Phytopathol. Mediterr. (2011) 50 (Supplement), S285–S299

Effects of grapevine applications of fosetyl-aluminium formulations for downy mildew control on "esca" and associated fungi

Stefano DI MARCO[1], Fabio OSTI[1], Francesco CALZARANO[2], Roberta ROBERTI[3], Annarita VERONESI[3] and Carmine AMALFITANO[4]

[1]Istituto di Biometeorologia, Consiglio Nazionale delle Ricerche, Via P. Gobetti, 101, 40129 Bologna, Italy
[2]Dipartimento di Scienze degli Alimenti, Università degli Studi di Teramo, Via C. Lerici 1, 64023 Mosciano S.A.,Teramo, Italy
[3]Dipartimento di Protezione e Valorizzazione Agroalimentare, Università di Bologna, Viale G. Fanin, 46, 40127 Bologna, Italy
[4]Dipartimento di Scienze del Suolo, della Pianta, dell'Ambiente e delle Produzioni Animali, Via Università, 100, 80055 Portici, Italy

Summary. Esca of grapevine is a fungal disease with a complex aetiology that is common in almost all regions of the world where grapes are cultivated. Despite much research, no effective control of the disease has been found. We investigated possible activity of fosetyl-aluminium (fosetyl-Al), an active ingredient in many fungicides against downy mildew, to inhibit development of esca in grapevine and the main pathogens linked to the disease, *Phaeomoniella chlamydospora* (Pch) and *Phaeoacremonium aleophilum* (Pal). In greenhouse experiments conducted on vines artificially inoculated with Pch or Pal, we found a reduction in necrosis in the woody tissue. In field experiments conducted over several years statistically significant reductions in the annual and cumulative incidence of the disease symptoms, and in cumulative vine mortality, were seen. The effect of fosetyl-Al treatments on leaf gas exchange, levels of resveratrol and ε-viniferin in the wood, and enzymatic activities were also studied. Hypotheses regarding the mechanism of action of fosetyl-Al against esca are outlined, and the possible use of products containing the chemical, applied on foliage to control downy mildew, is discussed as a strategy for control of esca.

Key words: *Phaeomoniella chlamydospora*, leaf gas exchange, foliar symptoms, grapevine stilbenes, leaf enzymatic activity.

Introduction

Esca of grapevine is a fungal disease of complex aetiology that is present in almost all areas where grapes are cultivated, affecting vines both in nurseries and in mature vineyards (Mugnai *et al*., 1999; Surico *et al*., 2006). Certain peculiarities that this disease shares only with the wood decay of kiwifruit (Di Marco and Osti, 2008), combined with our as yet incomplete understanding of the disease, have hampered efforts to develop an effective means controlling esca (Mugnai *et al*., 1999; Di Marco *et al*., 2000; Surico *et al*., 2006; Osti and Di Marco, 2010).

It is now believed that esca is a complex of at least two diseases: a white rot caused by *Fomitiporia mediterranea* (Fmed) and a tracheomycosis due to *Phaeomoniella chlamydospora* (Pch) and *Phaeoacremonium aleophilum* (Pal), or other species of *Phaeoacremonium*. Tracheomycosis comprises three different diseases that are distinguishable based on the symptomatology and age of the in-

Corresponding author S. Di Marco
Fax: +39 051 6399023
E-mail: s.dimarco@ibimet.cnr.it

fected plants: brown streaking, Petri disease, and young esca or leaf stripe disease, a term proposed recently by Surico (2009). The association in the same plant of white rot and tracheomycosis, as is commonly found in vineyards, has often been called "esca proper" (Mugnai *et al*., 1999; Calzarano and Di Marco, 2007).

Tracheomycosis is characterized by streaking and necrosis of varying extent in the wood of the trunk and principal branches of affected vines, interveinal chlorosis and necrosis of the leaves, and spotting of the berries (black measles). The foliar symptoms are only linked to vascular fungi, and it is most likely that these are caused by toxic substances (e.g., scytalone, isosclerone, polypeptides and polysaccharides) produced by these pathogens at the sites of mycelial colonization (Surico *et al*., 2006). Symptoms are intermittent and do not show up every year. In asymptomatic years the fruit produced by infected plants is indistinguishable in both quantity and quality from that produced by healthy plants (Calzarano *et al*., 2004b). Therefore measures that are able to reduce the symptom expression of the disease could help to limit crop damage.

Experiments using fungicides other than the banned sodium arsenite to control esca have already been conducted, and fosetyl-aluminium (fosetyl-Al) has yielded promising results when applied to vines on the foliage, or by injection in the soil or directly into the trunks (Di Marco *et al*., 2000; Laukart *et al*., 2001; Calzarano *et al*., 2004a). In particular, preliminary trials carried out in vines infected with esca proper sprayed with formulations containing fosetyl-Al for the control of downy mildew showed reductions in the incidence of symptomatic plants (Di Marco *et al*., 1999). Fosetyl-Al has been in use for some time as a fungicide. This active ingredient, which was developed in France at the end of the 1970s, is characterized by acropetal and basipetal translocation in plants, and triggers host plant defense mechanisms (Derks and Creasy, 1989; Nemesthoty and Guest, 1990). Further studies showed that, once absorbed by the plant, the molecule is rapidly converted into a phosphonate that has a mild effect on downy mildew pathogens (Fenn and Coffey, 1985; Sukarno *et al*., 1998). Formulations containing fosetyl-Al have been widely used to control various diseases, especially downy mildew or late blight (Cohen and Coffey, 1986).

The aim of the present study was to ascertain the reduction in esca foliar symptoms in the field due to fosetyl-Al commercial formulations, and to understand the mechanisms that caused such a reductions, evaluating effects of the chemical on associated pathogens and on their colonization ability. In order to understand how fosetyl-Al treatments acted towards this disease the effects of treatments on the biochemistry and plant physiology were also evaluated. In particular, given the capacity of fosetyl-Al to stimulate the production of certain pathogenesis-related (PR) proteins (Chuang *et al*., 2003), tests were conducted in the leaf on enzymatic activities that can interfere with fungal phytotoxins or their effects. The variation of *trans*-resveratrol and ε-viniferin content in wood tissue following treatment with fosetyl-Al was also verified, based on previous studies showing the activation of a defence mechanism consisting of the *ex novo* synthesis of resveratrol and viniferin. These are the only stilbenes shown to accumulate in wood tissue attacked by esca pathogens (Amalfitano *et al*., 2000; Agrelli *et al*., 2009). Finally, in the light of the correlation between rainfall and the appearance of foliar symptoms and the possible involvement the host transport system (Marchi *et al*., 2006; Surico *et al*., 2006), and also because preliminary data have indicated reduction in photosynthesis in plants treated with fosetyl-Al, effects of the chemical on net photosynthesis, transpiration rate, and stomatal conductance in field-grown vines were monitored.

Materials and methods

Field study of effects of fosetyl-Al on esca proper incidence and mortality

Studies were conducted over several years in three DOC vineyards located in a zone of low hills in the region of Emilia-Romagna (central-northern Italy): between 1993 to 1997 in a vineyard planted with cv. Lambrusco grasparossa (vine age 12 y at first application); from 1995 to 2000 in a vineyard planted with cv. Sangiovese (vine age 13 y at first application); and between 1998 and 2007 in a vineyard of cv. Albana (vine age 8 y at first application). In each case two plots (A and B) comprising 5 or 7 rows of vines making up a total of approximately 300 (Lambrusco), 500 (Sangiovese), and 1200 (Albana) plants were designated for use in each study. All plots received the same treatments

against pests and diseases including systemic fungicides for control of downy mildew. In addition, Plot A in each case was treated with formulations containing fosetyl-Al while Plot B (the experimental control) was treated with formulations that did not contain fosetyl-Al.

This part of the trials was performed in order to offer a practical, ready to use, side effect of downy mildew control strategies as they are applied by the growers. It was therefore important that the effect of adding fosetyl-Al could be visible whatever the level of downy mildew control achieved, as in the field control strategies always include different types of chemicals depending on the season. In all the remaining experiments aimed at obtaining possible explanations for effects of fosetyl-Al observed in the vineyard, all treatments were comparable.

