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CROP PROTECTION

RESEARCH PAPER

Evaluation of the use of wound-protectant fungicides and biological control agents against stem canker (*Neofusicoccum parvum*) of blueberry

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Abstract

B.A. Latorre, R. Torres, T. Silva, and K. Elfar. 2013. Evaluation of the use of wound-protectant fungicides and biological control agents against stem canker (*Neofusicoccum parvum*) of blueberry. Cien. Inv. Agr. 40(3): 537-545. Economically, blueberry (*Vaccinium corymbosum*) has become a very important fruit crop in Chile, and stem canker (*Neofusicoccum parvum*) has frequently been observed as a major disease. The symptoms are characterized by partial or total death of the foliage associated with extensive reddish-brown canker lesions at the base of the stems. Pruning wounds appear to be the main infection route. In this study, fungicide pastes and biological control agents were evaluated for their effectiveness as pruning wound-protectants against *N. parvum*. The mycelium of *N. parvum* was highly sensitive to benomyl, tebuconazole, and iprodione in vitro, with median effective concentrations (EC_{50}) of 0.15-0.25, 0.26-0.33, and 0.52-0.68 $\mu g \cdot mL^{-1}$, respectively. The pastes formulated with 0.1% benomyl, 0.5% tebuconazole, and 0.06% iprodione also provided considerable protection of pruning wounds against *N. parvum* on the stems of Duke blueberries under field conditions. However, pyraclostrobin, with relatively high EC_{50} values (>2 $\mu g \cdot mL^{-1}$) in vitro, was largely ineffective in vivo at a 0.1% concentration, and 75% citrus extract (Citrus SL), *Bacillus subtilis* QST713 (Serenade Max), and *Trichoderma* spp. (Trichonativa) were also ineffective. Additionally, pastes formulated with 5% boric acid, although effective, were phytotoxic.

Key words: *Botryosphaeria* canker, biological control, boric acid, ascorbic acid, *Vaccinium corymbosum*

Introduction

Blueberry (*Vaccinium corymbosum* L.) orchards extend from northern to southern Chile, currently comprising more than 12,000 ha. Among the blueberry diseases reported in Chile, stem canker caused by *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips (tel. *Botryosphaeria parva* Pennycook & Samuels) and other species of the family Botryosphaeriaceae has frequently been observed (Espinoza *et al.* 2009). These fungi are also very destructive pathogens on other hosts, *e.g.*, grapevines, in Chile (Díaz *et al*., 2013; Morales *et al*., 2012) and worldwide (Úrbez-Torres, 2011).

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The symptoms of the disease are characterized by partial or total death of the foliage, always associated with the presence of extensive reddish brown canker lesions at the base of the stems and the crowns of the plants. Internally, a vascular discoloration of the xylem characterizes diseased stems.

Pruning wounds are considered to be the main infection route (Úrbez-Torres, 2011; Úrbez-Torres and Gubler, 2011), such that protecting the wounds has been proposed as the only way to prevent infection. Although the effect of several fungicides against Botryosphaeriaceae species has been studied in perennial hosts (Bester *et al*., 2007; Brown-Rytlewski and McManus, 2000; Díaz and Latorre, 2013; Li *et al*., 1995; Luque *et al*., 2008; Ma *et al*., 2002; Pitt *et al*., 2012), there is little or no information on the efficacy of fungicide and biocontrol treatments against species of Botryosphaeriaceae that attack blueberries. Previously, Espinoza *et al.* (2009) demonstrated that isolates of *N. parvum* from blueberries were sensitive in vitro to fludioxonil and iprodione, but these fungicides were not tested under field conditions. The aim of this study was to evaluate the effectiveness of fungicide pastes and biological control agents as pruning wound protectants against *N. parvum* in blueberry.

Materials and methods

Isolates

N. parvum isolates B1-06 originally obtained from blueberry were used. For inoculation purposes, a mycelium of isolate B1-06 was obtained from a 10 day-old culture on potato dextrose agar acidified with 0.5 mL per liter of 90% lactic acid plus 0.005% tetracycline, 0.01% streptomycin, and 0.1% Igepal CO-630 (Sigma-Aldrich, MO, USA) (APDA). For in vitro studies, *N. parvum* isolates B1-06 and 5.5.4R1(2), both from blueberry, were used. The isolates were maintained on APDA at 5°C.

