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Effectiveness of conidia of *Trichoderma harzianum* produced by liquid fermentation against Botrytis bunch rot of table grape in Chile

B.A. Latorre*†, E. Agosín‡, R. San Martín‡ and G.S. Vásquez†

†*Facultad de Agronomía y Ciencias Forestales, Pontificia Universidad Católica, Casilla 306-22, Santiago, Chile; and* ‡*Facultad de Ingeniería, Pontificia Universidad Católica, Casilla 306-22, Santiago, Chile*

Over 100 isolates of *Trichoderma harzianum* Rifai were obtained from soil samples and from the phylloplane of kiwifruit (*Actinidia chinensis* Planchon), grape (*Vitis vinifera*), orange (*Citrus sinensis* (L.) Osbeck), eucalyptus (*Eucalyptus globulus* Labill.), and apricot (*Prunus armeniaca* L.) in Chile. A subsample of 48 isolates were tested and found to be antagonistic to *Botrytis cinerea* Pers. ex Fr. on apple fruits. Isolate S10B from soil in Chile provided similar control of Botrytis bunch rot under field conditions to reference isolate P1 (ATCC 74058) and T39 (Trichodex 25 WP). However, field trials conducted during four growing seasons (1992–1995) with preparations of conidia of formulated or non-formulated *T. harzianum* P1 provided only partial control of Botrytis bunch rot of 'Thomson Seedless' table grape. Disease incidence was significantly different ($p < 0.05$) from untreated controls, but equal to or less than the control achieved with vinclozolin (Ronilan 50 WP, 1.5 kg ha^{-1}) and similar to captan (Captan 80 WP, 4 kg ha^{-1}). This level of control is insufficient considering that tolerance for *B. cinerea* is very low (< 0.5%) on table grapes. Nevertheless, the antagonistic activity of *T. harzianum* may be effective if it is integrated with other control practices, and may result in acceptable levels of disease control with reduced levels of pesticide use. © 1997 Elsevier Science Ltd

Keywords: biological control; *Botrytis cinerea*; fungicides, gray mold; *Trichoderma harzianum*; *Vitis vinifera*

Introduction

Botrytis bunch rot (*Botrytis cinerea* Pers. ex Fr.) is the most important disease of table grape (*Vitis vinifera* L.) in Chile. Severe outbreaks occur when prolonged periods of moisture and cool temperatures (15–20°C) prevail at blossom time or during ripening. Control measures include canopy management, aimed at reducing bunch-rot conducive microclimate conditions and the application of fungicides. In Chile, growers commonly make 1–3 fungicide applications at flowering and 2–4 applications between veraison and harvest. The main fungicides used at present are captan, iprodione, and vinclozolin. However, *B. cinerea* readily develops resistance to the dicarboximide fungicides and they are becoming progressively less effective in Chile (Latorre *et al.*, 1994).

Several biological control agents are potentially useful in controlling diseases caused by *B. cinerea*. Of these, strains of *Trichoderma harzianum* Rifai, active against *B. cinerea*, have been studied on grape and other hosts (Elad, 1993; Harman *et al.*, 1996; Tronsmo, 1991). An integrated disease management program including mixtures or alternation of biological agents and fungicides has been suggested, particularly where resistance problems to fungicides have developed (Elad and Kirshner, 1993; Elad, *et al.*, 1993, 1995; Gullino and Garibaldi, 1987). However, effective and reliable biocontrol preparations with good shelf life are needed before biocontrol will be used extensively. The purposes of the research described here were (a) to obtain isolates of *T. harzianum* potentially useful as biocontrol agents, and (b) to produce and evaluate the effectiveness of conidia of *T. harzianum* obtained by liquid fermentation against Botrytis bunch rot of table grape. A short report has already been published (Latorre *et al.*, 1996).

Materials and methods

Isolation

Soil samples taken from rhizospheres and samples from aerial plant parts were seeded on: (a) potato dextrose agar acidulated with 0.5 mL L^{-1} of 1N lactic acid

*To whom all correspondence should be addressed.

