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RESEARCH PAPERS

Evaluation of fungicides to protect pruning wounds from Botryosphaeriaceae species infections on almond trees

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Summary. *In vitro* efficacy of ten fungicides was evaluated against four Botryosphaeriaceae spp. (*Diplodia seriata*, *Neofusicoccum luteum*, *N. mediterraneum* and *N. parvum*) associated with branch cankers on almond trees. Cyproconazole, pyraclostrobin, tebuconazole, and thiophanate-methyl were effective for the inhibition of mycelial growth of most of these fungi. An experiment on 3-year-old almond trees evaluated boscalid, mancozeb, thiophanate-methyl, pyraclostrobin and tebuconazole for preventative ability against infections caused by the four pathogens. Five months after pruning and fungicide application, lesion length measurements and isolation percentages showed no significant differences among the four pathogens after they were inoculated onto the trees, and also between the two inoculation times tested (1 or 7 d after fungicide application). Thiophanate-methyl was the most effective fungicide, resulting in the shortest lesion lengths and the lowest isolation percentages from artificially inoculated pruning wounds. This chemical is therefore a candidate for inclusion in integrated disease management, to protect pruning wounds from infections caused by species of Botryosphaeriaceae. This study represents the first approach to development of chemical control strategies for the management of canker diseases caused by Botryosphaeriaceae fungi on almond trees.

Key words: chemical control, *Prunus dulcis*, thiophanate-methyl.

Introduction

Fungi belonging to the Botryosphaeriaceae are widely recognized as severe trunk pathogens of many economically important woody crops occurring worldwide (Úrbez-Torres, 2011; Phillips *et al*., 2013). Characteristic symptoms caused by these pathogens following wood infection of fruit and nut trees include stem and branch dieback, cankers, and the eventual death of affected hosts (Damm *et al*., 2007; Cloete *et al*., 2011; Phillips *et al*., 2013; Adesemoye *et al*., 2014; Chen *et al*., 2014; González-Dominguez *et al*., 2017).

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Almond trees are well-recognized hosts of Botryosphaeriaceae species. In South Africa, Slippers *et al*. (2007) identified *Neofusicoccum australe* associated with diseases of almond trees. In Spain, Gramaje *et al*. (2012) reported the presence of *Botryosphaeria dothidea*, *Diplodia olivarum*, *D. seriata*, *N. australe* and *N. parvum* associated with diseased almond stems, branches or twigs on the island of Mallorca. Later, in a more detailed study conducted from 2009 to 2014 in 31 almond orchards located in the same region, *D. olivarum*, *D. seriata*, *N. luteum*, *N. mediterraneum* and *N. parvum*, were shown through pathogenicity tests to be the prevalent species associated with branch cankers on this crop (Olmo *et al*., 2016). In California, Inderbitzin *et al*. (2010) found *B. dothidea*, *D. seriata*, *N. parvum*, *N. mediterraneum*, and *N. nonquaesitum*

from almond trunks expressing bark canker, and *B. dothidea*, *Dothiorella sarmentorum*, *N. mediterraneum* and *N. parvum* from twig and branch cankers. Later, *Lasiodiplodia theobromae* (Chen *et al*., 2013) and *Do. iberica* (Doll *et al*., 2013, 2015) were also reported as canker causing pathogens, with infections developing above the graft unions of almond trees, producing amber sap and eventually girdling the trunks.

In 2013, Botryosphaeriaceae spp. associated with almond trunk cankers were indicated as a potential threat to the almond industry in California (Doll *et al*., 2013). A recent publication has also discussed the risk posed by the emergence of fungal trunk pathogens, including Botryosphaeriaceae spp., to the sustainability of tree nut industries worldwide (Gramaje *et al*., 2016).

One of the main courts for infections caused by fungal trunk pathogens and, more specifically, by species of Botryosphaeriaceae, is likely to be through pruning wounds (Serra *et al*., 2008; Úrbez-Torres, 2011; Úrbez-Torres and Gubler, 2011; Phillips *et al*., 2013). On almond trees, Doll *et al*. (2013) indicated that pruning wound and wind-caused cracks within the scaffolds are the preferred areas for infection by Botryosphaeriaceae spp. The cankers then enlarge throughout the growing season, girdling branches and leading frequently to the eventual death of infected trees. It is therefore very important to protect pruning wounds with fungicide applications as one of the primary strategies to control trunk diseases (Rolshausen *et al*., 2010).

