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Experiments on the control of esca by *Trichoderma*

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Summary. *Trichoderma harzianum* T39 (Trichodex®) and *T. longibrachiatum* strain 6 were applied on grapevine to determine their effectiveness against *Phaeomoniella chlamydospora* on vine cuttings and pruning wounds. Cuttings were dipped in a *Trichoderma* suspension either before or after callusing. Pre-callusing dips were carried out for 3 years and yielded contradictory results. By contrast, post-callusing *Trichoderma* dips led to significant growth of hairy roots and a reduction in the longitudinal discolorations caused by *P. chlamydospora* inoculated into the rootstock after dipping. *Trichoderma* spp. were also applied to pruning wounds of grafted potted vines, which were then inoculated by placing drops of a conidial suspension of *P. chlamydospora* on the wound surface. *Trichoderma* application here prevented black goo and necrosis in the wood below the wound. In the vineyard, *T. harzianum* T39 was sprayed after pruning for two consecutive years. The biocontrol agent was reisolated from the wood close to the sprayed pruning wounds for up to 2 months after spraying. Although further investigations are necessary, our findings suggest that *Trichoderma* could be one of the steps in the control of esca.

Key words: grapevine, biocontrol, *Phaeomoniella chlamydospora*, nursery, pruning wounds protection.

Introduction

Although all the causes and epidemiological factors of esca are not yet fully understood, recent studies have made clear that *Phaeomoniella chlamydospora* and/or *Phaeoacremonium* species are very likely to be involved in infecting young vines with esca in the nursery or in causing Petri disease (Edwards *et al.*, 2001; Gatica *et al.*, 2001; Surico, 2001; Zanzotto *et al.,* 2001). In the light of these findings, the search for control methods should focus on strategies for disease prevention and/or disease severity reduction (Di Marco *et al*., 2000).

Attempts to control esca with several chemicals have shown that complete prevention of esca is very difficult and that the outright eradication of esca fungi once they have colonized a vine plant is impossible (Mugnai *et al.*, 1999; Di Marco *et al.*, 2000). A more promising approach therefore seems to be to make vines more resistant to esca by using chemicals (e.g. fosetyl Al), or to apply biological control agents in the nursery and/or on young established vines (Di Marco *et al.*, 1999; Di Marco *et al*., 2002; Mazzullo *et al.*, 2000; Fourie *et al.*, 2001).

Species of the genus *Trichoderma* have become the most widely investigated of all potential mycofungicides that may represent an alternative to chemical control. Although their mode of action is not fully understood, they seem to be associated with mycoparasitism, the production of inhibitory compounds, competition for nutrients and space with pathogenic fungi, stimulation of plant growth and enhanced host resistance (Calderon *et al.*, 1993; Haram *et al.*, 1996; Zimand *et al.*, 1996; De Meyer

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et al., 1998; Elad *et al*., 1998; Harman, 2000; Woo *et al*., 2002). In a study on the control of esca, Di Marco *et al*. (2002) found that 22 strains of *Trichoderma* were active *in vitro* against *P. chlamydospora*. Some strains completely inhibited this fungus. Hunt *et al*. (2001) reported that *Trichoderma harzianum* exhibited various degrees of lytic action towards isolates of grapevine pathogens including *P. chlamydospora*. These authors also provided evidence suggesting that *Trichoderma* spp. protected pruning wounds against infection. Finally, Fourie *et al*. (2001) demonstrated that nursery applications of some strains of *T. harzianum* improved plant and total root mass development. That study suggested that *Trichoderma* treatments could lead to the development of stronger vines with lower rates of esca.

The present study was undertaken to determine whether applications of *Trichoderma* spp. on grapevines in the nursery carried out either before or after callusing would have beneficial effects on the plant and would be effective against *P. chlamydospora*, the fungus primarily involved, alone or with *Fomitiporia punctata*, in causing esca. Whether *Trichoderma* colonized pruning wounds in the vineyard when it was applied after pruning was also examined. Finally, *Trichoderma* was applied to pruning wounds on potted vines artificially inoculated with *P. chlamydospora*.

