Efficacy of fungicides against brown spot of pear in Argentina

Marisa Andrea Aluminé Tudelaa,b\*, María Cecilia Lutza,c, Gustavo Nestor Giménezd, Susana Noemí Di Masib,Graciela Noemí Posea,e,f, Dolores Del Bríoa,b,d , Molina, Juan Pablo Edwardsg

aConsejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

bInstituto Nacional de Tecnología Agropecuaria (INTA). Estación Experimental Agropecuaria Alto Valle (EEA).

cLaboratorio de Fitopatología, Instituto de Biotecnología Agropecuaria del Comahue (IBAC), Facultad de Ciencias Agrarias, Universidad Nacional del Comahue.

dDepartamento de Estadística, Universidad Nacional del Comahue (UNCo). Facultad de Economía y Administración.

eDepartamento de Ciencia y Tecnología, Universidad Nacional de Quilmes (UNQ).

fLaboratorio de Micología y Cultivo de Hongos Comestibles y Medicinales – INTECH, CONICET-UNSAM.

gEstación Experimental Agropecuaria - INTA, Balcarce, Argentina.

\*Corresponding autor [tudela.alumine@inta.gob.ar](mailto:tudela.alumine@inta.gob.ar) – ORCID: 0000-0001-8298-8064

Abstract

Brown spot of pear (BSP), a fungal disease of importance in Europe, has been recently detected for the first time in pear orchards in the Alto Valle of Río Negro (Patagonia), Argentina, South America. The disease is caused by *Stemphylium vesicarium* and its main symptoms are lesions in fruits and leaves. To assess counteracting measures against BSP, the effects of four fungicides were tested to evaluate *in vitro* efficacy against mycelial growth and spore germination of native *S. vesicarium* strains, and preventive and curative control of lesion development in pear fruit 'D'Anjou' var. In addition, the activities of two selected fungicides were determined through in-field assays. The fungicides tested were chosen according to their commercial availability and registration in pear crops and included: pyraclostrobin + boscalid (Bellis®); ziram (Ziram®); captan (Merpan®); and *Melaleuca alternifolia* extract (Timorex®). Bellis® presented the lowest EC50 values in germination and mycelial growth tests. Ziram® and Merpan® were also very effective in inhibiting germination. The plant-based biofungicide Timorex® did not achieve satisfactory effectiveness in *in vitro* trials, nor in bioassays after preventive and curative treatments. Bioassay results showed that preventive measures using Bellis®, Ziram®, or Merpan® were effective in reducing disease severity and suggested that BSP could be controlled by adequate selection of treatment time.

Keywords: Brown spot of pear; *Stemphylium vesicarium*; *In vitro* efficacy; Chemical control.

Declarations

Funding: This work was supported by Instituto Nacional de Tecnología Agropecuaria (INTA) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

Conflicts of interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical statement: All authors are fully aware of this submission and have declared that they have no competing interests. The results presented in this manuscript did not involve any protected and/or endangered species, field studies, human participants, specimens or tissue samples or vertebrate animals, embryos or tissues.

1. Introduction

"Brown spot of pear" (BSP), caused by *Stemphylium vesicarium* (Wallr). E.G. Simmons (teleomorph: *Pleospora herbarum* [Pers.] Rabenh, sin. *P. allii* [Rabenh.] Ces. & De Not.), is a very important disease in some areas of Europe. Several strategies are used to control BSP based on preventive sprays with fungicides. Recommended substances include dithiocarbamates (e.g. thiram and mancozeb), strobilurins (e.g. kresoxim-methyl, trifloxystrobin, and pyraclostrobin), and other products like captan and tebuconazole (Brunelli et al. 1984, 1986, 1997; Vilardell 1988; Ponti et al.1993, 1996; Llorente 1997; Llorente & Montesinos, 2006; Llorente et al., 2012). Traditionally, fungicide applications are carried out on a fixed schedule, with preventive spraying every 7 to 14 days from petal fall to harvest (Ponti et al. 1993; Ponti et al. 1996; Llorente, 1997; Llorente & Montesinos, 2006). This strategy involves however numerous applications and a large increase in production costs. Starting about a decade ago, the number of applications has been reduced due to the implementation of the BSPcast forecasting system. The latter proved similarly efficient compared to fixed-calendar applications, but led to 30-40% reduction in the amount of fungicide employed (Llorente et al., 2000).

In 2013, BSP was detected in the Valle Medio of Río Negro region on leaves and fruits of 'D’Anjou' pear trees (Dobra & Garcia, 2015). During the 2016/2017 fruit season, BSP was also detected in the Alto Valle of Río Negro and Neuquén region (Temperini et al., 2022), and was recurrent in the 2018/2019, 2020/2021, and 2021/2022 seasons (Tudela & Di Masi, 2022) with different levels of damage. Up to date, the infection has been diagnosed in the region in 'D'Anjou', 'Abate Fetel', and occasionally in 'Williams' and 'Packham's' pear cultivars. In 'D'Anjou' and 'Abate Fetel' BSP symptoms were detected on leaves and fruits, while in 'Williams' and 'Packham's' only fruit was affected (Tudela & Di Masi, 2022). Symptoms on fruit appeared as rounded, dark brown spots of a hard consistency, slightly depressed and of variable size, and occasionally surrounded by a reddish halo. Symptoms on leaves consisted of necrotic spots, rounded at first and growing afterwards, generally into a "V" shape.

