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Biocontrol of *Fusarium* spp. *in vitro* and in vine cuttings using *Bacillus* sp. F62

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Key words: Antagonism, bioagent, *Fusarium* wilt, vine rootstock.

Abstract: *Fusarium* spp., the causal agent of Fusarium wilt, cause substantial economic losses in viticulture, mainly in tropical regions. This study aimed to assess the biocontrol potential of *Bacillus* sp. F62 against *Fusarium* spp., both *in vitro* and in rootstock cuttings of the SO4 variety. To this end, the *in vitro* antagonism was evaluated through diffusible and volatile compounds synthesized by *Bacillus* sp. F62 on three *Fusarium* spp. isolates. Subsequently, the isolate FusA06-18 was selected for a rootstock cutting experiment. The vine cuttings underwent the following treatments: control, pathogen inoculation (Fus), bacterial inoculation (Bac), and bacterial followed by pathogen inoculation (Bac + Fus). Our findings revealed an average reduction of 39.1% in the mycelial growth of the pathogen through dual culture assay and a decrease of 11.6% in the *Fusarium* spp. radial growth due to the effects of volatile compounds. In the experiment with vine cuttings, applying *Bacillus* sp. F62 reduced the pathogen re-isolation frequency from 81.7% (Fus) to 63.3% (Bac + Fus). Therefore, *Bacillus* sp. F62 effectively suppressed the mycelial growth of *Fusarium* spp. and reduced the Fusarium wilt incidence in vine cuttings of the rootstock 'SO4'.

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All relevant data are within the paper and its Supporting Information files.

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The authors declare no competing interests.

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1. Introduction

In recent years, the young vine decline and death have affected many vineyards and nurseries worldwide (Gramaje and Armengol, 2011). This syndrome primarily affects vines exposed to stressful conditions, reducing plant productivity and survival in the field (Waite *et al*., 2015; Gramaje *et al*., 2018). Underperforming vines have been found to be affected by trunk and root diseases, disturbing physiological processes such as carbohydrate metabolism, defense responses, and photosynthetic rate (Fontaine *et al*., 2015; Akgül and Ahioğlu, 2019). In this context, *Fusarium* spp. have been associated with the failure or poor establishment of the vineyards, mainly in tropical regions (Halleen *et al*., 2003; Garrido *et al*., 2004; Król, 2006; Ziedan *et al*., 2011; Cruz *et al*., 2014; Abdullah *et al*., 2015; Markakis *et al*., 2017; Ghuffar *et al*., 2018; Reveglia *et al*., 2018; Akgül and Ahioğlu, 2019).

Fusarium spp. are soil-inhabiting pathogens that affect many plant

species, including grapevines (Sotoyama *et al*., 2016). These phytopathogens infect the vines through wounds in the root system, causing root rot. Subsequently, the pathogen promotes xylem obstruction, vascular injuries, and plant wilting due to the interruption of water and nutrients transportation to the shoots (Brum *et al*., 2012; Eljounaidi *et al*., 2016; Markakis *et al*., 2017). Besides, this pathogen can be transmitted through pruning and grafting, infecting the rootstock, graft union, and scion (Akgül and Ahioğlu, 2019). Fusarium wilt mainly affects susceptible vine rootstocks belonging to the *Berlandieri-Riparia* family, including the varieties SO4, Kobber 5BB, and Solferino. Although the 'SO4' rootstock exhibits high adaptability to different soils and climate conditions, ensuring good vineyard yield and fruit quality, it is highly susceptible to Fusarium wilt (Vilvert *et al*., 2016).

Given the difficulties in managing soil-borne pathogens, the limited efficacy, and the environmental risks of synthetic fungicides (Armengol and Gramaje, 2016; Gramaje *et al*., 2018), the use of antagonistic bacteria such as *Bacillus* spp. represent an alternative in the control of Fusarium wilt. These rhizobacteria can colonize plant tissues and vessels, suppressing the proliferation of vascular pathogens (Eljounaidi *et al*., 2016). In addition, rhizobacteria can promote plant growth and enhance crop yield (Legein *et al*., 2020; Morales-Cedeño *et al*., 2021).

In previous research, the rhizobacterium *Bacillus* sp. strain F62 demonstrated the potential to suppress black foot disease by 24.6% in 'SO4' (*Vitis berlandieri* x *V. riparia*) and by 29.5% in '1103P' (*Vitis berlandieri* x *V. rupestris*) rootstock plants obtained through micropropagation. Considering these findings, the present study aimed to evaluate the ability of *Bacillus* sp. F62 suspension to inhibit the mycelial growth of three isolates of *Fusarium* spp. and investigate its biocontrol activity against *Fusarium* sp. isolate FusA06-18 in stem wounds in the susceptible rootstock 'SO4'.