There have been no published reports of control of Botryosphaeriaceae spp. associated with canker diseases in almond wood. However, the control of these pathogens with fungicides has been studied, mainly on grapevine (Bester *et al*., 2007; Rolshausen *et al*., 2010; Amponsah *et al*., 2012; Pitt *et al*., 2012), and also on other crops such as apricot and peach (Li *et al*., 1995), avocado (Twizeyimana *et al*., 2013), blueberry (Latorre *et al*., 2013), cork oak (*Quercus suber*) (Luque *et al*., 2008), and *Protea magnifica* (Denman *et al*., 2004). In these studies, experiments conducted both *in vitro* and under field conditions assessed the efficacy of various fungicides to control cankers caused by Botryosphaeriaceae spp., obtaining satisfactory results for some of the fungicides tested such as thiophanate-methyl on apricot and peach (Li *et al*., 1995), azoxystrobin + propiconazole and metconazole on avocado (Twizeyimana *et al*., 2013), benomyl, tebuconazole and iprodione on blueberry (Latorre *et al*., 2013), carbendazim, fluazinam and tebuconazole on grapevines (Pitt *et al*., 2012), carbendazim and thiophanate-methyl on cork oak (Luque *et al*., 2008), or prochloraz alternated with mancozeb on *P. magnifica* (Denman *et al*., 2004).

In Spain, which is the third largest almond producer after California and Australia (FAOSTAT, 2014), the almond industry is concerned about the emergence of branch cankers caused by Botryosphaeriaceae spp. (Gramaje *et al*., 2012; Olmo *et al*., 2016), and demands recommendations for pruning wound protection with fungicide applications that could be incorporated into integrated disease management programmes. The objectives of the present study were to (i) evaluate the *in vitro* efficacy of ten fungicides against four species of Botryosphaeriaceae (*D. seriata*, *N. luteum*, *N. mediterraneum* and *N. parvum*) associated with branch cankers on almond trees: and (ii) determine the efficacy of some of these fungicides applied to pruning wounds of almond, to prevent infections caused by these pathogens.

Materials and methods

Fungal pathogens

One isolate each of *D. seriata* (BAL-10), *N. luteum* (BAL-30), *N. mediterraneum* (BAL-3) and *N. parvum* (BAL-7) were used. These isolates were recovered from branch samples with cankers collected in symptomatic almond orchards in the island of Mallorca (Spain) (Olmo *et al*., 2016). For isolation, necrotic wood fragments from characteristic V-shaped cankers were cut from the affected branches, washed under running tap water, surface-disinfected for 1 min in 1.5% sodium hypochlorite solution, and washed twice with sterile distilled water. Small pieces of lesion margins were plated onto malt extract agar (MEA) (Difco Laboratories), supplemented with 0.5 g L^{-1} streptomycin sulphate (MEAS) (Sigma-Aldrich Laboratories). Plates were incubated for 7–10 d at 25ºC in the dark, and all emerging colonies were transferred to potato dextrose agar (PDA) (Biokar-Diagnostics). The isolates were then hyphal-tip subcultured and identified by morphological and molecular methods as described by Olmo *et al*. (2016). Representative isolates were stored in 15% glycerol solution at -80ºC in 1.5 mL capacity cryovials. Prior to use, a small plug of the colonized agar from each cryovial culture was transferred to PDA, and grown at 25ºC in the dark for 10 d.

Determination of EC_{50} values

Commercial formulations of ten fungicides representing seven chemical groups, were evaluated *in vitro* for mycelial growth inhibition of the four Botryosphaeriaceae species (Table 1). Appropriate volumes of each fungicide were added to molten PDA at about 50ºC to obtain final concentrations of 100, 10, 1 or 0.1 mg active ingredient (a.i.) L^{-1} (ppm). Mycelial plugs (4 mm diam.), obtained from the margins of actively growing cultures on PDA, were transferred to fungicide amended plates (four replicate plates per fungicide concentration). Control PDA plates were prepared without the addition of fungicides. Colonies were incubated at 25ºC in the dark for 5 to 6 d until the control plates were 80–90% covered with mycelium. Colony diameter was measured in each plate across two perpendicular axes, averaged, and the diameter of the mycelial plug was subtracted from the average. The percent inhibition for each isolate was calculated in relation to the colony growth of the control (unamended) plates. The experiment was repeated once.

Protection of pruning wounds

This experiment was conducted in October 2014 on 3-year-old trees of the almond cultivar Ferragnes grafted on the hybrid rootstock G × N (Garnem) C-14. Plants were grown in 6 L capacity plastic pots filled with peat. Inoculum preparation, fungicide applications and pathogen inoculations were conducted following methods adapted from those described by Travadon *et al*. (2013) and Twizeyimana *et al*. (2013).