Materials and methods

Nursery treatment

Trials were carried out in a commercial nursery at Brisighella, in Emilia Romagna, north-central Italy. All plant material used (various scion/rootstock combinations as indicated below) was provided by the nursery.

Pre-callusing application

Trials were carried out in 2001–2003 on about 3600 grafted cuttings of different cv./rootstock combinations each year: 'Trebbiano'/SO4 in 2001, 'Sangiovese'/SO4 in 2002, and 'Lambrusco maestri'/Kober in 2003.

The scions and rootstocks were grafted following the routine nursery procedures. The graft union was then sealed with wax without fungicide, and the grafted cuttings were immersed for 30 min in a *Trichoderma* suspension (*T. harzianum* T39, Trichodex®, 10^9 cfu g^{-1}) at 400 g hl^{-1}, stirring the suspension in order to avoid the deposit sinking to the bottom of the container. Control grafted cuttings were immersed in water. Cuttings were then stacked with sawdust in separate callusing boxes, each containing approximately 900 vines, and stored at 10–20°C for 3 weeks. Two boxes per treatment were set up.

At the end of this period, the cuttings were grouped in three classes according to grafting callus formation: i) excellent: callus completely surrounding the trunk, ii) normal: callus partly surrounding the trunk, or iii) failed: non-callused cuttings to be discarded.

For each treatment, results were expressed as the mean percentage of excellent, normal and failed cuttings. Data were subjected to Chi-Square statistical analysis ($P \leq 0.05$) using SAS version 8.1 (SAS, 1990).

Post-callusing Trichoderma *application and* P. chlamydospora *inoculation*

Sixty grafted cuttings cv. Trebbiano (rootstock SO4) stored in callusing boxes prior to planting were used in these tests. The bottom ends (2–3 cm) of the cuttings were placed for 30 min in PVC containers with suspensions of *Trichoderma*: Trichodex® at 400 g hl^{-1}, or *T. longibrachiatum* strain 6 at 1.8×10^6 cfu ml^{-1} (collection of DIPROVAL, University of Bologna, Italy). Control grafted cuttings were dipped in water. Treated cuttings were planted in plastic pots (20 cm diameter) containing a 30% commercial planting mixture of non-decomposed sphagnum peat (Floragard GmbH, Oldenburg, Germany) and 70% soil collected from the nursery. Potted vines were grown outside in an open frame, watered and pruned to prevent excessive shoot growth, never fertilized or treated for pests or disease. Fifteen vines per treatment were set up.

Sixty days after treatment, vines were inoculated with *P. chlamydospora* by inserting a plug (4 mm diameter) from a two-week-old potato dextrose agar (PDA) culture into a hole made with a hand drill in the rootstock 20–25 cm from the ground. The site of inoculation was covered with Parafilm M (American National Can, Chicago, IL, USA). A sterile PDA plug was used to evaluate the reaction of vine to wounding. Ten vines per treatment were set up.

Fifteen months after the treatment, plants were uprooted, and roots and *P. chlamydospora* development was assessed.

The root diameter of each plant was measured with a caliper. Roots were collected and grouped into 3 categories: hairy roots (<0.05 mm diameter); secondary roots (0.05–0.2 mm); primary roots (> 0.2 mm). For each category, the projected root area was measured by video image analysis (Richner *et al.*, 2000). Image acquisition was with a CCD camera (model TK-880, JVC, Yokohama, Japan) interfaced with a computer by an ELVIS board and Chameleon software (Sky Instruments Ltd., Llandrindod Wells, Powys, Wales, UK).

The images of roots were set in a digitalized form consisting of a three-dimensional matrix. Two dimensions of the matrix corresponded to the width and length of the image in pixels, and the third represented the colour value assigned to each pixel. Subsequently, each image was binarized to create a picture in which all pixels had only two possible values, black or white. The threshold was the gray value (150 pixels) setting the dividing line between the pixels turned to white and those turned to black.

For each treatment, data were expressed as the percentage of the primary, secondary and hairy roots in the projected root area (cm^2).