2. Materials and Methods

Microorganism isolates

Three isolates of *Fusarium* spp. (FusA97-11, FusP08-10, and FusA06-18) were isolated from symptomatic grapevines from Brazilian vineyards (Table 1). The rhizobacterium *Bacillus* sp. strain F62 was obtained from the soil in Caxias do Sul, Rio Grande do Sul State, Brazil. All microorganisms were preserved in the collection of the Laboratory of Biological Plant Disease Control at the University of Caxias do Sul, Brazil. Molecular identification of the rhizobacterium was performed by amplifying the *16S rDNA* gene with primers for bacteria domains, according to Sterky and Lundeberg (2000). The sequence exhibited 100% similarity to a pre-existing sequence in the National Center for Biotechnology Information (NCBI) of *Bacillus* sp. F62 with accession number NR 102783.2.

Table 1 - Isolates of *Fusarium* spp. used in the assays

Isolates	Origin (city/country)	Grapevine variety
FusA97-11	Alto Feliz, Brazil	Isabella
FusP08-10	Caxias do Sul, Brazil	Isabella
FusA06-18	Caxias do Sul, Brazil	Yves

Antagonism on mycelial growth of the pathogen

The antagonistic effect of *Bacillus* sp. F62 against *Fusarium* spp. was assessed in two different assays: antagonism through volatile and diffusible compounds. These experiments followed the methodology described by Russi *et al.* (2020). Initially, a single colony-forming unit (cfu) of *Bacillus* sp. F62 was cultured in a flask containing 10 ml of Potato Dextrose (PD) broth. The incubation was conducted on a rotary shaker at 150 rpm and 30 ± 2°C for 12 h. Subsequently, this pre-inoculum was transferred to an Erlenmeyer flask with 100 ml of PD broth and maintained under the same incubation conditions for 24 h. Afterwards, the bacterial suspension was centrifuged (3,500 × g) at 23°C for 5 min. The supernatant was discarded, and the pellet was washed twice with sterile water and resuspended in a 0.85% NaCl solution. The bacterial concentration was adjusted to 1×10^6 cfu ml^{-1} for *in vitro* antagonism and 1×10^8 cfu ml^{-1} for *in vivo* assay. Mycelial discs (5 mm in diameter) of the pathogen isolates were obtained from 10-day-old colonies grown in Potato Dextrose Agar (PDA) medium at 25 ± 2°C, with a 12 h light/12 h dark cycle.

In the antagonism through diffusible compounds, a mycelial disc was placed in a PDA medium plate, and after 24 h, four drops of a bacterial suspension (1×10^6 cfu ml^{-1}) were inoculated around the fungal mycelium. For the antagonism through volatile

compounds, a mycelial disc of the pathogen colony was inoculated in the center of a plate containing PDA medium. In another plate with the same medium, 100 µl of *Bacillus* sp. F62 suspension (1×10^6 cfu ml^{-1}) was uniformly spread. Subsequently, the plates were affixed together and sealed to prevent the loss of the bacterial metabolites. Plates inoculated with the pathogen isolates served as a control. All plates were incubated at 25±2°C with a 12 h light/12 h dark cycle for 14 days. The experiment was performed using a completely randomized design, with ten replicates for each fungal isolate.

Measurements of the colony diameter were performed using a digital caliper, and the data were used to determine the mycelial growth rate (MGR), according to the formula:

$$MGR = \Sigma [(d - dp) / N]$$

where d represents the mean of the colony diameter at the present day, dp represents the mean of the colony diameter from the previous day, and N represents the number of days of plate incubation. The mycelial growth inhibition (MGI) was also determined on the 14^{th} day of the experiment according to

$$MGI = [(dc - dt) / dc] \times 100$$

where dc and dt represent the mean of the colony diameters of control and treated groups, respectively, as described by Oliveira *et al.* (2016).