Fungicides

Fungicides containing benomyl (Benex 50 WP, Arysta, Chile), iprodione (Iprodione 50 WP, Agrospec, Chile), pyraclostrobin (Podexal 5 AL and Comet 250 EC, Basf, Chile), tebuconazole (Podaspec 5Al and Tebuconazole 250 WP, Agrospec, Chile), *Bacillus subtilis* QST713 (Serenade Max, Arysta LifeScience Chile), *Trichoderma* spp. (Trichonativa, Bionativa, Chile), ascorbic acid (Citrus SL, 75% citrus extract, Agrospec, Chile), and boric acid were used. Additionally, paste formulations of benomyl (Benex 50 WP), iprodione, and boric acid were prepared using an aqueous suspension of vinyl acrylic paint (VAP) (Latex base, Sherwin Williams Chile, S.A, Santiago, Chile).

In vitro test

The sensitivity of the *N. parvum* B1-06 and 5.5.4 R1(2) isolates to benomyl, iprodione, pyraclostrobin, and tebuconazole was assessed on APDA amended with 0.025 to 2.0 $\mu g \cdot L^{-1}$ of the respective fungicide (added after autoclaving the APDA). A mycelium plug (5 mm in diameter) was placed in the center of each of four 9 cm Petri plates per treatment. Non-amended APDA plates were used as the controls. The plates were incubated at 25 ºC for 48 h to measure the radial mycelial growth.

To study the conidial sensitivity to benomyl and tebuconazole, a conidial suspension was prepared in Eppendorf tubes (1.5 mL), adjusting the final concentration to 0.025 to 2.0 $\mu g \cdot L^{-1}$ of the respective fungicide. Conidia were incubated for 18 h at 25ºC prior to randomly observe at least 50 conidia per fungicide concentration per each of four replicates. Conidia were counted as germinated when a normal germ tube of at least the length of the conidia was obtained.

Bioassay

Detached actively growing shoots (<1-yr-old) (n=4) of blueberry (cv. 'O'Neal') were surface-disinfected (75% ethanol, 5 min), then aseptically pruned. The pruning wounds were immediately sprayed with benomyl (0.5, 1.0 and 1.5%), iprodione (0.03, 0.006 and 0.1%), pyraclostrobin (0.05, 0.1 and 0.5%) and tebuconazole (0.1, 0.5 and 1.0%). After 24 h, a mycelial plug (5 mm in diameter) of either isolate B1-06 or isolate 5.5.4 R1(2) of *N. parvum* was placed on the pruned stub and wrapped with Parafilm. The stems were placed vertically in a humid chamber (>90% relative humidity) at 20 ºC. The length of the necrotic lesions was determined after 12 days of incubation. An equal number of inoculated but non-treated pruning wounds were included as a control.

Field trials

Field trials were conducted in a commercial blueberry planting of cv. Duke in Santa Cruz (Colchagua Valley) in 2011 and 2012. Blueberry Duke and blueberry O'Neal were equally susceptible to *N. parvum* (data not shown). The blueberry plants were managed according to standard methods except that fungicides were not used.

In Trial 1, blueberry stems at 1 year of age were pruned in October (spring), and three stems were selected in each of the four plants per treatment. A 3 mm mycelial plug from the APDA cultures of *N. parvum* was placed over the fresh pruning cut and wrapped immediately with Parafilm to avoid rapid dehydration. The pruning wounds were treated 24 h before inoculation with a paste containing (w/v) 1% benomyl, 0.06% iprodione, 0.1% pyraclostrobin, 0.5% tebuconazole, or 5% boric acid or liquid formulations of 10% *Bacillus subtilis* (QST 713), 0.5% *Trichoderma* spp., or 75% citrus extract (equivalent to 7.08% ascorbic acid). The fungicide concentrations were determined on the basis of the label recommendations. The paste fungicides were applied with the aid of a paintbrush using approximately 1 mL per pruning wound, and the liquid formulations were sprayed with a hand sprayer using a similar volume per pruning wound. An equal number of inoculated but non-treated pruning wounds were included as a control.

In Trial 2, the effect of conventional fungicides (benomyl, iprodione, pyraclostrobin, and tebuconazole) was further evaluated as paste and spray treatments. The fungicides were applied at the concentrations indicated above, and the pruning wounds were inoculated 24 h after the treatments. This trial was conducted in autumn (May).

