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Effect of biostimulant sprays on *Phaeomoniella chlamydospora* and esca proper infected vines under greenhouse and field conditions

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Summary. Biostimulants are compounds that influence physiological processes in plants, producing better growth and enhancing stress tolerance. The effect of some biostimulants on vines was investigated over a number of years to assess their effect both on the incidence of esca leaf symptoms in the vineyard and on the growth of *Phaeomoniella chlamydospora* artificially inoculated into potted vines. Field trials were carried out for 4 –7 years in five 15 – 20-year-old vineyards infected with esca proper. Potted plants were sprayed with biostimulants, after which the vine trunks were inoculated with *P. chlamydospora*, and then the vines were sprayed again with biostimulants in the following 2 or 3 growing seasons. On the whole, biostimulants in the field did not reduce foliar symptoms. The percentage of symptomatic vines that had shown symptoms in previous years was higher in the biostimulant-sprayed plots. In the greenhouse, a certain reduction of internal necrosis caused by *P. chlamydospora* was seen with three of the four biostimulants tested. Prospects for biostimulants as a means control esca are discussed.

Key words: grapevine, control, *Phaeomoniella chlamydospora*.

Introduction

Although many studies are currently evaluating different methods and strategies to combat esca, control of the esca disease complex is still lacking. Esca control in mature vineyards, characterized by vines with dark wood streaking (tracheomycosis) and white rot, is still based on cultural practices, such as the renewal of the trunks of symptomatic plants, excising all the necrotic wood, and removing infected plants and/or pruning debris (Di Marco *et al*., 2000; Surico *et al*., 2006a; 2006b). These practices reduce the inoculum load and the risk of new infections, but they do not cure already existing infections (Monstert *et al*., 2006).

The esca complex is correlated with the physiology of the host plant. When the vine is physiologically healthy, esca pathogens live inside the plant without producing symptoms for several years. When stress conditions arise, however, infected vines also begin to suffer the negative consequences of these pathogens, consisting in potentially severe foliar symptoms and losses in yield. Esca is therefore a stress-related disease (Ferreira *et al*., 1999; Rooney-Latham *et al*., 2005; Surico *et al*., 2006b; Edwards *et al*., 2007).

Numerous studies on esca-infected vines have shown that the extent of wood deterioration is not correlated with the expression of foliar symptoms, but that foliar symptoms are correlated with toxins produced by the tracheomycotic fungi (Mugnai *et al*., 1999; Surico *et al*., 2000; Abou-Mansour *et al*., 2004; Marchi *et al*., 2006; Calzarano and Di Marco, 2007; 2008). There is, however, a relationship between foliar symptoms and the quality of both grape clus-

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ters and the wines pressed from them. Symptomatic vines have a lower quality, whereas asymptomatic vines, even if they are in fact diseased, produce grapes with quality-characteristics similar to the grapes of healthy vines (Calzarano *et al*., 2001; Calzarano *et al*., 2004b). Therefore, strategies aimed at reducing the incidence and/or severity of foliar symptoms would also limit loss in quality.

Biostimulants are non-nutritional compounds that promote favourable plant responses (Zhang, 1997; Miller and Gange, 2003; Schmidt *et al*., 2003), boosting physiological processes, producing faster growing and healthier plants, improving crop yield and quality, and enhancing stress tolerance (Blake, 2002; Fregoni and Fregoni, 2005). A recent Italian legislative decree published in the *Gazzetta Ufficiale* (2006) defined biostimulants as "products that are added to other fertilizers and/or to the soil and/or to plants, that promote or regulate the absorption of nutrients by plants, and that improve physiological anomalies". Phytohormones, or active ingredients with a specific phytosanitary function, are not biostimulants (Ciavatta and Cavani, 2006).

In some preliminary studies, biostimulants were sprayed on esca-infected vineyards in Emila Romagna and Abruzzo. The treatments were applied for only a short period (two years), and they did not lower esca severity (Di Marco and Osti, 2005a; Calzarano *et al*., 2007).

Over a considerable number of years, biostimulants were experimentally sprayed on vineyards affected with esca proper, in order to assess their effect on esca incidence in the field. These biostimulants were also tested in greenhouse trials on potted vines inoculated with *Phaeomoniella chlamydospora*, considered a representative tracheomycotic agent causing esca. This paper reports on the findings obtained in those experiments.

Materials and methods

Greenhouse trials

Two experiments were carried out over a five-year period, the first in 2003-2005, and the second in 2006–2007. Twelve replicates per treatment, each consisting of one potted vine, were set up.