No fungicide has been yet registered for BSP control in pear crop in Argentina. According to Argentina’s National Food Safety and Quality Service (Senasa, 2022), four types of fungicides have been approved to be used on pear trees: i) products with multisite activity (M), namely cupric sulfate pentahydrate, tribasic copper sulfate, mancozeb, thiram, ziram, captan, and folpet; ii) respiration inhibitors (C), e.g. boscalid and pyraclostrobin; iii) fludioxonil, an osmoregulation disruptor (E); and iv) pyrimethanil, which interferes with protein production and cell division by inhibiting methionine synthesis (D).

Given the recurrence of the disease in recent years in the Argentine Norpatagonia and the lack of prior local research about its control, the objective of this study was to evaluate through *in vitro* and in-field trials, the efficacy of four commercially available fungicides against S. vesicarium native strains. Assays included: i) *in vitro* evaluation of inhibitory activity on mycelial growth and conidial germination of *S. vesicarium* native strains; ii) evaluation of the effectiveness of preventive and curative treatments on detached fruits; and, iii) assessment of fungicides effectiveness against BSP in the field.

1. Materials and methods
   1. Pathogen

From the laboratory of Phytopathology’s ceparium at the Experimental Agricultural Alto Valle Station (EEA, by its Spanish Initials), three *S. vesicarium* strains identified as SF23, SF20, and SF8, were chosen for the fungicide sensitivity test. Molecular, morphological and pathogenic characteristics were described previously for each selected strain (Temperini et al., 2022). Single-spore cultures were grown on potato dextrose agar (PDA, Merck, 39 g in 1 L of distilled water) in Petri dishes and maintained at 25ºC for 7 days. The strains were also cultivated in media water- agar media (agar, Britania Lab., 20 g L-1) in Petri dishes incubated for 20 days at 20-25°C under a 12-h photoperiod of fluorescent ligth (Montesinos et al., 1995). Afterwards, the dishes were taken to a laminar flow chamber; covered with 9 mL of sterile distilled water and 50 µl of Tween 20, and gently rubbed with a sterile plastic loop. The suspension was then filtered through gauze and adjusted to a concentration of 5 x 104 conidia mL-1 using a Neubauer’s chamber.

* 1. Fungicides

Three single and one mixture fungicides (Galvez Patón, 2016; Gur et al., 2020) were used (Table 1). The single active ingredient fungicides were: i) Ziram® 76 WG (Agristar Argentina), which contains ziram, a non-penetrating protectant fungicide (FRAC Code M03); ii) Merpan® 83 WP (Adama Argentina), which contains captan, a non-penetrating protectant fungicide (FRAC Code M04); and ii) Timorex Gold® 22.25 EC (Syngenta Argentina), a preventive and curative fungicide of natural origin (FRAC code 46) which contains Melaleuca alternifolia extract at 22.25 % (W/V). The pre-packed fungicide mixture was Bellis® WG (Basf Argentina), a systemic, preventive, and curative fungicide containing pyraclostrobin (FRAC Code 11) at 12.8% (W/W) and boscalid (FRAC Code 7) at 25.2% (W/W).

* 1. Inhibition of mycelial growth of *S. vesicarium* in vitro

Five-mm PDA disks of each *S. vesicarium* strain were taken from the edge of freshly growing colonies and placed on PDA amended with different concentrations of each fungicide (Table 1) in 9-cm Petri dishes (one disc per dish) (Alberoni et al., 2005). Petri dishes containing PDA and sterilized water were used as controls. The dishes were incubated in a completely randomized design at 25ºC in the dark for 7 days and colony diameters (mm) were recorded. Three replicates (one Petri dish per replicate) were used per combination of fungicide and concentration, and the trial was carried out three times.

* 1. Inhibition of spore germination of *S. vesicarium* in vitro

Fresh conidial suspensions of the three *S. vesicarium* strains were placed in 9 mL tubes and mixed with an aqueous suspension of each fungicide to achieve the desired final concentrations, reaching a uniform concentration of 1 x 105 conidia mL-1 in a final volume of 4 mL. The fungicides and their concentrations are shown in Table 1. Conidial suspension mixed with water served as a control treatment. Three replicates (one tube per replicate) were used for each fungicide concentration, and the trial was carried out twice. The tubes were incubated at 25ºC, and the germination percentage of the control treatment was checked every hour by taking aliquots and counting 100 conidia. Once the control reached 100% of germination, germinating conidia were counted for each treatment under an optical microscope and the percentage of inhibition was calculated.

* 1. Bioassays in fruits

**Preventive treatments**. The fungicides were applied at recommended doses on 'D’Anjou' trees spaced at 4x4 m, applying 3.2 L per tree (2000 L/ha). They were located at the EEA of INTA, Río Negro, Argentina (39°01'45.1"S 67°44'33.5"W), with cold dry desert climate (Köppen: Bwk). Applications were carried out in a completely randomized design with a motorized sprayer (Still-SR 420), and included four trees per treatment. Twenty fruits per treatment (five per tree) were collected on days 1, 7, and 15 post-application, transferred to the laboratory, and inoculated. The inoculation was done by spraying single fruits with 3 mL of SF23 strain spore suspension (previously identified as highly pathogenic by Temperini et al., 2022) adjusted to 5 x 104 conidia mL-1 (adapted from Marchi et al., 1995) with a manual atomizer. Twenty untreated fruits were also collected, and the conidial suspension was similarly applied as control treatment.

