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First studies on the potential of a copper formulation for the control of leaf stripe disease within esca complex in grapevine

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Summary. An experimental formulation based on copper oxychloride and gluconates, which is able to penetrate vine foliar tissue, was tested for wood treatment against some of the fungi involved in the diseases included in the esca disease complex. *In vitro* trials were carried out to: i) test the effect of the formulation on the mycelial growth rate of *Phaeomoniella chlamydospora* (Pch), *Phaeoacremonium aleophilum* (Pal) and *Fomitiporia mediterranea* (Fmed), and on the conidial germination of Pch and Pal; and ii) conduct a preliminary investigation of the presence of the copper formulation on the ability of Pal to produce toxic metabolites. Trials were also conducted on potted vines to i) measure copper penetration into the trunk of potted vines using atomic absorption spectrophotometry; and ii) test the effect of the copper formulation on colonization of the fungus in trunks. Finally, the copper formulation was field-tested in vineyards showing leaf stripe. The implications of the tests in the control of the esca disease complex are discussed.

Key words: *Phaeomoniella chlamydospora, Phaeoacremonium aleophilum*, scytalone.

Introduction

Over the last ten years considerable research has been carried out on esca. This has led to a better understanding of the aetiology, epidemiology and hence control of the disease. It has also led to a tentative new definition of esca as being a complex of two distinct diseases: a vascular disease (grapevine leaf stripe [= young esca], associated with infections by vascular fungi, *Phaeomoniella chlamydospora* and often also *Phaeoacremonium aleophilum*) and a wood decay (true esca, caused by *Fomitiporia mediterranea* in Europe, and by many different basidiomycetes species in the other grape growing areas) (Surico *et al*., 2008; Fischer, 2006). The occurrence of both these diseases together in the same vine is now called esca proper (Surico, 2009). Despite many trials in both the nursery and the vineyard carried out after the sodium arsenite banning, there still is no chemical control method available against any of the diseases in the complex (Di Marco and Osti, 2007; 2009a; 2009b; Calzarano *et al*., 2004a; 2007; Fourie and Halleen, 2004; 2006; Fourie *et al*., 2001; Gramaje *et al*., 2009a; 2009b; 2010). Traditional cultural methods (including pruning wound protection) are recommended in order to limit yield loss and reduce the spread of the disease but remain not sufficient to ensure an effective control of esca proper, or of the two single diseases (Mugnai *et al*., 1999; Di Marco *et al*., 2000, 2004; Rolshausen *et al*., 2010).

Gravepine leaf stripe disease invariably cause a loss in yield and length of vineyard life (Calzarano and Di Marco, 2007; Calzarano *et al*., 2007). Reducing foliar symptoms may therefore limit such losses (Mugnai *et al*., 1999; Marchi *et al*., 2006;

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Calzarano and Di Marco, 2007; Calzarano *et al*., 2004b; Di Marco and Osti, 2008; 2009).

Application of fosetyl-Al on grapevine leaves to control downy mildew was observed to reduce the incidence of leaf stripe symptoms (Di Marco *et al*., 1999, 2011). The only known effective chemical to control the disease in the vineyard was the application of winter spraying with sodium arsenite (Desache *et al*., 1995; Larignon and Dubos, 1997; Di Marco *et al*., 2000). The mode of action of the arsenites, now banned because of their high toxicity, has not been explained, and they were not found to be really effective when tested *in vitro* against the esca complex associated fungi (Santos *et al*., 2006; Larignon, 2008). On the other hand the arsenites seem to accumulate in the vine wood following winter applications (Larignon, 2008). It was hypothesized that they interfered with the production of phytoxins (such as scytalone, isosclerone, polypeptides, exopolysaccharides) produced by the pathogens at the sites of mycelial colonization (Surico *et al*., 2006), and it is these metabolites that are mainly associated with the formation of the foliar symptoms (Surico, 2001; Bruno *et al*., 2007). Arsenite did not cure the disease in the vineyard but only alleviated or prevented the expression of leaf symptoms (Péros *et al*., 2008).