The control strategy for downy mildew was carried out using the following protocol, and fungicides were applied at the maximum doses recommended by the manufacturers and at the following growth stages (Coombe, 1995).

Plot A (fosetyl-Al)

Two applications of mancozeb at 7–10 d intervals (Dithane M-45®, Dow AgroScience, 200 g hL^{-1}), when the shoots attained a length of 10 cm (E-L 12).

Three treatments 10 to 12 d apart with a formulation containing fosetyl-Al + cymoxanil + mancozeb (R6 Triple Erresei®, or R6 Triple Blu®, Scam, 400 g hL^{-1}) between single flowers separated (E-L 17) and cup fall complete (E-L 26);

Three treatments applied at 10 to 12 d intervals between fruit-set (E-L 27) and pre-bunch closure (E-L 32) of fosetyl-Al + copper oxychloride (Aliette Bordeaux®, or R6 Erresei Bordeaux®, Bayer CropScience 400 g hL^{-1});

Two to three treatments with products containing copper oxychloride at 7–10 d intervals (Cupravit® or Cuprocaffaro®, Bayer Cropscience or Isagro, 300–400 g hL^{-1}) until veraison (E-L 35).

Plot B (experimental control)

The first and last treatments with mancozeb and copper oxychloride respectively, were the same as for Plot A.

Between single flowers separated (E-L 17) and cup fall complete (E-L 26), treatment with a formulation consisting of benalaxyl + mancozeb (Galben M 8-65 Blu®, Belchim Crop Protection, 250 g hL^{-1}) or metalaxyl + mancozeb (Ridomil Gold MZ®, Syngenta, 250 g hL^{-1}) was applied two or three times at 12 to 14 d intervals.

Between fruit-set (E-L 27) and pre-bunch closure (E-L 32), two to three treatments spaced 12 or 14 d apart with a formulation either of benalaxyl + copper (Galben R Blu®, Isagro, 400 g hL^{-1}) or metalaxyl + copper (Ridomil Gold R®, Syngenta, 400 g hL^{-1}), or at 10 to 12 d intervals with dimethomorph + copper (Forum R®, BASF, 350 g hL^{-1}).

Plants were assumed to be mainly affected by esca proper as confirmed by the presence of decayed and discoloured wood assessed in some vines with foliar symptoms uprooted from both plots (A and B) in the surveyed vineyards. In the vineyards planted with cv. Lambrusco grasparossa or Sangiovese, some vines had shown foliar symptoms in previous years, while the cv. Albana vines first began to show symptoms in 1999, the year after the beginning of the experiment.

The vineyards were monitored each year in the month of September, at the time of the maximum disease incidence (Marchi *et al.*, 2006). The number of symptomatic vines in each plot was recorded, as was the number of vines that had died in that particular year after showing foliar symptoms of esca during any previous year(s) of the study. The incidences of disease and mortality were calculated and expressed as percentages by dividing the number of vines with visible symptoms or the number of dead vines by the total number of vines in the plot (Di Marco and Osti, 2009). For each vineyard and year of investigation, the annual and cumulative incidences of symptomatic plants, and the cumulative vine mortality were recorded and a statistical analysis was carried out, comparing the results between treatment (plot A) and control plants (plot B) using the Chi-Square test (P=0.05) using SAS system software version 9.1 (SAS Institute, Inc., Cary, NC, USA).

Effects of fosetyl-Al on potted plants inoculated with Pch and Pal

Effects of treatments before inoculation

This experiment was conducted on 2-y-old cv. Montuni vines grafted onto SO4 rootstock and grown outdoors in pots containing 70% of a commercial mixture of peat with nutrients and ele-

ments at low concentrations (Floragard TKS2, Oldenburg, Germany) and 30% perlite. The plants were sprayed up to the point of incipient run off with a formulation containing fosetyl-Al (Aliette 80 WP, Bayer CropScience) at a rate of 20 g a.i. L^{-1} using an 8 L capacity Green pre-compression pump with a hand-held Spray/Get (Volpi Originale, Casalromano, Mantova, Italy). A total of six sprayings were carried out at 10 to 12 d intervals beginning in the last week of May. Two months after the final treatment (at the beginning of autumn), the vines were artificially inoculated. A hole (5 mm diam.) was made in the trunk of each vine 15 cm below the point of the graft using a hand-held drill, and a plug of potato dextrose agar (PDA, Difco Laboratories, Franklin Lakes, NJ, USA) colonized with *Phaeomoniella chlamydospora* (Pch) strain CBS 222.95 or *Phaeoacremonium aleophilum* (Pal) strain CBS 631.94 was inserted. The plants were kept in a greenhouse, where temperatures assessed over the years ranged from 4 to 35°C. The following spring, plants were divided into three groups for the study: (i) inoculated plants that were sprayed six times with fosetyl-Al; (ii) inoculated plants that received no fosetyl-Al treatment; and (iii) plants inoculated with sterile agar that received no fosetyl-Al treatment.

Each group consisted of ten plants, each replicate consisting of one potted vine. Six and 12 months after the inoculation, three plants were taken and analyzed from each group; the remaining four plants were analyzed 15 months after the inoculation. The trunk of each plant was cut along its length and the extent of necrosis was assessed by measuring the length of necrotic lesion developed from the inoculation site. Wood tissue was removed from the affected areas and cultured on PDA to establish the presence and viability of the pathogens.

The experiment was repeated with same set up and materials except that vines were inoculated with Pch only and plants were analyzed 18 months after inoculation.

Effects of treatment after inoculation

This experiment studied the effect of treatment with fosetyl-Al after infection. A similar protocol to that described above was used, except as follows: 2-y-old cv. Montuni vines were inoculated in January (i.e., when dormant) with Pch (Pal was not studied), and treatments were applied during the next vegetative season. Three groups of ten plants were studied: the first group was artificially inoculated and then treated with six sprays of Aliette 80 WP starting in May; the second was inoculated but received no fosetyl-Al treatment; and the third was inoculated with sterile agar and received no fosetyl-Al treatment. The plants were all inspected 12 months after the inoculation.

The following year the experiment was repeated following the same procedure but the vines were inspected at 22 months after inoculation.

The area of inoculation was analyzed as described above and the results, expressed as the average extent of the necrotized region, were compared using the Duncan test (P=0.05) using SAS system software version 9.1 (SAS Institute).

Field study on effects of fosetyl-Al on leaf gas exchange

Tests were carried out in a vineyard of 15-y-old cv. Riesling Italico / SO4, located in the Colli Bolognesi grape growing area. The parameters of gas exchange, i.e. net photosynthesis (P_n), transpiration rate (E) and stomatal conductance (g_s), were monitored in 2007 and 2009 on plants treated with two formulations containing fosetyl-Al: either fosetyl-Al + cymoxanil + mancozeb (after the appearance of visible clusters) or fosetyl-Al + copper oxychloride (after fruit-set). The experimental control plants were treated with formulations of metalaxyl + cymoxanil + mancozeb (Eucritt triplo NC, Siapa) or metalaxyl + copper oxychloride (Eucritt Rame WG, Isagro). Sampling was not possible in 2008 due to unfavourable weather conditions.

For each formulation only one spray of fungicide was applied to the plants. The parameters P_n, E and g_s were measured using a portable ADC LCA3 (Analytical Development Co., Hoddesdon Herts, UK) equipped with an infra-red camera and a Parkinson leaf chamber to collect the gases exchanged over a 6.2 cm^2 area on the surface of each leaf.

The plants were sprayed with the fungicides in the morning at the concentrations recommended on the labels. The parameters of interest were then measured daily between 14:00 and 16:00, beginning on the day the product was applied, under conditions of photosynthetically active radiation (PAR) ≥ 900 μmol photons m^{-2} s^{-1}; relative humid-

ity of about 35–40% and 40–45% for the periods of May and late June–early July respectively; and temperatures of 29–32ºC in May and 30–32ºC in late June–early July, recorded at the times of measurement.