Plant material

One-year-old vines, cv. Trebbiano, grafted on SO4, (first experiment) and cv. Cabernet Sauvignon Omega, grafted on 1103P, (second experiment), were obtained from a commercial nursery and planted in 20 cm diameter plastic pots containing 70% of a commercial mixture of peat with nutrients and elements at low concentrations (Floragard TKS2, Oldenburg, Germany) and 30% perlite. Potted plants were grown outdoors, watered normally, treated with copper or sulphur-based compounds, and protected from hail with screens.

Biostimulant spraying and fungal inoculation

Vines were sprayed on the leaves with a portable atomizer sprayer (capacity 5 l). They were sprayed four times every year at 10- to 14-day intervals, and following manufacturer's dosage instructions, for 3 years, except for Brotomax, for which the vineyard spraying schedule recommended by the manufacturer was followed (Table 1). In the first year, seven days after the last spraying, potted vines were inoculated with *P. chlamydospora* strain 56 (CBS 229.95). A hole 5 mm in diameter was drilled into the trunk and a plug from a 2-week-old PDA fungal culture was inserted (a sterile plug was inserted for the control). The holes were covered with Amojell lubricant (Sigma-Aldrich) and protected with Parafilm M (American National Can, Chicago, IL, USA) to prevent drying. The second experiment followed the same biostimulant spraying schedule but used Marvita rather than Fitostim as the biostimulant.

Evaluation of sprayings, fungal re-isolations, and data analysis

The effect of the biostimulant sprays was determined by assessing the vegetative characteristics of the sprayed vines during each growing season, the internal longitudinal spread of the necrosis through the vines, and the re-isolation of the pathogen from the discoloured wood. Necrotic lesions were measured during each growing season, and a final assessment (based on at least 7 vines per type of spray given) was made 30 months after inoculation in Experiment 1, and 18 months after inoculation in Experiment 2. For the final assessment, the trunks were cut lengthwise from the inoculation site and the length of the necrotic lesions was measured. The data were expressed as the average length of necrosis and statistically analyzed using Duncan's Multiple Test, P=0.05.

Soon after the lesions were measured, 28 fragments of necrotic wood (4 per plant) from each spray treatment for the first experiment, and 21 fragments (3 per plant) for the second experiment, were cut aseptically and placed on PDA amended with streptomycin sulfate (Merck) at 800 mg l^{-1} in order to assess the viability of *P. chlamydospora* from the inoculated plants. Plates were maintained at 25±2°C in the dark. The percentage of fertile fragments of *P. chlamydospora* was calculated.

Vineyard trials

The trial was conducted in 2001–2007 in 5 vineyards. Biostimulants were sprayed with a pressure atomizer. Half of the inspected vines in each vineyard was sprayed. Details on field spraying are summarized in Table 1.

In each vineyard, the trunks of some vines were cut and the wood inspected to determine wood discoloration and decay caused by esca.

Every growing season, between the end of August and the third week of September, at the time of the maximum expression of esca symptoms, the incidence of esca was calculated in each vineyard by dividing the number of vines with visible symptoms by the total number of inspected vines (excepted dead vines). Data were expressed as annual and as cumulated incidence (Calzarano and Di Marco, 2007).

The percent frequency of vines with foliar symptoms was also calculated, and expressed as the ratio in a given year of the number of symptomatic vines that had shown symptoms in previous years over

Table 1. Biostimulants used: composition, application rate and schedule, plant phenological stage and vineyard details.

Trade name	Composition	Plant phenological stage[a] (application schedule)	Application			Cultivar	Vines inspected (No.)
			Rate[b]	No.[c]	Years		
Brotomax	Aluminium lignin sulphate, gluconic acid, microelements	I: shoot 25 cm long II: 20 days after I III: berries beginning to touch IV: end of harvest	1 l hl^{-1}	4	2001–2007	Pignoletto	445
Fitostim	Aminoacids, peptides, peptones	From pre-flowering to majority of berries touching (14 day-interval)	150 ml hl^{-1}	4	2003–2005	Pignoletto	290
Kendal	Glutatione, oligosaccharine	From pre-flowering to majority of berries touching (10- to 14-day interval)	30 ml hl^{-1}	4	2004–2007 2003–2006	Albana Sangiovese	115 498
Marvita	*Aschophyllum nodosum* extract	From pre-flowering to majority of berries touching (10- to 14-day interval)	300 g ha^{-1}	5	2004–2007	Pignoletto	476

[a] The phenological stages are described according to BBCH scheme.
[b] The application rate refers to the commercial formulations.
[c] No. of sprayings per year.

the total number of symptomatic vines assessed in that year, and multiplying by 100. This percentage was first calculated the year after the biostimulant sprays were initiated.