Copper is one of the earliest active ingredients employed to control plant diseases. Copper-based fungicides are used against a broad spectrum of diseases to prevent fungal or bacterial infections. To control fungal wood pathogens, copper compounds are usually applied soon after a severe frost or in some cases hail, in order to protect any resulting wounds from infection (Mugnai *et al*., 1999; Di Marco *et al*., 2000). A common practice, even if never experimentally tested, is also to protect pruning wounds with copper after mixing it with an acrylic glue.

Recently, copper compounds have been developed in the form of copper chelates. This reduces the impact of copper on the environment, as they are applied at a much lower rate, and is more effective and persistent than traditional formulations. Copper chelate compounds are formulated with amino acids, and with peptides or gluconic acid, and enable the copper to be absorbed into the plant tissue as organic molecules (Maini, 2002).

The aim of this study was to determine whether a gluconate copper formulation applied to the vine trunk in winter has some effect against the esca pathogens, recalling the activity of sodium arsenite, which also was mainly applied to the vine trunks in winter. Specifically, laboratory, greenhouse and field trials were carried out to test the effectiveness of an experimental formulation consisting of copper oxychloride, dyes and gluconates.

Here are given the results of the preliminary experimentation.

Materials and methods

In vitro trials

Mycelial growth assay

The pathogens tested were *Phaeomoniella chlamydospora* strain CBS229.95 (Pch); *Phaeoacremonium aleophilum* strain CBS631.94 (Pal) and *Fomitiporia mediterranea* strain CBS201.03 (Fmed). The experimental copper formulation (CF), prepared by Productos Agrícolas Macasa S.L. (Igualada, Barcelona, Spain), consisted of copper oxychloride (3%), dyes (2%) and gluconates. It was tested against each pathogen at a variety of concentrations. The CF was dissolved in water and the solution added to a potato dextrose agar (PDA) medium at 40°C, and at final concentrations of 100, 10, 1, and 0.1 mg Cu L^{-1}. Plugs (5 mm) of each fungus were cut from the margin of growing cultures on PDA and placed at the centre of Petri dishes containing PDA plus CF (only PDA for the control). Petri dishes were incubated at room temperature (18 to 25°C) in the dark. After 20 days (for Fmed), or 25 days (for Pch or Pal), colony diameters were measured twice on each Petri dish and averaged. Treatments were replicated four times, each replicate consisting of one Petri dish. Probit analysis was performed to calculate the EC_{50} values using SAS system software version 9.1 (Anonymous, 1990).

Conidial germination assay

Conidial germination was assessed for Pch and Pal above mentioned strains, following Jaspers (2001). The CF was tested against each fungus at 5 concentrations of copper oxychloride 100, 10, 1, 0.1, and 0.01 mg Cu L^{-1}, in a water-agar medium. Unamended water agar was used as the control. Conidia were harvested in sterile distilled water from 3-week-old colonies grown on PDA in Petri dishes. Aliquots (1 mL) containing 2×10^6 conidia

of each fungus were spread on the Petri dishes plates containing the control medium or the medium amended with the CF at the various concentrations. Petri dishes were incubated for 48 h in the dark. Treatments were replicated four times, each replicate consisting of one Petri dish. A 1 cm^2 square agar plug was cut from each Petri dish, placed on slides, and the germinated conidia were counted under a microscope, assessing 100 conidia. A conidium was considered germinated if its germ tube was at least as long as the conidium itself.

Data were subjected to Probit analysis (95% confidence limit) using SAS system software version 9.1 (Anonymous, 1990). Results are expressed as EC_{50} values.