**Curative treatments**. One hundred fruits were randomly selected from five untreated trees in good nutritional and sanitary conditions from the same orchard. Fruits were taken to the laboratory and inoculated by spraying 3 mL of SF23 strain spore suspension per fruit, adjusted to 5 x 104 conidia mL-1, with a manual atomizer. After 24 h, the curative treatments were performed by spraying test fungicides with a manual atomizer. Fruit used as control did not receive any fungicidal treatment.

In both preventive and curative treatments twenty fruits were used per treatment and time, placed inside plastic containers (five fruits per container) with individual support for each fruit. To maintain humidity conditions, the bottom of each container was covered with paper towel soaked in 10 mL of distilled sterile water. The fruits were incubated for 7 days at 25°C., after which the percentage of affected fruit (disease incidence) and the number of spots that developed on each fruit (disease severity) were determined. The assays were carry out twice.

* 1. In-field assays

Subsequently, and based on their performance in vitro, the efficacy of Bellis®, a penetrating fungicide, and Ziram®, a non-penetrating fungicide, against BSP was evaluated through in-field assays with products applied at their recommended doses of use (Table 1). Treatments were applied to 'D’Anjou' trees spaced 4x4 m, in an orchard with previous reports of BSP symptoms located at 39°04'04.5"S, 67°36'15.1"W, applying 2000 L/ha with a sprayer (Arbus 2000 TF). Trees treated with water served as controls. Foliar sprays of each treatment were applied before rain according to the forecast of the national meteorological service. Applications were carried out in a completely randomized design, with ten replicates (one tree) per treatment, on two seasons, 2018/2019 and 2019/2020. For both seasons, BSP incidence was determined 15 days after predisposing conditions by examining 250 leaves and fruits per replicate.

* 1. Data analysis

Data were analyzed with R software version 4.2.3 (R Core Team, 2022), using the emmeans (Lenth et al., 2020), ggplot2 (Wickham, 2016), and nlme (Pinheiro et al., 2021), lme4 (Bates et al., 2015), DHARMa (Harting, 2022) and glmmTMB (Brooks et al., 2017) packages.

Effective doses of the different fungicides for 50% inhibition of germination and mycelial growth (based on colony growth at 7 days of incubation) were determined using drc (Ritz & Streibig, 2005) and ec50estimator (Alves, 2020) and medrc (Gerhard & Ritz, 2023) packages according to Kunova et al., 2014; Stewart et al., 2014; Saville et al., 2015; Wang et al., 2017; Noel et al., 2018; and Koppel, 2020. A nonlinear log-logistic model with three-parameters (LL.3) was fitted to generate dose-response curves for each isolate–fungicide combination. The relative potencies (EC50 values) for the tested strains and repetitions were compared between fitted dose-response curves using the EDcomp function of drc package.

For preventive treatments on detached fruits, a completely randomized design with factorial structure was used, including fungicide and time of treatment effects. In the severity analysis, a generalized linear mixed model was fitted using the number of spots per fruit was used as the response variable, fugicide and day as predictive variables, while repetition and replicate were used as random effects. The goodness of fit was evaluated through residuals test and analysis of variance (ANOVA) was carried out with *p* < 0.05 indicating significance. Tukey’s multiple means comparison test, slicing by time of treatment, was performed.

In curative treatments on detached fruits, a completely randomized design was used. A linear mixed model was fitted using the number of spots per fruit was used as the response variable, fungicide as the predictive variable and repetition and replicate as random effects. The goodness of fit was evaluated through residuals test. ANOVA was carried out with *p* < 0.05 indicating significance. The Tukey’s method was used for means comparisons.

In the incidence analysis of curative and preventive treatments, the proportion of affected fruits per fungicide was analyzed using generalized linear mixed-effects models with binomial distribution. The goodness of fit was evaluated through residuals test, and the Tukey’s method was used for means comparisons.

In field experiments, a generalized linear mixed-effects model was fitted, with the proportion of affected leaves as the response variable, fungicide and season as predictive variables and tree as random effect. The goodness of fit was evaluated through residuals test and the Tukey’s method was used for means comparisons.

1. Results
   1. Inhibition of mycelial growth of *S. vesicarium* in vitro

After 7 days of culture, the average colony diameter of the three fungal strains in control treatment was 57.56 ± 2.01 mm. All the fungicides studied showed a clear increase in effectiveness at progressively higher concentrations. Bellis® was the most effective fungicide against mycelial growth, showing EC50 values of 0.02 µg mL-1 for SF8 and 0.03 µg mL-1 for SF20 and SF23 strains. Ziram® and Merpan® were less effective than Bellis®, showing for the three strains mean EC50 values of 7.67 and 30.54 µg mL-1., respectively. Timorex® showed in turn the lowest inhibitory effect on mycelial growth, with a mean EC50 value of 143.22 µg mL-1 (Table 2).

Curve fitting analysis using the EDcomp function indicated no significant differences between EC50 values for each fungicide on individual *S. vesicarium* strains. Therefore, single dose-response curves per fungicide were plotted for all three strains (Fig. 1). The EC50 values among fungicides were statistically different according to the estimated ratios of effect doses, with *p* < 0.0001.

* 1. Inhibition of spore germination of *S. vesicarium* in vitro

Conidial germination reached 100% in the control treatment. For all three fungal strains, Bellis® exerted also the highest effect on conidial germination, with a mean EC50 of 0.017 µg mL-1. Ziram® and Merpan® were also effective, with mean EC50 values of 0.34 and 1.14 µg mL-1, respectively. Timorex® was the least effective product against conidial germination, with a mean EC50 value of 293.79 µg mL-1 (Table 2).

