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Bacillus sp. A8a reduces leaf wilting by *Phytophthora* and modifies tannin accumulation in avocado

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Objective / Background. The objective was to assess the biocontrol capacity of *Bacillus* sp. A8a in avocado (*Persea americana*) plants infected by *Phytophthora cinnamomi.*

Materials and Methods. A greenhouse experiment was implemented with four treatments: 1) control plants; 2) plants infected with *P. cinnamomi*; 3) plants inoculated with *Bacillus* sp. A8a; 4) plants infected with *P. cinnamomi* and inoculated with *Bacillus* sp. A8a. We evaluated several morpho-physiological variables during the experiment, which lasted 25 days after infection (dai). Moreover, we analyzed tannin density in stems at 25 dai to determine the plant defense response against the disease.

Results. Inoculation with strain A8a reduced wilting symptoms by 49 % at 25 dai, compared with non-inoculated plants. No differences were detected in morpho-physiological variables between treatments. However, a greater tannin accumulation was registered in the xylem of infected plants, whilst plants inoculated with strain A8a displayed a larger tannin density in the cortex.

Conclusion. Our results confirm the biocontrol activity of *Bacillus* sp. A8a in avocado plants and suggest that tannin differential accumulation in the cortex of plants inoculated with the bacteria may contribute to the enhanced tolerance of avocado plants against *Phytophthora* root rot.

Key words: Biocontrol; Chemical defense; *Persea americana*; *Phytophthora cinnamomi*; Water potential.

Introduction

Mexico is the main producer and exporter of avocado (*Persea americana*) in the world, with an annual production of nearly 2.4 million tons (FAOSTAT, 2020). Nationwide, Michoacán is the state with the highest production (SIAP, 2019). However, the incidence of pests and diseases has restricted the productivity of the crop (Tapia-Rodríguez *et al*., 2020). One of the most destructive diseases of avocado orchards worldwide is the wilting or root rot caused by the oomycete *Phytophthora cinnamomi* (Van den Berg *et al*., 2021). In Mexico, *P. cinnamomi* is found in the main avocado-producing areas, with an estimated incidence of 5 to 90 %, thus considered one of the main limiting factors of the production of this fruit (Agapito-Amador *et al*., 2022; Mondragón-Flores *et al*., 2022).

Phytophthora cinnamomi affects all avocado varieties and has a higher incidence in soils with poor drainage, due to its infection mechanism, which takes place through motile zoospores (Hardham, 2005) or chlamydospores (Hwang and Ko, 1978). *P. cinnamomi* causes necrosis in fine roots, thus limiting the uptake of water and nutrients, subsequently induces leaf wilting, and finally, leaf wilting, and finally, tree dieback (Hardham, 2005). Among the structural and/or biochemical defense responses of the host to the infection by *P. cinnamomi,* the accumulation of lignin or callose has been reported as a reinforcement of the cell wall, combined with the accumulation of phenolic compounds in roots and the production of reactive oxygen species (ROS) (van der Berg *et al*., 2018; Camisón *et al.*, 2019). In other hosts, the defense responses against *P. cinnamomi* include the accumulation of tannins (Camisón *et al*., 2019). However, further research on the defense mechanisms in *P. americana* varieties against the infection by *P. cinnamomi* are necessary to broaden our understanding of this pathosystem (van der Berg *et al*., 2021).

The management strategies of *Phytophthora* root rot usually include cultural practices aimed at reducing water saturation in soils, the use of pathogen-tolerant or resistant rootstocks and the use of fungicides (Belisle *et al*., 2019; Ramírez-Gil and Morales-Osorio, 2020). Factors such as the increase in the resistance of the phytopathogen to fungicides, as well as the restrictions surrounding the use of agrochemicals for the export of avocado, have favored the search for alternative, more eco-friendly control strategies (Cortazar-Murillo *et al*., 2023). In this context, the use of microorganisms as biological control agents of *P. cinnamomi* has gained increasing interest. Most of the available reports on the biocontrol of *P. cinnamomi* have focused on evaluating strains of *Trichoderma* spp., since these are fungi that

display different antagonism mechanisms (López-Herrera *et al*., 1999; Martins *et al*., 2022). Bacteria such as *Serratia* sp. ARP5.1 or *Pseudomonas fluorescens*, have also been suggested as promising biocontrol agents, due to the antagonistic activity of their metabolites against *P. cinnamomi* (Granada *et al*., 2018; Sumida *et al*., 2020). These previous reports show the potential of bacteria as biocontrol agents of *P. cinnamomi*.