For each treatment, five leaves of similar age, size and position in the canopy were selected from five different plants and three measurements were made on each leaf. The data were compared by analysis of variance (ANOVA) at P=0.05, using SAS software, version 9.1 (SAS Institute). For each year, the results are reported as the means for each daily sampling expressed as μmol CO_2 m^{-2} s^{-1} (P_n); mmol H_2O m^{-2} s^{-1} (E); and mmol H_2O m^{-2} s^{-1} (g_s).

Effects of fosetyl-Al on the level of *trans*-resveratrol and ε-viniferin in grapevine wood

Two-y-old cv. Sangiovese vines grafted onto SO4 and growing outdoors in pots, were inoculated with Pch in December (while dormant) with a plug from a fungal culture (or sterile agar for the experimental controls) following the procedure described above. The plants were then treated with fosetyl-Al (Aliette 80 WP, Bayer CropScience) in three applications to the leaves using a Green pre-compression pump (described above) at intervals of 10 d beginning in the first week of May. At 7 d from the last application, the plants were uprooted and analyzed.

The experiment was set up with the following treatments: (i) plants inoculated with Pch and not treated with fosetyl-Al; (ii) plants inoculated with Pch and treated with fosetyl-Al; (iii) plants inoculated with sterile agar and not treated with the fungicide; (iv) plants inoculated with sterile agar and treated with the fungicide; (v) plants not inoculated and not treated; and (vi) plants not inoculated but treated with fosetyl-Al. Each treatment was applied to five plants, replications represented by one plant in a pot.

For chemical analyses, wood shavings were obtained from pieces of the trunk of each plant, removed around the margin of the necrotic zone from the inoculation point. The shavings were lyophilized and then pulverized in liquid nitrogen in a blender. The powdered samples were extracted with petroleum ether in a Soxhlet extractor for 8 h and then shaken, first in methanol for 4 h in the dark at room temperature, and then in an equal volume of fresh solvent overnight. The methanol extracts were combined, dried using a Rotavapor at ≈35ºC, and re-dissolved in enough methanol for HPLC analysis in a C18 column 250×4 mm internal diam., containing 5 μm diam. beads (LiChrospher 100 RP-18), using a flow rate of 1 mL min^{-1} and elution with CH_3CN/H_2O along a linear gradient of CH_3CN from 20 to 33% over a period of 90 min, and again from 33 to 20% for 5 min. The column was re-equilibrated under isocratic conditions for 5 min before the next run. The detector was set at a wavelength of 290 nm. The standards used were commercial grade *trans*-resveratrol (Sigma-Aldrich, St. Luis, MO, USA) and ε-viniferin extracted and purified from wood samples taken from healthy vines, following the method described by Amalfitano *et al*. (2000).

The data obtained were expressed in mg of *trans*-resveratrol or ε-viniferin per g, and data were analyzed using the Duncan test (P=0.05) by SAS system software version 9.1 (SAS Institute).

Effects of fosetyl-Al on enzyme activity in leaves

Protein extraction

To analyze the enzyme activity induced by the fosetyl-Al treatments, leaves were harvested from 2-y-old potted vines (cv. Terrano on SO4) that had been treated with fosetyl-Al (or water as the experimental control) by means of a pre-compression pump as described above. Samples (ten leaves each time) were collected 3, 6, 9 and 13 d after treatment. Each leaf sample was weighed, quickly frozen in liquid nitrogen, and immediately ground to fine powder using a pre-chilled mortar and pestle. Proteins were extracted with chilled sodium acetate buffer 20 mM, pH 5.5 (1 mL g^{-1} fresh weight) containing 1% (w/v) polyvinylpolypyrrolidone (Sigma-Aldrich Co.). Extractions were carried out at 4°C for 90 min with continuous gentle stirring. Each crude extract was centrifuged twice at 12,000 rpm for 20 min at 4°C. The supernatant was filtered using a GV Millex® Syringe Filter Unit (Millipore Corporation, Billerica, MA, USA) to remove the solid particles, and concentrated and desalted using an Ultrafree® Centrifugal Filter Unit (Millipore Corporation). Protein concentration was determined by the protein-dye binding method of Bradford (1976), using BSA (BioRad Laboratories, Inc., Hercules, CA, USA) as the standard.

1,3-β-glucanase assay

Activity of 1,3-β-glucanase in the leaves was

evaluated by measuring the rate of production of reducing sugars (Sigma-Aldrich Co.), employing laminarin as the substrate and following a modified version of the procedure of Kauffmann *et al*. (1987). The reaction mixture was composed of 0.5 mL sodium acetate buffer 0.1 M, pH 5.2 containing 1 mg mL^{-1} laminarin and 90 μL enzymatic solution. After incubating each sample for 2 h at 37°C, 0.3 mL of alkaline copper reagent was added and the mixture was heated at 100°C for 20 min. After cooling, 0.2 mL of Nelson's chromogenic reagent was added and absorbance was measured at 660 nm (Ashwell, 1957). Glucose and enzyme standards and substrate blanks were included. One unit of 1,3-β-glucanase activity was defined as the amount of enzyme that released 1 mg glucose per min.

Lipoxygenase assay

Activity of lipoxygenase was determined at 25°C on the leaves by monitoring the increase in absorbance at 234 nm caused by the conversion of linoleate into the corresponding hydroperoxide, following the procedure of Doderer *et al*. (1992) with some modifications. Activity was measured in a reaction mixture consisting of 20 μL enzymatic solution, 5 μg total protein, and 1.480 mL substrate solution containing 0.035% linoleic acid (Sigma-Aldrich Co.) in 0.1 M buffer at three pH values (5.5, 6.5 and 9.0) in order to quantify the activity of the different lipoxygenase isoforms. The buffers consisted of sodium acetate, sodium phosphate, and Tris-HCl, respectively. One unit of lipoxygenase activity was expressed as the quantity that generated 1 μmol hydroperoxide per min at 25°C, using a molar extinction coefficient of 25,000 M^{-1} cm^{-1}.

Peroxidase assay

Peroxidase activity was assayed in the leaves by measuring at 30°C the increase in absorption at 470 nm due to the formation of tetraguaiacol from a reaction mixture containing 0.46% (v/v) guaiacol (Sigma-Aldrich Co.) and 13 mM H_2O_2 in Tris-HCl buffer 50 mM, pH 7.4 (Caruso *et al*., 1999). Crude protein extracts from samples of fosetyl-Al-treated or untreated leaves taken at different times were each added to 1.5 mL (final volume) of the reaction mixture and analyzed. One unit of peroxidase activity was expressed as the amount of enzyme required for the formation of 1 μmol tetraguaiacol per min.

Data on the activity of each enzyme were expressed as the mean of three independent experiments ± Standard Deviation (SD) at a 95% confidence interval, and the mean values for each time were analyzed for statistical significance by *t*-test (GraphPad Prism 3.0. software), comparing treated samples versus corresponding untreated experimental controls.

Results

Field study of effects of fosetyl-Al on the incidence of esca proper

The annual incidence of symptomatic esca in fosetyl-Al treated and control plants (plots A and B) was compared. It was found that the incidence of symptoms in cv. Albana (which appeared for the first time in 1999) was less in the vines treated with fosetyl-Al than in the control plants for each of the study years (1999–2007), and the difference was statistically significant by the Chi-Square test (P=0.05) in seven of the nine years (Figure 1a). Similar results were obtained for three of the six years of the study conducted between 1995 and 2000 on cv. Sangiovese (Figure 1b). The cumulative incidence showed a similar trend, with a constant and statistically significant (P=0.05) reduction in symptom appearance in the plots of cv. Albana treated with fosetyl-Al for all the years of the study except 2000, and for the last three years of the study in cv. Sangiovese, in comparison to the control plots (Figure 1a and 1b).

For both cv. Albana and cv. Sangiovese, the cumulative mortality was always less in the fosetyl-Al treated than in the control plants, and this difference was statistically significant (P=0.05) for each year except in the first (1996) for cv. Sangiovese and in 2005, 2006 and 2007 for cv. Albana (Figure 1a and 1b).

In the case of cv. Lambrusco grasparossa, the plots treated with fosetyl-Al showed less annual incidence of the disease, a less cumulative incidence, and a less cumulative mortality rate in comparison to the control plots (Figure 1c), although the differences were not statistically significant on the Chi-Square test.

