both after nine or six mixture applications.

The greatest activity of the mixture for reducing foliar symptom incidence and severity was recorded in the Abruzzo vineyards treated with nine full mixture applications.

The nine-application strategy significantly reduced ($P < 0.05$) mean GLSD incidence in 2013 in the Montepulciano d'Abruzzo-2 vineyard, from 18.8% in the untreated vines to 1.3% in the treated vines. In 2015 in the Trebbiano d'Abruzzo vineyard, mean GLSD incidence was 55.5% for the untreated vines and 8.4% for those treated with the mixture (Figures 1 and 3). In 2013 the same statistically significant reduction (18.0% in untreated vines and 3.2% in treated vines) was also recorded in the Montepulciano d'Abruzzo-1 vineyard (Figure 1; Table 4).

For each of these vineyards and trial years, severity of leaf symptoms was also significantly decreased: in the Montepulciano d'Abruzzo-2 vineyard, from 9.6% in the untreated vines to 0.6% in treated vines; in the Trebbiano d'Abruzzo vineyard, from 11.1 to 3.3%, and in the Montepulciano d'Abruzzo-1 vineyard, from 10.9 to 1.0% (Figures 1 and 3; Table 4).

In the Trebbiano d'Abruzzo vineyard in 2013 and 2014, the nine-application strategy gave less reduction of symptom expression, but this was still statistically different compared with the untreated plots (Figures 1 and 2; Table 4).

In the Emilia-Romagna trials, the difference in symptom incidence between the untreated plots and the nine-application treated plots was less, but still statistically significant ($P < 0.05$). In the Cabernet-Sau-

