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Original Article

Silviculture

Biological Control of *Ceratocystis fimbriata* by *Bacillus subtilis* on *Acacia mearnsii* Seedlings

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ABSTRACT

The aim of this study was to evaluate the effect of *Ceratocystis fimbriata* isolates, obtained from *Actinidia deliciosa* (kiwifruit), on black wattle (*Acacia mearnsii* De Wild.) seedlings, the potential biological control exerted *in vitro* by *Bacillus subtilis* on *C. fimbriata*, and the *in vivo* effect of *B. subtilis* against *C. fimbriata* on black wattle seedlings. Isolates of *C. fimbriata* obtained and identified from kiwi plantations were used in pathogenicity tests on *A. mearnsii* seedlings. *In vitro*, the antagonistic potential of *B. subtilis* on the pathogen was analyzed by direct confrontation tests. To evaluate the effect of *B. subtilis in vivo*, black wattle seedlings were inoculated with Rhizolyptus® seven days before and after pathogen inoculation. On black wattle seedlings, *C. fimbriata* isolates caused 44,15% to 100% severity. Direct confrontation tests revealed that the strains were effective in the biological control of *C. fimbriata* isolates *in vitro*. *In vivo*, Rizolyptus® was not efficient in controlling *C. fimbriata*.

Keywords: fungus, wilt, bioprotector.

Bacillus subtilis no Biocontrole de *Ceratocystis fimbriata* em Mudas de *Acacia mearnsii*

RESUMO

O objetivo deste trabalho foi avaliar o comportamento de isolados de *Ceratocystis fimbriata* obtidos de kiwi (*Actinidia deliciosa*) em mudas de acácia-negra (*Acacia mearnsii* De Wild.), o potencial de biocontrole *in vitro* de *Bacillus subtilis* sobre *C. fimbriata* e o comportamento *in vivo* de *B. subtilis* contra *C. fimbriata* em mudas de acácia-negra. Isolados de *C. fimbriata* obtidos e identificados em plantios de kiwi foram utilizados em testes de patogenicidade em mudas de *A. mearnsii*. O potencial antagônico *in vitro* de *B. subtilis* sobre o patógeno foi analisado em testes de confrontação direta. Para avaliar o comportamento *in vivo* de *B. subtilis*, as mudas de acácia-negra foram inoculadas com Rizolyptus® sete dias antes e após a inoculação do patógeno. Nas mudas de acácia-negra os isolados de *C. fimbriata* exibiram percentuais de severidade que variam de 44,15% a 100%. Nos testes de confrontação direta as cepas mostraram-se eficientes no biocontrole *in vitro* sobre os isolados de *C. fimbriata*. *In vivo*, o produto Rizolyptus® não foi eficiente no controle de *C. fimbriata*.

Palavras-chave: fungos, murcha, biocontrole.

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1. INTRODUCTION AND OBJECTIVES

Black wattle plantations (*Acacia mearnsii* De Wild.) are important mainly for family farming. Their economic importance relies on bark extracts that are used to produce tannin and timber and on their exportation for cellulose manufacturing (Schumacher et al., 2003). For the first time in Brazil, Ribeiro et al. (1988) reported the infection of black wattle by *Ceratocystis fimbriata* Ellis & Halsted, which resulted in wilting or death. It has been verified, since 2001, in commercial plantations of black acacia in Rio Grande do Sul (RS), the presence of *C. fimbriata* attack in isolated sites (Santos & Ferreira, 2003). In kiwifruit (*Actinidia deliciosa*) plantations in the region of Farroupilha, RS, symptoms of wilt, wood browning and reduced size fruits were caused by *C. fimbriata*. This pathogen has been causing damage to the producers, assuming economic importance, due to the high susceptibility of the grafted material, compromising the establishment of the crop (Piveta et al., 2013).