Biocontrol on rootstock cuttings

Four-year-old dormant cuttings of 'SO4' were obtained from vineyards at Embrapa Grape and Wine, Bento Gonçalves, Rio Grande do Sul State, Brazil. After hydration in distilled water for 24 h, the cuttings (30.0 cm in height) were subjected to hot water treatment at 50°C for 30 min, as described by Lerin *et al.* (2017). Four cuttings were arranged in each plastic pot containing 500 ml of autoclaved substrate (90% sphagnum peat and 10% vermiculite), pH 5.5, amended with 5 g l^{-1} of gradual release fertilizer (5-6 months). The isolate of *Fusarium* sp. FusA06-18 was selected for the *in vivo* assay due to its intermediate behavior in *Bacillus* sp. F62 antagonism.

The experiment was carried out using a completely randomized design, with 60 rootstock cuttings per treatment, according to Haidar *et al*. (2016 a), with modifications. Rootstock cuttings were subjected to surface disinfection with 70% (v/v) ethanol by rubbing with cheesecloth, and then cuttings were wounded with a scalpel above the first basal bud (4 mm in diameter). The trial consisted of applying the following treatments at the wounds: control (40 µl of sterile water), Bac (40 µl of *Bacillus* sp. F62 suspension containing 1×10^8 cfu ml^{-1}), Bac + Fus (40 µl *Bacillus* sp. F62 suspension and a mycelium disc of FusA06-18), and Fus (mycelium disc of FusA06-18). The wounds were covered with plastic film, and the cuttings were maintained in a growth chamber, at 26±2°C, under a 12 h light/12 h dark photoperiod provided by cool white fluorescent tubes. The relative humidity was maintained at 70%. During a 60-day experiment, the cuttings were watered three times a week with sterile water, at 80% of the maximum water holding capacity.

The following morphophysiological responses were assessed after 30 and 60 days: bud number (Budn), leaf number (Leafn), inflorescence number (Infln), and shoot length (Shootl, cm). The pathogen re-isolation frequency (FPR, %) was also determined at the experiment's end. The stems were debarked for pathogen re-isolation, and four fragments were collected 1 cm above and below the inoculation site. These fragments were surface disinfected by sequential immersion in 70% (v/v) ethanol for 30 sec and 3% (v/v) sodium hypochlorite for 1 min. Subsequently, the stem fragments were rinsed three times with sterilized water and then inoculated in plates containing PDA medium. The plates were incubated at 25°C for 10 days. The frequency of *Fusarium* spp. re-isolation was recorded compared to the total number of fragments obtained from each rootstock cutting.

Statistical analysis

The dataset was subjected to Shapiro-Wilk and Levene's tests to assess the normality and homoscedasticity, respectively. In the *in vitro* antagonism and the assay with rootstock cuttings, parametric data underwent one-way ANOVA followed by the Tukey test and non-parametric data were analyzed using the Kruskal-Wallis test followed by the Dunn-Bonferroni test. The frequency of pathogen re-isolation (FPR) between the treatments Fus and Bac + Fus was evaluated using the Mann–Whitney U-test. All analyses were performed with SPSS 22.0 software (SPSS Inc. Chicago, IL), and the threshold for statistical significance was set at $P<0.05$.

3. Results

Bacterial antagonism on mycelial growth

The inhibitory potential of *Bacillus* sp. F62 was determined against three isolates of *Fusarium* spp. (FusA97-11, FusP08-10, and FusA06-18) using diffusible and volatile compounds assays. In the antagonism through diffusible compounds, the bioagent exhibited statistically significant suppression of all isolates of *Fusarium* spp., reducing the mycelial growth rate compared to the control (Table 2, Fig. 1). Among the pathogenic strains evaluated, *Fusarium* sp. isolate FusA97-11 demonstrated the highest mycelial growth rate. The mycelial growth inhibition (MGI), determined on the last day of the assay, ranged from 30.4% (FusA06-18) to 47.1% (FusP08-10).

In the assessment of bacterial antagonism through volatile compounds, there was a statistically significant difference between the treatments (Fus and Bac + Fus). The volatile organic compounds led to a reduction in mycelial growth rate in all the *Fusarium* spp. isolates (Table 2, Fig. 2). The mycelial growth inhibition (MGI) ranged from 8.2% (FusA97-11) to 14.3% (FusP08-10). Although volatile compounds exhibited lower effectiveness in inhibiting the radial growth compared to diffusible compounds, these volatile metabolites not only affected the radial growth of the pathogen but also caused modifications in mycelial morphology (Fig. 3). Regarding the antagonism of *Bacillus* sp. F62 against *Fusarium* spp., both diffusible and volatile compounds promoted a higher suppression against the pathogenic isolate FusP08-10.