(PDA), modified with 0.1 mL L^{-1} of Igepal (Alltech Assoc., Inc. Deerfield, II, USA), streptomycin (100 mg L^{-1}) and chlortetracycline (50 mg L^{-1}) (MPDA); and (b) modified TSM medium (Elad 1993; Elad *et al.*, 1981; Smith *et al.*, 1990). Isolate were subcultured and stored in silica gel at 0°C.

The antagonistic capabilities of 48 isolates of *T. harzianum* were tested against *B. cinerea* on apple fruits ('Granny Smith', 10–12.5°Brix) as previously described (Tronsmo and Raa, 1977). For this purpose, a conidial suspension of *T. harzianum* (10^6 conidia mL^{-1}) and *B. cinerea* (10^4 conidia mL^{-1}) were prepared from 7–10 day old cultures on PDA. Five holes (0.5 × 2 cm) were aseptically made with a core borer on each of five surface-disinfected fruits (5 min in 75% ethanol). Three holes were treated with 0.5 mL of conidial suspension of either unknown isolate of *T. harzianum* alone; the unknown *T. harzianum* isolate plus *B. cinerea*, *T. harzianum* P1 plus *B. cinerea* and *B. cinerea* alone. The last hole was always treated with sterile distilled water. Fruits were incubated in humid chambers at 23°C for 4 days before measuring the radial growth of the lesions developed. Data were analyzed for variance and means were separated according to Duncan's multiple range test.

In order to select only isolates of *T. harzianum* able to grow at cool temperatures, a mycelial plug (0.5 cm in diameter), taken from 7 to 14 day old cultures in PDA of each of 48 isolates from Chile and isolate P1 (ATCC 74058), were transferred to PDA and incubated for 8 days at 5°C and 4 days at 10 and 15°C. Three plates were seeded per isolate.

Conidia production

Conidia of isolates P1 and S10B (from soil in Chile) were produced in a 300 L Fermenter Type P (Bio-engineering AC, Switzerland) in modified Richard's medium (Harman *et al.*, 1991) at 28°C for 60–70 h (aeration rate 0.4 vol air/vol medium per min, agitation: 180 rpm and constant illumination with a 40 W lamp).

Field evaluations

Experimental plots were established from the 1992–1993 through the 1995–1996 growing seasons with 'Thompson Seedless' table grapes in the Central Valley of Chile, trained on to a high trellis system with a horizontal plane of vegetation at 2 m height and a vine spacing of 4 × 4 m (625 vines ha^{-1}). Plants were sprayed at blossom (November) and between veraison and harvest (January–February) with a motor-driven 50 L sprayer (Briggs & Stratton) provided with a hand gun nozzle using between 1000–1500 L ha^{-1}. Non-treated controls and an equal number of applications of vinclozolin (Ronilan 50 WP, 1.5 kg ha^{-1}) were included in each trial. Captan (Captan 80 WP, 4.0 kg ha^{-1}) was included in 1994–1995.

Disease incidence (percentage of clusters infected) and severity (percentage of diseased berries) were estimated at harvest in a 200 cluster sample per replicate. Each cluster was examined when first symptoms appeared and were classified as diseased if at least one berry was rotten. The total number of rotten berries were counted. Data was analysed for variance and means were separated according to Duncan's multiple range tests.

Strain evaluation

The effectiveness of isolates S10B and P1 applied as non-formulated products (5×10^{12} conidia ha^{-1}) were evaluated and compared with 1×10^{13} conidia ha^{-1} of isolate T39 (commercially available as Trichodex 25 WP, Makhteshim Chemical Works, Ltd, Israel). Plants were treated three times at blossom and three times between veraison and a week before harvest. Treatments were distributed according to a randomized complete block design with four replicates and four plants as experimental units.

Efficacy of strain P1

The efficacy of non-formulated isolate P1 was evaluated in 1992–1993 and 1993–1994 growing seasons, and as formulated product (22% skim milk, 50% perlite, 4% saccharose) in 1994–1995 and 1995–1996 growing seasons. In 1995–1996, two trials were conducted, one in a location historically characterized by a moderate disease pressure (Buin) and the other in a location historically characterized by a high disease pressure (Rancagua). Grapevines were sprayed two or three times at blossoming followed by an equal number of treatments between veraison and harvest. Trials were designed as randomised complete blocks with four replicates and 5–6 plants as experimental units.