Detection of scytalone and isosclerone in Pal filtrates

The trial was carried out as a preliminary investigation using a Pal strain as the species naturally produces higher levels of scytalone than Pch (Bruno and Sparapano, 2006), and low levels of isosclerone. For phytotoxin detection, stationary liquid cultures of Czapek-Dox broth (Sigma-Aldrich, Milwaukee, WI, USA), enriched with 0.1% yeast extract and 0.1% malt extract were used. The medium was autoclaved and distributed into sterile flasks (150 mL per flask) (Sparapano *et al.,* 2000). Where indicated, 0.1 mg L^{-1} of copper was added using the commercial formulation. A 5-mm-diam. plug of agar mycelium taken from the edge of 2-week-old growing colonies of Pal (CBS631.94) was placed in each flask, which contained fresh medium. Stationary cultures were incubated at 25±2°C in the dark for 28 days. The medium was then filtered in tulle cloth and blotting paper to remove mycelium and conidia from the culture filtrate. The trial was replicated three times, one flask per replicate.

Culture filtrates were tested for the presence of scytalone and isosclerone by LC/MS-MS analysis by means of an agilent 1200 rapid resolution HPLC system equipped with an Agilent 6410 triple quadrupole MS detector operating in ESI negative mode. Chromatographic conditions were: column: C18 Phenomenex luna 15 cm 3 μm particle size, 100 A porosity. Solvent flow: 0.25 mL min^{-1}, water/acetonitrile (both acidified by 1‰ formic acid) linear gradient as follows: acetonitrile initial concentration 5%, then 15% at 8 min, then 90% at 15 min, hold until 18 min, then 5% at 21 min, hold until 25 min.

The detection of trace concentration of the two toxins was carried out at the Centro Interdipartimentale di Spettrometria di Massa, Sesto Fiorentino, Firenze, Italy. The source ionization conditions and collision induced dissociation (CID) condition were established with pure analytes in continuous flow injection mode. Source conditions were: gas (N_2), temperature 350°C, vaporizer temperature 250°C, gas flow 8 L min^{-1}, nebulizer gas pressure 30 psi, capillary voltage 4000 V, charging voltage 2000 V. The most suitable conditions for MS/MS detection were reported in Table 1.

Trials on potted vines

Effect of copper oxychloride on P. chlamydospora

The rootstocks of sixty 2-year-old potted vines (cv. Merlot/SO4) grown in an unheated glasshouse, were inoculated with Pch as main vascular pathogen involved in esca disease complex (Surico *et al.*, 2008); a plug of a PDA culture (sterile agar for the control) was inserted into a hand-drilled

Table 1. Conditions used for MS/MS detection of scytalone in *Phaeoacremonium aleophilum* culture filtrate. At these analytical conditions the limit of detection (LOD) and limit of quantitation (LOQ) were 1 μg L^{-1} and 5 μg L^{-1}, respectively, as established by spiking known amount of analytes in a previously analyzed (negative) blank sample pool.

Compound	Precursor ion (*m*/*z*)	1st quadrupole resolution	Product ion (*m*/*z*)	3rd quadrupole resolution	Dwell time (ms)	Fragmentor (V)	Collision energy (V)
Scytalone	193	unit	151	unit	50	100	22
Scytalone	193	unit	123	unit	50	100	28
Isosclerone	177	unit	159	unit	50	60	16
Isosclerone	177	unit	133	unit	50	60	16

hole (4–5 mm deep), (Di Marco and Osti, 2009). The holes were covered with Amojell lubricant (Sigma-Aldrich, Milwaukee, WI, USA) and sealed with Parafilm M (American National Can, Chicago, IL, USA) to prevent drying. The vines were uniformly sprayed with CF to incipient runoff using a using an 8 L capacity Green pre-compression pump with a hand-held Spray/Get (Volpi Originale, Casalromano, Mantova, Italy). The experimental formulation was sprayed at 0.1 g Cu L^{-1} in the following three phenological stages (except when stated differently): 1) at the start of leaf fall; 2) at the end of pruning; 3) at 5–6 cm shoot length, as recommended by the manufacturer.