This fungus initially colonizes the cambium between the bark and wood, which is a typical characteristic of the xylem pathogen. A typical symptom manifests in cross sections of woody organs and appears as dark radial striations (Baker & Harrington, 2004). The ascending colonization by the pathogens happens very fast in trees suffering from water stress as a consequence of the attack, which increases the occurrence of radial dark striations towards the base apex (Ferreira et al., 2006).

Information on the behavior of this pathogen is obtained through pathogenicity tests in cross inoculations and is important to support strategies for disease control and prevention. Genetic control has been used; however, results are obtained over the long term (Silva et al., 2004). Alternative techniques are important in the control of ceratocystis wilt, since the use of fungicides is restricted due to a lack of registration within the Ministry of Agriculture and Supply.

Studies involving biological control have been increasing and they include *in vitro* and *in vivo* tests with *B. subtilis*. Endophytic organisms, such as *B. subtilis*, present control mechanisms that involve the production of antibacterial and antifungal substances (Castillo et al., 2006). Therefore, the aim of this study was to evaluate the effect of *C. fimbriata* isolated obtained from kiwifruit in black wattle seedlings, and to investigate the biological control potential exerted by *B. subtilis* on *C. fimbriata* in black wattle seedlings *in vitro* and *in vivo*.

2. MATERIALS AND METHODS

Pathogenicity tests and *in vivo* experiments were carried out at the Forest Nursery of the Center of Rural Sciences (CRS), Federal University of Santa Maria (UFSM), Santa Maria, RS. *In vitro* experiments were performed in the Laboratory of Plant Pathology, Department of Plant Protection of UFSM.

2.1. *Isolates of* Ceratocystis fimbriata

Five isolates (PM08, PM29, PP08, PC03, and PB01) of *C. fimbriata*, belonging to the mycology collection of the Laboratory of Plant Pathology "*Elocy Minussi*" at UFSM were used. The isolates were obtained from soil samples and kiwi plant material (Monty, Elmwood, and Hayward cultivars), which presented typical symptoms of the disease in four orchards located in the region of Farroupilha, RS (Table 1) (Piveta et al., 2013).

Table 1. Geographic coordinates, date of collection, and access number for *Ceratocystis fimbriata* isolates obtained in the region of Farroupilha, RS.

Isolate	Coordinates	Date of collection
PB01	S 29°09'.022" W 51°24'.805"	Jul/2010
PC03	S 29°09'.505" W 51°22'.783"	Jul/2010
PM08	S 29°14'.242" W 51°23'.187"	Jul/2010
PM29	S 29°14'.242" W 51° 23'.187"	Jul/2010
PP08	S 29°11'.751" W 51° 24'.118"	Jul/2010

Source: Piveta et al. (2016).

2.2. *Pathogenicity test in black wattle seedlings*

Black wattle seedlings (*A. mearnsii*), aged approximately six months old and 0,5 m tall, were transplanted into 1,5 L plastic bags containing commercial substrate Carolina Soil®.

Five isolates of *C. fimbriata* were transferred to Petri dishes (90 mm) containing Potato Dextrose

Agar (PDA) medium and maintained in an incubator at 25°C for 10 days, with a 12 hours photoperiod. Subsequently, 10 mL of sterile distilled water were added to the plates. After scraping the colony surface with a Drigalski spatula, the spore suspension was filtered through a double layer of gauze and calibrated to $2{,}5 \times 10^6$ spores mL^{-1} (Laia et al., 2000).

The plants were inoculated by performing a cross superficial incision from the stem to approximately 5 cm above the collar region. The opening of the incision was completed with the aid of a utility knife, into which approximately 0,5 mL of spore suspension was inoculated. The wound was covered with plastic PVC film to prevent dryness and contamination by other microorganisms. For controlled plants, sterile distilled water was used instead of inoculum suspension. After inoculation, the plants were kept in a greenhouse for 30 days before evaluation (Mafia et al., 2011). Four repetitions of three seedlings were used.

2.2.1. Disease severity assessment

As described by Piveta et al. (2013), the disease severity percentages of the isolates were calculated with the equation:

$$S = LL \times HP^{-1} \times 100 \quad (1)$$

LL: lesion length; HP: plant height.