Effects of fosetyl-Al on potted plants inoculated with Pch and Pal

In the two experiments that involved treating plants: (i) with fosetyl-Al before inoculation, or (ii)

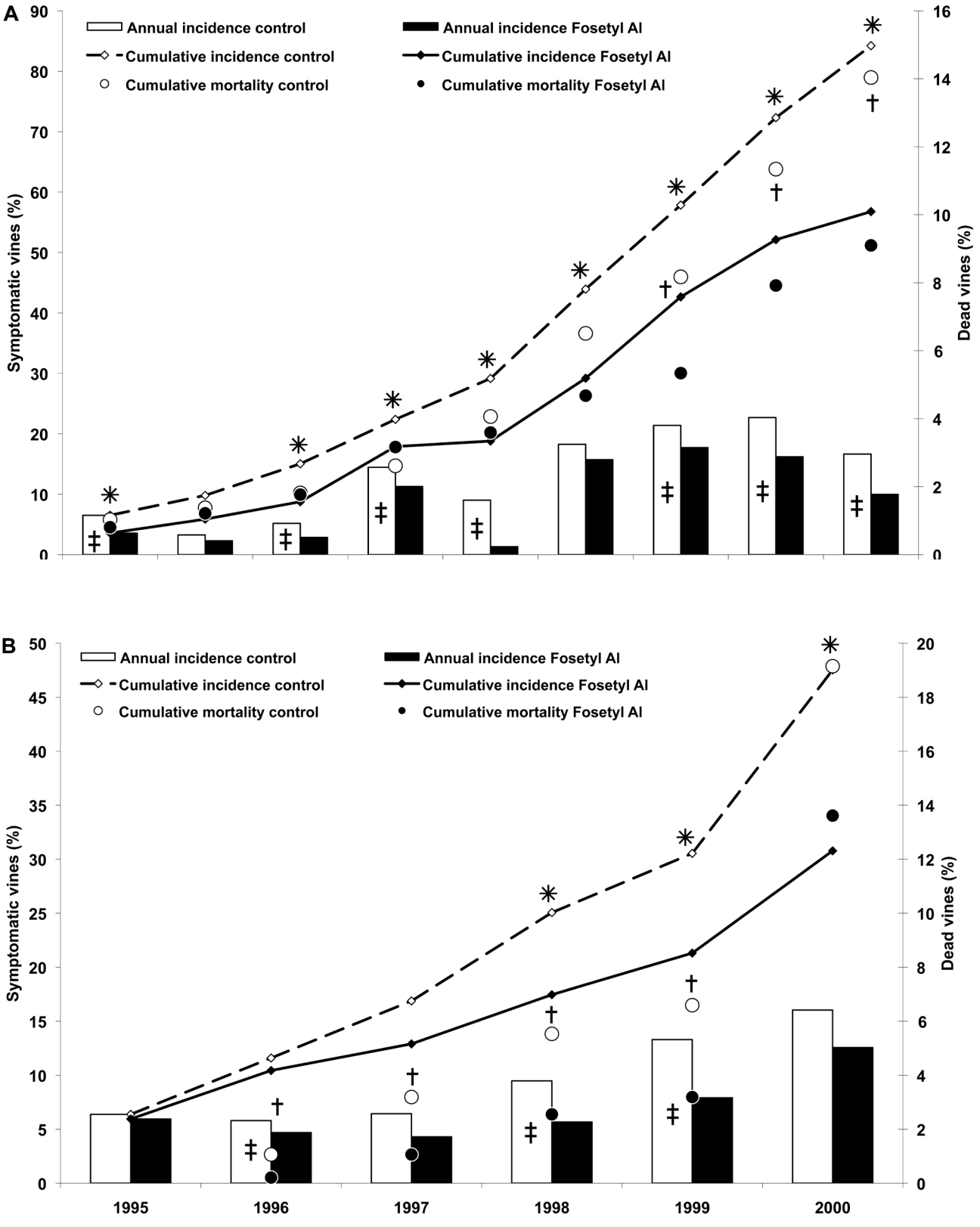


Figure 1. *Continued*

Figure 1. *Continues*

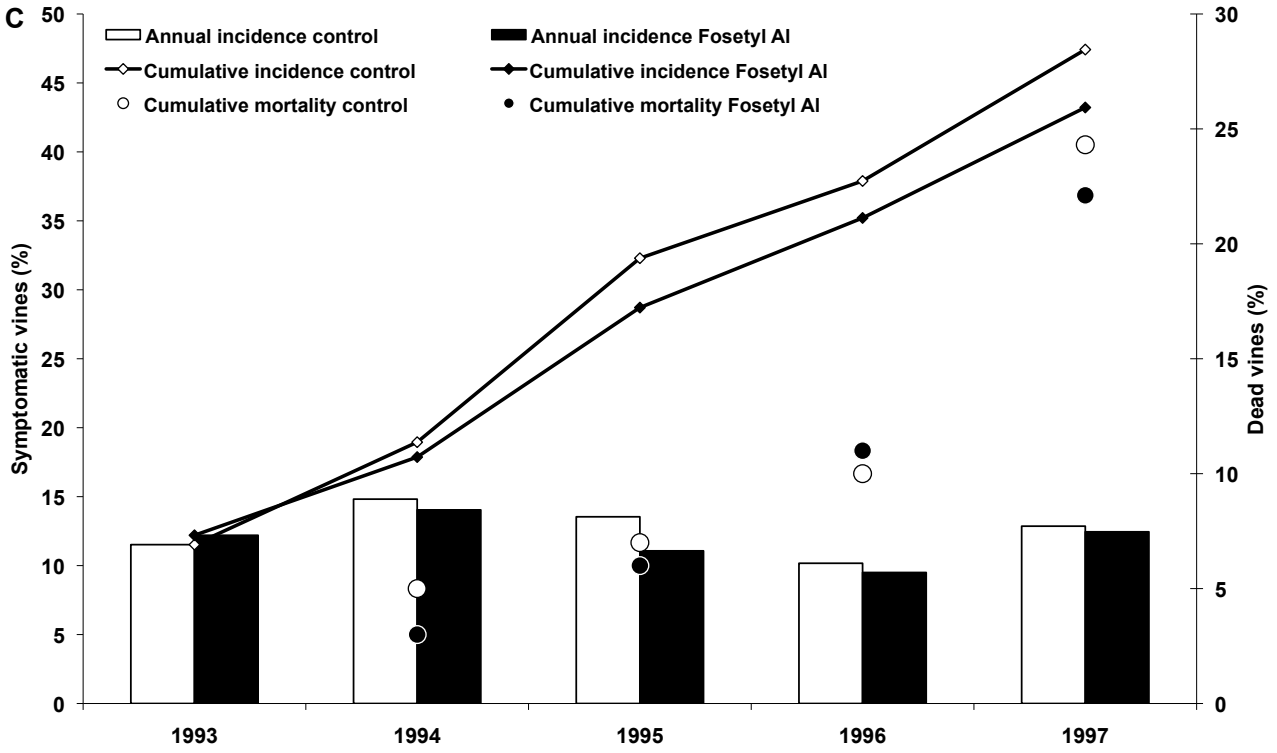


Figure 1. Effect of fosetyl-Al treatments on mean annual and cumulative esca incidence, and cumulative mortality due to esca, from surveys carried out in vineyards of grapevine cv. Albana (A), Sangiovese (B), and Lambrusco grasparossa (C), that were either treated or not with fosetyl-Al. For each year, annual disease incidence was calculated as the number of symptomatic vines over the number of all inspected vines; the cumulative disease incidence was calculated as the number of symptomatic vines plus diseased vines that did not show symptoms in that year over the number of all inspected vines; the cumulative mortality was calculated as the number of vines that had died in that particular year after showing foliar symptoms of esca during any previous year(s) of the study over the number of all inspected vines. For each year, means of fosetyl-Al and control marked with a symbol are different (P=0.05; Chi-Square test): annual incidence, ‡; cumulative incidence, ✳; or cumulative mortality, †.

after inoculation, both strategies led to reductions in the extent of necrosis caused by tracheomycotic fungi. The infections were never entirely eliminated from treated plants, however, as Pch and Pal were always re-isolated from the wood samples from treated plants.