The trials on potted vines were carried out over a 3-year period following one of two schedules. Schedule A (Pch inoculation after the first spraying): 1st year, two CF sprays, at leaf fall and end of pruning, followed by Pch inoculation in the following spring; 2nd and 3rd, three CF sprays (following recommendation). Schedule B (Pch inoculation before first year spraying): 1st year, Pch inoculation at the end of summer, followed by one CF spray at the end of leaf fall and one at 6–7 cm shoot length; 2nd and 3rd year, three CF sprays (following recommendation)

Each trial comprised twelve replicates, one vine per replicate. At the end of each year of treatment the length of the necrotic streaks was measured on three vines. A further *in vitro* assessment was made to verify fungal colonization of the necrotic streaks measured. Data were expressed as the average length of necrosis, and statistically analyzed using Duncan's multiple test, P=0.05.

Detection of copper inside the wood tissue of potted vines

The CF at a concentration of 0.1 g Cu L^{-1} was sprayed on a number of 2-year-old potted vines, as above, with controls sprayed with water only. Vines were sprayed to incipient runoff soon after the end of leaf fall. Samples were collected 2 or 10 days after spraying. Two replicates per treatment were set up: for each treatment six vines were uprooted and divided into 2 groups of 3 vines, each vine being a replicate. The central portion of the vine stem was cut and soaked for 30 min in a buffer phosphate solution (pH 4.5) to facilitate the removal of any copper residues from the surface (Ivan Portillo, DIPROVAL, University of Bologna, Bologna, Italy, personal communication). The stems were then washed with distilled water. The operation was repeated twice. To prevent any buffer solution from being absorbed by the cut ends of the stems, 3 cm of wood at either end was cut off and discarded. The sample was then carefully scrubbed with an iron brush to remove bark tissue. The brush was carefully cleaned between samples.

Copper in the vine samples was detected by inductively coupled plasma-optical emission spectrometry (ICP-OES) (Stilwell *et al*., 2003). Each vine sample consisted of wood from three vines, prepared as follows: the wood was ground to sawdust (particle size less than 0.25 mm) and dried at 105°C. Approximately 1.5 g of sawdust was placed into 20 mL of 70% HNO_3. Samples were mineralized for 120 min at 110–120°C in a DigiPREP Jr heating block (SCP Science, Quebec, Canada) equipped with a special temperature control probe DigiPROBE. The sample was then analyzed by ICP-OES (Perkin Elmer - Optima model 7300DV N0770796). A calibration curve was prepared from two acidic solutions: one containing 120 mg kg^{-1} of Ca, K, Mg, Na and P, plus 40 mg kg^{-1} of Cu, Fe, Al, Mn, Sn, and Zn; and one containing 80 and 20 mg kg^{-1} of these two groups of macro and micro-nutrients respectively. The solution containing macro and micro nutrients at 80 and 20 mg kg^{-1} was twice diluted successively to a final concentration of 20 and 5 mg kg^{-1} for macro and micro-nutrients respectively, to obtain the calibration curve. Errors due to the chemical and physical condition of the samples were corrected by adding an internal standard (Y at 2 mg kg^{-1}). The blank consisted of 20 mL HNO_3 70%.

The copper content was determined at a wavelength of 327.4 nm and validated by comparing it with both an external calibration curve and an internal standard. Data represent the mean of two replicates and were expressed as mg Cu kg^{-1} of dry weight.

Field trials

Vineyards of cv. Pignoletto and Riesling, belonging to the same producer and established in 1991, were used for field trials (Table 2). Both vineyards had been surveyed and mapped for incidence of grapevine leaf stripe symptoms (esca foliar symptoms) since 2001 (cv. Pignoletto) and 2003 (cv. Ries-

ling). Large plots were used to counteract symptom variation across the vineyards. In both vineyards, two plots containing 500 vines each were used for treatment, with CF sprayed during the 2006 to 2009 growing seasons at a concentration of 3.3 mL L^{-1}, corresponding to a copper concentration of 106 mg L^{-1}. In each vineyard, a 500-vine-plot was left unsprayed as a control. Three annual sprays were applied with the producer's sprayer using the following schedule: after harvest (end of September), at pruning time (January), and when the vine-shoot length reached 8–10 cm (May). Sprays were carried out following the above schedule for three application cycles from September 2006 until January 2009.