2.2.2. Reisolation of Ceratocystis fimbriata

For the reisolation of *C. fimbriata* isolates, host stem fragments were utilized using the carrot slice method described by Laia et al. (2000).

2.2.3. Isolation of monoascospore cultures

With a needle, a mass of ascospores was transferred from a single perithecium to another plate containing agar water (AW) medium (20 g agar and 1 L distilled water), supplemented with antibiotic (streptomycin), at a concentration of 100 $\mu L.mL^{-1}$. After transferring, the ascospore mass was spread onto a Petri dish with the aid of a Drigalski spatula. The plates were subsequently incubated in the dark, at 25°C for 24 hours and a needle was then used to extract the chain of endoconidia. The conidia attached to the needle were transferred to the center of another Petri dish, containing PDA medium supplemented with antibiotics (streptomycin), to be subsequently incubated under the same conditions for 15 days (Alfenas et al., 2007).

2.3. Evaluation of the antagonistic activity of Bacillus subtilis *on* Ceratocystis fimbriata *in direct confrontation tests*

For paired cultures tests, a commercial product based on *B. subtilis* (UFV 3918), i.e., Rizolyptus®, was used with the same *C. fimbriata* isolates used in the pathogenicity tests on black wattle seedlings.

For the tests, a 7 mm dish containing *C. fimbriata* mycelium in PDA medium was transferred to the center of a 90 mm Petri dish containing PDA medium. The plates were then incubated at 25°C for 7 days, with a 12 hours photoperiod. After this period, four discs containing *B. subtilis* (UFV 3918) bacterial cells, grown in PDA culture medium for 48 hours at 25°C, were placed equidistant from each other and from the center of the Petri dish. The plates were kept in an incubator for seven days under the same conditions and the antagonistic activities were evaluated after this period and compared to those observed on control plates containing only the pathogen (adapted from Sottero et al., 2006).

During the incubation, the plates were evaluated daily and the mycelial growth of the pathogen isolates was measured with the aid of a digital caliper. To calculate the percentage inhibition of mycelial growth, the following formula was used:

$$\%\ \text{inhibition} = ([\text{r.g.cont} - r.g.treat] \div \text{r.g.cont}) \times 100 \quad (2)$$

r.g.cont: radial growth of controls; r.g.treat: radial growth of treated samples (Menten et al., 1976). Each treatment consisted in four replicates.

2.4. In vivo *assay of* Ceratocystis fimbriata × Bacillus subtilis *on black wattle seedlings*

From the results obtained in the tests described under sections 2.2 and 2.3, the composition of the treatments (T) was established as follows: T1 – control seedlings, using only sterile distilled water; T2 – seedlings inoculated only with Rizolyptus®; T3 – Rizolyptus® inoculated seven days before the isolate PP08; T4 –

Rizolyptus® inoculated seven days before the isolate PM29; T5 – seedlings inoculated only with the isolate PP08; T6 – seedlings inoculated only with the isolate PM29; T7 – Rizolyptus® inoculated seven days after the isolate PP08; T8 – Rizolyptus® inoculated seven days after the isolate PM29.

With the aid of a utility knife, a small longitudinal incision was made up to approximately 5 cm above the collar region of black wattle seedlings into which approximately 1 mL of commercial Rizolyptus® was inoculated. The wound was covered with plastic PVC film to prevent dryness and contamination by other organisms.

After seven days, the spore suspensions of PM29 and PP08 of *C. fimbriata* isolates were inoculated as described under section 3.3, i.e., 1 cm longitudinally to the area where Rizolyptus® was inoculated (T3 and T4). Seedlings containing only spore suspension isolates (T5 and T6) or only Rizolyptus® (T2) were inoculated. Controlled plants were treated only with sterile distilled water (T1). The seedlings were inoculated with Rizolyptus® (T7 and T8), seven days after pathogen inoculation as previously described (Brooks et al., 1994). The plants were kept in a greenhouse for 30 days. The antagonist potential of *B. subtilis* was evaluated through the disease severity percentages of the isolates, the reisolation of the pathogen, and the isolation of monoascoporic cultures, which were performed according to the methodologies described under sections 2.2.1, 2.2.2, and 2.2.3, respectively. Four repetitions containing three seedlings each were used.