Curve fitting analysis using the EDcomp function indicated no significant differences between EC50 values for each fungicide among individual fungal strains. Therefore, single dose-response curves per fungicide were plotted for all three strains (Fig. 2). In turn, significant differences were observed among the EC50 values of each fungicide for the fungal strains combined according to the estimated ratios of effect doses (*p* < 0.0001).

* 1. Bioassays in fruits
     1. Preventive treatments

After storage for 7 days at 25 °C, fruits in the control group showed a 100% incidence of BSP, with an average of 76 spots per fruit. Fruits inoculated 24 h after Bellis® treatment (day 1) showed a 12.5% disease incidence and a mean of 0.13 ± 0.33 spots per fruit, while effectiveness declined in those inoculated 7 and 15 days later (disease incidences of 67.5 and 85%, and disease severities of 2.75 ± 3.16 and 4.23 ± 3.42spots per fruit, respectively).

Fruit inoculated on days 1 and 7 after Merpan® treatment showed a 45% and 72.5% disease incidence, respectively, and a disease severity <5 spots per fruit. Fruits inoculated on day 15 after Merpan® treatment showed a disease incidence of 80% and disease severity >5 spots per fruit, respectively.

Fruit inoculated on days 1 and 7 after application of Ziram® showed 60% and 57.5% of disease incidence, and disease severities of 1.13 ± 1.24 and 1.48 ± 2.06 spots per fruit, respectively. These values were different from those recorded for fruit inoculated on day 15, where 100% of fruit was affected with a mean of 6.18 ± 4.53 spots per fruit.

Similar to control fruit, all the fruits treated with Timorex® presented more than 5 spots at all inoculation times. Fruit inoculated on day 1 after Timorex® treatment showed a disease severity of 19.5 ± 9.39 spots per fruit, while fruit inoculated on days 7 and 15 after Timorex® treatment showed a disease severity of 54.30 ± 16.25 and 57.58 ± 16.67 spots per fruit, respectively.

The model fitted for de incidence of preventive treatments presented a good fit (algo mas para agregar?). According to the means test slicing by fungicide, the incidence on fruits treated with Bellis® and Merpan® was lower in those inoculated on day 1. In the case of Ziram®, the incidence was lower in fruits inoculated on days 1 and 7, while in Timorex® there were no differences between the proportions of affected fruits in the inoculation times.

According to the residuals analysis, the severity model presented a good fit (algo más). There were significant differences in the number of spots per fruit between the fungicides applied on different days (*p* < 0.0001 on ANOVA).

All combinations of treatments and inoculation timings were subjected to Tukey's test, slicing by day. When inoculations were administered on day 1, the most efficient treatment that decreased disease severity was Bellis®(a), followed by Merpan®(ab) and Ziram®(b), showing a reduction of between 99 and 97% of spots per fruit. Timorex® (c) showed in turn the lowest efficiency but still achieved significant control with respect to the control treatment, showing a reduction of 70% of the spots in the fruits inoculated on day 1 post-application. When inoculations were administered on day 7, Bellis®, Merpan®, and Ziram® (a) were the most efficient treatments, showing a reduction of approximately 95% of disease incidence, with significant differences with respect to both Timorex® (b) and control (c), but not between them. In this case, Timorex® was able to reduce the disease severity by 27%. Similar results were obtained for fruit inoculated on day 15 (Table 3).

* + 1. Curative treatments

In the curative treatments on detached fruits, applied 24 h after inoculation with the *S. vesicarium* SF23 strain, disease incidence was 90% for Bellis®, 95% for Merpan® and Ziram®, and 100% for Timorex® and control. The model fitted for de incidence presented a good fit (?), and the test of means indicated that there were no differences between the treatments.

All untreated control fruit was affected by BSP, with a mean of 28 spots per fruit. The highest proportion of fruit with a large number of spots was observed upon Timorex® treatment. The selected model presented a good fit according to the residuals test. According to ANOVA, all treatments had a significant effect (*p* < 0.0001 for all). On Tukey’s test, all treatments presented significant differences from the control, with Ziram®, Merpan®, and Bellis® being the most effective (Table 4).

* 1. In-field assays

In the first season, ten days after Bellis® and Ziram® treatments (performed at the end of January 2019), there was a 7.2 mm rain and 15 hours of wet leaf, with an average temperature of the wet period of 13ºC. In the second season, two days after the first treatment (performed at the end of December 2019), there was a rain of 8 mm and 13 hours of wet leaf, with an average temperature of the wet period of 18.1ºC. A second treatment was done at the end of January 2020 and one day after the application, an 18 mm rain event with 14 hours of wet leaf and a mean temperature of 15 ºC were recorded. Symptoms of natural infection by *S. vesicarium* in pear fruits were not detected due to the slightly predisposing environmental factors (although the minimum of 6 h of humidity necessary for infection was recorded, the average temperature was less than the optimum of 20-25ºC). Nevertheless, since symptoms were detected on leaves, the proportion of affected leaves could be determined. The model fitted for the proportion of affected leaves showed a significant interaction between treatment and season effects. In the first season (2018/2019), the control treatment presented 11.1% of incidence, which was reduced to 3.46% and 4.27%, respectively, upon treatment with Bellis® and Ziram®. In the second season (2019/2020), the control presented 9.8% of affected leaves, while incidence for Ziram® and Bellis® was 2.88% and 1.08%, respectively. The Tukey’s test, used to compare the treatments slicing by season, revealed that during the first season Ziram® and Bellis® had similar effect, while in the second season Bellis® had more efficacy than Ziram® in decreasing symptoms.