Leaf stripe symptoms were recorded on each vine each year in the first week of September. On the basis of these inspections the annual incidence of esca was calculated as a percentage of vines that exhibited leaf stripe in that year, and the cumulative incidence of esca each year was calculated as the sum of the annual incidence in that year plus the number of all those standing vines that had had leaf stripe symptoms in any previous year during the survey period (Marchi *et al.,* 2006). Surveys continued until the 2010 growing season.

For each vineyard and for each year of investigation, the annual and cumulative incidences of symptomatic plants were recorded in treated and untreated plots. A statistical analysis was carried out comparing the results between the two plots in each vineyard and in each year using the Chi-Square test (P=0.05) by SAS System Software version 9.1 (Anonymous, 1990).

Results

In vitro trials

Mycelial growth and conidial germination assay

The effect of the culture filtrate on the mycelial growth and the conidial germination of the main disease agents are shown in Table 3. The CF substantially reduced mycelial growth of Pch with 1.449 mg Cu L^{-1} compared with Pal and Fmed, with EC_{50} values at 11.610 and 11.242 mg Cu L^{-1} respectively. It also greatly reduced conidial germination of Pch, with an EC_{50} of 0.038 mg Cu L^{-1}, compared with Pal with an EC_{50} of 27.669 mg Cu L^{-1}

Detection of scytalone and isosclerone in Pal filtrates

The culture filtrate of the Pal strain grown in liquid culture was analysed for scytalone and isosclerone content, revealing that Pal produced a detectable amount of scytalone (24 μg L^{-1}) in the control medium, despite having no effect on the fungal growth. Isosclerone was below detectable level. On the contrary, when Pal was grown in a medium containing the CF, no scytalone (detection threshold = 2 μg L^{-1}) was detected in the culture filtrate.

Trials on potted vines

Effect of copper oxychloride on P. chlamydospora

The CF sprayed on potted vines either before or after Pch inoculation, did not reduce the size of the necrosis caused by the pathogen (Table 4).

Table 2. Details of vineyards in which field trials were carried out: fields were first sprayed with CF after harvest in 2006.

Cultivar	First year of assessment of leaf stripe symptoms	Sprayed plot			Unsprayed plot		
		Number of vines inspected	Rows	2006 Cumulative incidence[a]	Number of vines inspected	Rows	2006 Cumulative incidence[a]
Pignoletto	2001	466	6	32	518	5	29
Riesling	2003	470	5	16.4	505	6	16.1

[a] For each vineyard, cumulative incidence was recorded evaluating foliar symptoms since the first year of assessment.

Table 3. Effectiveness of the copper formulation against the mycelial growth and conidial germination of species in the esca complex fungi.

Fungal species	Mycelial growth		Conidial germination	
	EC_{50} (mg Cu L^{-1})	95% confidence limit	EC_{50} (mg Cu L^{-1})	95% confidence limit
Phaeomoniella chlamydospora	1.449[a]	0.763–2.652[a]	0.038[a]	0.025–0.057[a]
Phaeoacremonium aleophilum	11.610	6.694–21.224	27.669	24.832–30.867
Fomitiporia mediterranea	11.242	7.209–18.043	n.a.[b]	n.a.[b]

[a] EC_{50} and 95% confidence limit was calculated from probit analysis, SAS system software version 9.1 (Anonymous, 1990).
[b] n.a., not applicable.

Detection of copper inside the wood tissue of potted vines

Copper was detected inside the trunk of potted vines two (87 mg Kg^{-1}) and ten (58 mg Kg^{-1}) days after spraying, soon after the end of leaf fall. The values of copper detected on the trunk surface did not change over time (Figure 1).

The product penetrated at 1 to 3 mm below the bark.