2.5. Statistical Analysis

The analysis of variance was carried out by comparing the means with the Tukey's test, at 5% probability, using the software SISVAR 5.3 (Ferreira, 2010). To analyze the results, the data were transformed from percentages to arcsine, square root $(\times/100)^{0.5}$.

3. RESULTS

In the pathogenicity tests on black wattle seedlings, the isolates of *C. fimbriata* showed different levels of disease severity (Figure 1). Significant severity percentages were observed for PM29, PP08, PM08, and PC03 isolates, which were different from those obtained for the controlled seedlings, which were inoculated with sterile distilled water and did not present symptoms that resulted in drying or wilting. The PM29 isolate caused the death of all the seedlings (Figure 2) within 28 days and this was not statistically different from the observed for the isolate PP08, which presented severity of 94,03%. These isolates were responsible for the greatest number of plants presenting symptoms that resulted in wilting and death.

PC03 and PM08 isolates followed the same trend in the manifestation of symptoms, with a severity > 50%. These isolates did not statistically differ among themselves and PP08 and PB01; however, they were significantly different from the control. The PB01 isolate exhibited the lowest severity percentage (44,15%) and was not statistically different from the observed in the control and the PC03 and PM08 isolates of *C. fimbriata*.

The average percentage inhibition of mycelial growth of *C. fimbriata* isolates (Figure 3 and 4) indicated that Rizolyptus® was more efficient at controlling the PP08 isolate, followed by the PC03, PM08, and PM29 isolates with 32,2%, 29,77%, 28,58%, and 26,33% inhibition, respectively. The PB01 isolate presented the lowest percentage inhibition (14,12%) *in vitro*. In this isolate, the bacteria allowed the fungus to grow for up to 120 hours after the cultures were paired, opposite to that observed for other isolates of *C. fimbriata*, in which the bacteria inhibited mycelial growth 96 hours after the start of direct confrontation.

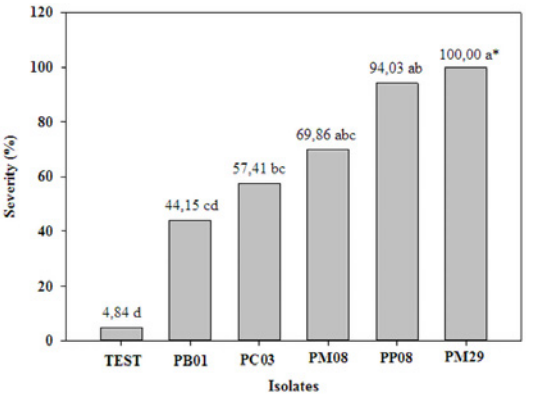

Figure 1. Disease severity (%) of ceratocystis wilt on seedlings of *Acacia mearnsii*, inoculated with *C. fimbriata* isolates obtained from kiwifruit. (*): averages followed by the same letter do not differ among themselves by the Tukey's test ($p \leq 0.05$). Coefficient of variation (%) = 19,08.

Figure 2. Pathogenicity test of *C. fimbriata* in *Acacia mearnsii* seedlings. (A): dead plant inoculated with isolated PB01 and the control treatment inoculated only with sterile distilled water; (B): control and dead plant inoculated with isolate PC03.