In an earlier study, Méndez-Bravo *et al*. (2018) isolated the strain *Bacillus* sp. A8a from the rhizospheric soil of avocado. This strain displayed antagonistic activity against *P. cinnamomi* in direct antagonism tests and through the emission of volatile compounds. In addition, the crude extracts of *Bacillus* sp. A8a completely inhibited the growth of *P. cinnamomi* (Cortazar-Murillo *et al*., 2023). The plant growth promoting ability of *Bacillus* sp. A8a was confirmed in the model plant *Arabidopsis thaliana* and in tomatillo (*Physalis ixocarpa*) (Méndez-Bravo *et al*., 2018, 2023). Therefore, the aim of this study was to assess the biocontrol ability of *Bacillus* sp. A8a, through the evaluation of the effect of its inoculation on morpho-physiological variables and percentage of wilt on avocado plants infected by *P. cinnamomi*.

Materials and Methods

Biological material

Bacterial strain *Bacillus* sp. A8a, which was isolated from the rhizospheric soil of an avocado tree with Phytophthora wilt symptoms in an orchard in Huatusco, Veracruz (Méndez-Bravo *et al*., 2018), was used as a potential biocontrol agent. The pathogenic agent used in this study was the strain TGR-1-5 of *P. cinnamomi*, donated by the Plant Pathology Laboratory of the Instituto de Investigaciones Agropecuarias y Forestales of the Universidad Michoacana de San Nicolás de Hidalgo— and isolated from rotting avocado roots in an orchard in Tinguindín, Michoacán. Its virulence was determined in tests performed on *Nicotiana benthamiana* leaves (Mondragón-Flores, 2022).

Preparation of bacterial inoculant and of the *P. cinnamomi* zoospore suspension

To produce the bacterial inoculant, the strain *Bacillus* sp. A8a was streaked on Luria Bertani (LB) agar medium and incubated for 24 h at 26 °C. After this time, a loopful of bacterial biomass was suspended in sterile water. The concentration of the inoculant was adjusted to approximately 1.5×10^8 CFU mL^{-1}, according to the 0.5 standard of the McFarland scale.

The *P. cinnamomi* zoospore suspension was prepared as described by Zentmyer and Chen (1969) with slight modifications. Briefly, the *P. cinnamomi* TGR 1-5 strain was cultivated in Petri dishes with cornmeal agar for six days at 25 °C. Later, mycelial squares of approximately 0.5 cm^2 were cut, evenly distributed into two new Petri dishes and 10 mL of clarified V8 liquid culture medium (0.01 g of $CaCO_3$, 10 mL of Campbell's® V8 juice and 990 mL of distilled water) and incubated for 24 h at 25 °C. After this time, the V8 medium was discarded, the mycelium was washed with 10 mL of Zentmyer solution (1.64 g of $Ca(NO_3)_2$, 0.05 g KNO_3 and 0.48 g of $MgSO_4$ in 1 L of distilled water) and incubated for 30 min at room temperature under fluorescent light. The wash procedure with mineral solution was repeated six times. The mycelium was then incubated at 23 °C with exposure to fluorescent light for 10 h, followed by 8 h in the dark. To promote the release of zoospores, the salt solution was removed and the mycelium was washed with 10 mL of distilled water at 4 °C. Subsequently, water was added again and the mycelium – water mixture was incubated for 20 min at 4 °C. After this time, the water with *P. cinammomi* zoospores was recovered. Finally, the zoospores were stained with lactophenol blue (2 drops in 750 mL of solution) to be able to count them in a Nuebauer chamber and the concentration of the suspension was adjusted to 2.5×10^4 zoospores mL^{-1}.

Implementation of the greenhouse experiment

To evaluate the potential of *Bacillus* sp. A8a as a biocontrol agent against *P. cinnamomi,* 90 avocado plants of the Méndez variety, of approximately two years-old, were acquired from the "Piedras Blancas" nursery (Lagunillas, Michoacán, Mexico). The experiment consisted of four treatments: 1) 30 "control" plants, treated only with water [C]; 2) 30 plants infected with *P. cinnamomi* [Pc]; 3) 15 plants inoculated with *Bacillus* sp. A8a [B]; and 4) 15 plants infected with *P. cinnamomi* and inoculated with *Bacillus* sp. A8a [BPc] (Reverchon *et al*., 2023). On day 1, defined as the first day of the experiment (t0), half of the plants (n=45) were infected with the *P. cinnamomi* zoospore suspension, as described by Aveling (1999) and Masikane *et al.* (2019). First, the plants were retrieved from their grow bags and the excess soil was removed from their roots. Later, their root system was submerged for two hours in a suspension with a concentration of 4.9×10^2 zoospores mL^{-1}, which was prepared by diluting 1.6 L of the starting zoospore suspension in 80 L of drinking water. The roots from the other half of the plants were submerged in 80 L of drinking water with 1.6 L of distilled water (non-infected plants). Each plant was transplanted into a 10 L black plastic bag, with a substrate mixture of peat (PRO-MIX®)-perlite-vermiculite (ratio 3:1:1, v/v/v) sterilized at 121 °C for 1 h, with 1 volume of plant nursery soil. After 48 h, the plants were placed under

waterlogging conditions (substrate saturation) for five days to favor the infectious process of *P. cinnamomi.* Next, the excess water was drained and plants were watered with drinking water throughout the experiment to maintain the humidity of the substrate. *Bacillus* sp. A8a was inoculated 15 days before the infection of the plants with *P. cinnamomi*. For this, 30 plants were inoculated with 250 mL of the *Bacillus* sp. A8a suspension, applied on the base of the stem. Fifteen of these plants were assigned to treatment [B], and the other 15 plants, to treatment [BPc]. The bacterial suspension was applied again on the day after the infection (day 2) and once again 15 days later (day 16). The avocado plants were distributed randomly over a metal bed in the greenhouse of the Escuela Nacional de Estudios Superiores (National School for Higher Studies) of the UNAM – Morelia. The experiment was carried out in March and April, 2021, at an average temperature of 31 °C recorded at 11:00 am.