Field trials

The CF applications at max. 1.5 kg Cu per hectare (Di Marco *et al.,* 2010) significantly reduced the annual incidence of leaf stripe symptoms in both vineyard 1 (inspected since 2001) and vineyard 2 (inspected since 2003) for the three years surveyed (Figure 2). In the season following each CF application, there was a significant reduction in incidence of symptoms compared with untreated control vines with the exception of vineyard 1 (cv. Pignoletto) in 2008 (Figure 2A).

On both cultivars the rate of cumulative incidence of symptoms was significantly less in each season following treatment with CF, with the exception of vineyard 1 in 2007 (Figure 2A). For both vineyards in 2010, when the CF was no longer sprayed, there was no significant difference in the annual disease incidence (Figure 2).

Discussion

The activity of several fungicides has been tested in *in vitro* studies on Pch strains, but none of the tested products had an appreciable effect on both mycelial growth and conidial germination: systemic fungicides such as DMI, triazole and, in particular, benzimidazole have been effective on mycelial growth but not on conidial germination, whereas the contact fungicides folpet, chlorothalonil and anylopyrimidine were effective only on conidial germination (Jaspers, 2001; Groenwald *et al*., 2000; Gramaje *et al*., 2009b). The CF tested

Table 4. Effect of copper formulation sprays on the length of necrosis caused by *Phaeomoniella chlamydospora* (Pch) and on extent of colonization 3 years after inoculation in the trunk of potted grapevines.

Spray treatments	Schedule A		Schedule B	
	Necrosis average length (cm)	Fertile fragments for Pch (%)	Necrosis average length (cm)	Fertile fragments for Pch (%)
Sprayed	5.2 a[a]	4.9 a[a]	4.5 a[a]	4.9 a[a]
Unsprayed	4.2 a	6.7 a	4.4 a	6.7 a

[a] Values in column followed by the same letter do not differ significantly according to Duncan's multiple range Test (P =0.05).