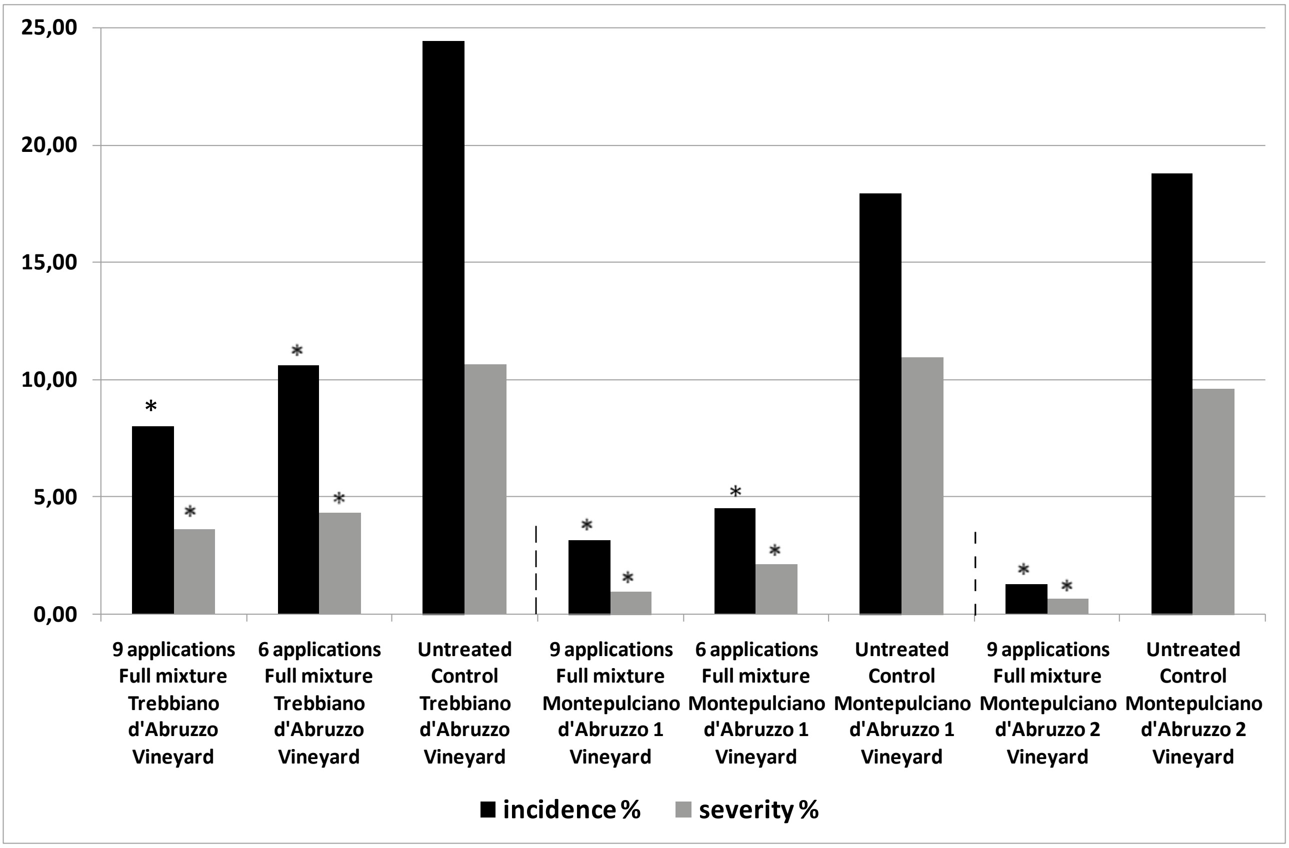

Figure 1. Mean incidence and severity of GLSD leaf symptoms in a Trebbiano d'Abruzzo and two Montepulciano d'Abruzzo vineyards, after applications of the mixture of $CaCL_2$, $Mg(NO_3)_2$ and seaweed extract in 2013. Chi-square statistics compared the full mixture treatments to the untreated controls in three trials. In the Trebbiano d'Abruzzo and Montepulciano d'Abruzzo 1 vineyards, the nine applications treatment was also statistically compared with the six applications treatment. For symptom incidence or severity, * indicates statistically significant differences ($P < 0.05$) between treatments. Bars labelled with the same symbol are not statistically different.

vignon vineyard, the mean incidence of symptomatic vines was, in 2014, 54.5% in the untreated plots and 36.6% in the treated plots, and in 2015, 45.8% for untreated and 27.9% for treated plots. In the Sangiovese vineyard in 2014, mean incidence in untreated plots was 39.6% and 17.3% for treated plots, and in 2015 was 35.7% for untreated plots and 19.7% for treated plots (Figures 2 and 3; Table 4).

Although the effects of mixture applications on the incidence of GLSD were less, the reductions in severity of the disease were greater in the Emilia-Romagna vineyards compared to the Abruzzo vineyards. For the Cabernet Sauvignon vineyard in 2014, the mean severity indices were 31.1% for the untreated vines and 13.0% for treated vines, and in 2015 were 23.9% for untreated and 11.2% for treated vines. Similar results were obtained in the Sangiovese vineyard (Figures 2 and 3; Table 4).

The differences in incidence of symptomatic vines between untreated and treated plots were less where in the six-application strategy was applied compared to the nine-application strategy. The mean incidence of symptomatic vines in the Trebbiano d'Abruzzo vineyard was, in 2013, 24.5% for untreated vines and 10.6% for treated vine, and in 2014 was 30.4% for untreated and 12.2% for treated. In the Montepulciano d'Abruzzo-1 vineyard, in 2014, mean incidence of symptomatic vines was 18.0% for untreated vines and 4.5% for the treated vines (Figures 1 and 2; Table 4).