Measurement of morphological and physiological variables in avocado plants

The measurement of morphological and physiological variables was carried out at days 1 (t0), 4 (t1),7 (t2), 13 (t3) and 25 (t4) after inoculation (dai) with *P. cinnamomi*. The morphological variables that were recorded were height (cm), diameter (cm), and number of leaves, each measured from six individuals for treatments C and Pc, at each time; for treatments B and BPc, measurements were taken from three individuals. The percentage of wilting of each plant was calculated using the following formula:

$$\text{Wilting (\%)} = \frac{\text{No. of wilted leaves}}{\text{No. of total leaves}} \times 100$$

as reported by Wang *et al.* (2006) and Azil *et al*. (2021).

For physiological variables, three plants were measured per treatment. Stomatal conductance (mol $CO_2/m^2/s$), photosynthetic rate ($\mu mol/m^2/s$) and transpiration ($mmol/m^2/s$) were measured using the portable gas exchange system Li-Cor 6400 (LI-COR, Lincoln, NE, USA). The measurements were performed on two extended leaves with no apparent damage per selected plant, between 8:00 h and 11:00 h, with a light intensity of 1500 $\mu mol/m^2/s$ and environmental CO_2. The water potential (MPa) of the plant was measured at pre-dawn (5:00 h) and at mid-day (14:00 h) with a Scholander 1000 pressure chamber (PMS Instrument Co., Albany, OR, EE.UU.), in two leaves per plant, which were covered with aluminum foil half an hour before measuring to allow the water potential of the leaf and of the stem to become homogenized (Aguilar-Romero *et al.,* 2017).

Histochemical analyses of stem sections and determination of the presence of callose and tannins

To determine callose and tannins in the avocado stems at the end of the infection process (25 dai), a 1.5 cm-long fragment of stem was cut from the basal part of three plants per treatment using pruning shears. The fragments were processed following the methodology described in Johansen (1940). The fragments were fixed for 24 h in an FAA solution (70 % alcohol, formaldehyde and distilled water in a 5:5:90) ratio. After the fixation period, the FAA solution was removed and an ethanol-glycerol solution was added (1:1, v/v) to preserve the samples. Later, the samples were washed with tap water, dehydrated in increasing concentrations of ethyl alcohol (30, 50 and 70 %) and were passed through a series of increasing concentrations of tert-butyl alcohol (Sandoval, 2005). The stem fragments were placed in histological paraffin (Hycel) and 15-20 µm-thick cuts were made on the transverse and radial faces, using a rotary microtome (Leica RM2125 RTS). The sections were stained with a combination of 1 % tannic acid (in water); 3 % ferric chloride (in water) and 3 % lacmoid (in a 50 % ethanol and 50 % water solution), according to Gurr (1965). The sections were then washed with a 1% sodium bicarbonate solution (in 50 % ethanol), dehydrated in gradual concentrations of alcohol at 70, 80, 90 and 100 % and the tissues were clarified with absolute xylene. Finally, the sections were mounted on synthetic resin and observed under a compound microscope (Leica DM750M). The tannins were quantified using images captured with a 20× lens in bright-field microscopy. Images were taken of the transversal sections of avocado stems of the four treatments at 25 dai. The obtained images were analyzed using the Software ImageJ (https://imagej.nih.gov/ij/index.html).

Statistical analyses

The results were analyzed in RStudio, v. 2022.12.0. Data normality was verified using the Shapiro-Wilk test and the variance homogeneity, with the Bartlett test. The data obtained from the measurements of morphological and physiological variables, as well as from the quantification of the content of tannins, were analyzed using two-way and one-way analyses of variance, respectively, followed by Tukey's test for normally distributed data. The data that did not exhibit a normal distribution were analyzed using a Kruskal-Wallis test and a Wilcoxon rank-sum test with a Bonferroni-Holm adjustment. Results were considered significant with a $P \leq 0.05$.