Phaeomoniella chlamydospora development in the vine wood was assessed in longitudinally split vine trunks. The length of the internal necrotic streaks was measured. Data were expressed as the average length of the internal streaks and subjected to statistical analysis using Duncan's multiple range test, at P=0.05.

Further *in vitro* investigations were performed in order to assess the viability of the pathogen isolated from the inoculated plants.

Pruning wound application

Greenhouse trials

One pruning wound per plant was made on two–year–old grafted potted vines cv. Montuni (rootstock SO4). The wounds were immediately sprayed with *T. harzianum* (Trichodex, 400 g hl^{-1}) or *T. longibrachiatum* strain 6 (1.8×10^6 cfu ml^{-1}) suspension (10 plants per treatment). Twenty-four hours after spraying, the wounds were inoculated by placing drops of a *P. chlamydospora* conidial suspension (1.7×10^7 conidia ml^{-1}) on the wound surface. Control vines were treated with sterile water. Vines were grown in a non-climatized greenhouse and watered at regular intervals.

Twelve months after spraying, the pruned shoots were cut transversely into approx. 3-mm-thick disks. The first disk, which had received the spray directly, was discarded, and the following three were examined under a stereomicroscope, to determine the percentage of vessels showing tyloses or black goo (a tar-like sap in the xylem vessels) on their surface (Morton, 2000). Fungal isolations were made by placing wood fragments from each disk (3–4 fragments per disc) onto PDA + 300 mg l^{-1} streptomycin sulphate.

Vineyard trials

Two-year trials were performed in two vineyards, one with cv. Riesling and one with cv. Sauvignon. In order to reduce the susceptibility of wounds to infection (Larignon, 1998), pruning was delayed as much as possible (till end of winter) and was immediately followed by *Trichoderma* (Trichodex 400 g hl^{-1}) spraying. In 2001, the biocontrol agent was mixed with an emulsified pine resin (Vapor Gard® 100 ml hl^{-1}) to improve adhesion. Spraying was done on 8 rows, each with about 75 vines. At 7, 15, 30 and 60 days after spraying in 2001, and at 5, 30 and 60 days in 2002, approximately 30 samples of wood close to pruning wounds were collected from sprayed and control vines. The samples had an area of about 1 cm^2 each and were placed in a selective substrate containing 1.2% potato dextrose broth, 1.5% agar, 50 mg l^{-1} Rose–bengale, and 300 mg l^{-1} streptomycin sulphate, to assess the viability of the *Trichoderma*. At each survey date, the percentage of wood samples yielding *Trichoderma* spp. was determined.

Results

Nursery treatment

Pre–callusing application

Trichoderma applications on graft calli gave different results in the 3 years of the trial (Table 1). In 2001, the distribution of cuttings over the three classes differed significantly, with a greater number of excellent and failed cuttings in treated plants than in untreated plants. In 2002 treated and untreated plants differed slightly but significantly only in the "failed cuttings" category (24% treated vs. 20% untreated). In 2003 there were no significant differences between treated and untreated cuttings in any class of cuttings.

Post–callusing application and P. chlamydospora *inoculation*

Cuttings treated with *T. harzianum* T39 or *T. longibrachiatum* 6 developed significantly more hairy roots than did the untreated plants (Fig. 1), while there were no differences in the development of primary and secondary roots (Fig. 2).

The effects of *Trichoderma* post–callusing treatment on *P. chlamydospora* infection are summarized in Table 2. Necrotic streaks caused by the pathogen 15 months after inoculation were significantly longer in the *P. chlamydospora*-only control (10.3 cm) than in the *Trichoderma*-treated vine trunks. Streaks length was significantly reduced both with Trichodex (2.1 cm) and with *T. longibrachiatum* 6 (3.4 cm).

Table 1. Effect of the pre-callusing application of *Trichoderma harzianum* T39 (Trichodex) on graft callus formation.

Year	Treatment	Number of cuttings per graft callus			□Chi–square*P*[a]
		Excellent	Normal	Failed	
2001	*T. harzianum*	1137	99	578	< 0.0001
	Untreated	44	1385	392	
2002	*T. harzianum*	71	1299	431	0.0064
	Untreated	60	1370	355	
2003	*T. harzianum*	91	1330	390	0.0524
	Untreated	70	1370	345	

[a] For each year, distribution of cuttings into classes differs statistically according to Chi–Square test ($P \leq 0.05$).

