Applications of *Trichoderma* to prevent *Phaeomoniella chlamydospora* infections in organic nurseries

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Summary. In order to prevent or reduce infection in grapevine nurseries, greenhouse and nursery trials were carried out to evaluate the effects of *Trichoderma harzianum* (Rootshield®) on the morpho-physiological characteristics of grapevine and on *Phaeomoniella chlamydospora* artificially inoculated on potted cuttings. The long-distance activity of *Trichoderma* against *Botrytis cinerea* was also examined. The study was performed in a commercial nursery where plants were grown organically. Results greatly depended on the vine-growth stage at which *Trichoderma* was applied. Treatment at rooting was the most effective, whilst callusing-box application or treatments at both rooting and callusing gave inconsistent but generally negative results. Treatment at rooting further improved the quantitative and qualitative characteristics of the root system, and increased the percentage of certifiable vines produced. Moreover, *Trichoderma* application also reduced the necrotic area caused by *B. cinerea* inoculated on the leaves collected from *Trichoderma*-treated plants and the extent of necrosis of *P. chlamydospora*-inoculated cuttings. This reduction in necrosis was significantly higher 15 months after inoculation. On the other hand, *Trichoderma* increased vine mortality at the end of the growing season compared with untreated plants. On the whole, only when it was applied at rooting did *Trichoderma* produce positive effects on the morpho-phisiological characteristics of grapevine and increased its tolerance to stress–related diseases, such as forms of esca found in the nursery.

Key words: grapevine, *Trichoderma harzianum*, Petri disease.

Introduction

Studies have recently demonstrated that *Phaeomoniella chlamydospora* and *Phaeoacremonium* species can produce latent infections in nursery cuttings and that they cause Petri disease (Edwards *et al.*, 2001; 2004; Zanzotto *et al.*, 2001). Infection with these fungi occurs at all stages of the grapevine propagation process, from infection of the rootstock cane vessels to the end of the nursery period. A large number of wounds are produced during all these nursery stages (Fourie and Halleen, 2002; 2004; Edwards *et al.*, 2004). *P. chlamydospora* and *Phaeoacremonium* spp. spread through infected propagation material and cause Petri disease, a serious disease of young grapevines (Surico, 2001; Fourie and Halleen, 2004). *Phaeomoniella chlamydospora* tends to be isolated from cuttings more often than *Phaeoacremonium* spp. and is the most important fungal pathogen associated with Petri disease (Edwards and Pascoe, 2004; Fourie and Halleen, 2004; Gaforio *et al.*, 2005; Whiteman *et al.*, 2005; Ridgway *et al.*, 2005; Retief *et al.*, 2006).

In recent years, several studies have been carried out to develop procedures and products to prevent or reduce *Phaeomoniella* infection of the

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woody tissues of grapevine propagation material (Di Marco *et al.*, 2000; Fourie *et al.*, 2001; Fourie and Halleen, 2006). A number of biological control agents and fungicides were tested, but only benomyl, and *Trichoderma* associated with hot-water treatment were at all effective (Fourie and Halleen, 2004).

The potential of nursery applications of *Trichoderma* to control *P. chlamydospora* and *Phaeoacremonium* species (Fourie *et al.*, 2001; Hunt *et al.*, 2001; Di Marco *et al.,* 2002; 2004; Fourie and Halleen, 2004; 2006; Veronesi *et al.*, 2006) was investigated. *Trichoderma harzianum* formulations at various nursery stages produced a significant increase in root development, and a slightly reduced level of pathogen infection on cuttings uprooted from field nurseries or on potted vines (Fourie *et al.*, 2001; Di Marco *et al.*, 2004; Fourie and Halleen, 2004). These beneficial effects are probably due to a multiple role of *Trichoderma,* which acts directly against the pathogen and at the same time also interacts with the grapevine physiology (Harman *et al.*, 2002; 2004a). The aim of this ongoing study was to better understand the mode of action and effectiveness of *Trichoderma* formulations in the control of the Petri disease pathogens. This paper reports on results obtained from 2 combinations of scion/rootstock graftlings grown and managed under organic conditions and treated with *Trichoderma harzianum* at various nursery stages for the control of *P. chlamydospora*.

Materials and methods

Nursery treatment

Field trials were carried out during the 2005 growing season in a commercial nursery at Faenza, Emilia Romagna, north-central Italy. Cuttings came from material certified under Council Regulation (EEC) N° 2092/91 of 24 June 1991, on the organic production of agricultural products. Scion/rootstock combinations Trebbiano romagnolo (TR3T) grafted on K5BB or 1103P were used in the trials. Treatments were performed using Rootshield® granules, a *T. harzianum* T 22 commercial formulation (1×10^7 CFU g^{-1}) (Intrachem Bio Italia S.p.A., Bergamo, Italy). The grafting was done with an Omega-cut grafting machine, and cuttings were sealed with wax without fungicide.