1. Discussion

BSP is a disease of recent appearance in Argentinian pear orchards, caused by *S. vesicarium* (Dobra & Garcia, 2015; Temperini et al. 2022). Due to the high economic significance of pear production in the Patagonian region, and since studies on BSP in Argentina are fairly scarce, there is a pressing need to study the pathogen in affected growing areas as to identify effective fungicides for its control. Therefore, in the present work fungicides with different modes of action registered in Argentina were evaluated on their ability to inhibit conidia germination and mycelial growth and to reduce rotting caused by *S. vesicarium* infection in pear fruit.

Among many statistical software packages available to estimate dose-response relationships, the current analyses relied on an open source R package ("drc") that has been widely implemented in similar studies over the last decade (Kunova et al., 2014; Stewart et al., 2014; Saville et al., 2015; Wang et al., 2017; Noel et al., 2018; Koppel, 2020). According to the EC50 values obtained, and following the criteria defined by Edgington et al. (1971), we concluded that for the inhibition of mycelial growth of *S. vesicarium*, the only highly toxic fungicide was Bellis®. Comparatively, Ziram® was moderately toxic, Merpan® was slightly toxic, and Timorex® had no toxic effect. Regarding spore germination inhibition, Bellis®, Ziram®, and Merpan® were highly toxic, while Timorex® showed no toxicity. The fungicides evaluated in this work have different modes of action. Pyraclostrobin (a component of Bellis®) is a quinone outside inhibitor (QoI) that inhibits mitochondrial respiration by blocking electron transport at the cytochrome bc1 complex (complex III) (FRAC, 2021). QoIs control a range of fungal pathogens by inhibiting spore germination and mycelial growth (Anke, 1995; Bartlett et al., 2002). Boscalid (the other component of Bellis®) is a succinate dehydrogenase inhibitor (FRAC, 2021) effective against different stages of fungal development, mainly spore germination and germ tube elongation, although it inhibits also other stages such as appressoria formation and mycelial growth (Stammler, 2008). The penetrating effect of this fungicide could explain the high sensitivity exhibited by *S. vesicarium* strains during conidial germination and mycelial growth *in vitro*. Cases of resistance to strobilurins such as kresoxim-methyl, trifloxystrobin, and pyraclostrobin have been reported in Italian pear orchards (Collina et al., 2007; Alberoni et al., 2010). However, our results in 'D’Anjou' fruit showed that the *S. vesicarium* strains studied were sensitive to pyraclostrobin. Thus, further studies would be required to assess potential resistance factors in different pear cultivars. Merpan® (containing captan) is a nonpenetrating fungicide with multisite activity (FRAC, 2021) whose mode of action is based on reaction with thiols (Gordon, 2010). This product inhibits conidial germination by interrupting respiration through inhibiting the glycolytic conversion of glucose into pyruvate (Hochstein & Cox, 1956; Gur et al., 2020). This mechanism may thus explain the differences observed in this work between Merpan® EC50 values for spore germination and mycelial growth. Of note, these results agree well with those obtained by Gur et al. (2020) for conidia germination and mycelial growth of *Alternaria alternata f.sp. mali.* Ziram® is another nonpenetrating fungicide with multisite activity (FRAC, 2021) which acts also by interfering with enzymes involved in the respiration process and inhibiting spore germination (Dattatray et al., 2020). In the present study, Ziram® achieved lower EC50 values ​​than Merpan® in both conidia germination and mycelial growth assays. These two fungicides were included in the present study due to their multisite action and the fact that fungi do not easily develop resistance to them. Timorex® (containing extract of *Melaluca alternifolia*) disrupts the fungal cell membrane (FRAC, 2021) and is recommended against a broad spectrum of ascomycete and bacterial plant diseases. The motivation to include this product in the present study was based on its organic and non-resistance-generating nature; however, in our study Timorex® showed limited control over spore germination and mycelial growth of the *S. vesicarium* strains assayed.

The efficacy of preventive and curative treatments against BSP development in detached fruit was tested with inoculation trials. Whereas all nonpenetrating fungicides represent preventive o protectant agents, some penetrating fungicides can also have preventive or protective action. Penetrating fungicides like Bellis® act mainly during the incubation period paralyzing the infectious process and are able to inhibit fungal growth inside plant tissues before symptoms and signs are observed (Bartlett et al., 2002; Amaro et al., 2020; Carmona et al., 2020). In this work, the preventive treatments were able to significantly reduce the incidence and severity of BSP infection, while curative treatments were not as effective. The most effective treatment was Bellis®, applied one day before fungal inoculation. This result was reasonable, considering the penetrating mode of action of the fungicide. In turn, the high effectivity showed by Merpan® and Ziram® could be attributed to their multisite activity and proved ability to inhibit conidial germination. Regarding BSP incidence, there were no differences between fruit treated with Timorex® and control fruit. However, disease severity analysis indicated that fruit treated with Timorex® had fewer spots than control fruit. Overall, our results showed that although all the treatments were able to reduce disease level, their effectiveness decreased as time elapsed after application, possibly due to natural degradation of the products. According to Llorente (1997), fungicides applied curatively are ineffective for the control of the disease. This can be explained by the release of toxins that occurs after *S. vesicarium* germination (Singh et al., 1999). Consequently, if the fungicide is applied once the infection has already occurred, germination or lengthening of the germinative tube is inhibited; however, if the toxin has already been released, it will inevitably cause necrosis (Llorente 1997; Llorente & Montesinos, 2006; Singh et al. 1999). Nevertheless, if preventive treatments could not be carried out, the possible usefulness of a curative treatment should be evaluated, even if timing is not optimal. Coinciding with Llorente & Montesinos, 2006, the levels of disease reduction attained by curative treatments in this work were modest, as they failed to reduce incidence but still achieved a severity reduction of ~60%. If we consider the importance of inoculum management, curative applications might achieve a considerable reduction in disease levels. The speed of *S. vesicarium* spore germination depends on both temperature and availability of water for the plant tissue (Montesinos et al. 1995; Llorente et al. 2006). Therefore, if temperatures are low, germination takes longer and curative treatments may have significant effects in reducing disease. In this work, the high levels of infection obtained in the bioassays in detached fruit were due to the favorable conditions and high levels of inoculum used. Ponti et al. (1993) showed that under very predisposing climatic conditions and with a high level of inoculum it is very difficult to achieve 100% effective control, even with the best fungicide management strategies.