Vineyards were also inspected for esca-related mortality (occurrence of discoloration and decay in dead vine wood). Vines that died in the first year of the study were excluded because it was not certain whether these plants died in that year or in a previous year. Data were expressed as annual and as cumulated incidence of mortality.

For each year of assessment, the annual cumulated incidence of mortality, the incidence of foliar symptoms, and vine mortality were subjected to Chi-Square statistical analysis (P=0.05) using SAS version 8.1 (Anonymous, 1990).

Results

Greenhouse trials

Results of greenhouse trials are summarized in Table 2.

At the end of the first experiment (30 months after inoculation) biostimulant-sprayed vines showed a certain reduction in the necrotic streaks caused by *P. chlamydospora*. This reduction was significant (P<0.05) only with vines sprayed with Fitostim (7.4 cm), as compared with unsprayed inoculated control plants (11.0 cm). At the end of the second experiment (18 months after inoculation) vines sprayed with Brotomax and Marvita showed a significant (P<0.05) reduction in necrotic lesions from *P. chlamydospora* (4.9 cm and 4.1 cm respectively) in comparison with the unsprayed inoculated control vines (6.7 cm).

Phaeomoniella chlamydospora was isolated from the necrotic lesions of both sprayed and unsprayed vines. However, vines sprayed with biostimulants had lower percentages of fragments with *P. chlamydospora*: ranging from 60.7 % (with Kendal and Brotomax, first experiment) to 76.2% (second experiment) compared with the control (75.0% - 85.7%).

Vineyard trials

Both vineyards sprayed with Kendal had a higher annual incidence of esca than the unsprayed vineyards (P>0.05), but the difference

Table 2. Effects of sprays of biostimulants on *Phaeomoniella chlamydospora* (Pch) inoculated in the trunk of potted grafted grapevines.

Treatment	Experiment 1 (30 m.a.i. [a])		Experiment 2 (18 m.a.i.[a])	
	Average necrosis length (cm)	Fertile Pch fragments (%)	Average necrosis length (cm)	Fertile Pch fragments (%)
Brotomax	9.8 a[b]	60.7	4.9 b[b]	71.4
Fitostim	7.4 b	64.3	nt	nt
Kendal	10.0 a	60.7	6.7 a	76.2
Marvita	nt	nt	4.1 b	76.2
Untreated control	11.0 a	75.0	6.7 a	85.7

[a] Months after inoculation.
[b] Values in column followed by the same letter do not differ significantly according to Duncan's Multiple Range test (P=0.05).
nt, Not tested.

was not significant (Fig. 1A, B). The cumulated incidence gave contradictory results: the percentage of symptomatic 'Albana' vines in the Kendal-sprayed plot increased, though not significantly ($P>0.05$), whereas the percentage of 'Sangiovese' vines decreased, as compared with the unsprayed plot. In the first four years of Brotomax spraying (2001–2004), the percentage of symptomatic vines