Results

Changes in the morpho-physiological variables of avocado plants in response to the inoculation of *Bacillus* sp. A8a and infection by *P. cinnamomi*

Our results show that the inoculation of *Bacillus* sp. A8a in plants infected with *P. cinnamomi* (treatment BPc) reduced the percentage of wilting by up to 49 % at 25 dai, in comparison to infected plants that were not inoculated with the bacteria (treatment Pc) (Figure 1). In addition, wilting in plants infected with *P. cinnamomi* increased with time, going from 2.9 % (1 dai) to 50 % at the end of the experiment (25 dai). In contrast, the percentage of wilting decreased in infected plants inoculated with *Bacillus* sp. A8a throughout the experiment, going from 40.4 % at 13 dai to 7.1 % at 25 dai (Figure 1A). Nevertheless, these differences were not significant, most likely due to the intra-treatment variation.

No significant differences were observed in plant height and stem diameter between treatments for a given time, nor in the interaction between time and treatment (Figures 1B, C). Moreover, no significant differences were detected in the stomatal conductance, photosynthetic rate and transpiration between treatments, regardless of the time of measurement (Table 1). However, the water potential at 25 dai was significantly lower in plants infected with *P. cinnamomi* (treatments Pc and BPc) than in non-infected plants (treatments C and B), both in their measurement at 5:00 h (Figure 2A) and at 14:00 h (Figure 2B). No significant differences were observed between treatments Pc and BPc in terms of water potential (Figure 2).

Accumulation of tannins in avocado stems in response to the infection by *P. cinnamomi* and to the inoculation of *Bacillus* sp. A8a

The percentage of the area covered by tannins at 25 dai was quantified. The infection caused by *P. cinnamomi* tripled the area of tannins in the inner and outer xylem, compared to the control plants (Table 2). In contrast, the inoculation of *Bacillus* sp. A8a induced a greater accumulation of tannins in the cortex, although not significantly. The inoculation of *Bacillus* sp. A8a doubled the accumulation of tannins in the cortex of the uninfected plants (37.5 % in treatment B), and induced an increase of 16.9 % in the area of tannins in the cortex of infected plants (treatment BPc), in comparison with infected plants that were not inoculated with the bacteria. In addition, in infected plants, the inoculation of strain A8a reduced the accumulation of tannins in the inner and outer xylem (Table 2; Figure 3), displaying a shift in the accumulation pattern of tannins induced by the biocontrol strain. Finally, callose was not found in any of the analyzed tissues.

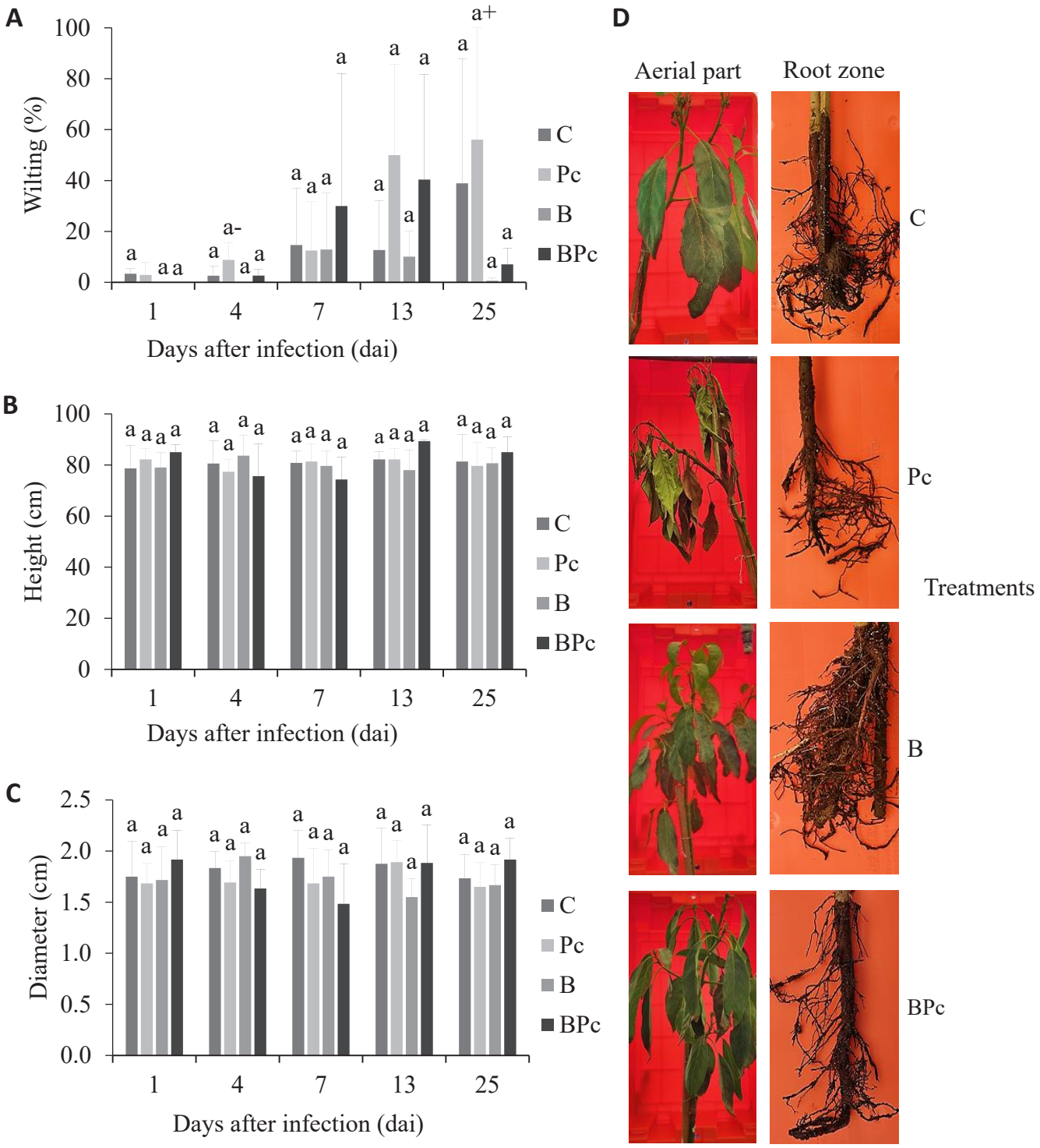
A
Wilting (%)
Days after infection (dai)
B
Height (cm)
Days after infection (dai)
C
Diameter (cm)
Days after infection (dai)
C
Pc
B
BPc
D
Aerial part
Root zone
C
Pc
B
BPc
Treatments