For each of the four fungi, inoculum was prepared as a suspension of mycelium fragments from potato dextrose broth (PDB; Sigma-Aldrich Laboratories) cultures. Mycelium fragments were used be-

Table 1. Fungicides assessed for *in vitro* activity against four Botryosphaeriaceae pathogens of almond.

Chemical group	Active ingredient	Trade name	Manufacturer	Formulation[a]	Registered concentration in Spain[b]
Copper	Copper oxychloride	Cuprosan	Bayer (Valencia, Spain)	500 g Kg^{-1} WG	3-4 g L^{-1} (almonds)
Triazole	Cyproconazole	Caddy Pepite	Bayer (Valencia, Spain)	100 g Kg^{-1} WG	0.01-0.02 g L^{-1} (fruits)
	Tebuconazole	Folicur	Bayer (Valencia, Spain)	250 g Kg^{-1} WG	0.5-1 g L^{-1} (peaches)
Phthalimide	Captan	Merpan	Aragonesas Agro (Madrid, Spain)	800 g Kg^{-1} WP	1.5-2.5 g L^{-1} (almonds)
	Folpet	Folpec	Sapec Agro (Valencia, Spain)	500 g Kg^{-1} WP	2.5-3 g L^{-1} (almonds)
Dithiocarbamate	Mancozeb	Micene	Sipcam Inagra (Valencia, Spain)	800 g Kg^{-1} WP	2-3 g L^{-1} (almonds)
	Thiram	Deepest	Lainco (Barcelona, Spain)	500 g L^{-1} SC	3.5-5 mL L^{-1} (almonds)
Methoxy-carbamate	Pyraclostrobin	Cabrio	BASF (Barcelona, Spain)	250 g L^{-1} EC	0.3-0.4 mL L^{-1} (grapevines)
Pyridine-carboxamide	Boscalid	Cantus	BASF (Barcelona, Spain)	500 g Kg^{-1} WG	1-1.2 g L^{-1} (grapevines)
Thiophanate	Thiophanate-methyl	Cercobin	Certis (Elche, Spain)	450 g L^{-1} SC	1-1.5 mL L^{-1} (peaches)

[a] WP, wettable powder; WG, water dispersible granule; EC, emulsifiable concentrate; SC, suspension concentrate.
[b] Registered concentrations in Spain were determined following De Liñán-Carral and De Liñán-Vicente (2016).

cause of difficulty to produce spores from some species in culture (Twizeyimana *et al*., 2013). For each isolate, a starter culture was first produced using ten 2 cm diam. agar plugs from a 7-d culture grown on PDA. These were inoculated into a 250 mL capacity Erlenmeyer flask containing 100 mL of PDB and incubated at 25°C on a shaker at 150 rpm for 3 d. These PDB cultures were then homogenized using a blender (Moulinex LM233A) (1 min, speed 5), and 2 mL of the homogenized starter cultures were inoculated into a 125 mL capacity Erlenmeyer flask containing 40 mL of PDB. After 3 d incubation at 25°C and 150 rpm, the entire liquid cultures were homogenized and adjusted with sterile water to 3×10^4 mycelial fragments mL^{-1}. Mycelial fragments were primarily less than 0.5 mm in length.

Five fungicides, selected after evaluation of the the *in vitro* experiments, were applied at label rates indicated for almonds or for similar pathogens in other crops. The rates were: boscalid at 1.2 g a.i. L^{-1}), mancozeb at 3 g a.i. L^{-1}, thiophanate-methyl at 1.5 mL a.i. L^{-1}, pyraclostrobin at 0.4 mL a.i. L^{-1}, and tebuconazole at 0.75 g a.i. L^{-1}.

In each tree, the main stem (of approx. 2 ± 0.5 cm circumference) was pruned at 50–60 cm distance from the grafting point and the fungicides were immediately sprayed onto the wound to runoff, using a handgun sprayer (Solo). The treated or control (no fungicide application) wounds were then each inoculated with a mycelial suspension of each Botryosphaeriaceae spp. at two different times, either 1 or 7 d after fungicide application. Pruning wounds were inoculated by pipetting 100 µL of inoculum onto the surface of each wound, and then sealing the inoculation site with Parafilm (Bemis Company Inc.). Non-inoculated controls were each treated with 100 µL of non-colonized PDB.

The pots were arranged in a randomized complete block design with three blocks in a temperature controlled greenhouse (20 ± 2ºC). Fungicides, fungal pathogens, and inoculation times (1 or 7 d after fungicide application) were all factors in the experiment, each combination represented by one plant per block. Additionally, inoculated controls without fungicide treatment (one per block) were also included for each fungal pathogen. The experiment was repeated once.