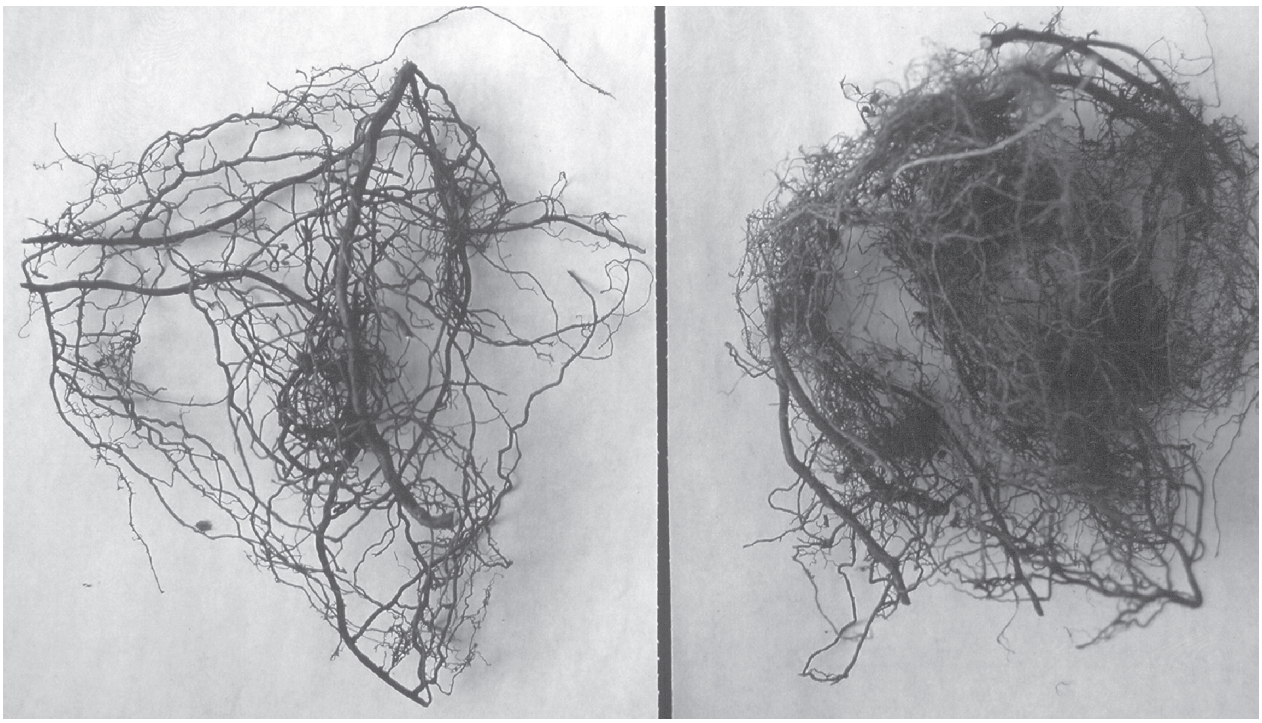

Fig. 1. Grapevine cuttings treated with *T. harzianum* T39 (Trichodex) (right) by dipping the bottom end (2–3cm) for half an hour in a spore suspension (400 g hl $^{-1}$) before rooting. Control cuttings (left) were dipped in water. Extent of rooting was assessed 15 months after treatment.

Table 2. Effect of post-callusing application of *Trichoderma* on *Phaeomoniella chlamydospora* inoculated in the grapevine trunk 60 days after *Trichoderma* treatment.

Treatment	Average length of internal necrosis (cm)
T. harzianum T39 (Trichodex)	2.1 c[a]
T. longibrachiatum 6	3.4 b
P. chlamydospora control	10.3 a
Sterile control	1.2 c

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

Trichoderma did not however completely stop the development of *P. chlamydospora*, which was still reisolated from the necrotic tissue of inoculated vines.