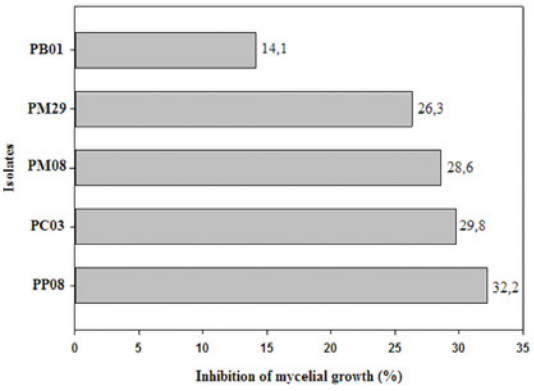

Figure 3. Percentage of mycelial growth inhibition (%) of PP08, PM29, PM08, PC03, and PB01 *C. fimbriata* isolates, obtained from kiwifruit and paired with the commercial form of *Bacillus subtilis* Rizolyptus®.

All seedlings inoculated with PM29 and PP08 isolates and the biological control agent presented symptoms that resulted in wilting and death (100% severity). The disease severity percentages of seedlings inoculated only with Rizolyptus® (T2) (2,67%) or controls (T1) (3,04%) inoculated only with sterile distilled water were not statistically different (Figure 5).

Seedlings inoculated with *B. subtilis* (Rizolyptus®) seven days before (T3 and T4) or seven days after the pathogen (T7 and T8), showed the same characteristics as plants inoculated with *C. fimbriata* isolates alone (T5 and T6).

The first wilt symptoms were observed 15 days after inoculation with the fungus. The symptoms evolved gradually, regardless of the presence of bacteria when inoculated before and after the pathogen. On day 28 of the experiment, all plants inoculated with *C. fimbriata* died after reaching a severity of 100% (Figure 6).

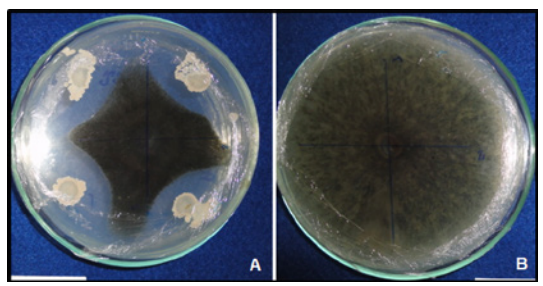

Figure 4. Culture pairing for *B subtilis* in the commercial form Rizolyptus® and isolated PC03 *C. fimbriata*. (A): Rizolyptus® x PC03; (B): control treatment with isolated PC03 only.

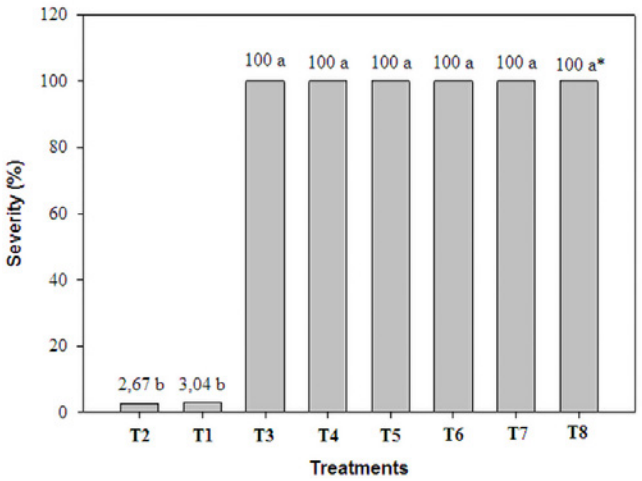

Figure 5. Disease severity (%) on the *in vivo* biological control test of the commercial form of *B. subtilis* Rizolyptus® on PM29 and PP08 *C. fimbriata* isolates, obtained from kiwifruit on *Acacia mearnsii* seedlings. T1: seedlings inoculated only with sterile distilled water; T2: seedlings inoculated only with Rizolyptus®; T3 and T4: seedlings inoculated with Rizolyptus® seven days before PP08 and PM29 isolates; T5 and T6: seedlings inoculated only with PP08 and PM29 isolates; T7 and T8: seedlings inoculated with Rizolyptus® seven days after PP08 and PM29 isolates; (*): averages followed by the same letter do not differ among themselves by the Tukey's test ($p \leq 0.05$). Coefficient of variation (%) = 0,38.