Effects of treatment before inoculation

A statistically significant reduction (P=0.05) in necrosis in the plants infected with Pal was detected after treatment with fosetyl-Al (Table 1).

Less necrosis was also found in the plants infected with Pch (Table 1), but the reduction was not statistically significant (P>0.05) due to the wide variability in the values recorded in this experiment. A second trial was carried out, inoculating plants with Pch but using a larger number of replications in order to reduce the effect of the variability in the data. Infected wood samples were studied 18 months after the inoculation, and the levels of necrosis were significantly less (P=0.05) in the treated compared to the untreated plants, and similar to the amount of necrosis found in plants inoculated with sterile agar.

Effects of treatment after inoculation

Necrosis was always more extensive in the experimental controls than in the plants treated af-

ter inoculation with Pch, and this difference was statistically significant (P=0.05). Analogous results were recorded 12 months after inoculation (experiment 1) and 22 months after inoculation (experiment 2) (Table 1).

Field study of effects of fosetyl-Al on leaf gas exchange

Data were recorded in two different years. In each year net photosynthesis (P_n), transpiration rate (E) and stomatal conductance (g_s) were significantly altered (P=0.05) for a period of 3 days following the application of fosetyl-Al + cymoxanil + mancozeb and for a period of 4 days following the application of fosetyl-Al + copper oxychloride, respectively compared to control plants treated with a formulation containing metalaxyl instead of fosetyl-Al (Figure 2).

Spraying with fosetyl-Al + cymoxanil + mancozeb decreased average values for all three parameters on the day of the application. For example, in 2007 P_n diminished by ≈ 6.6 μmol CO_2 m^{-2} s^{-1} in the treated plants on day 1 compared to the control value of ≈11.8 μmol CO_2 m^{-2} s^{-1} (Figure 2a). Over the following 2 d P_n values gradually increased, but on the third day were still significantly below the control values. In 2009 the mean g_s values measured on day three after treatment were 127 mmol H_2O m^{-2} s^{-1} in the controls (treated with combinations of fungicides that did not include fosetyl-Al) compared with 116 mmol H_2O m^{-2} s^{-1} in the plants treated with formulations containing fosetyl-Al; this difference was statistically significant (Figure 2b). Transpiration rate was also greater in the controls than in the plants treated with fosetyl-Al + cymoxanil + mancozeb (Figure 2c).

In both years, treatment with fosetyl-Al + copper oxychloride had a more constant and lasting effect than fosetyl-Al + cymoxanil + mancozeb (Figure 2a–c).

Effects of fosetyl-Al on the level of *trans*-resveratrol and ε-viniferin in grapevine wood

The application of fosetyl-Al formulations did not modify (P>0.05) the levels of *trans*-resveratrol and ε-viniferin in the trunks of young potted vines. Inoculation both with Pch and sterile agar plugs increased (P=0.05) in the levels of *trans*-resveratrol and ε-viniferin compared with to the wood of non-inoculated vines regardless, of fosetyl-Al application (Table 2).

Effects of fosetyl-Al on enzyme activity in leaves

To determine whether 1,3-β-glucanase, peroxidase or lipoxygenase activities were increased in response to fosetyl-Al treatment, total protein extracts from control and treated leaves respectively (i.e., leaves sprayed with either sterile water or with fosetyl-Al) were analyzed 3, 6, 9 and 13 days after treatment using a spectrophotometer.

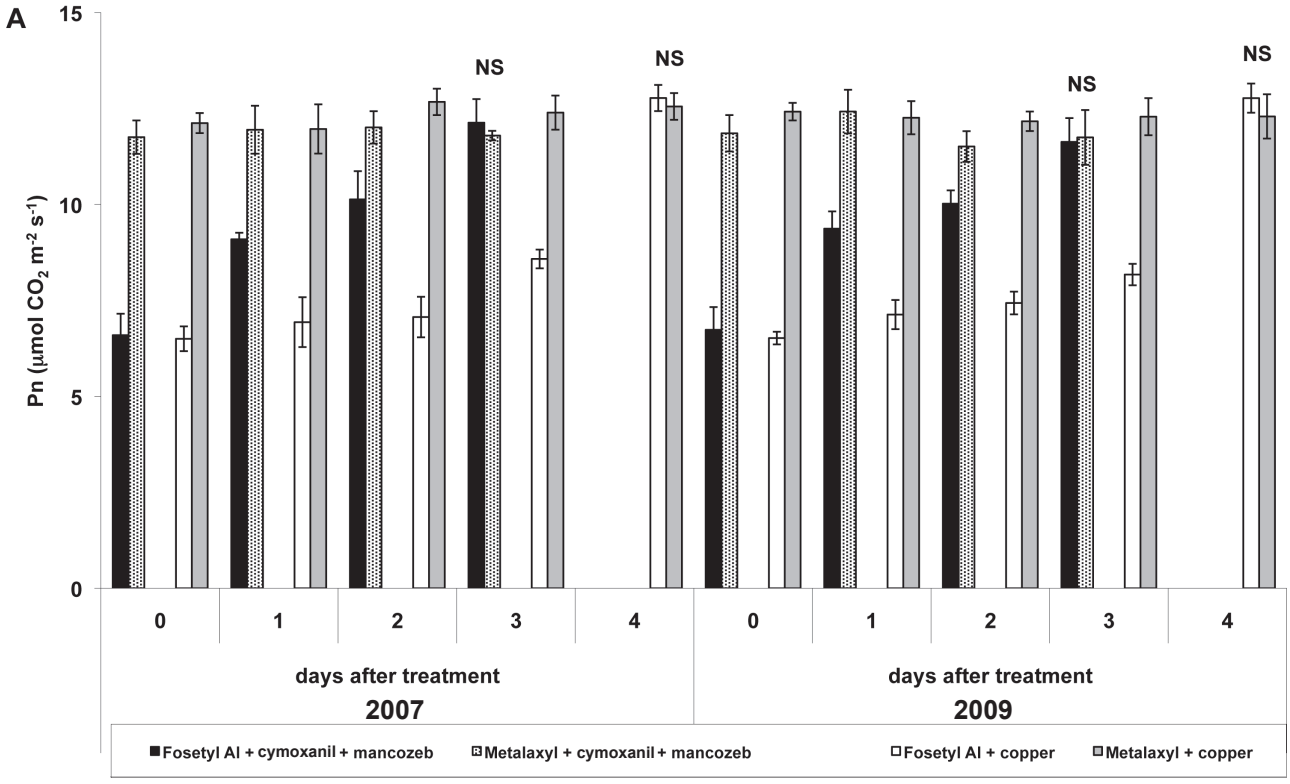
As shown in Table 3, 1,3-β-glucanase activity increased in the protein extracts from treated leaves

Table 1. Mean internal necrosis lengths in wood of potted grapevine plants, either untreated or treated with fosetyl-Al, and inoculated with Pal or Pch. Pre-inoculation application: for each treatment four plants 15 months (first experiment) and 18 months (second experiment) after the inoculation were inspected. Post-inoculation application: for each treatment ten plants were inspected 12 months (first experiment) and 22 months (second experiment) after the inoculation.

Treatment	Necrosis length (mm)				
	Pre-inoculation application			Post-inoculation application	
	Pal	Pch		Pch	
	1st experiment	1st experiment	2nd experiment	1st experiment	2nd experiment
Fosetyl-Al	38.7 b[a]	37.7 ab	29.5 a	25.3 a	35.7 b
Untreated	56.7 c	44.3 b	79.2 b	36.6 b	73.5 c
Sterile control[b]	20 a	24.7 a	24.5 a	22.5 a	19.2 a

[a]Means in each column followed by the same letter do not differ (P=0.05), Duncan's multiple range test.

[b]Plants each inoculated with a sterile agar plug.