Figure 1. Effect of the inoculation of *Bacillus* sp. A8a and *P. cinnamomi* in avocado trees at 1, 4, 7, 13 and 25 dai (days after infection). A) Percentage of wilting caused by *P. cinnamomi*; B) tree height (cm); C) stem diameter (cm). Bars show the average of the data (n = 6, treatments C and Pc; n = 3, treatments B and BPc) ± s.d. Different letters indicate significant differences (two-way ANOVA, $P \leq 0.05$). In Figure 1A, (+) indicates significant differences between times, compared with (-), for the Pc treatment (ANOVA, $P \leq 0.05$). D) Representative photographs of the aerial parts and root systems of avocado trees at 25 dai, in the four treatments. C: control; Pc: infected with *P. cinnamomi*; B: inoculated with *Bacillus* sp. A8a; BPc: infected with *P. cinnamomi* and inoculated with *Bacillus* sp. A8a.

Table 1. Photosynthetic rate, stomatal conductance and transpiration in avocado trees infected with *P. cinnamomi* and inoculated with *Bacillus* sp. A8a.

Physiological variables	Treatments	Days after infection (dai)				
		1	4	7	13	25
Photosynthetic rate (µmol/m^2/s)	C	0.7 ± 1.1a	2.8 ± 4.4a	4.1 ± 3.6a	1.1 ± 0.6a	4.4 ± 1.9a
	Pc	0.7 ± 0.7a	1.8 ± 3.0a	0.9 ± 1.7a	1.5 ± 1.8a	3.7 ± 1.9a
	B	0.8 ± 1.1a	1.7 ± 2.7a	3.4 ± 2.8a	2.4 ± 2.0a	0.7 ± 1.0a
	BPc	1.0 ± 0.9a	2.1 ± 1.1a	4.3 ± 3.3a	1.8 ± 2.1a	1.8 ± 2.2a
Stomatal conductance (mol CO_2/m^2/s)	C	0.5 ± 0.5a	5.2 ± 6.9a	5.0 ± 4.5a	0.9 ± 0.4a	3.7 ± 2.2a
	Pc	0.3 ± 0.1a	2.9 ± 3.6a	1.1 ± 1.1a	1.3 ± 1.8a	2.8 ± 1.8a
	B	0.2 ± 0.1a	2.3 ± 2.4a	4.5 ± 3.8a	3.5 ± 4.6a	0.7 ± 0.8a
	BPc	0.4 ± 0.1a	2.0 ± 0.8a	3.6 ± 2.9a	1.4 ± 1.5a	2.0 ± 2.3a
Transpiration (mmol/m^2/s)	C	0.2 ± 0.2a	1.2 ± 1.6a	1.2 ± 1.0a	1.0 ±1.0a	1.1 ± 0.6a
	Pc	0.1 ± 0.0a	0.7 ± 0.8a	0.3 ± 0.3a	0.4 ± 0.1a	0.8 ± 0.5a
	B	0.0 ± 0.0a	0.6 ± 0.6a	1.4 ± 1.1a	1.6 ± 1.3a	0.2 ± 0.2a
	BPc	0.1 ± 0.0a	0.6 ± 0.2a	1.0 ± 0.8a	0.4 ± 0.4a	0.6 ± 0.6a

Values show the average of three replicates ± e.e. Stomatal conductance data are expressed as their original value multiplied by 100. Different letters indicate significant differences between treatments within each time (ANOVA, $P \leq 0.05$).