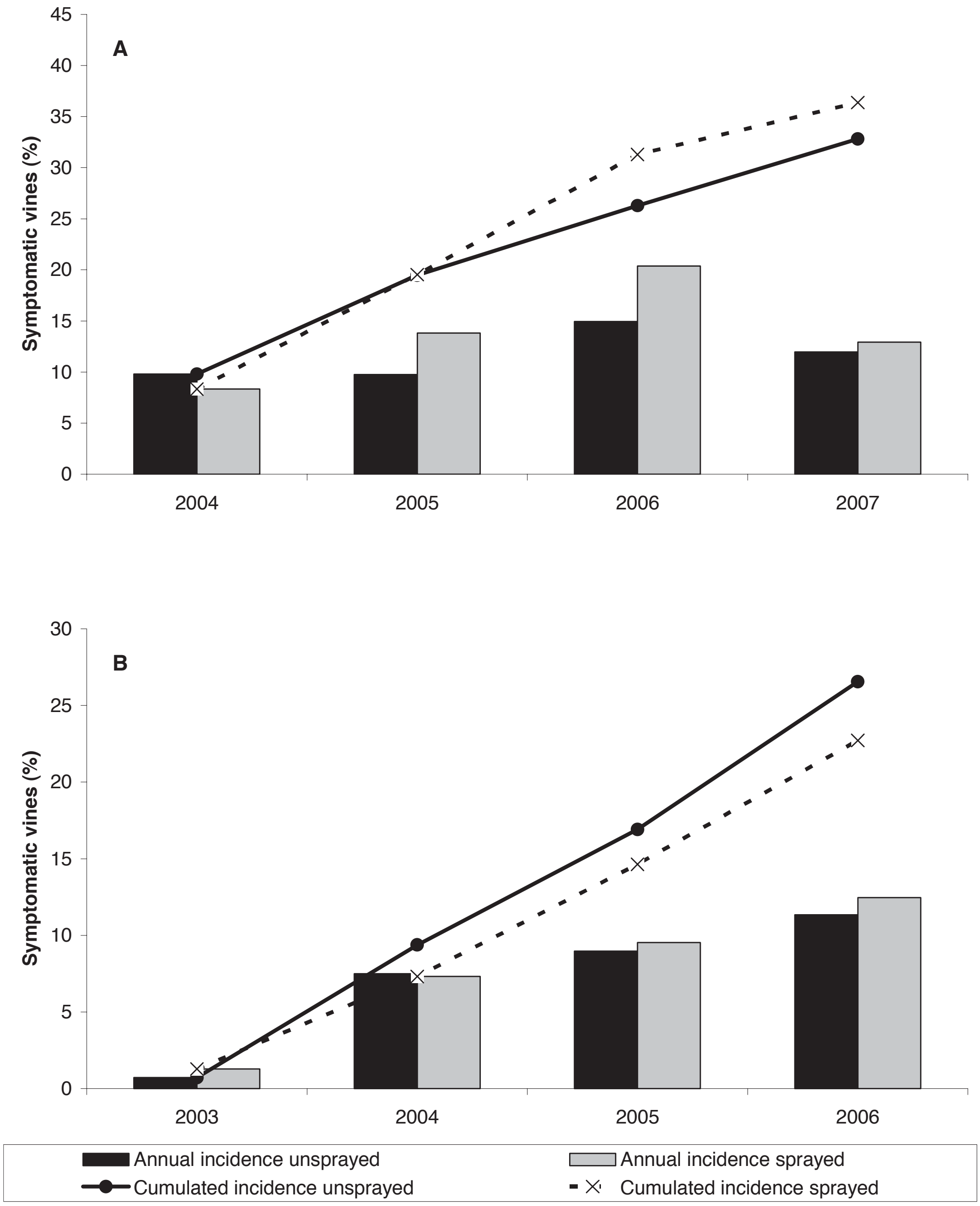

Fig. 1. Effects of applications of Kendal on annual incidence (bars) and cumulated incidence (lines) assessed in vineyards cv. Albana (A) and Sangiovese (B). For each treatment and year of assessment, values of annual incidence or cumulated incidence are not significantly different according to Chi–Square test ($P>0.05$).

in the sprayed plot marginally increased and the cumulated esca incidence was statistically higher than in the unsprayed plot (Fig. 2A). Starting from 2005, the annual incidence in the sprayed plots slightly decreased, and this decrease became significant ($P<0.05$) in 2007. The cumulated incidence