Finally, a trial was conducted to evaluate integrated control of Botrytis bunch rot. For this purpose, approximately $1–1.7 \times 10^{13}$ conidia ha^{-1} of formulated isolate P1 were sprayed at the beginning of flowering, full flowering, and at veraison (8.5 °Brix) followed by a single application of vinclozolin 48 h after high-risk infection periods were determined with a disease predictor (Envirocaster, Neogen Co., MI, USA) (Avilés *et al.*, 1995; Broome *et al.*, 1995).

Population dynamics of *T. harzianum*

The total population of non-formulated *T. harzianum* isolates S10B and P1, and Trichodex (T39) on flowers and berries of 'Thompson Seedless' table grape, treated as described for strain comparison, were monitored after three applications at blossom time and later after three other applications between veraison and harvest. Similarly, the population of *T. harzianum* P1 was monitored on leaves and clusters of 'Thompson Seedless' table grapes sprayed twice at flowering and twice between veraison and a week before harvest with the 10^{13} conidia ha^{-1} of either formulated or non-formulated products. Approximately 20–30 g of flowers and 21–47 g of leaves were collected weekly after the third and second application at blossom, respectively, and later about 99–106 g of berries were sampled weekly after the second application before harvest. Samples were washed in sterile distilled water (1:1 w:v) by vigorously shaking for 2 min. The resulting suspension was diluted serially, and 0.1 mL was plated on MPDA. Cultures were incubated for 5–7 days at 23°C. Four replicates of at least two plates each were included.

Results

Isolation and biocontrol assay

Over 100 isolates of *T. harzianum* were isolated from soil samples and aerial plant parts of kiwifruit (*Actinidia chinensis* Planchon), grape (*V. vinifera*), orange (*Citrus sinensis* (L.) Osbeck), eucalyptus (*Eucalyptus globulus* Labill.), and apricot (*Prunus armeniaca* L.). A subsample of 48 isolates were tested and found to be antagonistic to *B. cinerea* on apple fruits; 18 of these (37.5%) exhibited a degree of control equal to or better than reference isolate P1.

Most of the isolates were unable to grow at 5°C, but all of them grew at 15°C. Isolates S10B and P1 grew at 10°C but not at 5°C and were highly antagonistic against *B. cinerea*. Thus, isolate S10B, recovered from soil, was selected for further studies.

Table 1. Relative effectiveness among isolates of *Trichoderma harzianum* against Botrytis bunch rot of 'Thompson Seedless' table grapes

Treatments and rates (ha^{-1})	Botrytis bunch rot[a] Incidence (%)	Severity (%)
T. harzianum[b]		
P1 (5×10^{12} conidia)	10.1 b[c]	1.70[ns]
S10B (5×10^{12} conidia)	9.5 b	0.75
T39 (1×10^{13} conidia)	8.0 ab	1.20
Vinclozolin (750 g)[d]	5.8 a	0.80
Untreated control	14.2 c	2.40

[a]Three applications at flowering and three between veraison and harvest. Incidence = percentage of diseased clusters and severity = percentage of diseased berries.
[b]P1 = ATCC 74058, S10B = Chilean isolate from soil, and T39 = Trichodex.
[c]Means of four replicates followed by different letters were significantly different according to Duncan's multiple range test at $p < 0.05$. ns = not significant for variance at $p < 0.05$.
[d]Ronilan 50 WP.

Table 2. Effectiveness of *Trichoderma harzianum* against Botrytis bunch rot of 'Thompson Seedless' table grape, under field conditions in the Central Valley of Chile

Control agents[a] and sprays (flowering + pre-harvest)	Botrytis bunch rot[b] Incidence (%)	Severity (%)
1992–1993		
T. harzianum P1 (3 + 2)	2.5 a[c]	0.26 a[c]
Vinclozolin (3 + 2)	3.0 a	0.19 a
Untreated control	7.5 b	1.18 b
1993–1994		
T. harzianum P1 (3 + 3)	10.1 b	1.70 [ns]
Vinclozolin (3 + 3)	5.8 a	0.80
Untreated control	14.2 c	2.40
1994–1995		
T. harzianum P1 (2 +2)	17.2 ab	1.46 ab
Vinclozolin (2 + 2)	15.0 b	1.29 b
Captan (2 + 2)	17.6 ab	1.20 c
Untreated control	23.6 a	2.06 ab
1995–1996		
Buin		
T. harzianum P1 (2+2)	16.5 b	0.30 b
Vinclozolin (2 + 2)	14.5 b	0.37 b
Untreated control	29.5 a	1.26 a
Rancagua		
T. harzianum P1 (2+2)	40.3 [ns]	1.36 b
Vinclozolin (2 + 2)	37.8	1.21 b
Untreated control	62.5	2.53 a