Discussion

Using a greenhouse time-course experiment, we found that the inoculation of *Bacillus* sp. A8a in avocado plants that were infected with *P. cinnamomi* reduced wilting symptoms by up to 49 %, 25 days after infection, in comparison with infected plants that were not inoculated with the bacteria. In previous studies, we reported the potential of *Bacillus* sp. A8a to inhibit *P. cinnamomi* in *in vitro* tests and its ability to produce volatile antimicrobial compounds from the group of pyrazines and ketones, as well as diffusible compounds such as macrolactin, difficidin, bacillaene and bacilysin (Méndez-Bravo *et al.,* 2018; Cortazar-Murillo *et al.,* 2023). This study helped verify the ability of strain A8a to control wilting by *Phytophthora* in its natural host, *P. americana*. Our results emphasize the potential

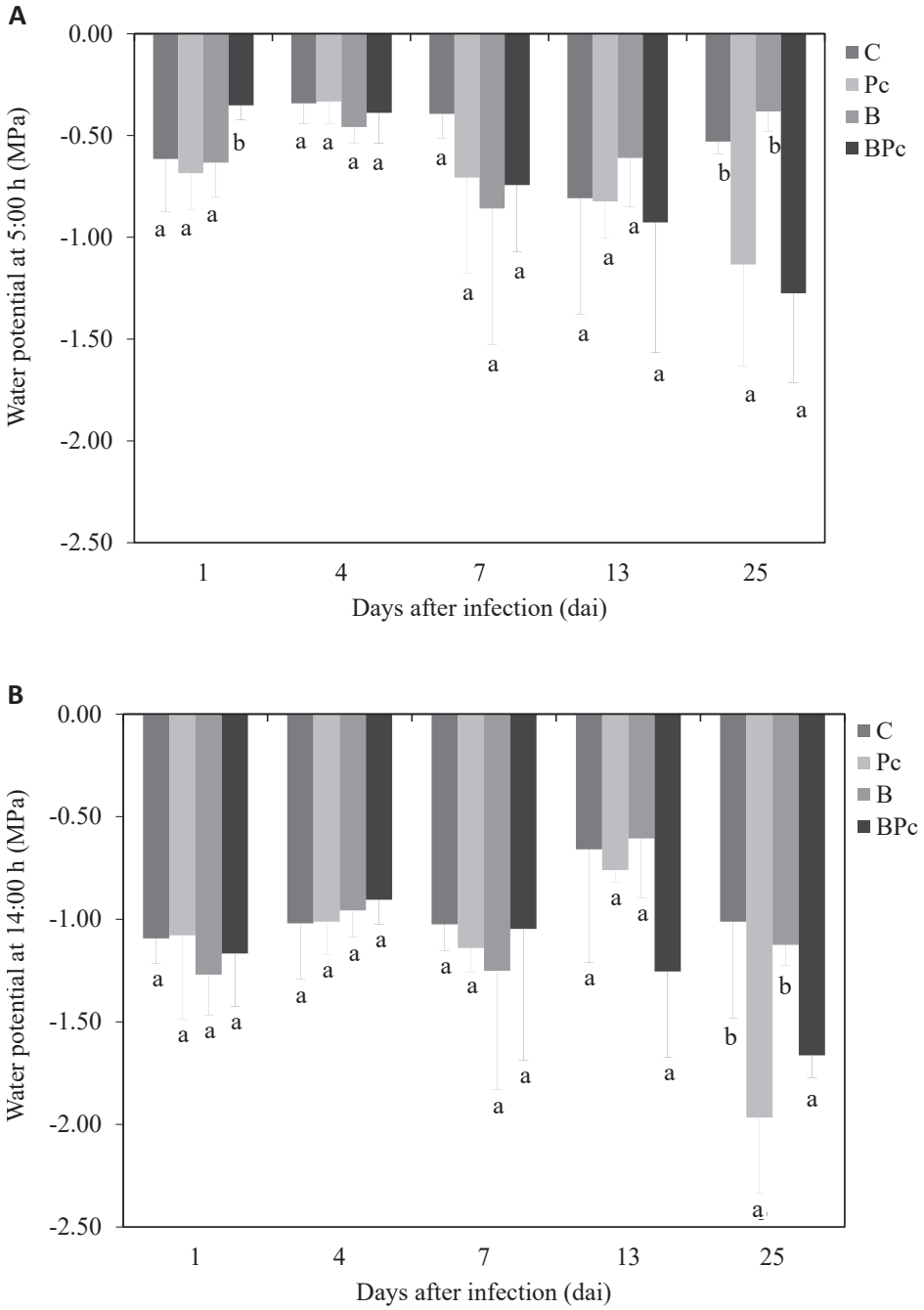

Figure 2. Leaf water potential in avocado trees at 1, 4, 7, 13 and 25 dai. A) Leaf water potential (MPa) at 5:00 am; B) foliar water potential at 14:00 pm. Bars show the average of the data (n = 3 ± s.d.). Different letters indicate significant differences between treatments at the same time. The water potential measured at 1, 4 and 25 dai was analyzed with a one-way ANOVA and Tukey's test ($P \leq 0.05$). Non-parametric data recorded at 7 y 13 dai were analyzed with Kruskal-Wallis and Wilcoxon rank sum tests ($P \leq 0.05$).