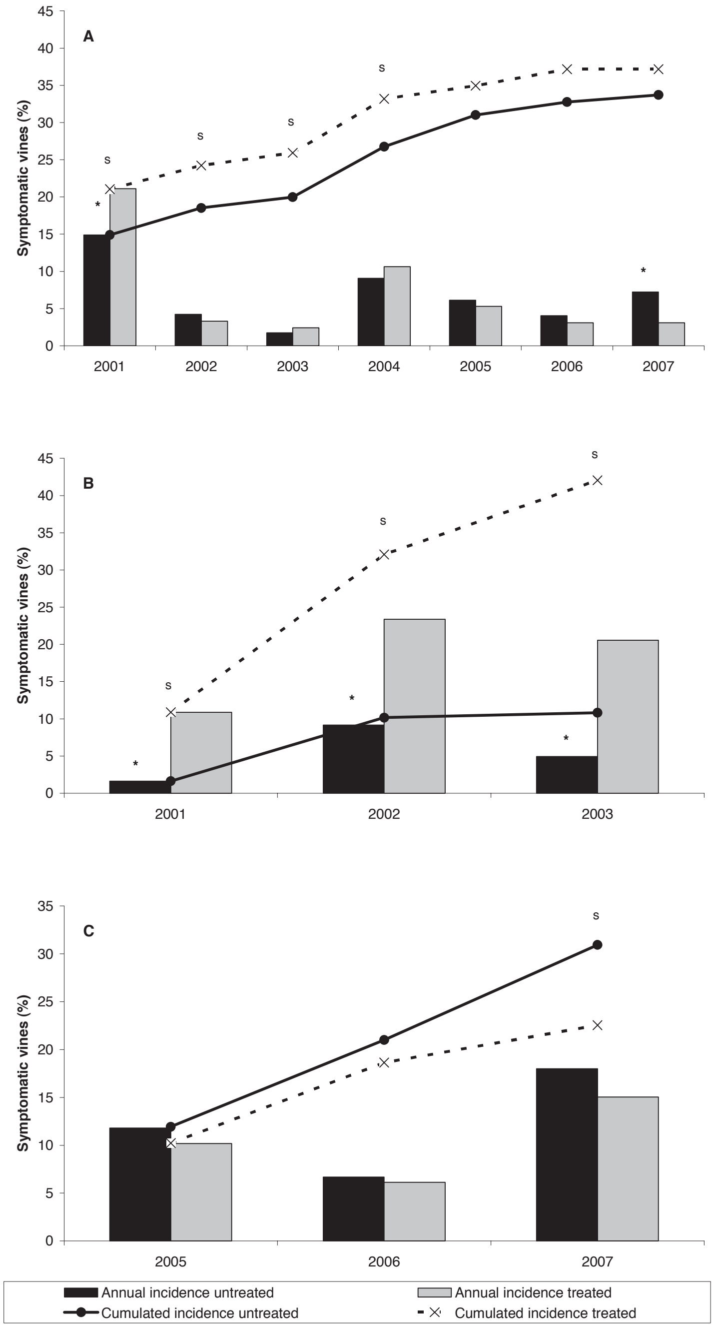

Fig. 2. Effects of applications of Brotomax (A), Fitostim (B) and Marvita (C) on annual incidence (bars) and cumulated incidence (lines) assessed in three different vineyards cv. Pignoletto. For each treatment and year of assessment, values of annual incidence or cumulated incidence marked with asterisk or "S" respectively, differ significantly according to Chi–Square test ($P<0.05$).

increased less strongly in the sprayed plot than in the unsprayed plot. The percentage of annual and cumulated esca incidence calculated for the period of 2003–2005 was significantly greater (*P*<0.05) in the plot sprayed with Fitostim than in the unsprayed plot (Fig. 2B). Both the annual and the cumulated incidence of esca in the Marvita-sprayed plot was lower than that in the unsprayed plot (Fig. 2C). In 2007 the percent cumulated incidence was significantly different (*P*<0.05) between the sprayed and the unsprayed plots.

In each year of the study, symptomatic vines that had shown symptoms in previous years were more frequent in sprayed plots than in unsprayed plots. This was found with all biostimulants sprayed, except Kendal in 2005 ('Albana' vineyard trial), and Brotomax in 2004 and 2007 (Fig. 3 and 4).