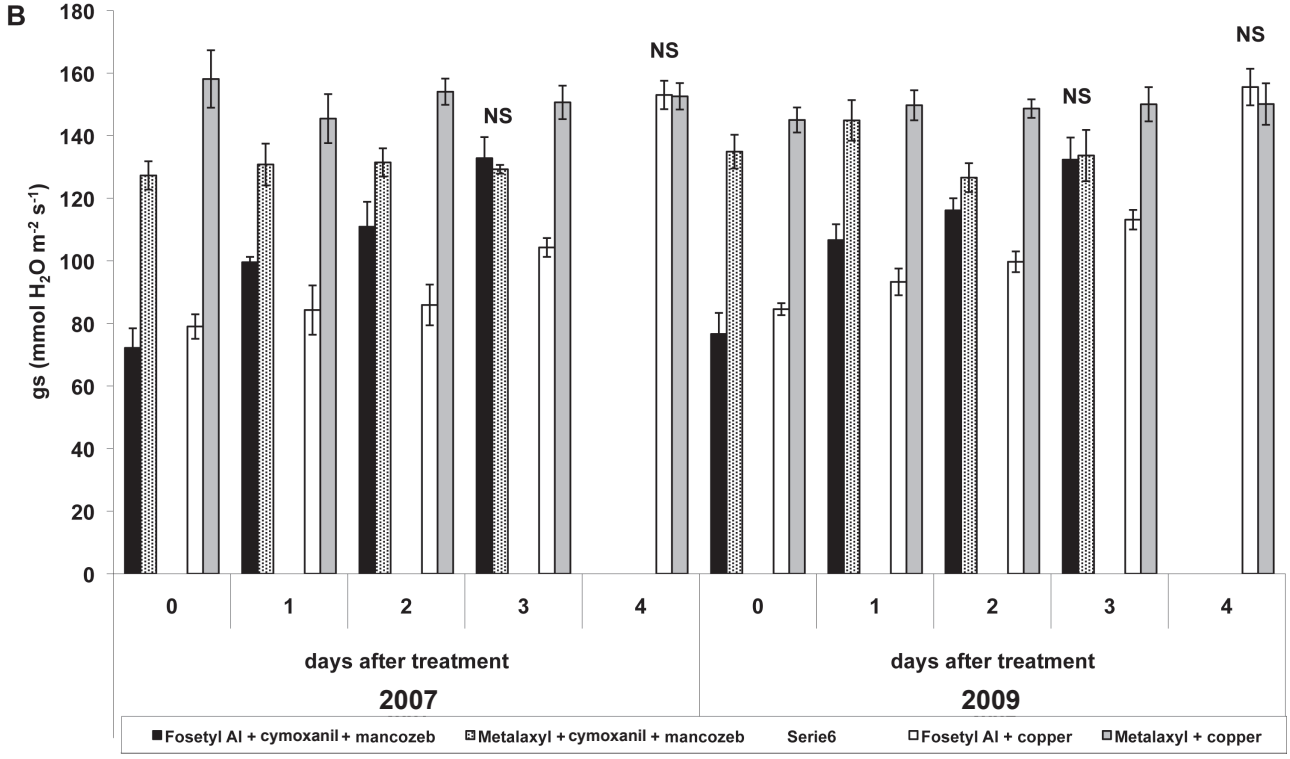


Figure 2. *Continued*

Figure 2. *Continues*

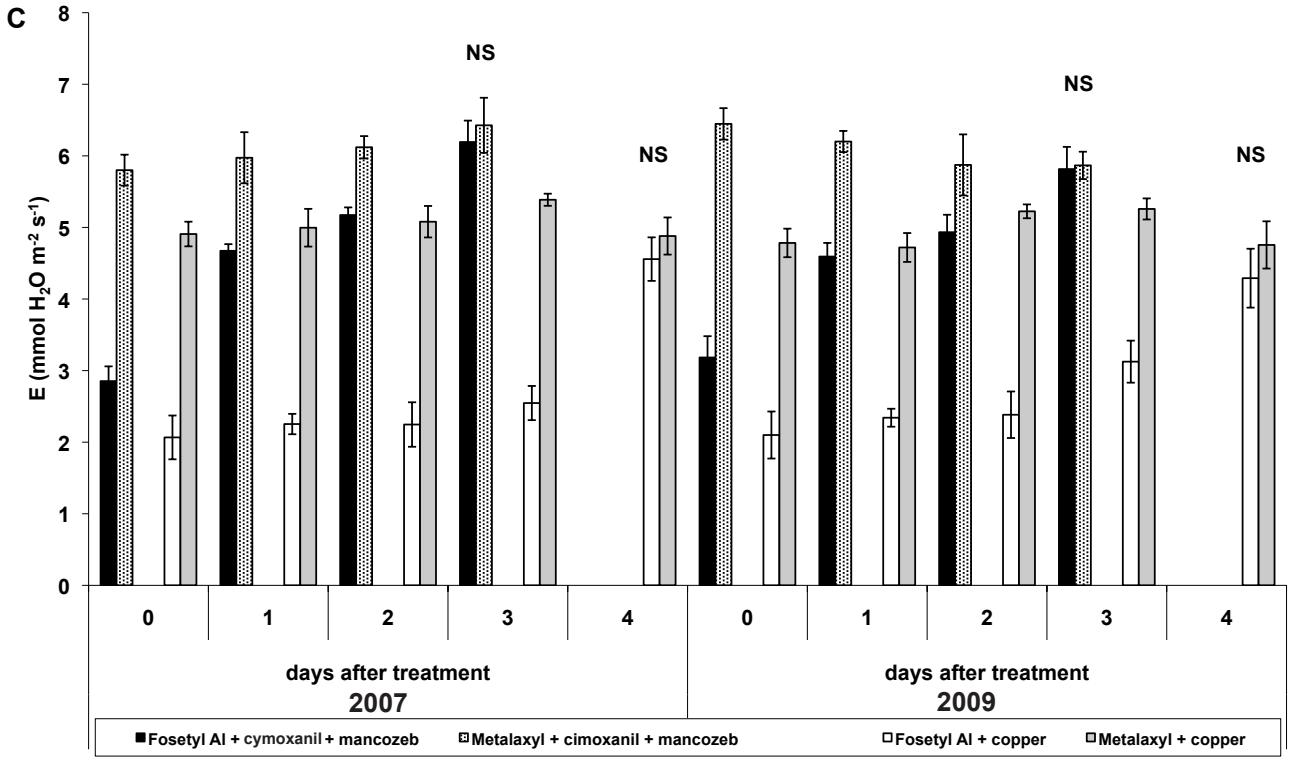


Figure 2. Effect of treatments on Mean net photosynthesis (Pn) A, transpiration rate (E) B, and stomatal conductance (gs) C for grapevine plants either untreated or treated with fosetyl-Al. These parameters were monitored in 2007 and 2009. Bars indicate standard errors of the means for each day of inspection in the surveyed period; for day of inspection, means of fosetyl-Al and control marked with asterisk (*) are different (P=0.05; analysis of variance).

at 3 and 9 days after the treatment compared with the control leaves, while by day 13 activity had decreased. A basal level of 1,3-β-glucanase activity was detected at all sampling times in the experimental controls.

Basal lipoxygenase activity was recorded in the leaves treated with water over the entire pH range, with no appreciable differences were detected between the sampling times (Table 3). Over the pH range studied, leaves that had been treated with fosetyl-Al had increased lipo-oxygenase activity at 3 d and lasting through to 9 d after treatment. This increase was statistically significant at 6 d (at pH 5.5), from 6 to 13 d (at pH 6.5), and at 9 d (pH 9). On the other hand, lipoxygenase activity was less at 13 d at pH 5.5 and pH 9.

Peroxidase activity measured in the protein extracts was completely inhibited by fosetyl-Al and no differences in the expression of the basal level ($3.95\ 10^{-6}$ U mL^{-1}) was ever recorded.

Discussion

This study has shown that formulations containing fosetyl-Al applied against downy mildew can limit the extent of necrosis in the wood of potted grapevine plants inoculated with Pch and Pal, and that these treatments can reduce both esca leaf symptom expression and mortality to the disease in field situations.