Table 2. Percentage of the area occupied by tanniniferous cells at 25 dai in different stem sections of avocado trees infected by *P. cinnamomi* and inoculated with *Bacillus* sp. A8a.

Treatments	Area occupied by tannins (%)		
	Cortex	Outer xylem	Inner xylem
C	17.9 ± 16.0a	16.5 ± 0.9a	16.0 ± 4.2a
Pc	24.5 ± 10.6a	55.0 ± 26.2a	50.3 ± 25.1a
B	37.5 ± 9.0a	26.1 ± 16.1a	17.1 ± 3.8a
BPc	41.4 ± 1.4a	15.1 ± 1.1a	27.4 ± 0.7a

Values represent the average of two measurements ± e.e. Different letters indicate significant differences between treatments for the same stem area (Kruskal-Wallis, $P \leq 0.05$).

of rhizobacteria to inhibit the growth of *P. cinnamomi* and reduce the damage caused by wilting by *Phytophthora,* confirming previous reports by Ramírez-Gil and Morales-Osorio (2020) with strain *Bacillus* sp. BC3 or by Ramírez-Restrepo *et al.* (2021) with *Serratia plymuthica* AED38. Although the reduction of the incidence of wilting symptoms in treatment BPc was not statistically significant, the high intra-treatment variation, regardless of the biocontrol activity of *Bacillus* sp. A8a, may have led to the lack of significant differences. This high variation may be related to the phenotypical plasticity that is usually found in avocado and in its rootstocks (Ashworth and Clegg, 2003), and may be controlled by increasing either the number of replicates or the duration of the experiment. A longer monitoring of the effects of strain A8a on inoculated plants is therefore necessary, since, as a woody plant, the responses of avocado to biological treatments aimed at improving its growth and productivity may take time to appear (Barra *et al*., 2016). In this case, it was not possible to extend the duration of the experiment due to the death of the plants in treatment Pc at 25 dai. *Phytophthora cinnamomi* attacks the feeder roots of the avocado trees, which are responsible for the absorption of nutrients and water, causing wilting, defoliation, loss of productivity and, ultimately, the death of the tree (Hardham, 2005; Méndez-Bravo *et al.,* 2018). These symptoms may be partly related to the development of a severe water stress in the infected plant. In this study, the infection by *P. cinnamomi* produced greater water stress in infected plants (Pc y BPc) than in uninfected plants (C and B) at 25 dai, in agreement with results obtained by Sterne *et al.* (1978). Interestingly, wilting in infected plants (Pc) was up to 49 % higher than that of infected plants which were inoculated with *Bacillus* sp. A8a (BPc), suggesting that strain A8a reduces the damage caused by *P. cinnamomi* without compensating the water stress. In addition, no significant differences were

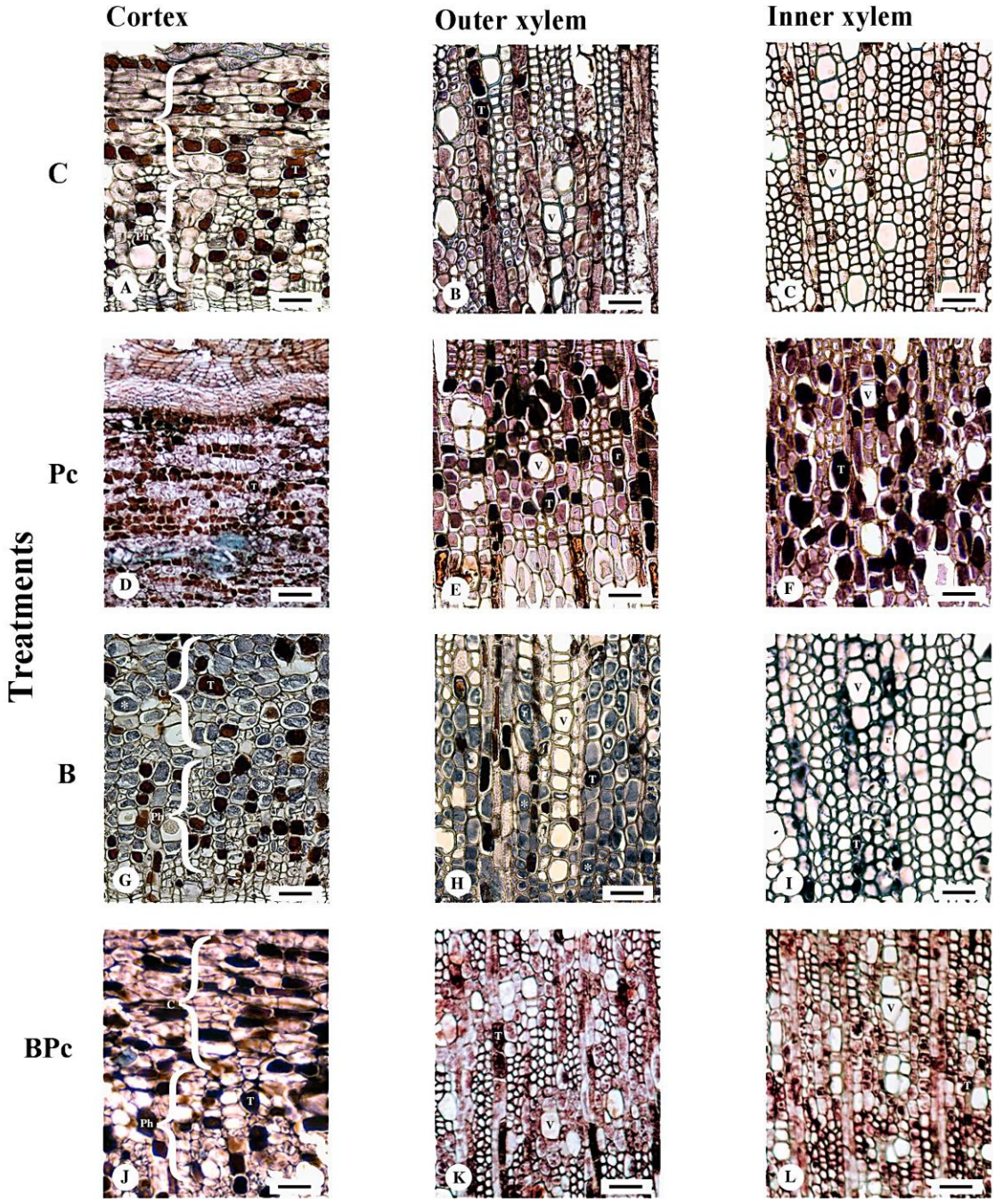
Cortex
Outer xylem
Inner xylem
Treatments
C
Pc
B
BPc
A
B
C
D
E
F
G
H
I
J
K
L
T
V
Ph
C