Nursery vine-growth stages for *Trichoderma* application

Callusing

Grafted cuttings were drenched for 30 min in PVC boxes containing the *Trichoderma* formulation suspension at 15 g l^{-1} (water for the control). For each treatment, cuttings were divided into four groups of 750–800 plants each; each group was stacked with sawdust in separate callusing boxes and stored at 10–20°C for 3 weeks.

At the end of this period all cuttings were inspected, visually assessed for grafting callus formation (Di Marco *et al.*, 2001), and the percentage of cuttings that were plantable in the nursery field was calculated. For each treatment, results were expressed as the mean percentage of plantable cuttings over the total number of inspected vines.

Rooting

The bottom ends (2–3 cm) of grafted cuttings stored in callusing boxes were placed for 30 min in PVC buckets with a suspension of the *Trichoderma* formulation at 15 g l^{-1} (water for the control). Graftlings were then planted in a nursery field at 7–8 cm in-row spacing and about 70 cm between rows. Two hundred cuttings per treatment with 4 replicates of 50 cuttings each were set up. Five weeks after planting, cuttings were treated by soil drenching the *Trichoderma* suspension at 0.5 g l^{-1}.

Callusing+rooting

Grafted cuttings were treated both at callusing and at rooting. The experimental design was performed following the same method as that used for rooting applications (200 cuttings per treatment with 4 replicates of 50 cuttings each).

Ratings during the nursery field period and after uprooting

During the nursery-field period, the number of growing cuttings with each treatment was assessed at 7, 15, 22, 38 and 122 days after planting. At each time of assessment, data were expressed as the mean percentage of growing plants, and subjected to statistical analysis using Duncan's multiple range test, $P=0.05$.

Twenty-eight weeks after planting plants were uprooted following commercial nursery procedures and "certifiable plants" were selected according to EU directive 2005/43. For each treatment, 4 randomly selected plants were carefully uprooted with

the soil around the roots and taken to the laboratory. Root development was determined by video image analysis as described in Di Marco *et al.* (2004). The percentage of certifiable cuttings with each treatment was calculated.

Inoculation of potted grapevines with *Phaeomoniella chlamydospora*

Eighty TR3T/K5BB graftlings treated at callusing and/or at rooting in the nursery as described above were planted in plastic pots (20 cm diameter) containing a soil mixture (30% commercial mixture of non-decomposed sphagnum peat and 70% soil from the nursery). Potted vines were grown outdoors in an open frame, regularly watered and treated with a copper fungicide to control downy mildew. Twenty plants per treatment were set up. Ten weeks after planting, potted plants were inoculated with *P. chlamydospora* by inserting a plug (4 mm diameter) from a three-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. A plug of sterile medium was used for the control.

Five and again 15 months after inoculation, 8 vines were uprooted, their trunks split lengthwise, and *P. chlamydospora* development was assessed. The length of the necrotic streaks was measured. Data were expressed as the average length of the necrotic lesion and subjected to statistical analysis using Duncan's multiple range test, P=0.05. Further *in vitro* tests, putting the necrotic woody streaks on PDA plates, were carried out in order to assess the viability of the pathogen isolated from the inoculated plants.

Inoculation with *Botrytis cinerea* on the leaf surface of nursery plants

For each treatment, 20 leaves were collected from 5 nursery plants at 4 months after planting. Leaves were immersed for 30 sec in 15% ethanol, rinsed with de-ionized sterilized water and dried on sterilized blotting paper. Leaves were then placed in Petri glass dishes (15 cm diameter) on a plastic grid positioned over moist filter paper. Leaves were inoculated with conidia from a *B. cinerea* colony (strain PVFi-Bc72) supplied by the Dipartimento di Biotecnologie Agrarie, University of Florence, Italy. The fungus was grown on PDA at 21±2°C with a 16-h day. Three to five droplets of a *B. cinerea* conidial suspension (20 μl and 10^5 co-

nidia per droplet) were placed on the upper surface of each leaf. The inoculated leaves were maintained in a climatic chamber at 21±2°C with a 16-h day for 7 days and then assessed for leaf necrosis (Derckel *et al.*, 1999; Aguero *et al.*, 2005).

Leaf necrosis was measured 7 days after inoculation using video image analysis. 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 were set in a digitalized form consisting of a three-dimensional matrix to distinguish between healthy and necrotic leaf tissue. 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 (177 and 56 pixels for leaf surface and necrosis, respectively) setting the dividing line between the pixels that turned white and those that turned black. Data were expressed as the percentage of necrotic surface over the whole leaf surface.