Although the etiology of the disease is still controversial, our research was focused on Pch and Pal, because recent studies have indicated their main role in all esca syndromes, and in the occurrence of foliar symptoms (Bruno and Sparapano, 2006; Mostert *et al*., 2006; Andolfi *et al*., 2009; Calzarano and Di Marco, 2007).

Tests on grapevine plants grown in pots showed a reduction in the necrosis caused by Pch and Pal after treatment with fosetyl-Al. This is consistent with the findings from a study by Laukart *et al*. (2001) who reported lower incidence of necrosis in

Table 2. Mean amounts of *trans*-resveratrol and ε-viniferin detected from wood of potted grapevine plants either treated with fosetyl-Al or untreated. Five plants were inoculated (December) sprayed with fosetyl-Al in three applications (May), and the wood around the margins of the necrotic zones was analysed 7 days from the last application.

Treatment	*Trans*-resveratrol (mg g^{-1})	ε-Viniferin (mg g^{-1})
Pch inoculated treated	2.66 a[a]	5.33 a
Pch inoculated untreated	2.90 a	5.00 a
Sterile plug treated	2.15 a	4.12 a
Sterile plug untreated	2.05 a	4.52 a
Healthy[b] treated	0.05 b	2.70 b
Healthy untreated	0.83 b	2.34 b

[a]Means in each column followed by the same letter do not differ (P=0.05), Duncan's multiple range test.
[b]Samples from healthy plants were collected from the same part of the plant trunks as from wounded vines.

Table 3. Mean activities of 1,3-β-glucanase and lipoxygenase detected in leaves of potted grapevine plants at different intervals after treatment with fosetyl-Al. Ten leaves were collected 3, 6, 9 and 13 days after fosetyl-Al application.

Day[a]	1,3 β glucanase (U ml^{-1} 10^{-2} ± SD[b])		Lypoxygenase pH 5.5 (U mg protein $^{-1}$ ± SD[b])		Lypoxygenase pH 6.5 (U mg protein $^{-1}$ ± SD[b])		Lypoxygenase pH 9.0 (U mg protein $^{-1}$ ± SD[b])	
3	5.88±0.45a[c]	2.92±0.04	0.15±0.01N	0.13±0.02	0.19±0.04N	0.15±0.01	0.19±0.04N	0.14±0.01
6	4.28±0.15N	4.20±0.40	0.28±0.02b	0.15±0.01	0.17±0.01a	0.11±0.03	0.15±0.04N	0.15±0.01
9	4.41±0.32b	3.45±0.27	0.17±0.05N	0.11±0.03	0.92±0.16b	0.13±0.03	0.68±0.09c	0.18±0.01
13	2.63±0.10b	3.97±0.52	0.08±0.01b	0.14±0.01	0.45±0.10a	0.18±0.01	0.10±0.01a	0.14±0.01

[a]Days after treatment.
[b]Means are followed by SD, standard deviation (95%) of the mean of data recorded in three separate experiments.
[c]For each enzymatic activity, means of fosetyl-Al and untreated control in the same row followed by the letters a, b or c are different (P=0.05, P=0.01, and P=0.001 respectively) according to t-tests. N = non significant (P>0.05).

nursery plants naturally infected with Pch, after treatment with fosetyl-Al. The present study indicates that reduction of the extent of necrosis was not linked to variation in the levels of stilbenes, since high levels of stilbenes were been found in wood wounded and treated both with sterile agar plugs and plugs of the fungal esca pathogen cultures, and this increase also occurred after fosetyl-Al application (Amalfitano *et al*., 2000). Our previous laboratory studies showed that phosphorous acid could hamper the ability of Pal and Pch to metabolize and inactivate resveratrol and pterostilbene (Mazzullo *et al*., 2000).

The control of esca pathogens in grapevine nurseries is still limited: often hot water treatment (HWT) or, to a lesser extent, *Trichoderma* treatments, give reductions of infection levels (Di Marco and Osti, 2007). The reduction of the severity of Pch and Pal infections in young potted plants shown in this study leads to the hypothesis that foliar fosetyl-Al formulations applied against downy mildew in nurseries could be proposed to integrate with HWT and/or *Trichoderma* in order to improve the management of infections caused by esca pathogens.

The reduction of the foliar symptoms by fose-

tyl-Al applications in the field is likely to be due to a combination of interacting effects.

Assays of enzyme activity in the present study showed no peroxidase in the leaves treated with fosetyl-Al, findings which are consistent with a report by Lopez Serrano *et al*. (1996). On the other hand, elevated levels of lipo-oxygenases were recorded in the treated leaves. This implies that increased peroxide levels and increased oxidative capacity in treated leaves provides an unfavourable environment for fungal toxins such as scytalone and isosclerone, or for their mechanisms of action (Bruno and Sparapano, 2006). Furthermore, the observed rise in 1,3-β-glucanases could contribute directly to the degradation of the polysaccharide toxins produced by Pch (Andolfi *et al*., 2009). Dercks and Creasy (1989) and Creasy and Qi (2005) showed that grapevine leaves treated with fosetyl-Al had increased resveratrol content, with greatest levels 4 d after treatment. It is thus possible that, in the present trial, enhanced production of resveratrol enhanced leaf reaction to the effects of the infection (i.e. toxin accumulation).

Our study also demonstrated significant effects of fosetyl-Al formulations on the physiology of grapevine: i.e. reduced photosynthesis, transpiration rate and stomatal conductance detected for up to 3 or 4 d after treatment. Since the experimental control plants were treated with products containing the same active ingredients as the formulations applied to the treated plants, with the exception of fosetyl-Al, this indicateds the fosetyl-Al plays a major role in inducing these changes in leaf physiology. The ability of fosetyl-Al or phosphonates to induce a misfunctioning of plant stomata has been demonstrated in other studies (Vitagliano and Hoad, 1978; Cali, 2009). Moreover, increased esca foliar symptoms in vineyards treated with biostimulants was supposed to be due to increased plant physiological process favouring movement of fungal toxins to leaves (Surico *et al*. 2006; Di Marco and Osti, 2009).

To summarize, it is possible that fosetyl-Al can produce conditions in grapevine leaves unfavourable for toxin action, and can also reduce translocation of the toxins or reduce their levels below those sufficient to cause typical esca symptoms.

In our field study in vineyards, all formulations containing fosetyl-Al reduced incidence of symptomatic plants regardless of the different non-fosetyl-Al formulation used in one year or another. Although the reduction of symptomatic plants observed in each year of investigation in plots treated with fosetyl-Al was in many cases statistically significant, such reduction was not so remarkable. However, after many years of treatment, the much greater cumulative incidence of disease generally observed in untreated plots clearly demonstrated that adding fosetyl-Al as a long-term downy mildew control method, could form an improvement for the esca management, also making more convenient to adopt the traditional cultural practices. Integrated esca management has been previously advocated as the best approach for management of this disease, to improve vine health and longevity (Di Marco *et al*., 2000).

The trend in the mortality rates of symptomatic plants was similar for the three cultivars studied, with a reduction in mortality in the plots that had been treated with mixtures containing fosetyl-Al. On the basis of data gathered so far, this suggests that the enhancement of host defence systems can play a role in such mortality reduction. Similarly, a reduction of sudden oak death caused by *Phytophthora ramorum* treated with phosphonate has been reported, and is probably linked to increased of plant resistance to the pathogen (Garbelotto *et al*., 2008).

Despite the large number of studies on the development of esca control strategies for vineyards, none of the treatments tested proved to be effective against the disease (Mostert *et al*., 2006; Di Marco and Osti, 2009). After the banning of sodium arsenite treatments, fosetyl-Al is the only chemical treatment that provided decreases in foliar symptom expression, which is strictly correlated with the reduction in quantity and quality of the yield (Calzarano *et al*., 2004b).

In conclusion, the present study investigating the nature of the relationship between pathogens and host physiology or resistance mechanisms has clearly shown that fosetyl-Al can reduce the effects of tracheomycotic pathogens on grapevine plants. Moreover, the activity of the chemical against esca has to be considered as side effect of treatment strategies against downy mildew, and therefore the added benefit could be achieved at no extra cost. Evidence presented in this study also suggest that products containing fosetyl-Al could have additional value contributing to esca management, combined with other esca control methods.