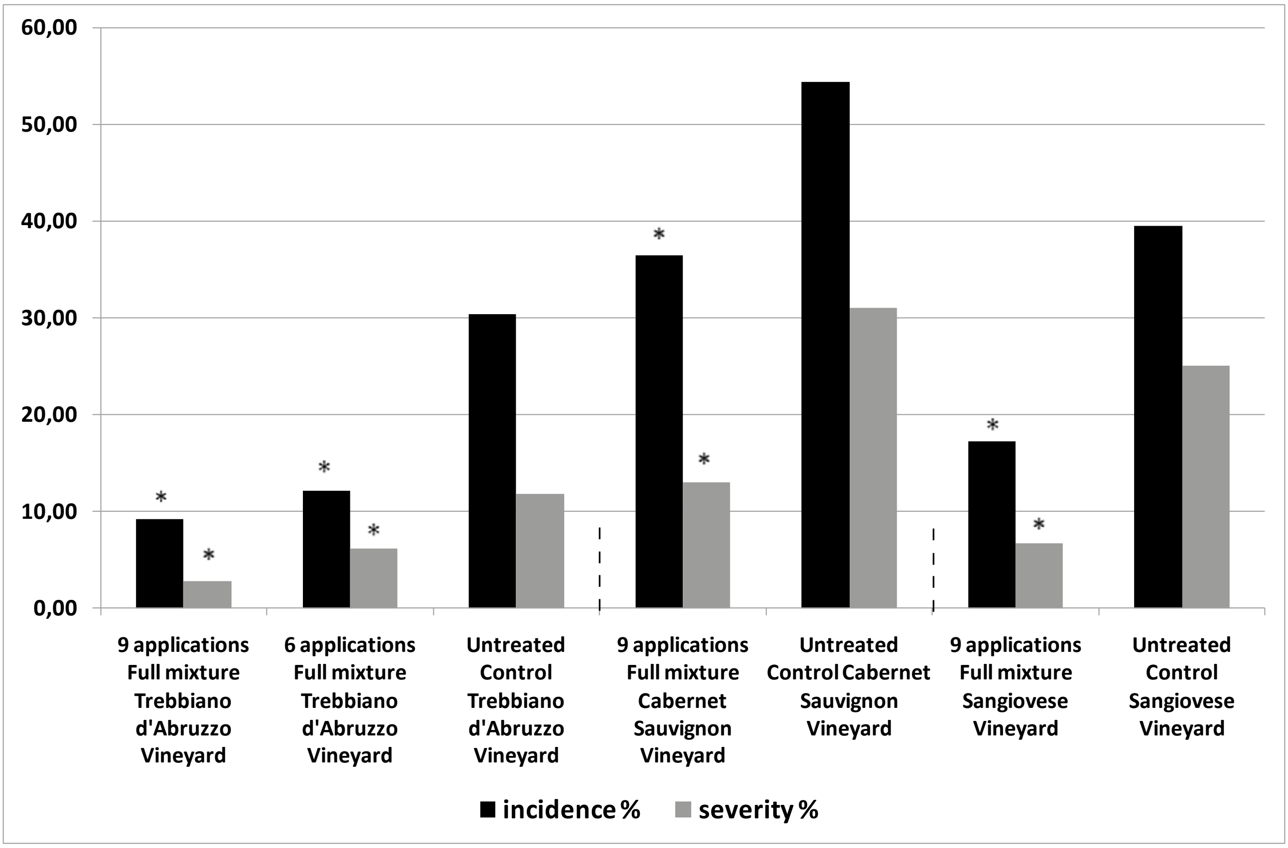

Figure 2. Mean incidence and severity of GLSD leaf symptoms in Trebbiano d'Abruzzo, Cabernet Sauvignon and Sangiovese vineyards after applications of the mixture of $CaCL_2$, $Mg(NO_3)_2$ and seaweed extract in 2014. Chi-square statistics compared the full mixture treatments to the untreated controls in three trials. In the Trebbiano d'Abruzzo vineyard the nine applications treatment was also statistically compared with the six applications treatment. For symptom incidence or severity, * indicates statistically significant differences ($P < 0.05$) between treatments. Bars labeled with the same symbol are not statistically different.

As with incidence of GLSD, the difference in the mean severity indices were less from the six-application strategy compared to that with nine applications. However, the differences between the six-application strategy and the untreated controls were statistically significant ($P < 0.05$) (Figures 1 and 2). No statistically significant differences were measured between the nine- and six-application strategies (Figures 1 and 2; Table 4).