According to Llorente & Montesinos (2006), a minimum of 6 h of wetness is required for infection by *S. vesicarium* at optimum temperatures between 20-25°C. The results obtained in our field trials indicated adequate control level for treatments with Bellis® and Ziram®, with significant differences relative to untreated fruit. The control achieved by the contact and the penetrant fungicides was similar in the first and second season. In the first season, the predisposing conditions occurred 10 days after the treatments were applied. Similar to the results of *in vitro* preventive treatments, it thus appears that preventive treatments with penetrating and non-penetrating fungicides had similar efficiency. In the second season, the disease level in the control treatment was higher than in the first season, and Bellis® achieved a greater decrease in disease incidence than Ziram®. In the two seasons analyzed, the average temperature was lower than the ideal temperature required for high disease incidence. For this reason, the incidence on leaves was low and no damage was recorded on fruits. However, differences in control efficiency among the tested fungicides could be established by assessing the proportion of affected leaves in the different treatments.

1. Conclusions

Our study presents a first account on the control efficacy of different fungicides against regional isolates of *S. vesicarium*, the causal agent of BSP. Based on our in vitro and in-field assays, Bellis®, Ziram®, and Merpan® were identified as potential BSP management tools. The most efficient fungicide in preventing spore germination and mycelial growth was Bellis®, which consistently required lower effective concentrations impeding 50% fungal growth compared to the other three fungicides tested. Ziram®, which prevented 50% of spore germination at dosages below 0.26 µg mL-1 and 50% of mycelial development at 7.7 µg mL-1, was the second-best fungicide. Preventive treatments were most effective in reducing the incidence and severity of BSP. It is suggested that if preventive applications cannot be made, treatments should be applied as soon as possible after the occurrence of predisposing conditions for the disease. To determine more clearly the efficacy of the evaluated fungicides, as well as of other active principles, in future trials field treatments should be repeated under climatic conditions more predisposing to disease development.

**Tables**

**Table 1.** Fungicide concentrations (µg mL-1) used in experiments with *S. vesicarium.*

**Table 2.** Half-maximaleffective concentration (EC50; μg mL-1) of test fungicides on mycelial growth and spore germinationof *S. vesicarium* strains.

**Table 3.** Efficacy of preventive treatments on 'D’Anjou' fruit, applied 1, 7, and 15 days before inoculation with *S. vesicarium* SF23 strain. Means with different letters are significantly different (*p* < 0.05) according to Tukey’s test.

**Table 4**. Efficacy of curative treatments on 'D’Anjou' fruit, applied 1 day after inoculation with *S. vesicarium* SF23 strain. Means with different letters are significantly different (*p* < 0.05) according to Tukey’s test.

**Figures**

**Fig. 1.** Dose-response curves of Bellis®, Merpan®, Timorex®, and Ziram® for mycelial growth inhibition at 7 days.

**Fig. 2.** Dose-response curves of Bellis®, Merpan®, Timorex®, and Ziram® fungicides for germination at 8 h.

Acknowledgments: This work was supported by the Instituto Nacional de Tecnología Agropecuaria (INTA) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). We acknowledge Dr. Isidre Llorente for critically reviewing this article.

References

* Alberoni, G., Cavallini, D., Collina, M., Brunelli, A. (2010). Characterisation of the first *Stemphylium vesicarium* isolates resistant to strobilurins in Italian pear orchards. European Journal of Plant Pathology 26(4), 453–457, https://doi.org/10.1007/s10658-009-9559-3.
* Alberoni, G., Collina, M., Pancaldi, D., Brunelli, A. (2005). Resistance to dicarboximide fungicides in *Stemphylium vesicarium* of Italian pear orchards. European Journal of Plant Pathology,113(2), 211–219, https://doi.org/10.1007/s10658-005-2332-3.
* Alves, K. (2020). ec50estimator: An Automated Way to Estimate EC50 for Stratified Datasets\_. R package version 0.1.0. https://CRAN.R-project.org/package=ec50estimator.
* Amaro, A., Baron, D., Ono, E., Domingos, R. (2020). Physiological effects of strobilurin and carboxamides on plants: an overview. Acta Physiologiae Plantarum, https://doi.org/10.1007/s11738-019-2991-x.

Anke, T. (1995). The antifungal strobilurins and their possible ecological role. Canadian Journal of Botany, https://doi.org/10.1139/b95-342.