Five months after pruning and fungicide applications, the main plant stems were cut off for disease assessment and fungal isolations. The stems were sectioned longitudinally from the site of inoculation and necrotic lesion lengths were measured. The stems were then surface disinfected for 1 min in 1.5% sodium hypochlorite solution, and washed twice with sterile distilled water. Isolations from each stem were conducted by plating seven small pieces of necrotic tissue from the edge of each lesion (or just below the area of inoculation if no lesion was visible) onto MEAS. Plates were incubated at 25ºC for 7–10 d in the dark. Fungi in these cultures were identified by morphological and molecular methods as described by Olmo *et al*. (2016).

Statistical analyses

In vitro mycelium growth inhibition (%) for all tested fungicides was converted to probits and plotted against $\log_{10}$ values of the different concentrations evaluated. Probit regression analysis was used to calculate the effective concentration to reduce mycelial growth by 50% (EC_{50} value). Analysis of variance (ANOVA) was performed using Statistix 10.0 (Analytical Software). The effects of fungicide, Botryosphaeriaceae spp., and experiment (performed twice), together with the two-way interactions, were analyzed. Mean EC_{50}values were compared using the Fischer´s least significant difference (LSD) at P=0.05.

In the pruning wound protection experiment, lesion lengths and isolation percentages were analyzed by ANOVA. Isolation percentages were arcsine square root transformed prior to analysis. The effects of fungicide, Botryosphaeriaceae spp., experiment (performed twice), and time, together with their interactions, were analyzed. Mean values were compared using the Fischer´s LSD at P=0.05.

Results

Fungicide EC_{50s}

In the *in vitro* experiment, the effects of fungicide, Botryosphaeriaceae spp., and their interactions were all statistically significant (P<0.05). Mean EC_{50} values for the fungicides are presented in Table 2. In general, the most effective fungicides for inhibition of mycelium growth for all four fungus species were tebuconazole (mean EC_{50}s from 0.09 to 0.25 mg L^{-1}), and pyraclostrobin (mean EC_{50}s from 0.05 to 0.99 mg L^{-1}). Cyproconazole was also effective for reducing mycelium growth of all four fungi, with mean EC_{50}s between

0.27 and 1.89 mg L^{-1}, except for *N. parvum* (mean EC_{50} = 4.32 mg L^{-1}. Thiophanate-methyl, reduced mycelium growth with mean EC_{50}s between 0.65 and 0.77 mg of L^{-1}, except for *N. luteum* (mean EC_{50} = 8.33 mg L^{-1}). Boscalid, captan, folpet, mancozeb, copper oxychloride and thiram all gave greater mean EC_{50}s (Table 2).

Among the four fungi, the results were variable. The most effective fungicides for *D. seriata* were pyraclostrobin (mean EC_{50} = 0.09 mg L^{-1}), tebuconazole (0.15 mg L^{-1}), thiophanate-methyl (0.67 L^{-1}) and cyproconazole (1.87 mg L^{-1}), without significant differences with mancozeb (mean EC_{50} = 7.05 mg L^{-1}).

Regarding *N. luteum*, the most effective fungicides were pyraclostrobin (mean EC_{50} = 0.05 mg L^{-1}), tebuconazole (0.09 mg L^{-1}), cyproconazole (0.27 mg L^{-1}), boscalid (0.34 mg L^{-1}), thiophanate-methyl (8.33 mg EC_{50} L^{-1}) and thiram (13.81 mg L^{-1}).

The most effective fungicides against *N. mediterraneum* were tebuconazole (mean EC_{50} = 0.21 mg L^{-1}), thiophanate-methyl (0.65 mg L^{-1}), cyproconazole (0.79 mg L^{-1}) and pyraclostrobin (0.99 mg L^{-1}).

Regarding *N. parvum*, pyraclostrobin, tebuconazole and thiophanate-methyl, were the most effective fungicides for inhibiting mycelium growth, showing mean EC_{50} values less than 1.0 mg L^{-1}, but these values were not significantly different from those for with captan (mean EC_{50} = 3.83 mg L^{-1}), cyproconazole (4.32 mg L^{-1}), and boscalid (6.86 mg L^{-1}).