In 2015, the active ingredients used for the control of other pests and diseases in combination with the nine applications of the full mixture did not cause any differences in the efficacy of the mixture towards GLSD symptoms in the Trebbiano d'Abruzzo vineyard, compared to the symptoms recorded for the nine applications with the full mixture alone (Figure 3; Table 4).

In 2015, the applications of the seaweed extract as a single component did not significantly reduce either incidence or severity of GLSD (Figure 3; Table 4).

Mechanical properties of berries

In the Trebbiano d'Abruzzo vineyard, in the first sampling carried out at the 79 BBCH growth stage, mechanical properties of grape berries were not affected ($P > 0.05$) by the mixture treatments, because the measured parameters were highly variable (Table 5).

The applications of the full mixture gave differences in the mechanical properties of the berries from

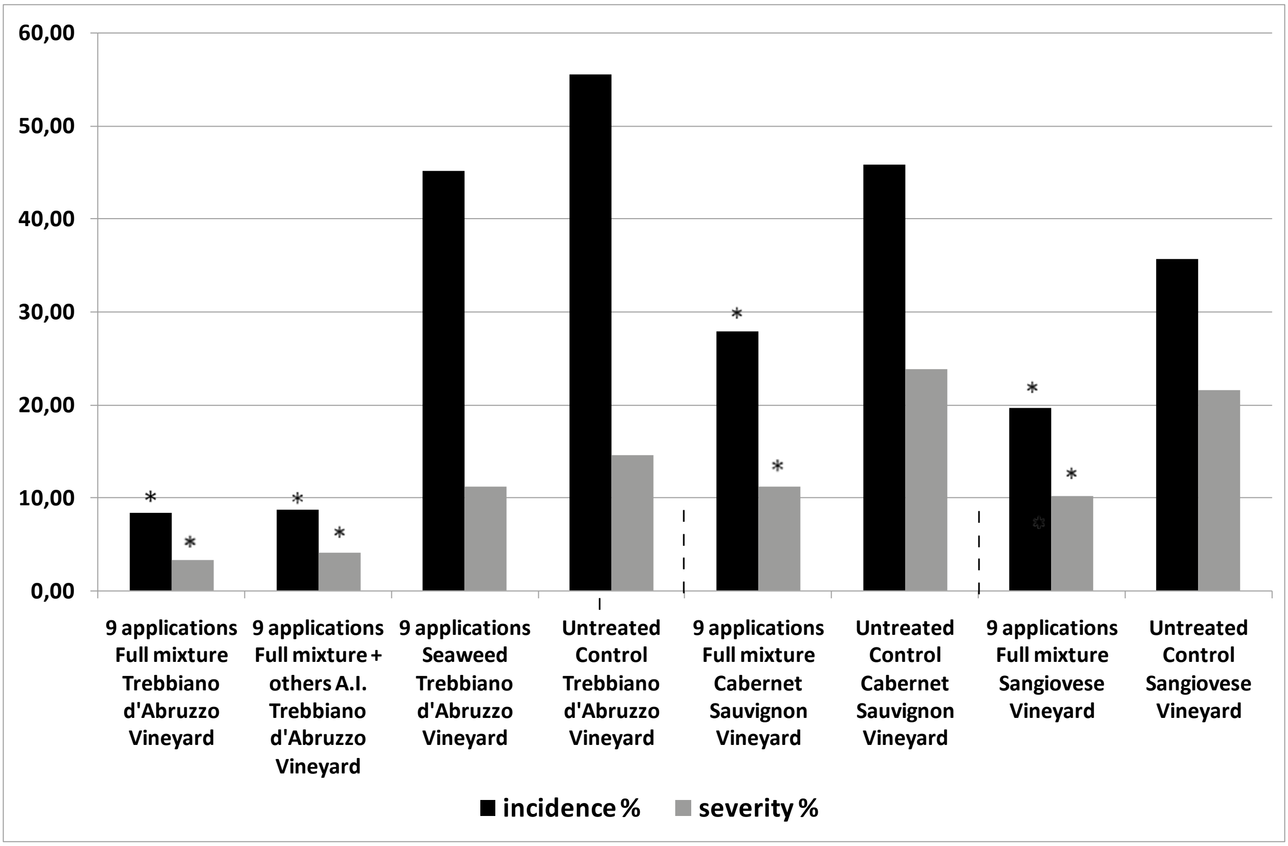

Figure 3. Mean incidence and severity of GLSD leaf symptoms in Trebbiano d'Abruzzo, Cabernet Sauvignon and Sangiovese vineyards after applications of the mixture of $CaCL_2$, $Mg(NO_3)_2$ or seaweed extract in 2015. Chi-square statistics compared the full mixture treatments to the untreated controls in three trials. In the Trebbiano d'Abruzzo vineyard, the full mixture, alone or in tank mix with active ingredients used for control of other diseases or pests, or seaweed alone treatments, were compared to the untreated control. For symptom incidence or severity, * indicates statistically significant differences ($P < 0.05$) between each treatment and the untreated controls.

healthy and asymptomatic vines compared with the untreated vines, starting from the second sampling assessment (81 BBCH) (Table 5).

In all the samples collected from healthy and asymptomatic vines, the mean maximum load measurements (N) were always significantly greater in the berries from untreated vines than those from full mixture treated vines, and were more evident in asymptomatic grapevines, except in the first sampling. In all of the trials, in berries from untreated healthy grapevines, the maximum load values decreased during the season up to harvest maturity, as occurred for the berries from untreated asymptomatic vines.