Trichoderma sprays on pruning wound

Greenhouse trials

Isolations from the vine-shoot sections of vines yielded a range of fungi: *Alternaria*, *Penicillium*, *Epicoccum*, *Botryosphaeria,* and *Fusarium*. No clear evidence of *P. chlamydospora* growing out of inoculated tissue was obtained. However, *Trichoderma* was isolated from samples of sprayed vines.

Phaeomoniella chlamydospora-inoculated, unsprayed vines, clearly showed black goo in the vessels (54%) and, in some cases, necrosis of the parenchyma. By contrast, *Trichoderma*-sprayed plants never showed necroses or black goo, although tyloses-like formation was found in 10–12% of the vessels (Table 3).

Vineyard trials

Trichoderma sprayed on fresh pruning wounds in the field remained persistent and viable for up to 60 days. Over a period of 8 weeks after spraying, recovery of *Trichoderma* gradually decreased. The addition of pine resin had no effect on the biocontrol agent (Fig. 3).

Discussion

Over the last few years, *Trichoderma* treatment to control fungi associated with esca have been tested at the nursery and on pruning wounds (Messina, 1999; Fourie *et al*., 2001; Hunt *et al*., 2001; Di Marco *et al*., 2002).

Messina (1999) reported that *Trichoderma* treatments in callusing boxes produced a stronger graft union and a shorter callusing period. Our trials gave conflicting results. There was clear beneficial effect on the graft union only in the first year. This variability of *Trichoderma* activity may reflect the complex set of interactions between the biocontrol agent and the host–plant. At the end of the callusing period, *Trichoderma* treatment generally led to the production of both more failed cuttings and more excellent cuttings than were produced in untreated plants, possibly due to a kind of selective effect during the grafting process.

As regards the effect of *Trichoderma* on vine rooting, Fourie *et al*. (2001) found that the fresh root mass of grapevines increased by 41.7% after monthly *Trichoderma* treatments to the soil in the nursery. In our experiment, hairy root development was four times as great in cuttings dipped in *Trichoderma* at the bottom end soon after graft callusing as in cuttings not so treated. Although no differences in vegetative growth between these plants were visually noted, vines probably benefited from a more developed hairy root system, which improved water and nutrient uptake and increased tolerance to stress-related diseases

Table 3. Effect of *Trichoderma* on pruning wounds in potted vines inoculated with *Phaeomoniella chlamydospora*.

Treatment	Necrosis	Black streaking (%)	Tyloses-like formation (%)
T. longibrachiatum 6 + *P. chlamydospora*	No	0	10
T. harzianum T39 + *P. chlamydospora*	No	0	12
T. longibrachiatum 6	No	0	0
T. harzianum T39	No	0	0
P. chlamydospora control	Yes	54	0
Wound control	No	0	0