Development of the root system

In order to assess root development, 4 plants treated at callusing and grown in the same row were uprooted at the end of the field nursery period and taken to the laboratory. Four TR3T/K5BB plants for each treatment, planted and grown in pots, were uprooted 7 months after planting. A further 4 potted plants treated at rooting were uprooted 19 months after planting. The root diameter was measured with a caliper and grouped into 3 categories: hairy (<0.05 mm diameter); secondary (0.05–0.2 mm); primary (>0.2 mm). For each category, the projected root area was measured by video image analysis following the method described by Di Marco *et al.* (2004). For each treatment, data were expressed as projected root area (cm^2).

Results

Nursery treatment

Selection of cuttings after callusing period

The percentage of plantable plants at the end of callusing was lower in treated plants than in

untreated plants for the TR3T/K5BB scion-rootstock combination (Fig. 1). No significant differences in grafting callus formation were noticed between treated and untreated cuttings.

Ratings during the nursery field period

Inspections during the growing season showed a decrease in the percentage of growing plants treated with *Trichoderma*. No further losses in growing plants were observed over 120 days after planting. Treatments generally caused a delay in the onset of vegetation. The difference between the percentage of growing plants among the ones treated with *Trichoderma* and the control one was significantly higher (*P*=0.05) when surveyed at the beginning (K5BB) or at the end (1103P) of the nursery period. The decrease in the percentage of growing plants was mainly observed on plants treated at callusing (Fig. 2).

Certifiable plants after uprooting

For each scion-rootstock combination the percentage of certifiable plants recorded from uprooted plants was higher in plants treated with *Tri-*

choderma at rooting than in the control plants. By contrast, application of *Trichoderma* at callusing yielded a lower percentage of certifiable plants than did the other treatments (Fig. 3).

Effect of *Trichoderma* on rootstocks of potted plants inoculated with *Phaeomoniella chlamydospora*

By the end of the growing season (5 months after inoculation) *Trichoderma* treatments had not reduced the necrotic streaks caused by *P. chlamydospora*, except for a slight reduction when *Trichoderma* was applied at rooting. Fifteen months after inoculation necrosis was significantly lower with *Trichoderma* applied at rooting (3.6 cm) than with the other treatments, in which the trunk streaks averaged from 6.2 to 7.0 cm. *Phaeomoniella chlamydospora* was still re-isolated from the necrotic tissue of inoculated vines (Table 1).

Effect of *Trichoderma* on the leaf surface of plants grown in the nursery and inoculated with *Botrytis cinerea*

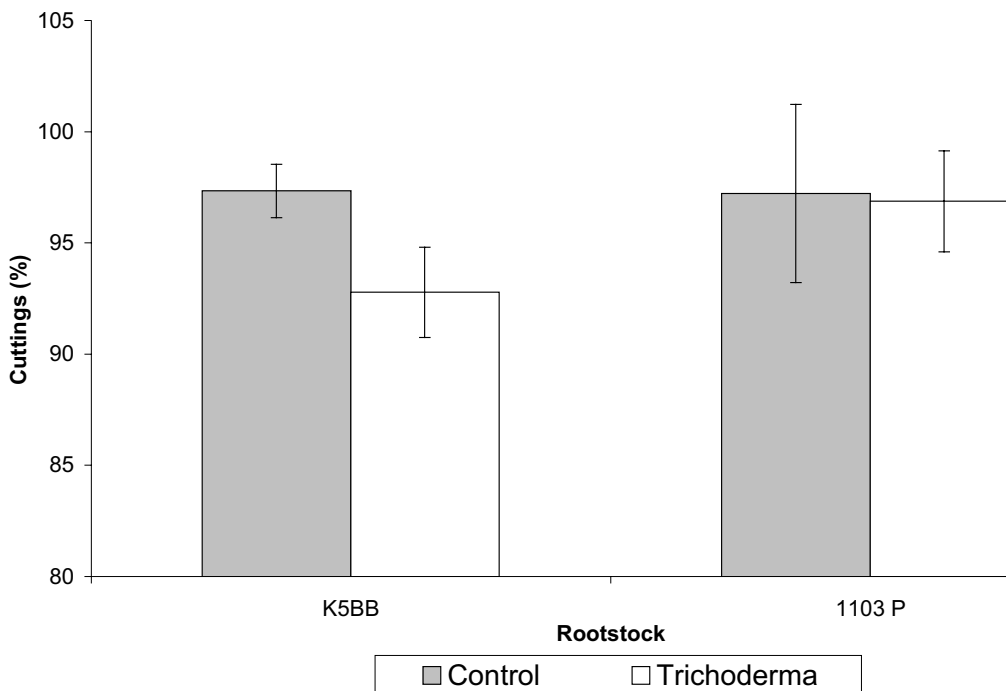The effect of *Trichoderma* on necrotic lesions from *B. cinerea* on leaves of nursery plants gave