[a]10^{12}–10^{13} conidia ha^{-1} of *T. harzianum* P1, non-formulated (1992–1994) or formulated (1994–1996); vinclozolin (Ronilan 50 WP, 1.5 kg ha^{-1}); captan (80 WP, 4 kg ha^{-1}).
[b]Incidence = % diseased clusters. Severity = % diseased berries.
[c]Means of four replicates followed by different letters were significantly different at $p < 0.05$, by Duncan's multiple range test, ns = not significant for variance at $p < 0.05$.

Table 3. The effect of formulation on the efficacy of *Trichoderma harzianum* against Botrytis bunch rot of 'Thompson Seedless' table grapes

Treatments[a]	Botrytis bunch rot[b] Incidence (%)	Severity (%)
T. harzianum, P1		
Formulated	16.5 ac[c]	0.3 ac
non-formulated	26.5 b	0.7 ab
Vinclozolin	14.5 a	0.4 a
Untreated control	29.5 b	1.3 b

[a]Rates: 10^{12} conidia ha^{-1}, and Ronilan 50 WP, 1.5 kg ha^{-1}, applied twice at flowering and once at veraison and before harvest.
[b]Incidence (% diseased clusters) and severity (% diseased berries) determined at harvest (17 °Brix) in a 200 cluster sample per treatment.
[c]Means of four replicates followed by different letters were significantly different according to Duncan's multiple range test ($p < 0.05$).

Table 4. The effectiveness of a single application of *Trichoderma harzianum* against Botrytis bunch rot of 'Thompson Seedless' table grape, followed by a single application of vinclozolin 48 h after an infection period[a]

Control agents and spray timing[b] A	B	C	D	Botrytis bunch rot[c] Incidence (%)	Severity (berries/cluster)
T	n	n	V	31.5 a[d]	0.9 a[d]
n	T	n	V	38.8 a	1.2 ab
n	n	T	n	57.8 bc	2.2 c
n	n	n	V	51.0 b	1.7 bc
n	n	n	n	62.5 c	2.5 c

[a]Infection periods were determined with an infield disease predictor (Noegen Co., MI, USA).
[b]A = beginning of flowering; B = full flowering; C = veraison (8.5 °Brix) and D = ripening (10.3 °Brix). T = 1–1.7 × 10^{13} conidia ha^{-1} of formulated product based on *T. harzianum*, isolate P1 (ATCC 74058). V = vinclozolin (Ronilan 50 WP, 1.5 kg ha^{-1}), applied 48 h after a single infection period recorded during ripening. n = no treatment.
[c]Incidence (% diseased clusters) and severity (% diseased berries) were determined in a 400 cluster sample per treatment. 10 days after the infection period.
[d]Means followed by different letters were statistically different according to Duncan's multiple range test ($p < 0.05$).

Effectiveness of *T. harzianum* under field conditions

An average yield of 1 $\times 10^8$ conidia mL^{-1} were obtained after 60–70 h of cultivation for isolates P1 and S10B. These isolates exhibited a similar degree of control, significantly ($p < 0.05$) greater than the untreated control, but less than vinclozolin. No significant differences were found between non-formulated isolates P1 or S10B and isolate T39, applied as a formulated product (*Table 1*).

During four seasons, regardless of disease pressure, isolate P1 provided an effective control of Botrytis bunch rot, reducing incidence and severity significantly ($p < 0.05$). The efficacy was similar to or less than vinclozolin, and similar to captan (*Table 2*). The effectiveness was significantly better ($p < 0.05$) for formulated products than for non-formulated preparations of *T. harzianum* P1 (*Table 3*).