In Trial 3, the persistence of benomyl and tebuconazole, the two best fungicides according to the previous trials, was studied against *N. parvum* on blueberry stems. For this purpose, the blueberry plants were pruned and inoculated as indicated above at 1 and 10 days after the fungicide treatments in the spring (October).

The length of the internal discoloration of the wood developing from the inoculation site was evaluated at 18 and 30 days after inoculation in Trials 1 and 2 and at 36 days after inoculation in Trial 3. The control efficacy (CE) was determined as CE = 100-(100 × MNL-T)/MNL-UT, where MNL-T = the mean necrotic lesion of the treated stems and MNL-UT = the mean length of the untreated control.

Re-isolations were performed on APDA. Three samples (approximately 5 mm in length) of diseased tissue per replicate were collected from the margins of necrotic lesions. The percent re-isolation was calculated as the number of positive isolations over the total number of samples plated on APDA.

Experimental design and statistical analysis

The effective concentration required to obtain a 50% (EC_{50}) inhibition of the mycelial growth was estimated with a linear regression analysis

in which X = the log fungicide concentration and Y = the probit % of the control.

In the bioassay, the fungicide treatments were randomly distributed in a complete randomized design with a 4 × 2 (fungicide concentration × isolates) factorial structure, with four replicates of one stem each. The data were Ln (x + 1) transformed before the statistical analysis, although the non-transformed data are presented.

The fungicide treatments in Trial 1 were arranged as a complete block design, with four replicates of three stems each as the experimental units. In Trial 2, the treatments were distributed as a complete 2 × 4 factorial design (application method x fungicide) with four replicates. The re-isolation data were transformed as the square root of x + 0.5 prior to the statistical analysis, although the non-transformed data are presented. The data were subjected to an analysis of variance, and the means were evaluated with Tukey's test ($P \leq 0.05$) with the aid of SigmaStat 3.1 (Systat Software Inc., San Jose, CA).

Results

The mycelium of *N. parvum* isolates B1-06 and 5.5.4 R1(2) was highly sensitive to benomyl, with EC_{50} values of 0.15-0.25 $\mu g \cdot mL^{-1}$, followed by tebuconazole, with EC_{50} values of 0.26-0.33 $\mu g \cdot mL^{-1}$, and iprodione, with EC_{50} values of 0.52-0.68 $\mu g \cdot mL^{-1}$. The EC_{50} values obtained for pyraclostrobin were >2 $\mu g \cdot mL^{-1}$ (Table 1). The germination of the conidia was also highly sensitive to benomyl and tebuconazole, with EC_{50} values of 0.19-0.27 and 0.75-0.62 $\mu g \cdot mL^{-1}$, respectively (Table 1).

In the bioassay, using detached blueberry shoots, an analysis of variance showed that the isolates and the interaction between fungicide concentration and the isolates were not significant. However, a significant ($P \leq 0.001$) fungicide effect was obtained. The mean length of the necrotic lesions was reduced from 26.0 mm in the non-treated controls to 1.0 to 6.1 mm with benomyl, 1.4 to 7.5 mm with tebuconazol, 4.5 to 11.9 mm with iprodione and 3.2 to 18.8 mm with pyraclostrobin. Independently of the fungicide, the best control was obtained using the highest concentration (Figure 1).

In the initial screening of protectant compounds against *N. parvum* in blueberry stems, 0.5% tebuconazole (Podaspec), 0.1% benomyl, and 0.06% iprodione were, in order of efficacy, the most effective fungicide treatments. These fungicides completely prevented the re-isolation of *N. parvum* and provided greater than 90% control efficacy. The treatments had a significant ($P \leq 0.001$) effect on the mean length of the necrotic lesions (Figure 2), varying from 30.1 mm in the non-treated controls to 0.6, 1.2, and 3.2 mm for the pruning wounds treated with tebuconazole, benomyl, and iprodione, respectively. Pyraclostrobin, applied at 0.1%, provided a relatively weak control (CE = 44.5%), with a mean necrotic lesion length of 16.7 mm, and *N. parvum* was re-isolated from the pruning wounds treated with pyraclostrobin (Table 2).

Table 1. Sensitivity of *Neofusicoccum parvum* isolates obtained from stem cankers of blueberries to fungicides with different modes of action.