Fig. 1. Mean percentage of plantable Trebbiano romagnolo cuttings on 1103P or K5BB at the end of the callusing period, 28 weeks after *Trichoderma* application. Bars represent standard deviation.
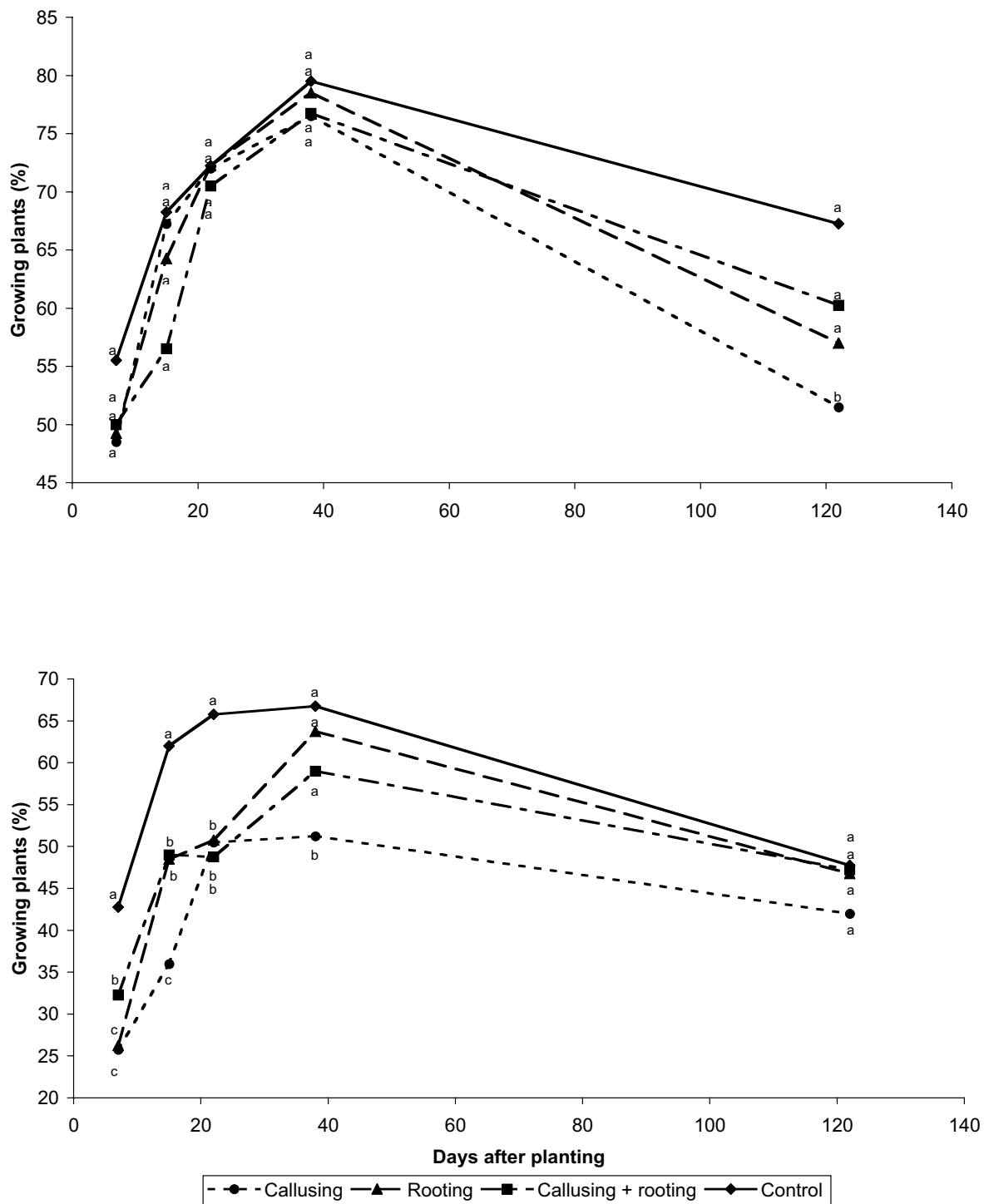
Fig. 2. Mean percentage of growing Trebbiano romagnolo plants on 1103P (top) and K5BB (bottom) treated with *Trichoderma* at various vine-growth stages during the nursery period. At each time of inspection, values followed by the same letter do not differ significantly according to Duncan's Multiple Range Test (*P*=0.05).
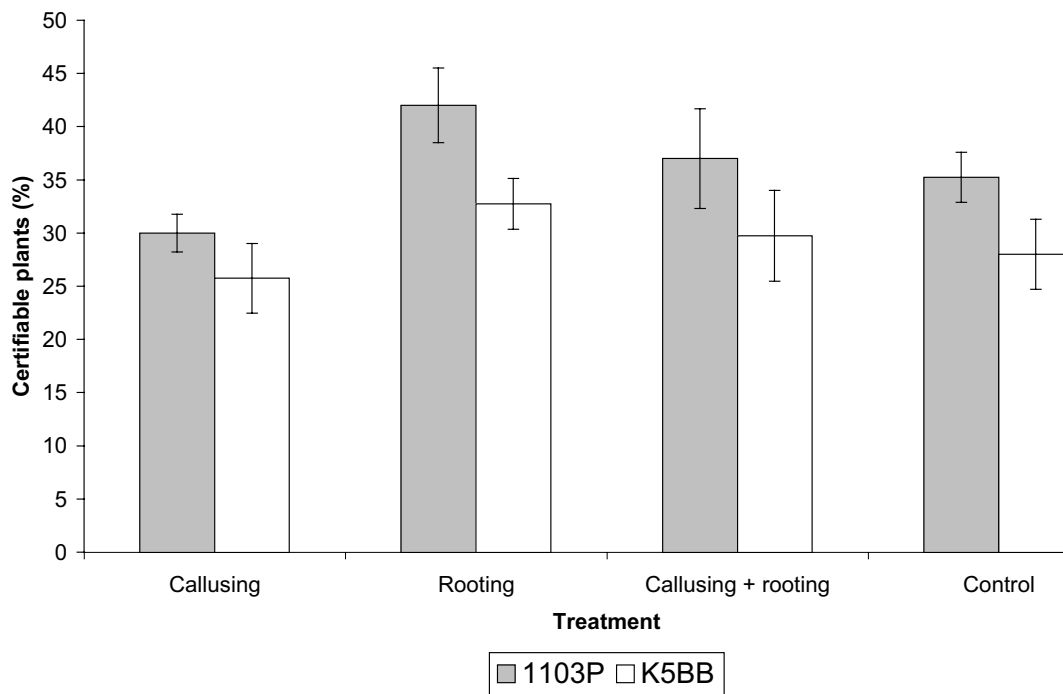
Fig. 3. Mean percentage of certifiable Trebbiano romagnolo plants on 1103P or K5BB treated with *Trichoderma* at various nursery-vine growth stages. Bars represent standard deviation.