Protection of pruning wounds

Statistical analysis of lesion lengths showed no significant differences among the four fungi, between inoculation times, or the interactions of these factors. However, significant differences (P<0.0001) were found among the fungicides (Table 3). Only the mean lesion lengths for the different fungicides tested are shown and compared in Figure 1. All the fungicides reduced the canker lesions compared with those resulting from the non-treated pruning wounds. The pruning wounds treated with thiophanate-methyl had the shortest lesion lengths (mean lesion length = 0.91 cm), although with no significant differences when compared with wounds treated with mancozeb (3.48 cm), boscalid (3.33 cm) or tebuconazole (2.70 cm). Pruning wounds treated with pyraclostrobin (4.85 cm) had the longest lesions among fungicide treatments, but with no significant differences from those treated with mancozeb, boscalid or tebuconazole.

Table 2. Mean EC_{50} (mg L^{-1}) s for *in vitro* inhibition of mycelium growth of *Diplodia seriata, Neofusicoccum luteum*, *N. mediterraneum* and *N. parvum* on potato dextrose agar amended with fungicides.

Fungicide	*D. seriata*	*N. luteum*	*N. mediterraneum*	*N. parvum*	L.S.D.(1)[2]
Boscalid	40.04 B c[1]	0.34 A a	>100 C d	6.86 A ab	21.12
Captan	21.04 A b	64.25 B c	45.66 B b	3.83 A ab	19.92
Cyproconazole	1.89 B a	0.27 A a	0.79 AB a	4.32 C ab	1.53
Folpet	44.43 A c	66.96 B c	81.38 B c	40.91 A d	19.31
Mancozeb	7.05 A ab	37.16 B b	80.97 C c	22.88 AB c	23.37
Thiophanate-methyl	0.67 A a	8.33 B a	0.65 A a	0.77 A a	1.80
Copper oxychloride	78.03 AB d	92.72 B d	82.38 AB c	65.38 A e	20.79
Pyraclostrobin	0.09 A a	0.05 A a	0.99 B a	0.10 A a	0.26
Tebuconazole	0.15 AB a	0.09 A a	0.21 BC a	0.25 C a	0.09
Thiram	25.63 A bc	13.81 A a	56.65 B b	15.76 A bc	21.18
L.S.D. (2)[3]	18.96	15.86	15.72	12.05	

[1] Means followed by the same letter do not differ significantly (P<0.05), as indicated by least significant difference (LSD). Capital letters are for comparison of means in the same row. Small letters are for comparison of means in the same column.
[2] $LSD_{0.05}$(1) is for comparison of means among pathogens for each fungicide.
[3] $LSD_{0.05}$(2) is for comparison of means among fungicides against each pathogen.

Table 3. Analysis of variance for effects of experiment, inoculation time, fungicide or pathogen, on lesion length and isolation percentage, resulting from inoculations with *Diplodia seriata*, *Neofusicoccum luteum*, *N. mediterraneum* or *N. parvum* on pruning wounds of almond trees cv. Ferragnes 5 months after pruning and fungicide application.

Experiment variable	Lesion length (cm)				Isolation percentage			
	df[1]	MS[2]	F	$P<F$[3]	df[1]	MS[2]	F	$P<F$[3]
Experiment	1	67.183	1.66	0.1990	1	0.25341	1.17	0.2810
Moment of inoculation	1	4.475	0.11	0.7398	1	0.78282	3.61	0.0589
Fungicide	5	531.689	13.14	<0.0001	5	1.83614	8.46	<0.0001
Pathogen	3	82.420	2.04	0.1098	3	0.18599	0.86	0.4641
Fungicide × pathogen	15	52.629	1.30	0.2038	15	0.30097	1.39	0.1556
Experiment × fungicide × pathogen	15	22.114	0.55	0.9116	15	0.12695	0.59	0.8845

[1] Degrees of freedom.
[2] Mean squares.
[3] Probabilities associated with individual F tests.