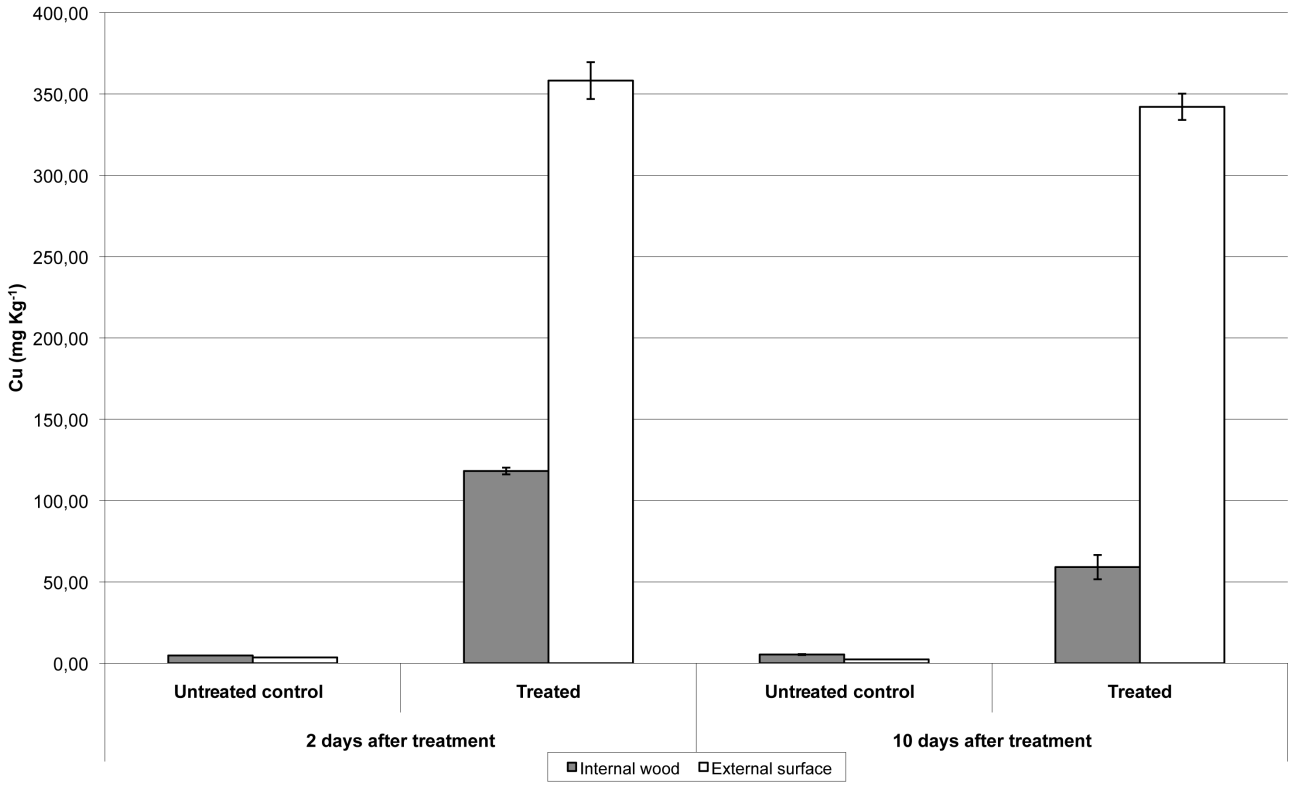

Figure 1. Amount (mg Cu kg^{-1}) of copper sprays detected on the surface or in the wood of stems of potted vines 2 or 10 days after spraying. Copper in the wood samples was detected by inductively coupled plasma-optical emission spectrometry.

in this study reduced conidial germination more than mycelial growth, as shown by other copper formulates (Gramaje *et al*., 2009b). The CF inhibited conidial germination of Pch similarly to the most efficient fungicides tested by Jaspers (2001), and better than another commercial copper oxychloride formulation, Kocide DF. What differentiates the application of the CF tested here in respect to other copper formulations is its ability to penetrate into the wood tissue, as was also shown for arsenites applied in winter treatments against "esca" disease (Larignon, 2008).

As with sodium arsenite, despite the ability to penetrate the wood, when the CF was applied to artificially inoculated young potted vines it did not reduce Pch colonization. Nevertheless field trials confirmed the activity of CF in reducing significantly the development of foliar symptoms over three years of application. Similarly, the application of sodium arsenite to grapevines with esca reduced foliar symptoms but had no effect on fungal colonization (Larignon, 2008). Therefore, the effectiveness of CF and sodium arsenite on foliar symptom expression does not appear to be strictly linked with their fungicidal or fungistatic activity on the fungi associated with the disease.

A possible hypothesis to explain this effect was provided by a preliminary investigation on the interactions of the product with fungal metabolism. In *in vitro* growth tests, CF inhibited the production of scytalone by Pal, a toxic metabolite associated with foliar symptom development (Abou-Mansour *et al*., 2004; Surico *et al*., 2006). Although more research is required to confirm this, our data may provide information for the understanding of the formation of grapevine leaf stripe foliar symptoms.

Further studies are needed to evaluate this and other copper formulations, for their ability to penetrate the wood of adult vines in the field, to interfere with fungal metabolism, and limit the pro-