		Median effective concentration EC_{50}, $\mu g \cdot mL^{-1}$			
		Mycelium		Conidia	
Fungicide	Fungicide group	B1-06	5.5.4 R1(2)	B1-06	5.5.4 R1(2)
Benomyl	Benzimidazole	0.25	0.15	0.19	0.27
Iprodione	Dicarboximide	0.68	0.52	nd	nd
Pyraclostrobin	QoI[1]	>2.0	>2.0	nd	nd
Tebuconazole	DMI[1]	0.26	0.33	0.75	0.62

[1]QoI = quinone outside inhibitor. DMI = sterol demethylation inhibitor. nd = not determined.

A moderate control, CE = 53.8%, was obtained using 5% boric acid. Ascorbic acid (Citrus SL), *Bacillus subtilis*, and *Trichoderma* spp. provided weak protection (CE <30.9%) of pruning wounds against infection by *N. parvum* on blueberry stems (Table 2). Similar results were obtained in a second experiment using these biological products (data not shown).

On the basis of the necrotic lesions obtained on the blueberry stems in Trial 2, an analysis of variance showed that the differences between the paste and liquid applications were not significant (P = 0.582). However, the fungicide factor and the interaction between the application method and fungicide were significant (P≤0.0001) (Table 3). Based on the re-isolations of *N. parvum* from diseased tissues, a significant (P≤0.0001) effect was obtained for the application method and fungicide, and the interaction between the application method and fungicide was significant (P≤0.0001) (Table 3).

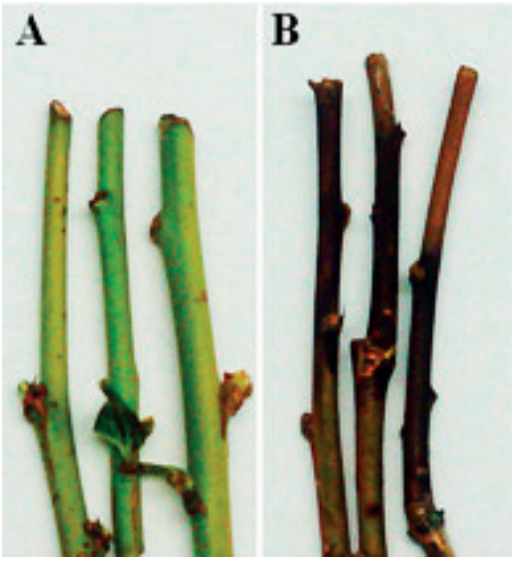

Figure 2. Control of stem canker (*Neofusicoccum parvum*) on inoculated stems of blueberry (*Vaccinium corymbosum*) cv. Duke. A. Inoculated pruning wounds protected with benomyl. B. Non-protected pruning wounds that developed reddish-brown lesions after 36 days under field conditions.
Discussion