The percentage of vines that died from esca after

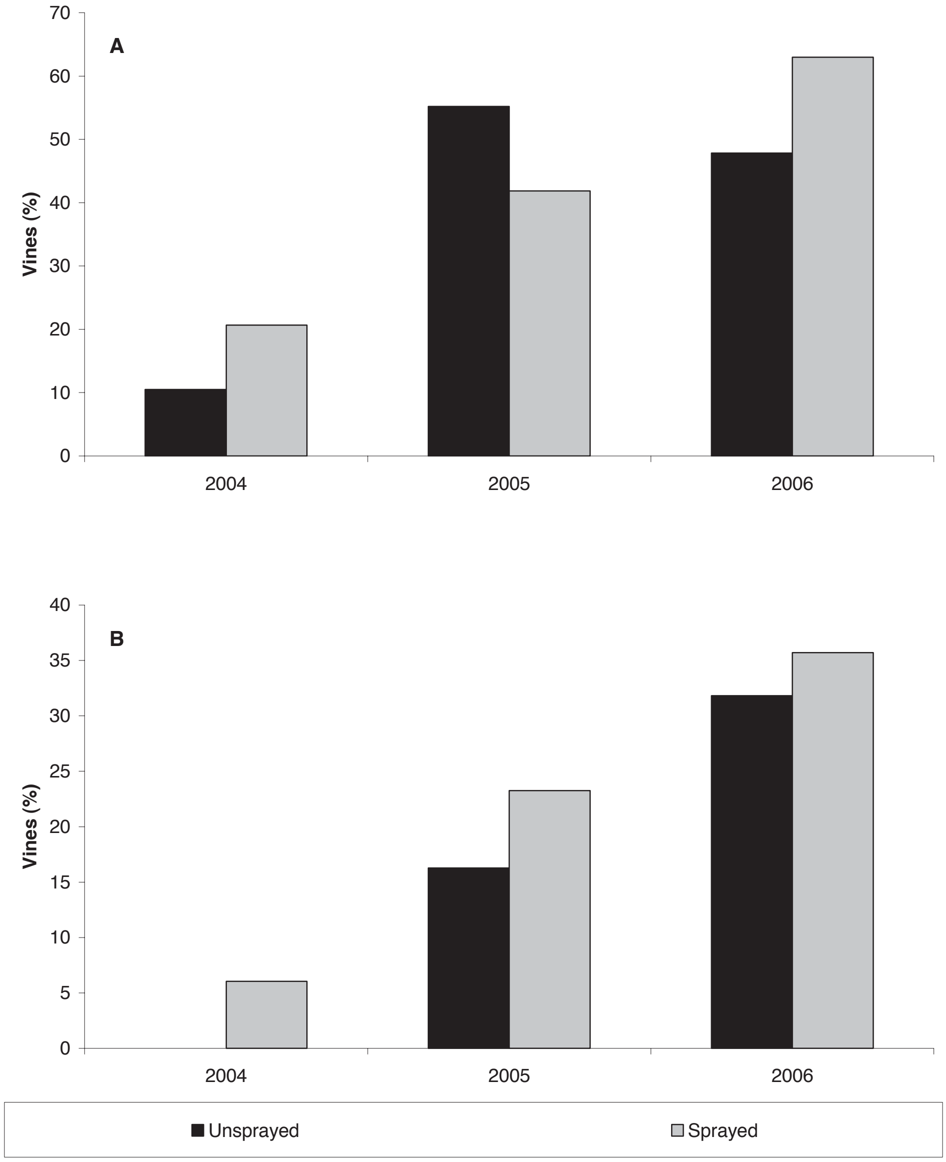

Fig. 3. Effects of applications of Kendal on the frequency of symptomatic vines that showed symptoms in previous years assessed in vineyards cv. Albana (A) and Sangiovese (B). For each treatment and year of assessment, values of frequency are not significantly different according to Chi–Square test (*P*>0.05).