The effectiveness of a single application of vinclozolin after a high-risk infection period was significantly

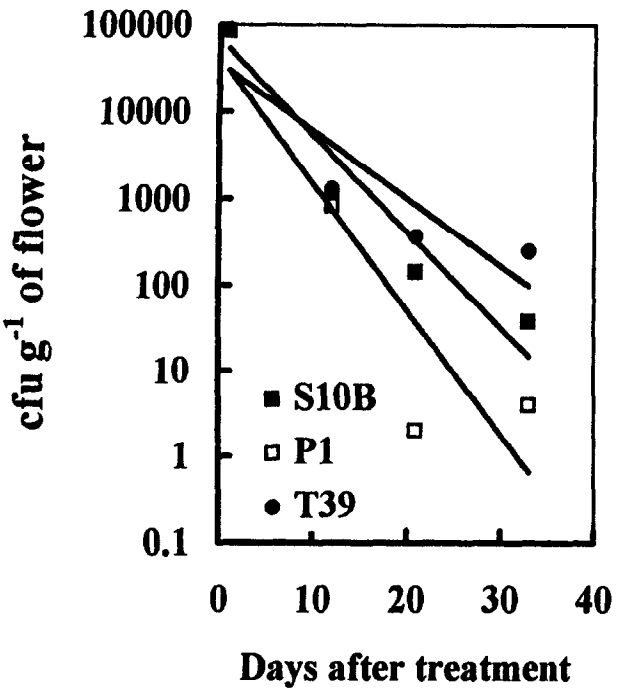

Figure 1. Population dynamics of *Trichoderma harzianum*, isolates S10B (non-formulated), P1 (ATCC 74058, non-formulated) and T39 (Trichodex 25 WP) on flowers of 'Thompson Seedless' table grape. Each point is the mean of four replicates. $Y_{S10B} = 68435e^{-0.255X}$, $R^2 = 0.8962$; $Y_{P1} = 42118^{e-0.335X}$, $R^2 = 0.8962$; $Y_{T39} = 35995^{e-01786X}$, $R^2 = 0.8113$

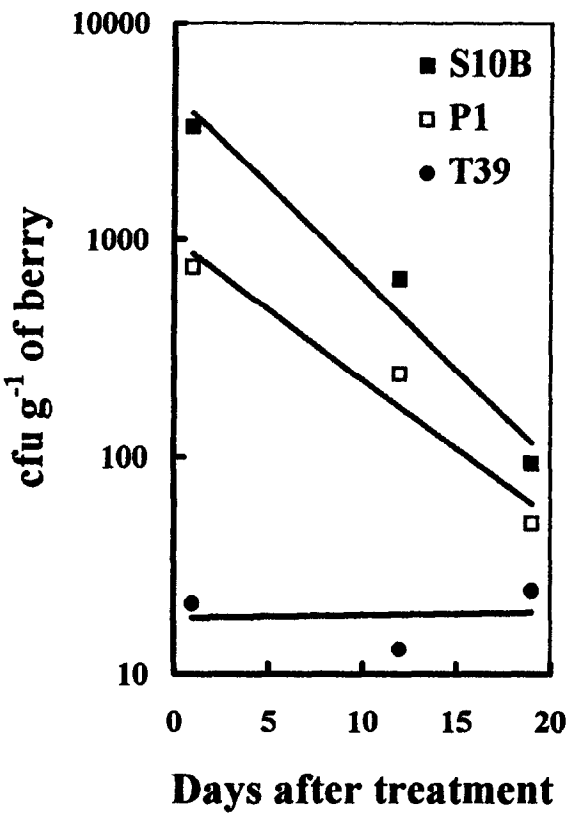

Figure 2. Population dynamics of *Trichoderma harzianum*, isolates S10B (non-formulated), P1 (non-formulated) and T39 (Trichodex 25 WP) on clusters of 'Thompson Seedless' table grape. Each point is the mean of four replicates. $Y_{S_1O_B} = 4651.4^{e-0.1939X}$, $R^2 = 0.9667$; $Y_{P1} = 998.33^{e-0.1472X}$, $R^2 = 0.9483$; $Y_{T39} = 18.147^{e0.0029X}$, $R^2 = 0.0065$