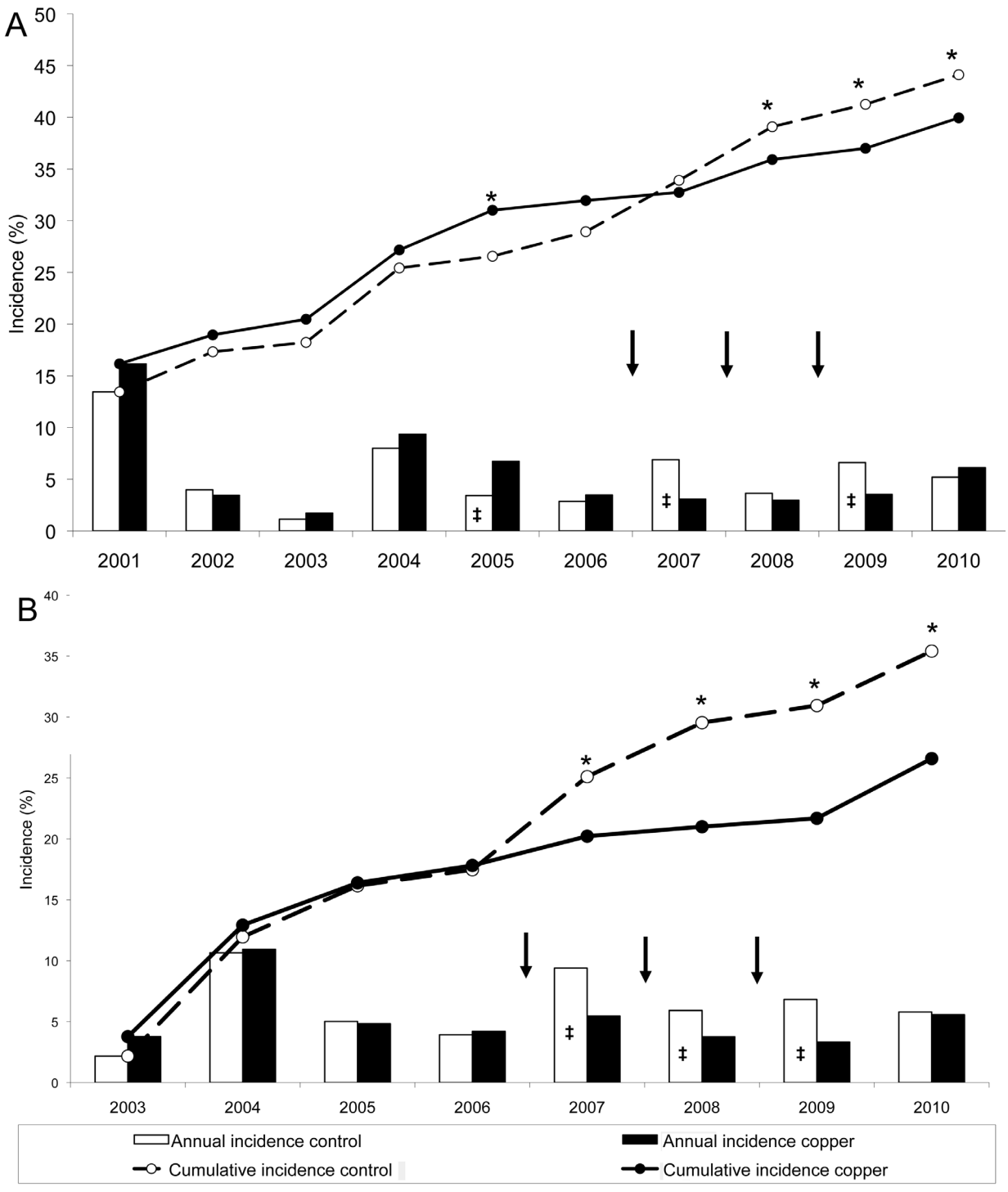

Figure 2. Effects of applications of the copper formulation on vineyards cv. Pignoletto (A) and Riesling (B). For each year of assessment, values of annual tiger stripe symptoms incidence (bar) or cumulative incidence (line) marked with * (cumulative incidence) or with ‡ (annual incidence), differ significantly according to Chi–Square test ($P<0.05$). Arrows indicates the years in which the applications were made.

duction of toxins associated with foliar symptoms. Copper formulations may also have the potential to reduce the inoculum load by acting as contact fungicides, given the amount that remains on the bark surface.

This study offers important new insights into the potential use of CF for the control of the esca disease complex, and may provide an alternative to sodium arsenite, which is banned for use on grapevine. The results of this study also open new possibilities for research into the factors leading to foliar symptoms.

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