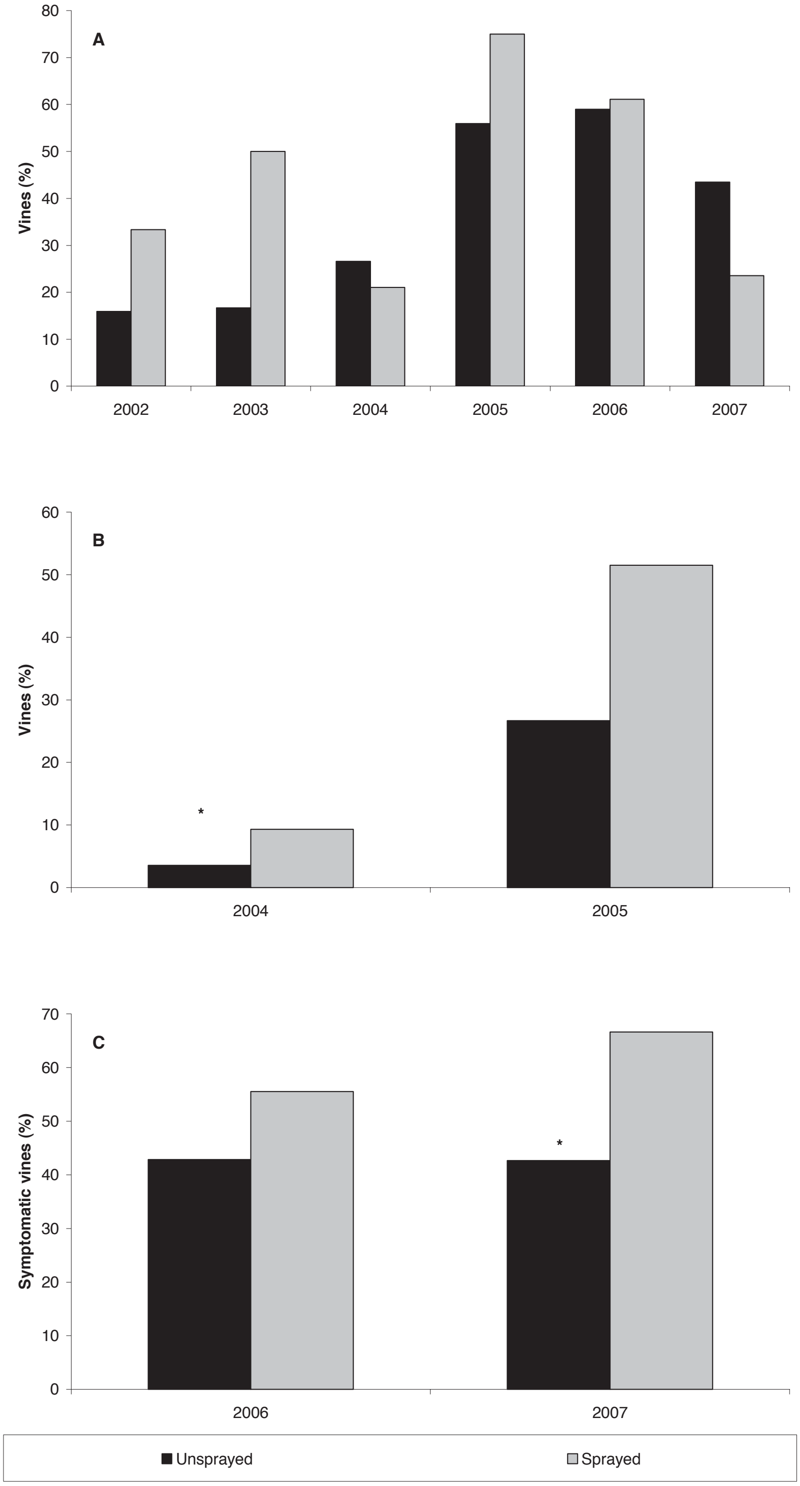

Fig. 4. Effects of sprays of Brotomax (A), Fitostim (B) and Marvita (C) on the incidence of symptomatic vines that had shown symptoms in previous years calculated in 3 different vineyards cv. Pignoletto. For each biostimulant sprayed and each year, bars marked with an 'S' differ significantly according to the Chi-Square test (*P*=0.05).

Table 3. Effects of biostimulant (Brotomax, Kendal and Marvita) sprays on the percentage of esca-related mortality on different grapevine cultivars. Top: vines that died in a given year (annual mortality). Bottom: vines that died in one of the years of the study (cumulated mortality). Data on the vineyard treated with Fitostim are not given because the number of vines that died from esca in this vineyard was too low.

Year of study	Annual mortality							
	Brotomax		Kendal				Marvita	
	Cv. Pignoletto		Cv. Albana		Cv. Sangiovese		Cv. Pignoletto	
	Unsprayed	Sprayed	Unsprayed	Sprayed	Unsprayed	Sprayed	Unsprayed	Sprayed
2002	0.8	0.4	nt[a]	nt	nt	nt	nt	nt
2003	0.2	0.0	nt	nt	nt	nt	nt	nt
2004	0.6	0.6	nt	nt	0.6	0.2	nt	nt
2005	0.2	0.0	0.0	0.5	0.2	0.0	nt	nt
2006	0.5	0.5	0.9	1.0	1.5	0.4	0.5	1.2
2007	0.9	1.1	0.9	0.5	nt	nt	0.5	0.5

Year of survey	Cumulated mortality							
	Brotomax		Kendal				Marvita	
	Cv. Pignoletto		Cv. Albana		Cv. Sangiovese		Cv. Pignoletto	
	Unsprayed	Sprayed	Unsprayed	Sprayed	Unsprayed	Sprayed	Unsprayed	Sprayed
2002	0.8	0.4	nt	nt	nt	nt	nt	nt
2003	1.0	0.4	nt	nt	nt	nt	nt	nt
2004	1.6	1.0	nt	nt	0.6	0.2	nt	nt
2005	1.8	1.0	0.0	0.5	0.8	0.2	nt	nt
2006	2.2	1.5	0.9	1.5	2.3*[b]	0.7*	0.5	1.2
2007	3.1	2.6	1.8	2.1	nt	nt	1.3*	2.7*

[a] For each treatment and cultivar, values in rows followed by an asterisk differ significantly according to the Chi-Square test (P=0.05).
nt, Not tested.

the first year of biostimulant spraying is shown in Table 3. The incidence of annual mortality in Brotomax-sprayed vines was slightly lower than in unsprayed vines. Spraying with Kendal gave contradictory results: in the 'Sangiovese' vineyard it reduced the percentage of annual and cumulated mortality, though the decrease was significant (P<0.05) only in 2006, but in the 'Albana' vineyard, it increased vine mortality, though not significantly, as compared with the unsprayed vines. An increase in percent mortality was also found with Marvita-sprayed vines. This increase was statistically significant in 2007 (P<0.05), but only for cumulated mortality.