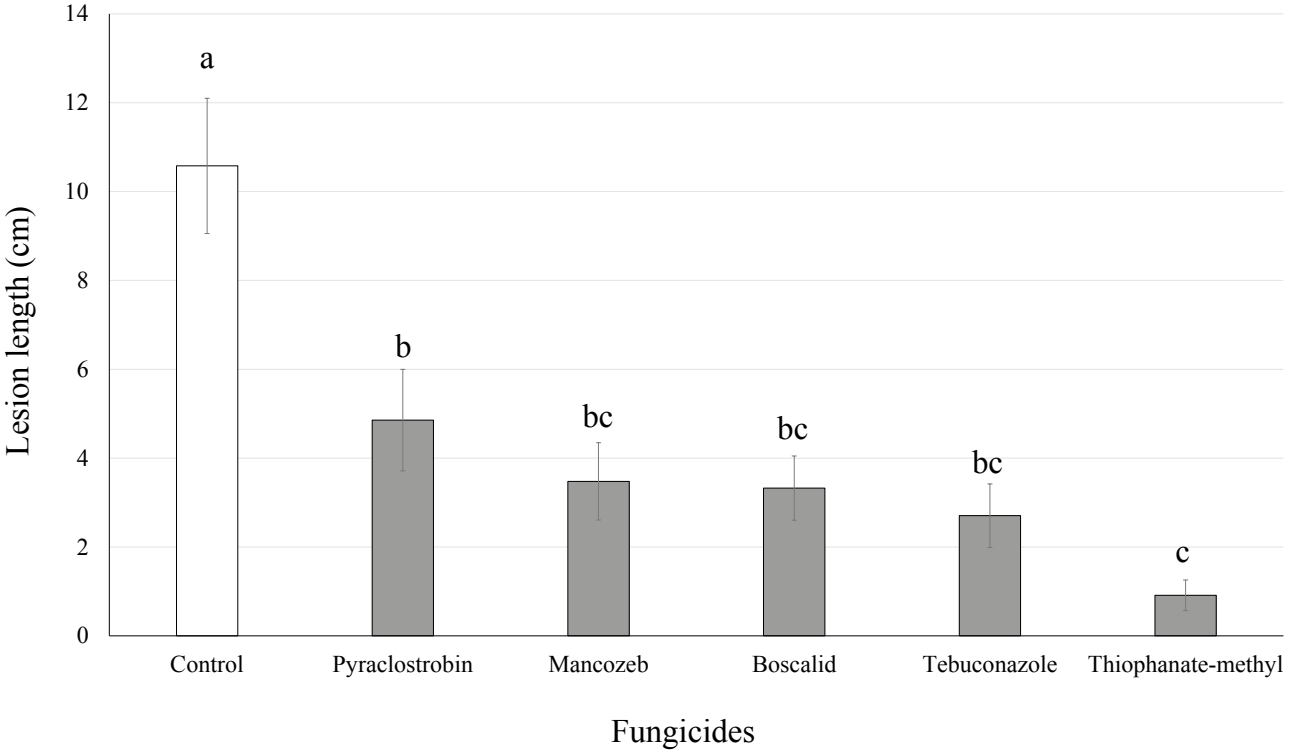

Figure 1. Mean lesion lengths (cm) caused by four Botryosphaeriaceae spp. on pruning wounds of almonds cv. Ferragnes, 5 months after pruning and fungicide application. Mean lesion lengths are based on 48 replicates per fungicide (three replicates per each of the four fungi and two inoculation times, and the experiment was conducted twice). Means followed by the same letter are not significantly different ($P > 0.05$). Bars represent the standard errors of the means.