The mean N values of the berries from untreated diseased asymptomatic vines were always greater than in berries from untreated healthy vines (Table 5). On the other hand, in the treated vines the maximum load values of the berries from healthy and asymptomatic vines were not statistically different ($P \leq 0.05$) (Table 5). Therefore, in the treated vines, the maximum load values were not different ($P > 0.05$) between healthy and asymptomatic vines at harvest maturity.

Significant decreases in the values of mean relative relaxation load (%) were detected, commencing from the second sampling (BBCH 81) in the berries collected from treated healthy and asymptomatic vines compared to the berries of the corresponding controls (Table 5). Similar to the N values, in the treated vines healthy and asymptomatic berry values did not dif-

Table 4. Results of Chi-square ($P < 0.05$) statistical analyses of data from trials carried out in 2013, 2014 and 2015.

Year	Vineyard	Treatment comparison	Incidence (*P*)	Severity (*P*)
2013	Trebbiano d'Abruzzo	9 FM applications / Untreated control	<0.0001	0.0013
		6 FM applications / Untreated control	0.0007	0.004
		6 FM applications / 9 FM applications	0.3840	0.6341
	Montepulciano d'Abruzzo-1	9 FM applications / Untreated control	<0.0001	<0.0001
		6 FM applications / Untreated control	0.0037	0.0019
		6 FM applications / 9 FM applications	0.1333	0.069
	Montepulciano d'Abruzzo-2	9 FM applications / Untreated control	0.0445	0.0022
2014	Trebbiano d'Abruzzo	9 FM applications / Untreated control	<0.0001	<0.0001
		6 FM applications / Untreated control	<0.0001	0.0226
		6 FM applications / 9 FM applications	0.3448	0.2777
	Sangiovese	9 FM applications / Untreated control	<0.0001	<0.0001
	Cabernet Sauvignon	9 FM applications / Untreated control	<0.0001	<0.0001
2015	Trebbiano d'Abruzzo	9 FM applications / Untreated control	<0.0001	<0.0001
		9 FM applications + a.i.s / Untreated control	<0.0001	<0.0001
		9 Seaweed applications / Untreated control	0.1233	0.1873
	Sangiovese	9 FM applications / Untreated control	<0.0001	<0.0001
	Cabernet Sauvignon	9 FM applications / Untreated control	<0.0001	<0.0001

FM = full mixture ($CaCl_2$ + $Mg(NO_3)_2$ + seaweed extract).
All the applications were carried out with the full mixture, except in 2015 in the Trebbiano d'Abruzzo vineyard, where the full mixture in tank mixes with active ingredients (a.i.s) used for other disease or pest control, and the seaweed extract alone, were applied.

fer statistically from the second sampling (BBCH 81) through to harvest maturity (Table 5).