Discussion

The primary objective of this study was to determine whether biostimulant sprays over a number of years would limit foliar symptoms in grapevines suffering from esca proper. On the whole, the biostimulants tested did not limit foliar symptoms. Similar unsatisfactory results were also recently obtained in Australia, where nutrient fertilizers and Brotomax sprayed on esca-infected grapevines did not limit disease symptoms or progress (Edwards and Pascoe, 2005).

In our trials, sprayed vines often showed a certain increase in the annual and cumulated incidence of esca. This finding is consistent with Calzarano *et al*. (2007), who found that frequent spraying of foliar fertilizers and bioactivators (eleven per year) on esca-proper infected vines during the growing season increased both the incidence and the severity of the disease.

In our study biostimulants were sprayed four or five times a year (except for Brotomax, which was sprayed following the manufacturer's spray schedule) on vines with most of their leaves asymptomatic and still photosynthetically efficient (Petit *et al*., 2006). The phenological growth stages at which biostimulants were sprayed, and the small number of sprayings, could not have caused any imbalances or excess of nutrients in the leaves, which generally make plants more susceptible to stress (Scheirs and De Bruyn, 2004). Calzarano *et al*. (2007) postulated that nutritional imbalances increased foliar symptom expression. Most likely, the increase in foliar symptoms from biostimulants found in this study was mainly due to an increase in plant physiological process: as a result of which fungal toxins moved to the leaves more readily and/or in greater amounts, thus raisng the incidence of foliar symptoms (Surico *et al*.). Further evidence for this increase in physiological processes was the fact that sprayed vines had a greater incidence of symptomatic vines that had shown symptoms in previous years . This higher incidence led to a clear reduction in the fluctuations of foliar symptoms from year to year.

The way in which Brotomax acts on esca and associated pathogens was investigated by Del Rio *et al*. (2001; 2004). In our study, after five years of spraying Brotomax in a vineyard without achieving any positive effect, the percent annual and cumulate esca incidence was reduced for three years. Another study likewise found that when Brotomax was sprayed on grapevines affected with Eutypa dieback the yield of the vines improved only after the biostimulant had been sprayed for three years (Sosnowski *et al*., 2005). It seemed that Brotomax in the field was only effective if it was sprayed for several years, and this limits its potential as a biostimulant to be used in infected vineyards. Moreover, the reduction in foliar symptoms found in our study may also have been favoured by the low percentage of symptomatic vines and by the proper management of the vineyard. Such conditions proved to be important also for the activity of fosetyl Al on foliar symptoms expression of esca (Di Marco *et al*., 1999; Di Marco and Osti, 2005b).

Biostimulants based on extract from the seaweed *Ascophyllum nodosum* (such as Marvita) were reported to have a positive effects when sprayed on cultivars of vegetable and flowering bedding plants, conifer and broadleaf trees, and turfgrass (Poincelot, 1993; Thompson, 2004; Erwin *et al*., 2004). In the present study, Marvita reduced esca symptoms in the vineyard. However, this is only a preliminary finding that will need some more years of investigations before its effect on esca in the vineyard can be known for certain.

The data obtained in this study made it impossible to ascertain the effect of biostimulants on esca-related mortality because of the low number of dead vines found in the vineyards studied.

Most biostimulants sprayed on the potted plants significantly reduced the necrotic lesions caused by the fungal pathogen. Lesions in artificially inoculated vines were also reduced by foliar, soil or trunk injection with the triazolic fungicides, fosetyl Al (Di

Marco *et al*., 1999; 2000) and biological control agents such as *Trichoderma* spp. (Di Marco *et al*., 2004; Di Marco and Osti, 2007). All these reductions were achieved only in simple and/or localized forms of infection occurring in young potted vines inoculated with *P. chlamydospora*, which are obviously different from esca proper as it occurs in the vineyard. Although the mode and mechanism of action of each biostimulant are different, and will be the object of further investigation, the reduction of necrosis in sprayed plants probably results from an interaction between the ingredients of the biostimulant and the physiological processes of the host plant. This interaction has a beneficial effect on the vine, in the same way as *Trichoderma* sprays have a beneficial effect on vines in the nursery (Fourie *et al*., 2001; Di Marco *et al*., 2004; Fourie and Halleen, 2004, 2006; Di Marco and Osti, 2007).

In conclusion, biostimulants were not effective against esca proper in infected vineyards. However, further studies are needed to assess the practical feasibility of biostimulants to control esca in plant material with no or low levels of infection.

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