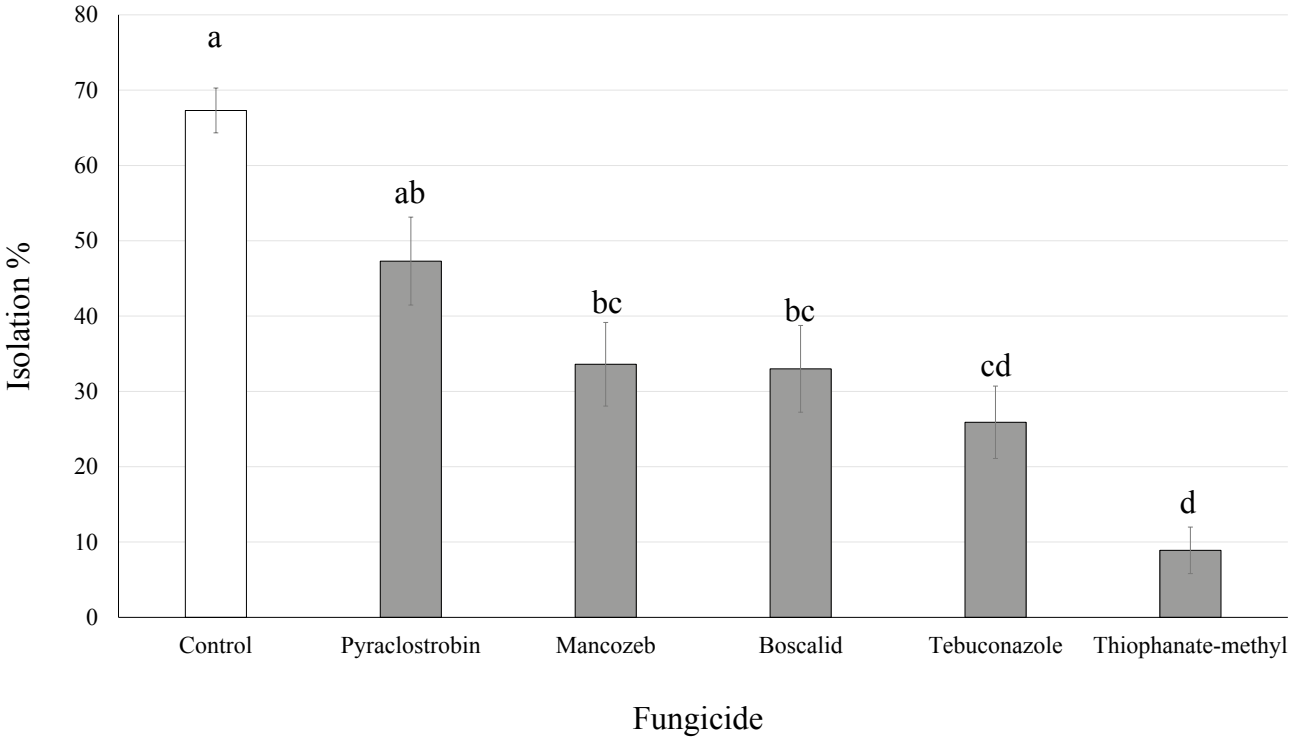

Figure 2. Mean isolation percentages of four Botryosphaeriaceae spp. on pruning wounds of almonds cv. Ferragnes, 5 months after pruning and fungicide application. Mean lesion lengths are based on 48 replicates per fungicide (three replicates for each of the four fungi and two inoculation times, and the experiment was conducted twice). Means followed by the same letter are not significantly different ($P > 0.05$). Bars represent the standard errors of the means.

Significant differences in isolation percentages were detected only among the fungicides ($P<0.0001$), but not among the four fungi, inoculation time (Table 3) or interactions between these factors. Only mean isolation percentages for the different fungicides tested are shown and compared in Figure 2. The greatest isolation percentage corresponded to the non-treated pruning wounds (mean = 67.3%), while the least was obtained for thiophanate-methyl (8.9%). Pruning wounds treated with mancozeb gave a mean isolation percentage of 25.9%, not significantly different from tebuconazole (33.6) or boscalid (33.0%). Pruning wounds treated with pyraclostrobin (47.3%) had the greatest isolation percentage among fungicides, showing no significant differences from the nontreated controls, or those treated with tebuconazole or boscalid.

Discussion

Species of Botryosphaeriaceae play major roles as causal agents of almond cankers (Slippers *et al*., 2007; Inderbitzin *et al*., 2010; Gramaje *et al*., 2012; Chen *et al*., 2013; Doll *et al*., 2013 and 2015; Olmo *et al*., 2016). The present study is the first report on the potential for fungicide control of the Botryosphaeriaceae species *D. seriata*, *N. luteum*, *N. mediterraneum* and *N. parvum*, which have been associated with this disease.

The *in vitro* screening experiments showed that tebuconazole and pyraclostrobin were the most effective fungicides for inhibition of mycelium growth of the four fungi studied. The results obtained with tebuconazole agree with the fungicide evaluations made by Denman *et al*. (2004) for the control of *N. protearum* on *P. magnifica* in South Africa, Latorre *et al*. (2013) for the control of *N. parvum* on blueberry, and with those of Bester *et al*. (2007) in South Africa, Amponsah *et al*. (2012) in New Zealand and Pitt *et al*. (2012) in Australia, for the control of different Botryosphaeriaceae species associated with Botryosphaeria dieback of grapevines. Our results with pyraclostrobin matched those obtained by Pitt *et al*. (2012). These authors, in an *in vitro* trial of 17 fun-

gicides to control four Botryosphaeriaceae spp. (including *D. seriata* and *N. parvum*) from grapevines, found that this fungicide was one of the most effective for reduction of mycelium growth. Twizeyimana *et al*. (2013) evaluated the effects of 12 fungicides on mycelium growth of several Botryosphaeriaceae spp. from avocado, including *N. luteum* and *N. parvum*, and concluded that pyraclostrobin was among those which were more effective.