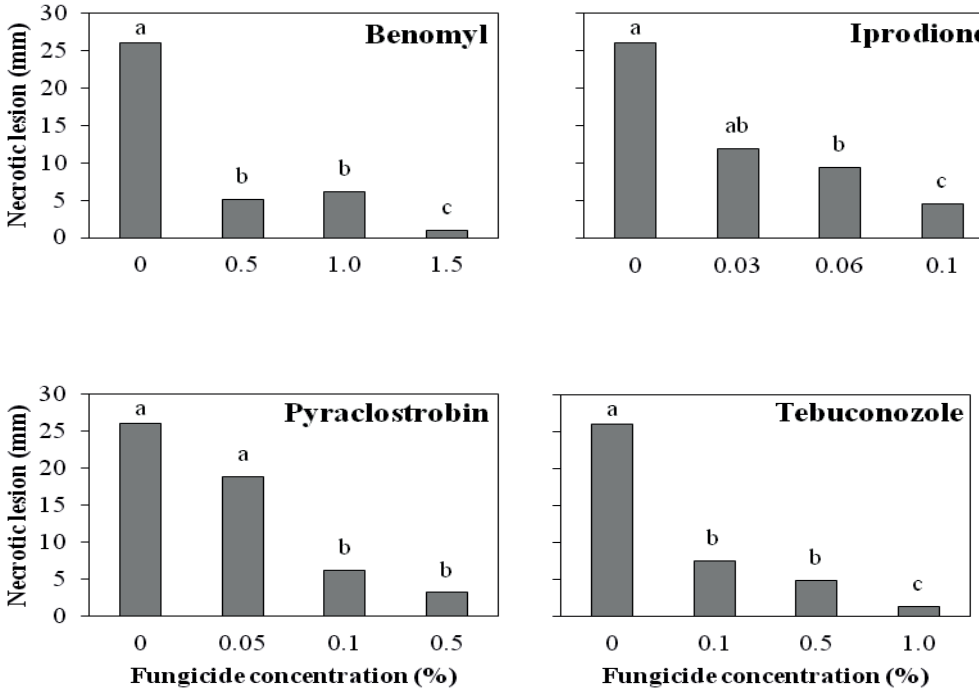

Figure 1. Effectiveness of fungicide spray applications as wound protectants against *Neofusicoccum parvum* infection in detached actively growing shoots of blueberry 'O'Neal'. The bars followed by the same letters are not significantly different according to Tukey's test (P = 0.05).

Table 2. Effect of fungicide treatments against *Neofusicoccum parvum* on pruning wounds of blueberry 'Duke' treated 24 h prior to inoculation.

Fungicide	Origin	Active ingredient	Rate[1] %	Necrotic lesions[2] mm	Re-isolation[3] (n = 3) no.
Podaspec, paste	Agrospec, Chile	Tebuconazole	0.5	0.6 a[6]	0.0 a[6]
Benomyl, paste[4]		Benomyl	0.1	1.2 a	0.0 a
Podexal, paste	Basf, Chile	Pyraclostrobin	0.1	16.7 b	1.0 b
Iprodione, paste[4]		Iprodione	0.06	3.2 a	0.0 a
Serenade WP	Arysta, Chile	*Bacillus subtilis* (QST 713)	10.0	24.4 c	3.0 c
Trichonativa	Bionativa, Chile	*Trichoderma* spp.	0.5	21.2 bc	1.5 bc
Citrus SL[5]	Agrospec, Chile	Ascorbic acid	7.1	20.8 bc	1.3 bc
Boric paste[4]		Boric acid	5.0	13.9 b	0.0 a
Non-treated			-	30.1 c	1.3 cb

[1]Rate = % concentration of active ingredients used in this study.
[2]Necrotic lesions were determined 18 days after the inoculation. Inoculations were performed using a mycelial plug at 24 h after the fungicide application.
[3]Re-isolations were conducted on APDA using three small samples of stem tissue collected from the margins of the necrotic lesions at 18 days after the inoculation.
[4]Paste formulations were prepared for this study using aqueous suspensions of vinyl acrylic paint (Latex base, Sherwin Williams Chile, S.A).
[5]Citrus SL is a 75% citrus extract product equivalent to 7.1% ascorbic acid.
[6]The means of four replicates followed by the same letters are not significantly different according to Tukey's test (P = 0.05). Re-isolation data were transformed via the square root of x+0.5 prior to the analysis, but the non-transformed data are presented here.

Table 3. Analysis of variance showing the effect of the application method and fungicide on the length of necrotic lesions on the stems of blueberry 'Duke' caused by *Neofusicoccum parvum*.

Treatment	Necrotic lesions mm	Re-isolation[1] no.
Application method (AP)		
Paste	2.1	1.2
Liquid	1.9	1.6
df	1	1
F	0.31	43.2
P	0.582	<0.001
SED[2]	0.33	0.09
Fungicide (F)		
Benomyl	0.06	0.0
Iprodione	1.95	1.5
Pyraclostrobin	3.06	2.5
Tebuconazole	0.00	0.0
Untreated	4.67	3.0
df	4	4
F	31.79	188.55
P	<0.001	<0.001
SED[2]	0.51	0.14
AM x F interaction		
df	4	4
F	23.93	43.95
P	<0.0001	<0.001
SED[2]	0.72	0.20

[1]Re-isolation = the number of samples yielding *N. parvum* on APDA using three samples per replicate.
[2]SED = standard error of the difference.

According to the length of the necrotic lesions, benomyl and tebuconazole effectively protected wounds against infection by *N. parvum* if the pruning wounds were inoculated 1 or 10 days after treatment (Figure 3). Inoculation performed 1 or 10 days after fungicide treatment provided greater than 97% CE. Independently of the application method, re-isolation was completely inhibited by benomyl and tebuconazole with inoculation at 1 and 10 days after fungicide application.