different results depending on the scion/rootstock combination. TR3T/1103P leaves from plants treated at callusing and those treated at rooting had significantly fewer necrotic lesions (0.6–1.9% of the

Table 1. Effects of *Trichoderma* applied at various nursery-vine growth stages on *Phaeomoniella chlamydospora* inoculated in the trunks of TR3T/K5BB grapevines 10 weeks after planting in PVC pots.

Treatment	Average length of internal necrosis (cm)	
	5 months[a]	15 months[a]
Callusing	1.7 a[b]	6.6 a
Rooting	1.4 a	3.6 b
Callusing + rooting	2.1 a	6.2 a
Untreated control	1.9 a	7.0 a

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

leaf surface) than did either untreated plants or plants treated at callusing and again at rooting (16–30% of the leaf surface). Effects on the TR3T/K5BB combination lesions of the leaves were only slight (Fig. 4).

Root development

The effect that *Trichoderma* applied at various nursery growth stages had on root development is shown in Fig. 5.

Seven months after planting, potted plants treated with *Trichoderma* clearly had more hairy roots than untreated plants. Of the treatments, *Trichoderma* application at rooting was the most effective. The development of hairy roots in plants treated at rooting was actually higher at 19 months after planting (rooting 139.3 cm^2; control 34.7 cm^2) than at 7 months (rooting 125.7 cm^2; control 62.4 cm^2) (Fig. 5, top).

The enhancement of hairy roots due to the rooting treatment was also seen in field-grown nursery plants (Fig. 5, bottom).