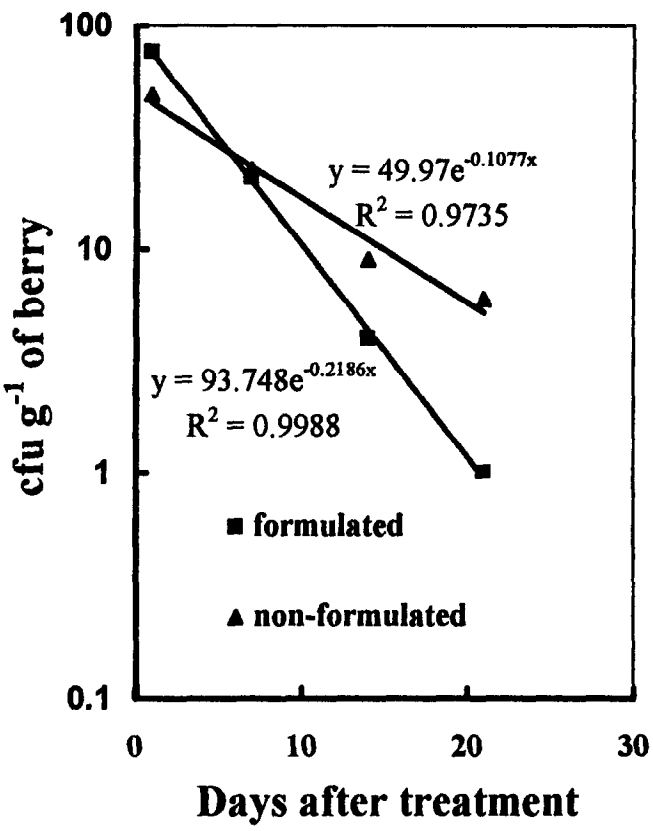

Figure 3. Population dynamics of formulated and non-formulated *Trichoderma harzianum* P1 (ATCC 74058) after two applications to flowers of 'Thompson Seedless' table grape. Each point is the mean of four replicates

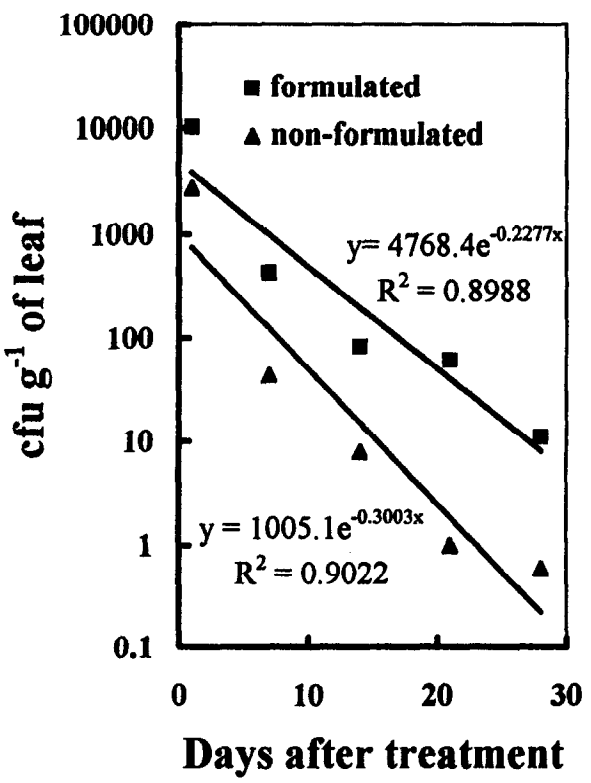

Figure 4. Population dynamics of formulated and non-formulated *Trichoderma harzianum* P1 (ATCC 74058) after two applications to leaves of 'Thompson Seedless' table grape. Each point is the mean of four replicates

($p < 0.05$) improved with a single application of formulated P1, applied at the beginning of flowering or full bloom. Latter applications of *T. harzianum* did not improve control significantly (*Table 4*).