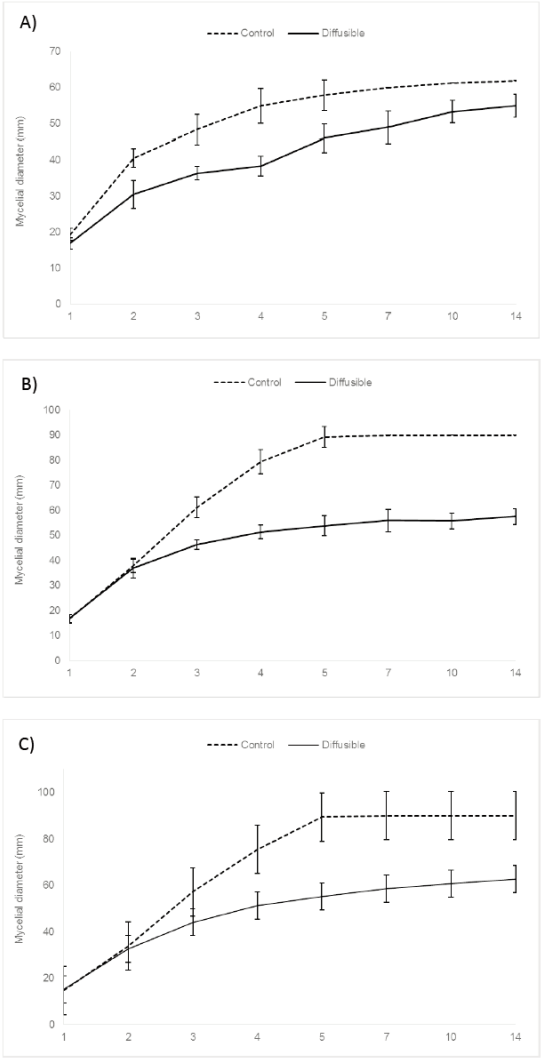

Fig. 1 - Mycelial growth of three *Fusarium* spp. isolates during 14 days of incubation in the antagonism assay through diffusible compounds. A) FusA97-11, B) FusP08-10, and C) FusA08-16

Table 2 - Mycelial growth rate (MGR, mm/day) of three *Fusarium* spp. isolates subjected to the following treatments: *Fusarium* spp. (Fus) and *Bacillus* sp. F62 + *Fusarium* spp. (Bac + Fus), in the antagonism through diffusible and volatile compounds. The mycelial growth inhibition (MGI, %) was determined on the last day of the experiment

Treatments	FusA97-11	FusP08-10	FusA06-18	Mean
		Antagonism through diffusible compounds		
Fus	10.8 ± 0.4 aA	10.4 ± 0.2 aB	10.7 ± 0.2 aAB	10.6 ± 0.3 a
Bac + Fus	6.5 ± 1.3 bA	5.5 ± 0.8 bA	6.1 ± 1.3 bA	6.0 ± 1.1 b
MGI (%)	39.8	47.1	30.4	39.1
		Antagonism through volatile compounds		
Fus	7.3 ± 0.1 aB	7.0 ± 0.2 aC	8.1 ± 0.2 aA	7.5 ± 0.2 a
Bac + Fus	6.7 ± 0.8 bAB	6.0 ± 0.6 bB	7.1 ± 0.5 bA	6.6 ± 0.6 b
MGI (%)	8.2	14.3	12.3	11.6

*Statistical analysis was performed separately in the antagonism through diffusible and volatile compounds.
**Equal lowercase letters indicate no statistically significant difference between the treatments (Fus and Bac + Fus) using t-test (P<0.05). Equal uppercase letters indicate no significant difference among the fungal isolates, using ANOVA followed by Tukey's test (P<0.05).