Bates D, Mächler M, Bolker B, Walker S (2015). “Fitting Linear Mixed-Effects Models Using lme4.” Journal of Statistical Software, 67(1), 1–48. doi:10.18637/jss.v067.i01.

Brooks, M.E., Kristensen, K., van Benthem, K.J., Magnusson, A., Berg, C.W., Nielsen, A., Skaug, H.J., Maechler, M., Bolker, B.M. (2017). “glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling.” The R Journal, 9(2), 378–400. https://doi:10.32614/RJ-2017-066.

Brunelli, A., Di Marco, G., Contarelli, G., Ponti, I. (1984). Prove di lotta contro la maculatura bruna delle pere. ATTI Giornate Fitopatologiche. I: 203-212.

Brunelli, A., Gherardi, I., Adani, N. (1997). Ridotta sensibilità di *Stemphylium vesicarium*, agente della maculatura bruna del pero, ai fungicidi dicarbossimidici. Informatore Fitopatologico. 9:44-48.

Brunelli, R., Rovesti, R., Di Marco, S., Ponti, I. (1986). Attivita di diversi fungicide contro la maculatura bruna del pero. Riv.Frutticoltura Ortofloricoltura 1:51-54.

Carmona, M., Sautua, F., Pérez-Hérnandez, O., Reis, E.M. (2020). Role of Fungicide Applications on the Integrated Management of Wheat Stripe Rust. Frontiers in Plant Science, 11, 733, https://doi.org/10.3389/fpls.2020.00733.

* Collina, M., Alberoni, G., Brunelli, A. (2007). First occurrence of strobilurin-resistant isolates of *Stemphylium vesicarium* in an Italian pear orchard. Communications in Agricultural and Applied Biological Sciences, *72*(4), 735–738.
* Cribari-Neto F, Zeileis A (2010). “Beta Regression in R.” Journal of Statistical Software, https://doi.org/10.18637/jss.v034.i02.
* Dattatray, K.P., Abasaheb, M.M., Suresh, P.R., Vuttal, V.B., Gulab, S.S., Schola, R. (2020). Effect of ziram on seed germination and seedling growth in mung bean. International Journal of creative research througths*,* https://ijcrt.org/papers/IJCRT2007468.pdf.

Dobra, A. C., Garcia, L. (2015). Presencia de mancha negra del peral, *Stemphylium vesicarium,* en el Valle Medio del Río Negro, Patagonia Argentina. XXXVIII Congreso Argentino de Horticultura. Bahía Blanca. Buenos Aires. pp 34(85):73.

Edgington, L.V., Khew, K.L., Barrow, G.L., (1971). Fungitoxic spectrum of benzimidazole compounds. Phytopathology, https://doi.org/10.1094/Phyto-61-42.

* FRAC. (2021). FRAC Code List ©\* 2021: Fungal control agents sorted by cross resistance pattern and mode of action (including coding for FRAC Groups on product labels). Fungicide Resistance Action Committee, 17. https://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2021--final.pdf?Sfvrsn=f7ec499a\_2
* Gálvez Patón, L., Gil-Serna, J., García-Díaz, M., Iglesias, C., Palmero, D. (2016). Stemphylium Leaf Blight of Garlic (*Allium sativum*) in Spain: Taxonomy and In Vitro Fungicide Response. The Plant Pathology Journal, 32, 388-395, https://doi.org/10.5423/PPJ.OA.03.2016.0063.
* Gerhard, D.; Ritz, C. \_Medrc: Mixed Effect Dose-Response Curves, R Package Version 1.1-0. 2018. Available online: https://rdrr.io/github/DoseResponse/medrc/ (accessed on 27 January 2023).
* Gordon, E., (2010). Captan and Folpet, in: Krieger R. (Ed), Hayes' Handbook of Pesticide Toxicology, Elsevier Science Publishing Co Inc, pp.1915-1949, https://doi.org/10.1016/B978-0-12-374367-1.00090-2.
* Gur, L., Reuveni, M., Cohen, Y. (2020). Control of Alternaria fruit rot in 'Pink Lady' apples by fungicidal mixtures. Crop Protection, 127, 104947, https://doi.org/10.1016/j.cropro.2019.104947.
* Hartig, F. (2022). DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models. R package version 0.4.6. http://florianhartig.github.io/DHARMa/
* Hochstein, P., Cox, C.E. (1956). Studies on the fungicidal action of N–(Trichloromethylthio)–4–cyclohexene–1,2–dicarboximide (captan). American Journal of Botany, 43, 437-441, https://doi.org/10.1002/J.1537-2197.1956.TB10514.X
* Koppel, D. (2020). Dose Response Modelling in R. https://alvesks.github.io/ec50estimator/articles/how\_to\_use.html
* Kunova, A., Pizzatti, C., Bonaldi, M., Cortesi, P. (2014). Sensitivity of nonexposed and exposed populations of Magnaporthe oryzae from rice to tricyclazole and azoxystrobin. Plant Disease. 98, 512-518, https://doi.org/10.1094/PDIS-04-13-0432-RE.
* Lenth, R., Singmann, H., Love, J., Buerkner, P., & Herve, M. (2020). Emmeans: Estimated marginal means, aka least-squares means. https://CRAN.Rproject.org/package=emmeans.

Llorente, I. (1997). Development of an infection forecasting model for *Stemphylium vesicarium*. Evaluation, validation and implementation on experimental plots in pear commercial orchards. Ph.D. Thesis. University of Girona, Girona, Spain.