Figure 6. *Acacia mearnsii* seedlings inoculated with *B. subtilis* in commercial form Rizolyptus® over isolated PP08 from *C. fimbriata*. (A): seedling inoculated only with Rizolyptus®; (B): control inoculated only with sterile distilled water; (C) seedling inoculated with Rizolyptus® seven days before the isolated PP08; (D): seedling only inoculated with the isolated PP08; (E) seedling inoculated with Rizolyptus® seven days after the isolated PP08.

4. DISCUSSION

The results suggest that the PC03, PM08, PP08, and PM29 isolates of *C. fimbriata* were pathogenic to black wattle. Symptoms of *C. fimbriata* infection on black wattle seedlings were initially observed as plants' closing their leaflets, which became yellow, wilted, and dry, leading to leaflets fall, and, consequently, death of the plant. According to Firmino et al. (2013), after 30 days inoculation of *C. fimbriata*, some plants of *Eucalyptus* spp. already had wilt symptoms, characteristic of this pathogen. Still according to these authors, from this

wilt, in a short period of time it was possible to observe pointer dryness and death of some plants. During the conduction of field experiments, it was observed that most progenies of *Eucalyptus* spp. inoculated did not present expressive symptoms of wilt, being these only noticed in hotter hours of the day or in the presence of water stress, but in a subtle way. However, 90 days after inoculation, the plants showed xylem discoloration and pathogen structures inside them. Thus, these apparently asymptomatic plants were susceptible to the isolates studied and would probably die if they were kept longer under greenhouse conditions.

According to Harrington et al. (2011), the isolates identified in Brazil do not have host specializations, since their levels of aggressiveness vary according to the species. In cross-inoculation tests, Johnson et al. (2005) observed that *C. fimbriata* isolates were able to induce symptoms in general that are different from those used to obtain the isolates. Therefore, *Quercus* isolates lead to discoloration of the xylem in *Prunus* plants. When inoculated with isolates obtained from *Prunus*, *Carya* and *Populus*, plants also showed symptoms of the disease.

B. subtilis proved to be effective for biological control *in vitro*, showing zones of inhibition even before direct contact with the pathogen, which can be attributed to the effect of antibiosis, characterized by the production of volatile metabolites released by the bacteria, in addition to the competition for nutrients and space, which thus interferes with the growth of *C. fimbriata* isolates. According to Kim et al. (2008), the inhibitory effect of bacteria, such as *B. subtilis*, may be attributed to the production of hydrolytic enzymes that are capable of degrading the cellular wall and impairing lipopeptide (LPP) production, thereby directly interfering with the mycelial growth of plant pathogenic fungi. In their studies, Seema & Devaki (2012) reported that *B. subtilis* strains could inhibit 50% *Rizoctonia solani* mycelial growth five days after the cultures were paired. According to these authors, the action of antibiotics, such as Iturin A and Surfactin, may have influenced mycelial growth and color of the pathogen colony.

Lee et al. (2008) found that in confrontation tests of 12 colonies of *B. subtilis*, isolated from different locations of the rhizosphere, on isolates of *Phytophthora capsici*, five colonies, R15, R22, R30, R32, and R33, presented maximum inhibition of the pathogen with zones of inhibition measuring 11,5 mm, 11 mm, 11,5 mm, 12 mm, and 12 mm, respectively. The same colonies were efficient in the biological control of *P. citrophthora*, *P. citricola*, and *Colletotrichum coccodes*. According to the authors of that study, the genus *Bacillus*, which was already established as a plant growth promoter, also plays an important role in controlling soil plant pathogens.