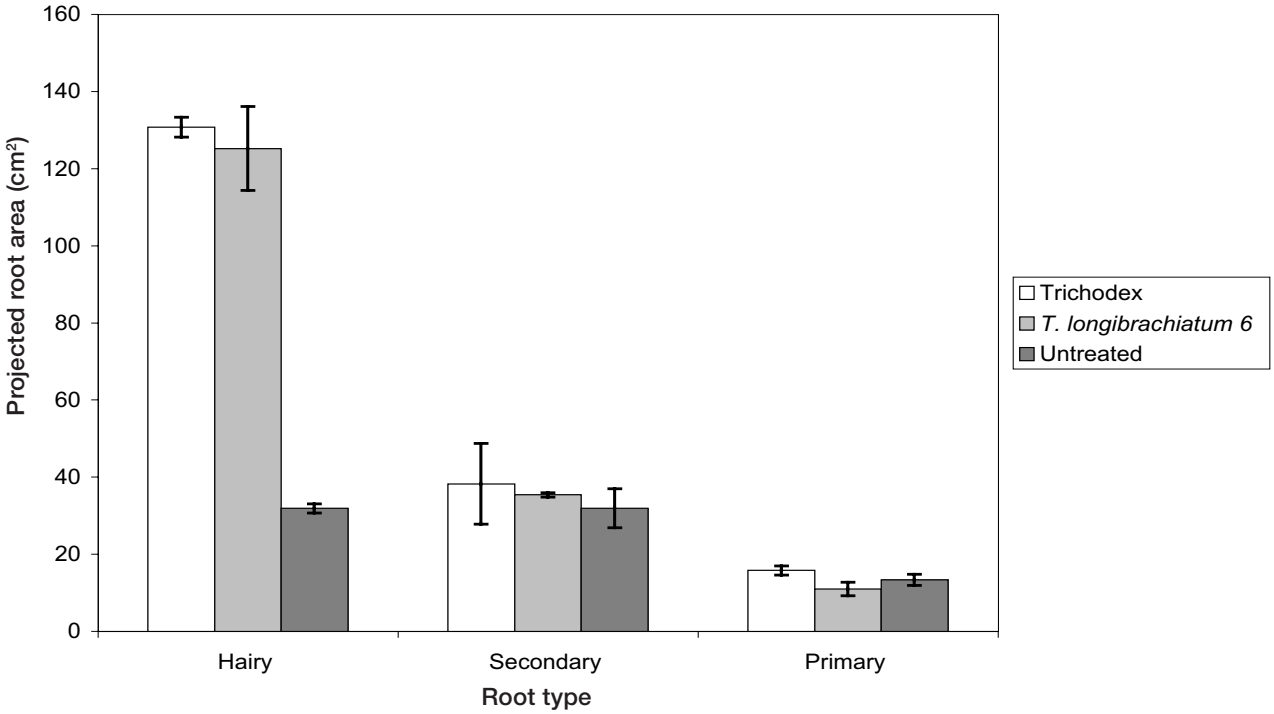

Fig. 2. Effect of post–callusing application of *Trichoderma* on the development of the root system. Bars represent standard deviation.

(Harman, 2000; Fourie *et al*., 2001). According to this hypothesis, our data demonstrate, for the first time to our knowledge, that post-callusing treatment with *Trichoderma* significantly reduces necrosis length caused by *P. chlamydospora* inoculated into the rootstock after the biocontrol agent. Since *Trichoderma* was applied far from the site of inoculation, the most likely explanation for this improved control of *P. chlamydospora* is that it resulted from a stronger plant defense reaction. Among studies on the defense reactions induced by *Trichoderma*, De Meyer *et al*. (1998) also reported that *T. harzianum* T39 was active against *Botrytis cinerea*, even when the antagonist was applied to plant parts that were spatially remote from the site of *B. cinerea* inoculation; they postulated the induction of systemic resistance.

Hunt *et al*. (2001) suggested that *Trichoderma* was potentially a strong agent protecting pruning wounds against infection from fungi involved in grapevine trunk disease and vine decline. Moreover, these authors were still able to isolate the biocontrol agent from infected samples 8 months after it had been applied on pruning wounds under controlled conditions. In the vineyard test of our study, *Trichoderma* sprayed on pruning wounds was recovered 60 days after spraying. The test could not be extended beyond this period because of the development of the canopy.

Initial attempts to control esca in the vineyard using *Trichoderma* were not very effective, probably because spraying was done on apparently healthy vines that were already affected with esca (Bisiach *et al*., 1996; Mugnai *et al*., 1999; Di Marco *et al*., 2000). Later results showed that pre-infection *Trichoderma* sprays on fresh pruning wounds prevented black goo and limited the effect of artificial infection of *P. chlamydospora* to a moderate Tyloses-like formation. This substantial degree of protection was achieved merely by spraying small pruning wounds in young vines. The results of this study therefore provide further evidence that *Trichoderma* is effective as a preventive agent used on vines that are not yet infected (Di Marco *et al*., 2000).

Numerous factors are involved in the complex interaction between *Trichoderma*, the vine and the

pathogen. An understanding of the mode of action and effectiveness of the biocontrol agent is needed to design an application protocol that is optimally suited for different nursery practices, or to use it as a protectant of pruning wounds. For this reason, further studies are needed to improve the biocontrol of *Trichoderma* and move towards its practical implementation.