Stem canker has been recognized as a major disease of blueberry and is caused by *N. parvum* and other species of the family Botryosphaeriaceae. In this study, the inoculated but non-treated stems developed a brown-reddish necrosis (Figure 2) extending downward from the site of inoculation, as described for this disease in a previous study (Espinoza *et al*., 2009). The length of the necrotic lesions was considerably affected by the date of the inoculation. The longest lesions resulted from spring inoculations. Therefore, blueberry stems were susceptible to infection by *N. parvum*, but their susceptibility varied considerably between spring

and autumn inoculations. However, it remains to be determined whether this effect was due to the physiological conditions of the host plant and/or the weather conditions, *e.g.*, the temperature at the time of the inoculation. Re-isolation of *N. parvum* was equally successful in blueberries inoculated in the spring and in the autumn.

According to the results obtained in three trials conducted in Colchagua Valley in two consecutive years, stem canker can be considerably reduced by protecting pruning wounds with fungicides. Pruning wounds, as has been suggested for grapevines (Rolshausen *et al*., 2010; Úrbez-Torres and Gubler, 2011), appear to be the main route of *N. parvum* infection in blueberry. In the present study, benomyl, tebuconazole, and iprodione were the most effective fungicides against *N. parvum*.

Previous studies have demonstrated that benzimidazole compounds are highly effective against species of Botryosphaeriaceae on grapevines (Kotze *et al*., 2011; Pitt *et al*., 2012), Japanese apricots, peaches (Li *et al*., 1995), and oaks (Luque *et al*., 2008). However, to our knowledge, this is the first report demonstrating the efficacy of benomyl as a pruning wound protectant against infection caused by *N. parvum* on blueberry stems.

The effectiveness of sterol demethylation inhibitors (DMI) against species of Botryosphaeriaceae varies considerably among DMI compounds, and in general conidia are less sensitive than mycelium in vitro (Torres *et al.,* 2013). In this study, the 0.5% tebuconazole treatments provided a strong control as a pruning wound protectant against *N. parvum* on blueberry stems. Similarly, tebuconazole has also been reported to be effective in reducing the infection caused by other Botryosphaeriaceae spp. on other hosts (Bester *et al*., 2007; Ma *et al*., 2002). However, other DMI compounds, *e.g*., penconazole, myclobutanil, and tetraconazole, were unable to protect pruning wounds from infection caused by Botryosphaeriaceae spp. (Pitt *et al.,* 2012).

The low efficacy (44.5%) obtained with the use of 0.1% pyraclostrobin in the field trials agreed with the relatively high EC_{50} (>2 $\mu g \cdot mL^{-1}$) obtained for the mycelial growth inhibition of *N. parvum* in vitro. A relatively low sensitivity of *N. parvum* isolates against pyraclostrobin has been reported previously (Pitt *et al*., 2012). However, it is still possible that considerable variation may occur among *N. parvum* isolates in nature, and it is also possible that the efficacy of pyraclostrobin can be improved if concentrations higher than 0.1% are used.

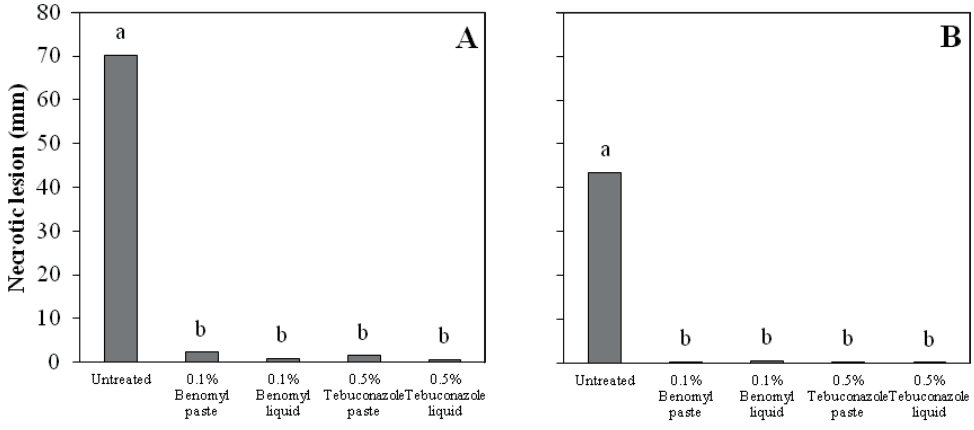

Figure 3. Effect of paste and liquid applications of fungicides as wound protectants for the prevention of *Neofusicoccum parvum* infection of blueberry pruning wounds. Pruning wounds were inoculated 1 day (A) and 10 days (B) after fungicide application. The bars followed by the same letters are not significantly different according to Tukey's test (P = 0.05).