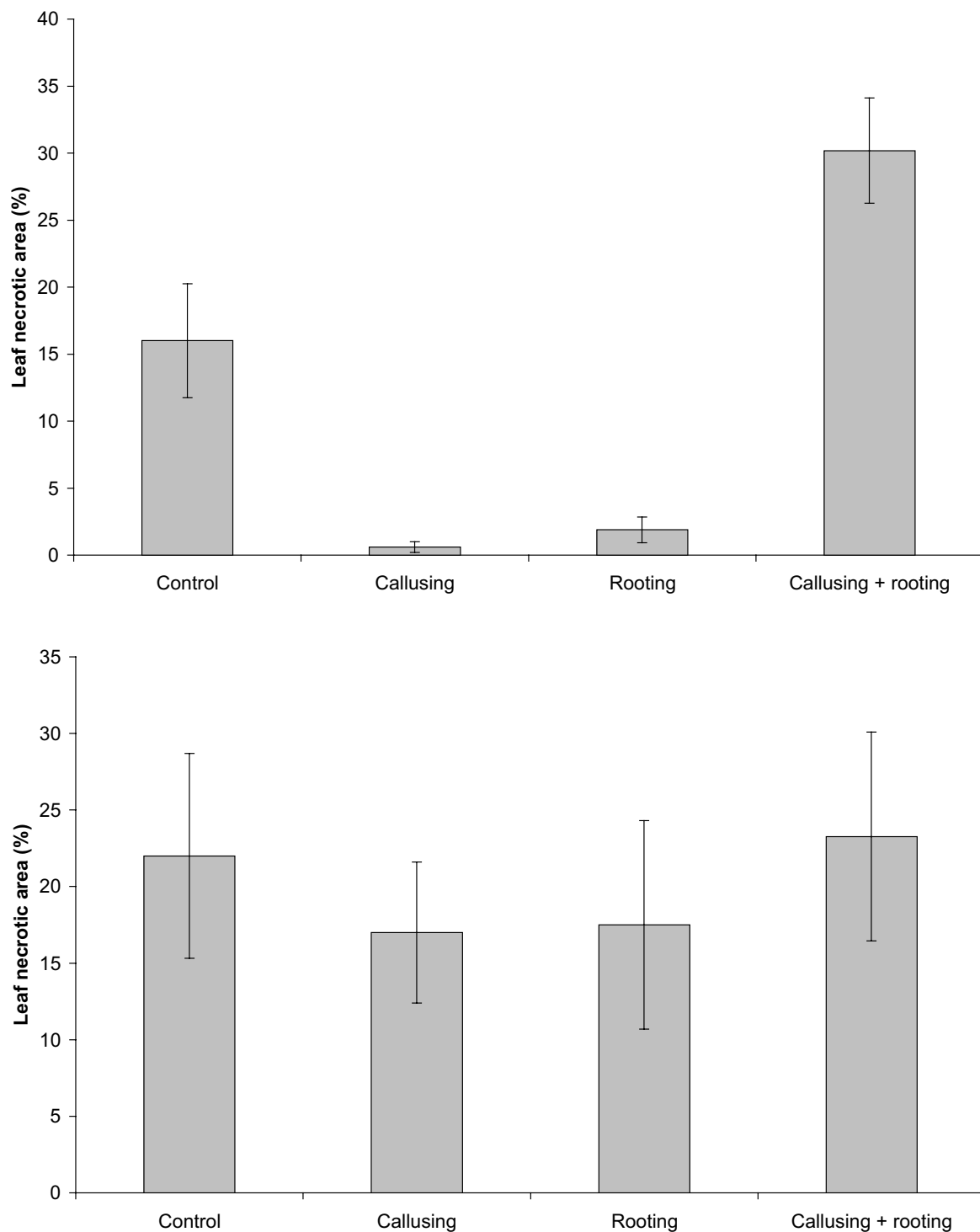No significant differences in between primary and secondary roots were observed.