Populations dynamics of *T. harzianum*

The total population of *T. harzianum* decreased rapidly after flowers were treated with non-formulated preparations of isolates S10B and P1 or Trichodex (T39), but detection was possible at least for 33 days after treatment (*Figure 1*). On berries, isolates S10B and P1 also decreased rapidly and isolate T39 was rather stable at low populations. All three isolates were detected on berries after 19 days of treatment (*Figure 2*). Similarly, the total population of *T. harzianum* decreased after leaves or berries were treated with formulated or non-formulated preparations of isolates P1 (*Figures 3* and *4*). Nevertheless, *T. harzianum* P1 was detected 21 and 28 days after treatment on berries and leaves, respectively.

Discussion

Control of diseases caused by *B. cinerea* using preparations based on isolates of *T. harzianum* are well documented for several crops, but the information regarding Botrytis bunch rot of grapevine is rather incomplete (Elad, 1993; Elad, Kirshner, and Gotlieb, 1993; Elad *et al.*, 1993, 1995; Gullino and Garibaldi, 1987; Harman *et al.*, 1996; Latorre *et al.*, 1996; Smith *et al.*, 1990). Our results demonstrate that isolates of *T. harzianum*, biologically active against *B. cinerea*, can be commonly isolated from soil samples, as well as from the phylloplane of several crops in Chile and most of them provided a similar or better degree of control of *B. cinerea* than reference isolates P1 or T39.

However, in agreement with previous reports (Gullino and Garibaldi, 1987), the disease control achieved under field conditions, with either formulated or non-formulated preparations alone, is insufficient considering that tolerance to Botrytis bunch rot of table grape is very low (disease incidence < 0.5% at final destination in Europe or USA) in Chile. Nevertheless, *T. harzianum* is compatible with dicarboximide fungicides (iprodione, vinclozolin) (Harman *et al.*, 1996). This would allow the development of an integrated control strategy combining biological and chemical control and cultural practices in order to reduce conditions that favor disease development as has been proposed (Broome *et al.*, 1995; Cohen *et al.*, 1996; Elad and Zimand, 1992; Elad *et al.*, 1993, 1995; Harman *et al.*, 1996). This control strategy aims to reduce the amount of chemicals employed, and consequently, lowers the risk of fungicide resistance, as well as concerns for human health derived from the exposure of fruits to chemicals.

Our results suggest that the earlier the application of *T. harzianum*, the better the performance. For instance, we found that disease incidence and severity were significantly ($p < 0.05$) reduced with a single application of *T. harzianum* P1 (formulated product) at flowering, followed by a single application of vinclozolin 48 h after the only warming period, determined by an infield disease predictor during ripening (*Table 3*).

Although isolates P1, T39, and S10B were isolated from very different sources than grape phylloplane, they were none the less able to survive on grape leaves, flowers, and clusters as previously described for other crops, providing an opportunity for control of *B. cinerea* in the field under commercial conditions (Elad and Kishner, 1993; Elad *et al.*, 1993; Tronsmo, 1991).

The total population of isolate P1 on leaves and berries decreased less rapidly when formulated preparations were sprayed. Both formulated and non-formulated preparations decreased less rapidly on grape leaves than on berries. Hence, grape leaves may provide a better environment than the surface of berries for establishment and colonization.

Consequently, an efficient and reliable formulation with long shelf life is needed to commercialize the use of *T. harzianum* against Botrytis bunch rot of table grape in Chile. Mass production of conidia with improved UV resistance, viability, and shelf life is critical. The latter might be obtained by formulating spores produced by submerged cultivation with a thicker and hydrophobic, instead of hydrophilic, outer wall, as shown before for aerial spores of *T. harzianum* (Muñoz *et al.*, 1995; Agosín *et al.*, 1996). Our formulation, using conidia of *T. harzianum* mass produced by liquid fermentation, and Trichodex (Cohen *et al.*, 1996), are at present alternatives that may be used despite the need for fungicides at certain specific phenological stages of grapevine development (e.g. blossoming or veraison). *Trichoderma harzianum* treatments should be complemented with fungicides under conditions highly favorable for disease development. A disease predictor (Avilés *et al.*, 1995; Broome *et al.*, 1995) or a decision support system for Botrytis management (Shtienberg *et al.*, 1996) may be very useful tools for the integration of biological control agents with other control practices for gray mold control.

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