Mechanical properties of berries from symptomatic vines were not modified by the treatments. Mean maximum load and relative relaxation load values of berries from asymptomatic and symptomatic shoots were not statistically different in the untreated or treated vines (Table 5).

Differences were detected between berries from symptomatic shoots compared to those from asymptomatic shoots, with berries from symptomatic shoots always having greater values for both mechanical parameters (Table 5).

The berries collected from asymptomatic shoots of GLSD-symptomatic vines showed no statistical differences ($P \leq 0.05$) in mechanical parameters from those sampled from asymptomatic vines (Table 5).

Mean displacement measurements (in mm) ranged between 2.68 and 3.32 mm. This indicated that the full mixture applications did not influence berry size. For each time of sampling and treatment, these values were not statistically different, although the berries from symptomatic shoots showed a tendencies reduced size compared to other treatments, noticeable from the third sampling (BBCH 83).

Measurements of berry absorbance difference

In 2015, in the Trebbiano d'Abruzzo vineyard the full mixture applications modified mean absorbance difference indices, recorded at harvest maturity on healthy and asymptomatic vines. The absorbance difference indices were significantly less ($P \leq 0.05$) in the treated vines compared the corresponding untreated vines (Figure 4). No differences in absorbance difference index was induced by the full mixture applications in berries from symptomatic vines (Figure 4).

Table 5. Mechanical properties recorded in 2015, and statistical analysis results (HSD at $P\leq0.05$) for berries of cv. Trebbiano d'Abruzzo treated with the mixture of $CaCl_2$, $Mg(NO_3)_2$, seaweed extract, and in the untreated control. HSD = Tukey's honest significant difference test.

Visually healthy berries of each group of vines	26/07/15 79 BBCH	09/08/15 81 BBCH	16/08/15 83 BBCH	30/08/15 85 BBCH	10/09/15 89 BBCH
Maximum Load (N)					
Healthy vines	9.2 a	4.9 b	4.1 b	3.7 b	3.1 b
Treated Healthy vines	7.5 a	4.1 a	3.4 a	3.1 a	2.7 a
Asymptomatic diseased vines	10.2 a	6.0 c	5.0 c	4.4 c	3.8 c
Treated asymptomatic diseased vines	7.6 a	4.1 a	3.5 a	3.1 a	2.8 a
Asymptomatic shoots of symptomatic vines	8.2 a	5.9 c	4.9 c	4.5 c	3.9 c
Asymptomatic shoots of treated symptomatic vines	7.8 a	6.0 c	5.0 c	4.5 c	3.9 c
Symptomatic shoots of symptomatic vines	9.1 a	6.0 c	5.9 d	6.0 d	5.0 d
Symptomatic shoots of treated symptomatic vines	10.0 a	6.1 c	6.0 d	6.1 d	5.0 d
Relative Relaxation Load (%)					
Healthy vines	44.6 a	36.9 b	35.8 b	34.5 b	33.0 b
Treated Healthy vines	42.7 a	36.0 a	34.2 a	32.8 a	32.5 a
Asymptomatic diseased vines	45.1 a	40.8 c	37.6 c	36.4 c	34.7 c
Treated asymptomatic diseased vines	42.1 a	35.8 a	34.1 a	32.9 a	32.6 a
Asymptomatic shoots of symptomatic vines	42.7 a	40.9 c	37.4 c	36.5 c	35.0 c
Asymptomatic shoots of treated symptomatic vines	42.3 a	40.8 c	37.3 c	36.4 c	34.9 c
Symptomatic shoots of symptomatic vines	44.0 a	41.1 c	41.1 d	40.2 d	37.3 d
Symptomatic shoots of treated symptomatic vines	46.0 a	40.8 c	41.1 d	40.1 d	37.7 d

BBCH 79: Majority of berries touching; BBCH 81: Beginning of ripening: berries begin to develop variety-specific colour; BBCH 83: Berries developing colour; BBCH 85: Softening of berries; BBCH 89: Berries ripe for harvest.