The average percentage inhibition of mycelial growth of *C. fimbriata* isolates revealed that Rizolyptus® was more efficient at controlling the PP08 isolate, followed by PC03, PM08, and PM29 isolates, which presented inhibition percentages of 32,2%, 29,77%, 28,58% and 26,33%, respectively. The PB01 isolate showed the lowest percentage inhibition *in vitro*, i.e., 12,14%. For this isolate, the bacteria permitted growth up to 120 hours after the cultures were paired, contrary to other isolates of *C. fimbriata*, in which the bacteria inhibited mycelial growth 96 hours after direct confrontation. According to Melo & Valarini (1995), 0G and 5G isolates of *B. subtilis* significantly inhibited mycelial growth of *Fusarium solani in vitro.* The percentage of inhibitions were 53,37 (5G) and 71.75% (0G). Corroborating the results obtained by the authors mentioned above, Shiomi et al. (2008) found that with the same 0G isolate of *B. subtilis*, satisfactory inhibition of *Fusarium moniliforme*, *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Exserohilum turcicum* were observed, with values of 53,84%, 48,97%, 42,68% and 46,68%, respectively. However, the percentages inhibition obtained in this study were lower than 50%, which may be related to the physiology of *C. fimbriata* isolates presenting lower mycelial growth than the pathogens previously cited.

Remuska & Dalla Pria (2007) confirms the biological control potential of the genus *Bacillus*. In their studies, it was found that *Sclerotinia sclerotiorum* was unable to form sclerotia, an important structure for their resistance when paired with *Bacillus thuringiensis*. The bacteria inhibited mycelial growth of the pathogen by 37,44% and the presence of an inhibition halo was also observed by the authors.

According to Kupper et al. (2003), *B. subtilis* isolates were efficient at controlling *Colletotrichum acutatum in vitro*. Antagonistic bacteria, such as *B. subtilis*, usually acts by antibiosis, competition and parasitism. Their antibiosis activity can inhibit the growth of a wide

variety of species, mostly via the production of toxic substances, which is the most efficient mechanism to control plant pathogens.

Marroni & Germani (2011) concluded that *B. subtilis* antagonized 9% of mycelial growth of *Macrophomina phaseolina* when paired *in vitro*. Based on their studies, the authors observed that the antagonistic potential of strains isolated from wild plants is higher when compared to strains isolated from cultivated plants.

Unlike the control observed *in vitro*, *B. subtilis* strains were not efficient at controlling *C. fimbriata in vivo*, as all seedlings inoculated with the PM29 and PP08 isolates presented symptoms that resulted in wilting and death. Bell et al. (1982) pointed out that biological control tests *in vitro* are used for prior mass selection of possible candidates as antagonists. They highlight that not all biological controls *in vitro* will display antagonist mechanisms *in vivo*.

Moreira & May De Mio (2015) obtained unsatisfactory results *in vivo* in the control exerted by *Bacillus* sp. on *Colletotrichum acutatum* in apple trees (*Malus domestica*). According to the authors, Serenade®, that is, the commercial form of *B. subtilis*, did not provide any significant protection against the number of spots caused by *Colletotrichum* spp. It is worth noting that the natural habitat of bacteria belonging to the genus *Bacillus* is the soil, although in this work and in the studies previously mentioned, the biological control was tested under different environmental conditions (stems and leaves), which might naturally have interfered with the mechanisms of action. Stockwell et al. (1998) point out that the satisfactory results obtained *in vitro* do not always reflect effectiveness in the field. For these authors, good results *in vitro* should be used as parameters to determine the viability of the control method under real conditions of infection. In this study, the failure of effective control *in vivo* supports the necessity of further investigations, especially referring to inoculation methods and bioprotector concentration.

5. CONCLUSIONS

PC03, PM08, PP08, and PM29 isolates of *C. fimbriata* obtained from kiwifruit are pathogenic to *Acacia mearnsii*.

B. subtilis is efficient in the biological control of *C. fimbriata* isolates *in vitro*, whereas it is not able to control the wilting of ceratocystis on black wattle seedlings, inoculated with PM29 and PP08 isolates of *C. fimbriata in vivo*.

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