It has been postulated that the healing process occurring in wounds reduces the susceptibility of pruning wounds to infection caused by several filamentous fungi in different host plants (Eskalen *et al*., 2007; Úrbez-Torres and Gubler, 2011). In the present study, the pruning wounds were very susceptible to *N. parvum* immediately after pruning in October, although they were still susceptible to infection caused by *N. parvum* when inoculation was performed at 10 days after pruning.

A wound-protectant paste prepared with boric acid has been tested in previous studies against species of Botryosphaeriaceae and *Eutypa lata* (Pitt *et al*., 2012; Rolshausen, and Gubler, 2005) on grapevines, providing good control under field conditions. Nevertheless, boric acid appears to be ineffective against Botryosphaeriaceae spp. in vitro (Pitt *et al*., 2012). In the present study, 5% boric acid completely inhibited *N. parvum* in vivo, and 0% re-isolation was obtained after 18 days of treatment. Therefore, the observed necrotic lesions were associated with phytotoxicity.

In conclusion, the results demonstrate that the occurrence of *N. parvum*-caused stem canker on blueberry can be reduced by protecting pruning wounds with fungicides (*e.g*., benomyl, iprodione, tebuconazole). Pruning wounds are susceptible to infection caused by *N. parvum* immediately after pruning, but additional research is needed to determine the efficacy of fungicides over time using conidia in addition to mycelia as the primary inoculum. The biological control agents and natural products tested were relatively ineffective against *N. parvum*.

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Resumen

B.A. Latorre, R. Torres, T. Silva y K. Elfar. 2013. Evaluación de fungicidas y agentes biocontroladores como protectores de heridas contra la cancrosis del tallo (*Neofusicoccum parvum*) del arándano. Cien. Inv. Agr. 40(3): 537-545. El arándano (*Vaccinium corymbosum*) se ha convertido en un cultivo de fruta económicamente muy importante para Chile. Entre las enfermedades que lo afectan, la cancrosis del tallo (*Neofusicoccum parvum*) ha adquirido gran importancia. Los síntomas se caracterizan por una muerte parcial o total del follaje, asociado a lesiones cancrosas café rojizas que afectan externa e internamente en la base de los tallos. Se acepta que la infección se inicia a través de las heridas de poda. Este estudio tuvo como principal objetivo evaluar la eficacia de pastas fungicidas para la protección de las heridas contra *N. parvum* en arándanos. Los resultados obtenidos demostraron que el micelio de *N. parvum* es muy sensible a los fungicidas benomilo, tebuconazol e iprodione *in vitro*, con concentraciones medianas efectivas (EC_{50}) de 0,15-0,25; 0,26-0,33 y 0,52-0,68 $\mu g \cdot mL^{-1}$, respectivamente. En condiciones de campo, las pastas fungicidas formuladas con 0,1% de benomilo, 0,5% de tebuconazol o 0,06% de iprodione otorgaron una protección considerable de las heridas de poda contra *N. parvum* en los tallos de arándanos cv. Duke. Pyraclostrobin, con valores de $EC_{50} > 2$ $\mu g\ mL^{-1}$ *in vitro*, fue relativamente ineficaz *in vivo* al utilizarlo en una concentración de 0,1%. Del mismo modo, 75% de extracto cítrico (Citrus SL), *Bacillus subtilis* QST713 (Serenade

Max) y *Trichoderma* spp. (Trichonativa) fueron ineficaces contra *N. parvum*. Además, las pastas formuladas con 5% de ácido bórico, aunque eficaces, resultaron fitotóxicas.

Palabras clave: Acido ascórbico, ácido bórico, *Botryosphaeria*, cancro, control biológico, *Vaccinium corymbosum.*

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