Fig. 4. Percentage of leaf area affected by necrotic lesions caused by *Botrytis cinerea* artificially inoculated on leaves collected from Trebbiano romagnolo cuttings on 1103P (top) and K5BB (bottom) grown in the nursery field. The assessment was carried out 7 days after inoculation. Bars represent standard deviation.
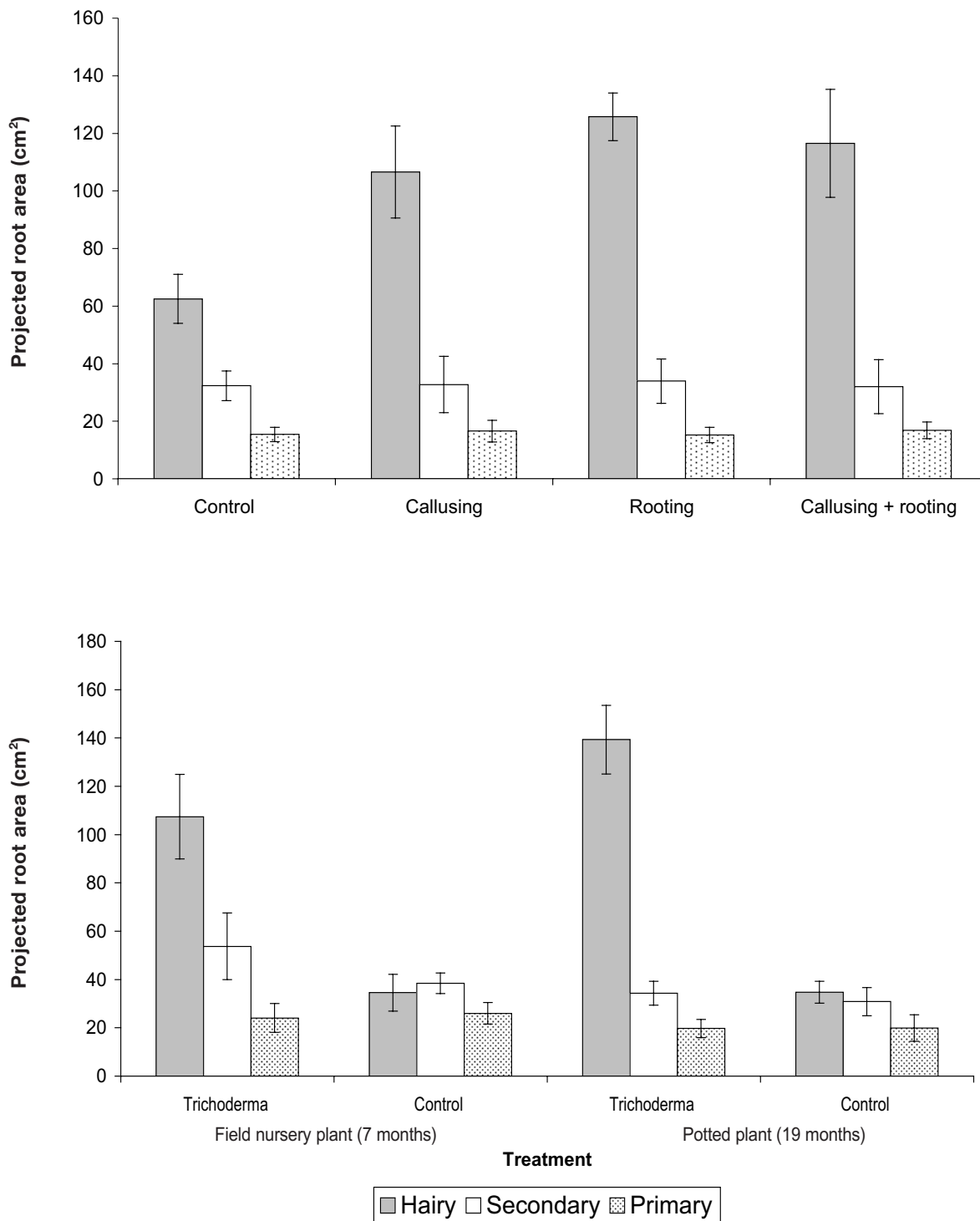
Fig. 5. Effect of *Trichoderma* applied at various nursery-vine growth stages on the development of the root type of potted Trebbiano romagnolo cuttings on K5BB 7 months after planting (top). The effect of the rooting treatment was assessed on Trebbiano romagnolo on K5BB plants collected from the nursery field at the end of the growing season and on Trebbiano romagnolo on K5BB potted vines 19 months after planting (bottom). Bars represent standard deviation.

Conclusions

The effect of *Trichoderma* on Petri disease pathogens and on vine cuttings was evaluated by applying the fungus directly to the plant and not to the pathogens. Consequently any results obtained in the study must be explained by taking account of the complex interaction between *Trichoderma*, vine-host, and pathogen (Chet and Baker, 1981; Chang *et al.,* 1986; Harman *et al.,* 2004a; Harman, 2006, Hoitink *et al.,* 2006). In addition, *Trichoderma* activity was assessed in an organic nursery, characterized by more natural vegetative conditions, to give a more accurate evaluation of *Trichoderma* treatment efficacy. The percentage of cuttings that grew, which was greatly compromised by severe infections of downy mildew, followed a similar dynamic for the two rootstocks, and did not vary starting from 4 months after planting in the nursery field. However, the take percentage of TR3T/1103P cuttings was consistently higher than that of the TR3T/K5BB cuttings, because of the greater vigour of that grafting combination (Ferroni and Scalabrelli, 1995).