In the present study, thiophanate-methyl and boscalid were also effective for inhibition of mycelium growth of most of the four fungi from almond. Thiophanate-methyl reduced mycelium growth of the four fungi evaluated, except for *N. luteum*. Twizeyimana *et al*. (2013) reported that this fungicide was ineffective for inhibiting mycelium growth of Botryosphaeriaceae spp. from avocado, so this chemical was not used in subsequent studies for wound protection in the field. However, other *in vitro* studies performed on apricot and peach trees (Li *et al*., 1995), cork oak (Luque *et al*., 2008) and grapevine (Amponsah *et al*., 2012), indicated that thiophanate-methyl was one of the most effective fungicides for inhibition of mycelium growth of Botryosphaeriaceae spp. Boscalid was one of the most effective fungicides against *N. luteum* in the present study, however it was not effective against *D. seriata* and *N. mediterraneum*, which agrees with the *in vitro* results reported by Bester *et al*. (2007) and Pitt *et al*. (2012) with Botryosphaeriaceae spp. isolated from grapevines. Cyproconazole was very effective for reducing mycelium growth of the fungi tested here except *N. parvum*, but it was not included in the pruning wounds protection experiment because tebuconazole, another triazole showing better results *in vitro*, was selected.

Botryosphaeriaceae spp. associated with almond cankers infect through wounds mostly caused by pruning activities (Doll *et al*., 2013). Thus, the use of fungicides, together with good cultural practices recommended on other woody crops, such as pruning out dead limbs and twigs, removal of pruning waste from affected orchards, and minimizing stresses, are all important for disease management (Bester *et al*., 2007; Rolshausen *et al*., 2010; Twizeyimana *et al*., 2013). The fungicides boscalid, mancozeb, pyraclostrobin, tebuconazole and thiophanate-methyl were selected for the wound protection experiment. Of the products tested, mancozeb and thiophanate-methyl are currently registered in Spain for control of other almond pathogens (MAGRAMA, 2016). Pesticide producers usually prefer to obtain label extensions for their products, which are more rapidly and less expensively obtained than full registrations of new fungicides for use in almond.

Our results for lesion size and pathogen isolation percentages, showed no significant differences among the four Botryosphaeriaceae spp. inoculated, and also between the two inoculation times evaluated. These results agree with those obtained by Twizeyimana *et al*. (2013) for pruning wound protection against Botryospheriaceae spp. on avocado. Their study indicated that the fungicide treatments remain effective until at least 7 d after application.

Among the fungicides selected for pruning wound protection, thiophanate-methyl gave the best results. With this treatment the pruning wound lesions were smallest and the pathogen isolation percentages were the least. This is similar to results of fungicide evaluations for the protection of pruning wounds against Botryosphaeriaceae spp. in field conditions conducted by Li *et al*. (1995) on apricot and peach, Luque *et al*. (2008) on cork oak, and Rolshausen *et al*. (2010) and Amponsah *et al*. (2012) on grapevines. The fungicide mancozeb, which was ineffective *in vitro*, gave good results regarding the isolation percentages. This fungicide was also one of the most effective treatments for control of Botryosphaeriaceae spp. in studies performed under field conditions on grapevines (Denman *et al*., 2004; Amponsah *et al*., 2012). The results of pruning wound protection with boscalid were similar to those obtained with mancozeb. Bester *et al*. (2007) and Pitt *et al*. (2012) did not include this fungicide in vineyard experiments because it had been ineffective *in vitro*.

In our study, tebuconazole, which was a very effective fungicide *in vitro*, provided better results in terms of lesion length than for isolation percentage. In studies conducted under field conditions by Bester *et al*. (2007), Amponsah *et al*. (2012) and Pitt *et al*. (2012), this fungicide was one of the most effective for control of Botryosphaeriaceae spp. on grapevines. However, in the study of Denman *et al*. (2004) on *P. magnifica*, it was not effective, although it showed good results *in vitro*.

Pyraclostrobin was the least effective fungicide for pruning wounds protection, which agrees with the results of Latorre *et al*. (2013) on blueberry and Twizeyimana *et al*. (2013) on avocado. However, Rolshausen *et al*. (2010) found that pyraclostrobin

was effective when applied to pruning wounds to control Botryosphaeriaceae spp. on grapevines.

Variable results when comparing our *in vitro* and wound protection trials with other published results with Botryosphaeriaceae spp. on other crops may have been due to virulence diversity of these species or variation in active ingredient efficacy (Elena *et al.*, 2015; Baskarathevan *et al.*, 2017). Taking into account all the results mentioned above, we consider thiophanate-methyl, as one of the potentially most interesting fungicides to control pathogens belonging to the family Botryosphaeriaceae on almond trees. This chemical was very effective for inhibiting *in vitro* growth of the fungal pathogens assessed, and for protecting pruning wounds. After the completion of this study, a commercial formulation of thiophanate-methyl has been authorized in Spain for use against almond diseases (MAGRAMA, 2016). This fungicide is therefore a good candidate for inclusion in integrated disease management programmes which aim to protect pruning wounds from Botryosphaeriaceae spp. infection.

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