* Llorente, I., & Montesinos, E. (2006). Brown spot of pear: An emerging disease of economic importance in Europe. Plant Disease, 90(11), 1368–1375, https://doi.org/10.1094/PD-90-1368.
* Llorente, I., Vilardell, A., Montesinos, E. (2006). Infection potential of *Pleospora allii* and evaluation of methods for reduction of the overwintering inoculum of Brown spot of pear. Plant Disease. 90, 1511-1516, https://doi.org/10.1094/PD-90-1511.

Llorente, I., Moragrega, C., Vilardell, P., Montesinos, E. (2000), STREP: a brown-spot disease predictor for scheduling fungicide sprays against *Stemphylium vesicarium* on pear. Bulletin European and Mediterranean Plant Protection Organization 30, 143–148.

* Llorente, I., Moragrega, C., Ruz, L., Montesinos, E. (2012). An update on control of brown spot of pear. Trees Structure and Function, 26(1), 239–245, https://doi.org/10.1007/s00468-011-0607-1
* Marchi, A., Folchi, A., Pratella, G. C., Caccioni, D. (1995). In vitro relationship between dithiocarbamate residue and *Stemphylium vesicarium* infection on pear fruit. Crop Protection, 14(4), 321–326, https://doi.org/10.1016/0261-2194(95)00001-3.
* Noel, Z. A., Wang, J., Chilvers, M. I. (2018). Significant Influence of EC50 Estimation by Model Choice and EC50 Type. Plant disease, 102(4), 708–714, https://doi.org/10.1094/PDIS-06-17-0873-SR.
* Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team. (2021). nlme: Linear and nonlinear mixed effects Models. https://CRAN.R-project.org/package=nlme
* Ponti, I., Brunelli, A., Tosi, C., Basaglia, M., Bevilacqua, T., Emiliani, G., Cont, C., Viccinelli, R. (1993). Verifica dell’attivita di diversi preparati contra la maculatura bruna del pero. Informatore Fitopatologico 5, 45-52 Porta-Puglia.
* Ponti, I., Brunelli, A., Tosi, C., Cavallini, G., & Mazzini, F. (1996). Aggiornamenti sull’attivita’ dei fungicide contro la maculatura bruna del pero. ATTI Giornate Fitopatologiche*.* 2, 165-172.
* R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.
* Ritz, C., Streibig, J. C. (2005). Bioassay analysis using R. J. Stat. Softw. 12. doi.org/10.18637/jss.v012.i05 Google Scholar
* Russell V. Lenth (2021). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.6.2-1. https://CRAN.R-project.org/package=emmeans
* Saville, A., Graham, K., Grunwald, N. J., Myers, K., Fry, W. E., Ristaino, J. B. (2015). Fungicide sensitivity of U.S. genotypes of *Phytophthora infestans* to six oomycete-targeted compounds. Plant Disease. 99, 659-666, https://doi.org/10.1094/PDIS-05-14-0452-RE.
* Senasa (2022). Límites máximos de residuos permitidos. https://www.argentina.gob.ar/senasa/programas-sanitarios/productosveterinarios-fitosanitarios-y-fertilizantes/registro-nacional-de-terapeutica-vegetal
* Singh, P., Bugiani, R., Cavanni, P., Nakajima, H., Kodama, M., Otani, H., Kohmoto, K. (1999). Purification and biological characterization of host-specific SV-toxins from *Stemphylium vesicarium* causing brown spot of European pear. Phytopathology, 89(10), 947–953, https://doi.org/10.1094/PHYTO.1999.89.10.947

Stammler, G. (2008). Mode of action, biological performance and latest monitoring results of boscalid sensitvity. Abstracts of the 18th Symposium of Research Committee on Fungicide Resistance.

* Stewart, J. E., Kroese, D., Tabima, J. F., Larsen, M. M., Fieland, V. J., Press, C. M., Zasada, I. A., Grünwald, N. J. (2014). Pathogenicity, fungicide resistance, and genetic variability of *Phytophthora rubi* isolates from raspberry (*Rubus idaeus*) in the western United States. Plant Disease. 98, 1702-1708, https://doi.org/10.1094/PDIS-11-13-1130-RE Link, ISI, Google Scholar
* Temperini, C.V., Tudela, M.A.A., Gimenez, G.N., Di Masi, S.N., Pardo, A.G., Pose, G.N. (2022). Brown spot of pear, an emerging disease in Argentina: identification and pathogenicity characterization of Argentinean *Stemphylium vesicarium isolates*. European Journal of Plant Pathology, https://doi.org/10.1007/s10658-022-02493-y
* Tudela, M.A.A., Di Masi, S. N. (2022). La mancha marrón del peral. Boletín Sanitario Nº 7. INTA Alto Valle. https://inta.gob.ar/documentos/presencia-de-la-enfermedad-mancha-marron-del-peral

Vilardell, P. 1988. *Stemphylium vesicarium* en plantaciones de peral. Fruticultura Profesional 18, 51-55.

* Wang, J., Bradley, C. A., Stenzel, O., Pedersen, D. K., Reuter-Carlson, U., Chilvers, M. I. (2017). Baseline sensitivity of Fusarium virguliforme to fluopyram fungicide. Plant Disease. 101, 576-582, https://doi.org/10.1094/PDIS-09-16-1250-RE Link, ISI, Google Scholar
* Wickham, H. (2016). ggplot2: Elegant graphics for data analysis. Springer-Verlag New York. https://ggplot2-book.org/