In the treated samples, the percentage of growing cuttings was lower with both rootstocks, suggesting that there was a kind of selection process driven by *Trichoderma* (Di Marco *et al.,* 2004). Lower percentages of growing cuttings occurred at different times depending on the rootstock combination: initially the percentage was lower only with the less vigorous TR3T/K5BB combination, but by the end of the growing season dead cuttings occurred with TR3T/1103P only.

The lowest mortality in growing plants occurred when vines were *Trichoderma*-treated at rooting, confirming previous studies (Di Marco *et al.,* 2004). By contrast, with both scion/rootstock combinations the highest mortality in growing cuttings was seen in cuttings treated at callusing only. The number of treatments, and hence the amount of *Trichoderma* used was thus not related to these differences. Otherwise, the choice of the nursery growth stage and the location may have influenced treatment efficacy, underscoring the complexity of the interaction between *Trichoderma* and the host plant, and its effects. Multiple effects of *Trichoderma* have already been described (MacKenzie *et al.,* 2000). Specifically, Héraux *et al.* (2005) reported that *Trichoderma* treatments of tomato plants reduced plant height and increased fruit yield.

The mortality of growing cuttings due to *Trichoderma*-treatment was however counterbalanced by an increase in certifiable cuttings obtained by the two *Trichoderma* treatments on the root callus (at rooting, and to a lesser extent at callusing+rooting). Especially rootstock 1103P, the most sensitive, had a higher final yield in certifiable cuttings with these treatments.

The stimulant effect of *Trichoderma* on root development, mainly on the hairy roots, was evident with all treatments, but especially with the treatment given at rooting. This phenomenon has been widely reported in the literature (Yedidia *et al.,* 2001; Harman *et al.,* 2004a; 2004b). Root growth promotion actually increased in the year after the treatment was applied, suggesting that *Trichoderma* became more active with time; this effect could be due to the active colonization of the roots by the fungus itself. Better growth of the hairy roots had a beneficial effect on the vine, with greater adsorption of nutrients from the soil and consequently a higher tolerance to stress, also from diseases such as esca (Fourie *et al.,* 2001; Yedidia *et al.,* 2001; Di Marco *et al.,* 2004; Fourie and Halleen, 2004). However, the enhancement of this beneficial effect must be considered putative since the study was carried out on cuttings left to overwinter in the soil, while usually in nurseries cuttings are uprooted and cold-stored in winter.

Finally, root proliferation, resulting as it does only from the treatment at callusing, could be due to the complete immersion of the cuttings in a *Trichoderma* suspension, followed by fungal colonization of the root-forming area. Probably, the *Trichoderma* strain persists and develops in this area, in a way similar to what happens when a basal wound is treated with a fungal suspension before planting (MacKenzie *et al.,* 1995; Paal and Banner, 2003). Furthermore, even a low spore count can colonize the whole root surface if a strain of *Trichoderma* adapted for the rhizosphere is used, as in this study (Harman *et al.,* 2004a; Harman, 2006).

The increased activity of *Trichoderma* with time also led to a reduction in the necrosis caused by *P. chlamydospora* in *Trichoderma*-treated plants; the data refer to the *Trichoderma*-treatment at rooting, which treatment did not become statistically significant until 15 months after pathogen inoculation.

Botrytis cinerea assays were performed in order to evaluate the ability of *Trichoderma* to act far from its application site; the ability of *Trichoderma* to act against *B. cinerea* has already been reported on tomato and grapevine (De Meyer *et al.*, 1998; John *et al.*, 2005). *Trichoderma* treatment at callusing and at rooting had a clear positive effect on 1103P, and to a lesser extent on K5BB, reducing the necrosis caused by *B. cinerea* inoculation. However, combining these two treatments gave results worse than those obtained with the control plants of both rootstocks. These results are a further indication of the complexity of the interaction between *Trichoderma*, the vine host, and the pathogen; the efficacy of the treatment cannot be reduced to a simple scheme of cause and effect but is the expression of a balance between the beneficial and the toxic effects of *Trichoderma* (Ousley *et al.*, 1993; Ahmed *et al.*, 2000; Bal and Altintas, 2006).

In conclusion, the type of *Trichoderma* application and its timing was examined in this study. Although the possibility cannot be excluded that further strains of *Trichoderma* may turn out to be harmful to grapevine varieties not tested here (Harman *et al.*, 2004a), *Trichoderma* application at rooting reduced the variability of results obtained in the organic nursery; this particular application is thus the best to achieve both high cutting quality and low disease incidence. The study also demonstrated that *Trichoderma* improves the condition of the roots and reduces the rate of *P. chlamydospora* infection over time; further investigation on this important topic is needed. Studies are under way to verify the effect of *Trichoderma* on natural infections of the causal agents of Petri disease in nursery vines.

Acknowledgements

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