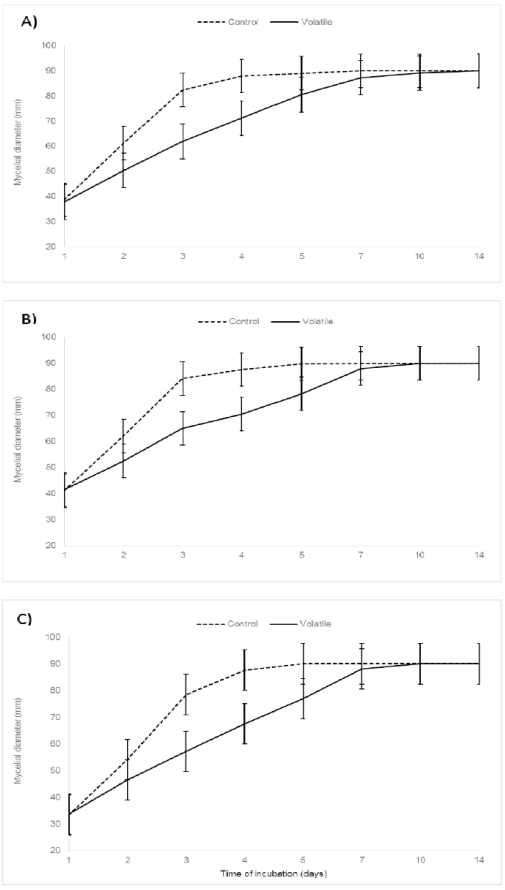

Fig. 2 - Mycelial growth of three *Fusarium* spp. isolates during 14 days of incubation in the antagonism assay through volatile compounds. A) FusA97-11, B) FusP08-10, and C) FusA08-16.

Fig. 3 - Morphology of colonies of *Fusarium* sp. isolate FusA06-18 in the antagonism assay with volatile compounds synthesized by *Bacillus* sp. F62 after 14 days of incubation. The control treatment is on the upper left side of the photograph.

Biocontrol on rootstock cuttings

The bioagent was applied for the biocontrol of *Fusarium* spp. isolate FusA06-18 in stem wounds of 'SO4' cuttings (Table 3). While the inoculation of *Bacillus* sp. F62 in wounds did not improve the growth promotion responses evaluated in rootstock cuttings, it reduced the frequency of the pathogen re-isolation from 81.7% in the Fus treatment to 63.3% in the Bac + Fus treatment (reduction of 22.5% in the Fusarium wilt incidence).

Table 3 - Morphophysiological responses in rootstocks cuttings of 'SO4': bud number (Budn), leaf number (Leafn), inflorescence number (Infln), shoot length (Shootl, cm), and frequency of pathogen re-isolation (FPR, %), subjected to the treatments: control, *Bacillus* sp. F62 (Bac), *Bacillus* sp. F62 + FusA06-18 (Bac + Fus) e FusA06-18 (Fus). The responses were assessed in two different periods: 30 and 60 days post-inoculation

Treatments	30 days post-inoculation				60 days post-inoculation				
	Bud number	Leaf number	Inflorescence number	Shoot length	Bud number	Leaf number	Inflorescence number *	Inflorescence number	FPR **
Control	0.5 ± 0.3	7.3 ± 0.9	2.3 ± 0.6	9.9 ± 4.4	0.6 ± 0.2	9.5 ± 2.3	1.9 ± 0.6	19.8 ± 7.2	-
Bac	0.6 ± 0.2	6.7 ± 1.3	2.2 ± 0.9	9.1 ± 2.5	0.6 ± 0.2	10.5 ± 1.6	1.9 ± 0.4	20.5 ± 4.2	-
Bac + Fus	0.6 ± 0.2	7.1 ± 1.9	2.2 ± 0.6	9.5 ± 2.9	0.6 ± 0.1	10.5 ± 1.3	1.70 ± 0.8	21.7 ± 5.2	63.3 ± 2.1 b
Fus	0.6 ± 0.1	6.9 ± 1.1	2.3 ± 0.9	10.3 ± 3.2	0.7 ± 0.1	10.3 ± 1.7	1.8 ± 0.5	22.7 ± 6.1	81.7 ± 1.5 a

* Different letters indicate statistically significant difference using ANOVA followed by the Tukey's test (P<0.05), except for inflorescence number (Infln) analyzed using the Kruskal-Wallis test followed by the Dunn-Bonferroni test (P<0.05).
** Frequency of pathogen re-isolation (FPR) was subjected to the Mann-Whitney U-test (P<0.05).

4. Discussion and Conclusions

The antagonism activity of the rhizobacterium *Bacillus* sp. F62 against *Fusarium* spp. was evaluated in two experiments, *in vitro* and *in vivo,* with vine cuttings of the rootstock 'SO4'. In the dual culture assay, the bioagent inhibited the growth rate of *Fusarium* spp. through the release of antimicrobial compounds and competition for space and nutrients. This finding is consistent with the observations of Nourozian *et al*. (2006), who reported that two strains of *B. subtilis* inhibited the mycelial growth of *Fusarium graminearum* by 97%. Similarly, Ziedan *et al*. (2010) found that seven strains of *Streptomyces* spp. exhibited notable antagonistic activity *in vitro* against *F. oxysporum*. Santos *et al*. (2016) also observed that a commercial product containing *B. subtilis* (Rizolyptus®) reduced the mycelial growth of six isolates of *Dactylonectria macrodidyma* by approximately 41%.