Discussion

The treatments with the full mixture based on calcium and magnesium salts and seaweed extract, carried out in the three-year period from 2013 to 2015 in different vineyards located in the Abruzzo and Emilia-Romagna Regions, proved to be effective for reducing GLSD symptom expression in grapevine leaves. The nine-application strategy for the full mixture showed the greatest activity, confirming the results previously obtained in 2010-2012 in the Abruzzo vineyards (Calzarano *et al*., 2014).

The six-application strategy was less effective than the nine-application strategy, but this difference was not statistically significant. Therefore, the adoption of a reduced number of applications (from nine to six) may lead to consistent reduction of symptoms expression. The most important point demonstrated in this study is the possibility of carrying out applications of the full mixture combined in tank mixes with other plant protection products normally used in vineyards, without decreasing the efficiency of the full mixture. This means that use of the mixture for management of GLSD will not impose economic impacts to normal management operations. However, trials on the efficiency of tank mixes of the mixture with other pesticides need to be repeated before these results can be confirmed. The applications of the seaweed alone were not effective for reducing incidence of GLSD symptoms or severity. The very low activity of the seaweed for suppressing symptoms was highlighted in a previous study on the effect of biostimu-

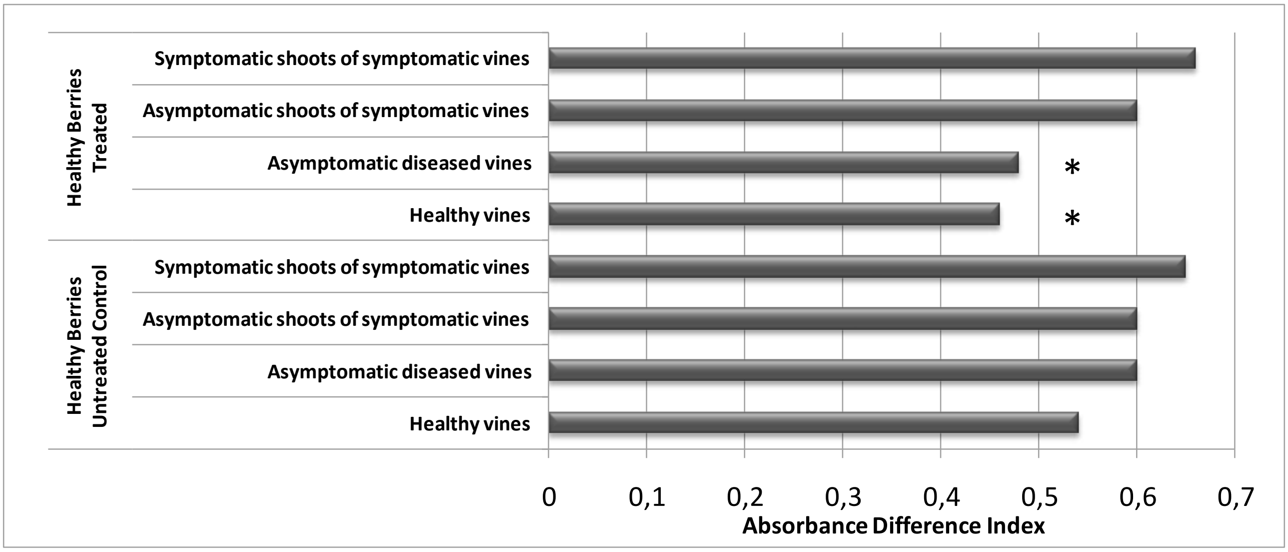

Figure 4. Mean absorbance difference values for grape berries from the Trebbiano d'Abruzzo vineyard recorded in 2015. Comparisons between treated and untreated vines were performed by analysis of variance (ANOVA). Values of the treated group labelled * are statistically different ($P \leq 0.05$) from the corresponding untreated group.

lants on vines affected by the esca complex (Di Marco and Osti, 2009). The present study confirms that the seaweed could play a role as a carrier of calcium and magnesium, thus enhancing the activity of these nutrients towards development of GLSD foliar symptoms (Calzarano *et al*., 2014).