Acknowledgments

This research was financially supported by the Inter-Regional Project "Grapevine esca: research and experiment in the nursery and in the field for prevention and cure" Ministero per le Politiche Agricole e Forestali (Ministry for Agriculture and Forestry Policy) and by the Centro Ricerche Produzioni Vegetali (CRPV). The authors are indebted to Professor G. Surico, Dipartimento di Biotecnologie Agrarie, Università degli Studi di Firenze, Italy, for suggestions and revision of the manuscript. We also thank Dr. M. Scannavini for helping to collect vineyard data.

Literature cited

Agrelli D., C. Amalfitano, P. Conte and L. Mugnai, 2009. Chemical and spectroscopic characteristics of the wood of *Vitis vinifera* cv. Sangiovese affected by esca disease. *Journal of Agriculture and Food Chemistry* 24, 11469–11475.

Amalfitano C., A. Evidente, G. Surico, S. Tegli, E. Bertellli and L. Mugnai, 2000. Phenols and stilbene polyphenols in the wood of esca-diseased grapevines. *Phytopathologia Mediterranea* 39, 178–183.

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, 680–684.

Ashwell G., 1957. Colorimetric analysis of sugars. *Methods in Enzymology* 3, 73–105.

Bradford M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248–254.

Bruno G. and L. Sparapano, 2006. Effects of three esca-associated fungi on *Vitis vinifera* L. I. Characterization of secondary metabolites in culture media and host responses to the pathogens *in calli*. *Physiological and Molecular Plant Pathology* 69, 209–223.

Cali Ö.I., 2009. The effect of fosetyl-Al application on stomata in tomato (*Lycopersicon esculentum* Mill.) plant. *Journal of Plant Breeding and Crop Science* 1, 45–48.

Calzarano F. and S. Di Marco, 2007. Wood discoloration and decay in grapevines with esca proper and their relationship with foliar symptoms. *Phytopathologia Mediterranea* 46, 96–101.

Calzarano F., S. Di Marco and A. Cesari, 2004a. Benefit of fungicide treatment after trunk renewal of vines with different types of esca necrosis. *Phytopathologia Mediterranea* 43, 116–123.

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

Caruso C., G. Chilosi, C. Caporale, L. Leonardi, L. Bertini, P. Magro and V. Buonocore, 1999. Induction of pathogenesis-related proteins in germinating wheat seeds infected with *Fusarium culmorum*. *Plant Science* 140, 87–97.

Coombe B.G., 1995. Adoption of a system for identifying grapevine growth stages, *Australasian Journal of Grape and Wine Research* 1, 104–110.

Chuang H.W., T.F. Hisieh, M. Duval and T.L. Thomas, 2003. Genomic analysis of *Arabidopsis* gene expression in response to a systemic fungicide. In: *Genomics of Plants and Fungi* (R. A. Prade, H. J. Bohnert, ed.), Marcel Dekker inc., New York, NY, USA, 237–253.

Creasy G.L. and G. Qi, 2005. Grapevine leaf and fruit tissue responses to fosetyl-Al and UV radiation. *American Journal of Enology and Viticulture* 56, 3.

Dercks W. and L.L. Creasy, 1989. Influence of fosetyl-Al on phytoalexin accumulation in the *Plasmopara viticola*-grapevine interaction. *Physiological and Molecular Plant Pathology* 34, 203–213.

Di Marco S., A. Mazzullo, F. Calzarano and A. Cesari, 1999. *In vitro* studies on the phosphorous acid - vitis stilbenes interaction, and *in vivo* phosetyl Al activity towards *Phaeoacremonium* spp. grapevine wood decay agents. In: *Modern Fungicides and Antifungal Compounds II* (H. Lyr, P.E. Russel, H.W. Dehene, H.D. Sisler, ed.), Intercept, Andover, UK, 171–178.

Di Marco S., A. Mazzullo, F. Calzarano and A. Cesari, 2000. The control of esca: status and perspectives. *Phytopathologia Mediterranea* 39, 232–240.

Di Marco S. and F. Osti, 2007. Applications of *Trichoderma* to prevent *Phaeomoniella chlamydospora* infections in organic nurseries. *Phytopathologia Mediterranea* 46, 73–83.

Di Marco S. and F. Osti, 2008. Foliar symptom expression of wood decay in *Actinidia deliciosa* in relation to environmental factors. *Plant Disease* 92, 1150–1157.

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

Doderer A., I. Kokkelink, S. van der Veen, B.E. Valk, A.W. Schram and A.C. Douma, 1992. Purification and characterisation of two lipoxygenase isoenzymes from germinating barley. *Biochimica and Biophysisica Acta* 1120, 97–104.

Fenn M.E. and M.D. Coffey, 1985. Further evidence for the direct mode of action of fosetyl-Al and phosphorous acid, *Phytopathology* 75, 1064–1068.

Garbelotto M., T.Y. Harnik and D.J. Schmidt, 2008. Efficacy of phosphonic acid, metalaxyl-M, and copper hydroxide against *Phytophthora ramorum in vitro* and *in planta*. *Plant Pathology* 58, 111–119.

Kauffmann S., M. Legrand, P. Geoffroy and B. Fritig, 1987. Biological function of 'pathogenesis-related' proteins: four PR proteins of tobacco have 1,3-β-glucanase activity. *EMBO Journal* 6, 3209–3212.

Laukart N., J. Edwards, I.G. Pascoe and N.K. Nguyen,

2001. Curative treatments trialed on young grapevines infected with *Phaeomoniella chlamydospora*. *Phytopathologia Mediterranea* 40, S459–S463.

Lopez Serrano M., M.A. Ferrer, R. Munoz, M.A. Pedreno and A.R. Barcelo, 1996. Antagonistic effects of fosetyl-Al and ethylene on peroxidase from grapevine cells. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz* 103, 200–205.

Marchi G., F. Peduto, L. Mugnai, S. Di Marco, F. Calzarano and G. Surico, 2006. Some observations on the relationship of manifest and hidden esca to rainfall. *Phytopathologia Mediterranea* 45, S117–S126.

Mazzullo A., S. Di Marco, F. Osti, A. Cesari, 2000. Bioassays on the activity of resveratrol, pterostilbene and phosphorous acid towards fungi associated with esca of grapevine. *Phytopathologia Mediterranea* 39, 1–9.

Mostert L., F. Halleen, P. Fourie and P.W. Crous, 2006. A review of *Phaeoacremonium* species involved in Petri disease and esca of grapevines. *Phytopathologia Mediterranea* 45, 12–29.

Mugnai L., A. Graniti and G. Surico, 1999. Esca (Black Measles) and brown wood-streaking: two old and elusive diseases of grapevines. *Plant Disease* 83, 404–418.

Nemesthoty G.S. and D.I. Guest, 1990. Phytoalexin accumulation, phenylalanine ammonia lyase activity and ethylene biosynthesis in fosetyl-Al treated resistant and susceptible tobacco cultivars infected by *Phytophtora nicotianae* var *nicotianae*. *Physiological and Molecular Plant Pathololog*y 37, 207–219.

Osti F. and S. Di Marco, 2010. 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

Sukarno N., S.A. Smith, E.S. Scott, G.P. Jones and S.E. Smith, 1998. The effect of fungicides on vesicular-arbuscular mycorrhizal symbiosis. III. The influence of VA mycorrhiza on phytotoxic effects following application of fosetyl-Al and phosphonate. *New Phytologist* 139, 321–330.

Surico G., 2009. Towards a redefinition of the diseases within the esca complex of grapevine. *Phytopathologia Mediterranea* 48, S5–S10.

Surico G., L. Mugnai and G. Marchi G, 2006. Older and more recent observations on esca, a critical overview. *Phytopathologia Mediterranea* 45, S68–S86.

Vitagliano C. and G.V. Hoad, 1978. Leaf stomatal resistance, ethylene evolution and ABA levels as influenced by (2-chloroethyl)phosphonic acid. *Scientia Horticulturae* 8, 101–106.

Accepted for publication: November 1, 2011