However, the volatile metabolites produced by *Bacillus* sp. F62 did not suppress fungal growth *in vitro*. This is in line with the findings of Nigris *et al*. (2018), who reported that *B. licheniformis* GL174 did not control the mycelial growth of *Phaeoacremonium aleophilum*, *Botryosphaeria* spp., and *Botrytis cinerea* through volatile compounds, while diffusible compounds inhibited the colonies growth by 60%. Likewise, Gao *et al*. (2018) observed that volatile molecules synthesized by *B. subtilis* CF-3 did not suppress the development of *Macrophoma kuwatsukai* and *Penicillium expansum*, causal agents of apple diseases. In contrast, Rocha and Moura (2013) observed that volatile compounds of *Streptomyces* sp. DFs1315 and *B. subtilis* reduced the colony diameter of *Fusarium oxysporum* f. sp. *lycopersici* by 18.1% and 17.5%, respectively.

Regarding the biocontrol potential of *Bacillus* sp. F62 against *Fusarium* sp., our experiments demonstrated a reduction in the percentage of pathogen re-isolation. Likewise, Haidar *et al*. (2016 a) reported that eight bacterial strains isolated from French vineyards effectively controlled *P. chlamydospora*, reducing the frequency of pathogen re-isolation from 31.4 to 38.7% compared to the control. Additionally, several bacterial strains, especially *Pantoea agglomerans*, significantly reduced the length of necrosis caused by *N. parvum* by 32.3% and 43.5% on grapevine cuttings (Haidar *et al*., 2016 b). Wicaksono *et al*. (2017) also observed that two isolates of *Pseudomonas* sp. inoculated onto wounds in grapevine cuttings cv. Sauvignon Blanc inhibited two botryosphaeriaceous species, *Neofusicoccum luteum* and *N. parvum*, and reduced lesion length caused by 32-52% compared to the untreated control.

Numerous studies have reported the ability of rhizobacteria to improve plant growth through nutrient solubilization, production of siderophores and phytohormones, such as auxins, gibberellins, and cytokinins (Olanrewaju *et al*., 2017). Rolli *et al*. (2017) tested the potential of fifteen rhizobacteria obtained from grapevines, olive trees, and pepper plants to enhance the growth of 'Syrah' grafted on '1103P' rootstock and 'Cabernet Sauvignon' grafted on 'SO4' rootstock in the field. The results demonstrated rapid colonization of the rhizoplane and root system of grapevine by the rhizobacteria. Moreover, bacterized plants showed longer shoots, larger diameters, and higher number of nodes on shoots.

In the current study, the application of *Bacillus* sp. F62 in artificially induced injuries did not increase plant growth of 'SO4' cuttings. Nevertheless, the inoculation of this same bacterium by soil drenching in cuttings of 'SO4' improved plant development by increasing the length of the primary shoot, the number of nodes in the primary shoot, and the total number of nodes (Russi *et al*., 2020). Wicaksono *et al*. (2017) evaluated the efficacy of two methods for bioagent inoculation: stem wounding and soil drenching. The authors found that *Pseudomonas* sp. colonized internal tissues of 'Sauvignon Blanc' cuttings when inoculated by wounding, but the bacterial proliferation failed when soil inoculated. As a result, plant morphological barriers and released toxins can prevent tissue colonization by some bacterial strains, reducing their effect in the phyllosphere (Balmer *et al*., 2012). According to Compant *et al*. (2010), tissue colonization is influenced by several factors, such as the pattern of plant exudates, nutrient availability, rhizobacteria growth rate, bacterial-host interactions, stress conditions, and plant genotype, which may explain the differences observed among these studies. Furthermore, the plant tissue inoculated and the phytopathogen strain can influence the antagonistic potential of rhizobacteria (Haidar *et al*., 2016 b).

In summary, this study demonstrated the effectiveness of *Bacillus* sp. strain F62 in controlling three isolates of *Fusarium* spp., inhibiting mycelial growth through volatile and diffusible compounds. Moreover, the rhizobacterium reduced the incidence of Fusarium wilt in 'SO4' vine cuttings that were

artificially infected with the pathogen. Consequently, *Bacillus* sp. F62 holds promising potential as a biocontrol agent for suppressing *Fusarium* spp. in susceptible vines.

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