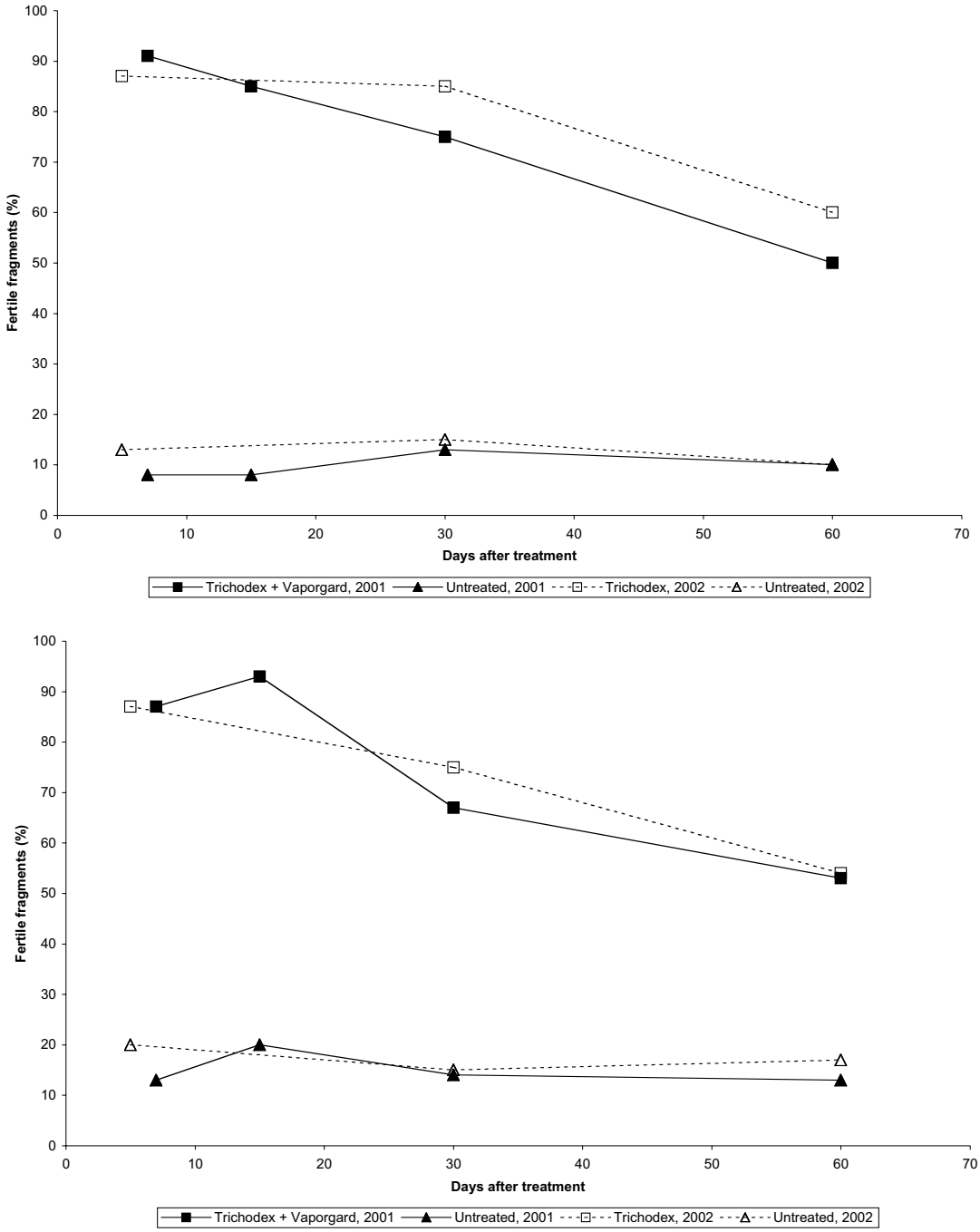

Fig. 3. Isolation of *Trichoderma* from wood samples of grapevine cv. Riesling (top) and Sauvignon (bottom) collected at different times after treatment with Trichodex®. Data represent the percentage of fertile fragments.

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