The lack of activity of seaweed for reducing GLSD symptoms strengthens the hypothesis of the roles of calcium and magnesium salts in symptom development. These compounds probably interfere with oxidative burst, which causes formation of leaf lesions (Andolfi *et al*., 2009). The assumption is that the host reaction would be triggered by toxic metabolites produced by esca pathogens in grapevine wood, and these metabolites are then translocated to leaves (Evidente *et al*., 2000; Tabacchi *et al*., 2000). The defense response in vines treated with the full mixture was associated with increased amounts of *trans*-resveratrol, flavonoids, and calcium oxalate druse crystal (as morphological barriers), in grapevine leaves (Calzarano *et al*., 2014).

Berries from healthy and asymptomatic vines treated with the full mixture had improved ripening characteristics, indicated as reduced firmness (N) and increased elasticity (%), compared to berries from untreated vines (Bernstein and Lustig, 1981; Lang and During, 1990; McQueen-Mason and Cosgrove, 1995; Sessiz *et al*., 2017). In addition, at harvest maturity, the full mixture treatments gave similar ripening levels in berries from asymptomatic and healthy vines, thus improving the berry quality compared to untreated asymptomatic vines, in which higher N and % values were recorded.

These results are consistent with those obtained by Calzarano *et al*. (2014). In that study, the treated vines, both healthy and asymptomatic, produced increased grape quantity in comparison to untreated vines. The sugar contents were greater in the treated asymptomatic vines compared to the untreated asymptomatic vines, but this difference was not detected between treated and untreated healthy vines.

Berries from GLSD asymptomatic vines had lower sugars contents than berries from healthy vines, with the greatest content of sugars in the Trebbiano d'Abruzzo cultivar (Calzarano *et al*., 2001; 2004). Therefore, in the asymptomatic vines the decrease in sugars was counteracted by the applications of the full mixture, which did not act on healthy vines (Calzarano *et al*., 2017a). The lower absorbance difference indices assessed in the treated vines, indicating decreased chlorophyll content, and confirmed greater ripening levels on the treated healthy and asymptomatic vines (Ziosi *et al*., 2008).

The applications of the full mixture did not improve the ripening of healthy berries on symptomatic vines, both in asymptomatic and symptomatic shoots. Mechanical and absorbance difference measurements did not show differences between treated and untreated berries from symptomatic vines. This indicates that the physiological processes of vines with foliar symptoms are too altered to benefit from the mixture applications.

Berries from symptomatic shoots of symptomatic vines were firmer and less elastic (higher N and relative relaxation load levels) compared to the berries of the other vine groups. While increased firmness indicates reduced ripening, reduced elasticity highlights the incoherent and watery structure of the berries from symptomatic vines. This reflect physiological alterations and an anomalous ripening trend (Bernstein and Lustig, 1981; Lang and During, 1990; McQueen-Mason and Cosgrove, 1995; Sessiz *et al*., 2017).

This research further highlights the correlation between symptom expression of this type of trunk diseases and host physiology (Magnin-Robert *et al.*, 2011). Nutrients and mineral elements could play roles in stress responses initiated by pathogen infections (Bertsch *et al*., 2013), affecting fungal growth (Amorabe *et al*., 2005) or pathogenic mechanisms (Osti and Di Marco, 2010; 2014).

Improvements of yield quality previously assessed by Calzarano *et al.,* (2014) as berry sugar content, were further demonstrated in the present study, as indicated by the mechanical and absorbance difference measurements.

The use of biocontrol agents such as *Trichoderma* spp. (Gramaje *et al*., 2018; Di Marco *et al*., 2004) and the application of the calcium, magnesium and seaweed represent important tools with proven effects, for reducing GLSD symptom expression in vineyards.

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