Figure 3. Tannin accumulation in cross-sections of the cortex (C), outer xylem and inner xylem of avocado stems from the four treatments at 25 dai. A – C: Control treatment. A. A few tanniniferous cells (T) are observed interspersed in the tissue. B. Tanniniferous cells (T) are observed in the ray parenchyma. Vessels (V) and rays (r) are indicated. C. A few tanniniferous cells (T) are observed in the axial parenchyma and in some radial parenchyma (r). Ph = secondary phloem. Scale: A - C. 30 µm. Figures D – F: treatment Pc. D. Abundant tanniniferous cells (T) are observed. E. Tanniniferous cells (T) are in the radial as well as in the axial parenchyma. V = vessels. F. Tanniniferous cells (T) are mostly concentrated in the radial cells. Scale: D. 60 µm. E and F. 30 µm. Figures G – I: treatment B. G. Cross-section through the secondary phloem (Ph) and cortex (C). Some tanniniferous cells are interspersed amid parenchyma cells full with starch (asterisks). H. A long radial chain of vessels (V) can be observed in the center of the image. Only a few tanniniferous cells (T) are observed in the radial parenchyma. I. Tanniniferous cells (T) are even scarcer than in the outer xylem. Scale: G. 25 µm. H and I. 30 µm. Figures J – L: treatment BPc. J. Tanniniferous cells (T) are observed. K. Grouped or solitary vessels (V) and some tanniniferous cells (T) are observed in the radial parenchyma. L. Few tanniniferous cells (T) are observed in the axial parenchyma. Vessels (V) are grouped in radial chains or in groups of four. Scale: J - L. 35 µm.

found in the stomatal conductance and transpiration, nor in the photosynthetic rate, between healthy and infected plants. Ploetz and Schafeer (1989) suggest that the effect of *P. cinnamomi* on CO_2 assimilation, transpiration and stomatal conductance in avocado trees is not consistent, unless another stress factor is added, such as waterlogged conditions, which consistently induces water stress in infected trees. The lowest values of the physiological variables were observed 7 dai in the Pc treatment (Table 1), although not significant, may confirm this hypothesis, since waterlogged conditions were maintained for five days in our experiment.

The infection by *P. cinnamomi* and the inoculation with *Bacillus* sp. A8a induced modifications in the accumulation of tannins in the different sections of the avocado stem at 25 dai. In the Pc treatment, the accumulation of tannins was observed in the outer and inner xylem. Phenolic compounds, such as tannins, play an important part in the plant defense mechanisms against biotic stress (Gallardo *et al.,* 2019), and are involved in the inhibition of extracellular fungal enzymes, the reduction in nutrient availability and the inhibition of oxidative phosphorylation (Rashad *et al*., 2020). The accumulation of tannins in the xylem in response to the infection by *P. cinnamomi* is probably part of the plant's defense and contributes to its resistance to wilting by *Phytophthora* (Phillips *et al*., 1987). On the other hand, plants inoculated with strain A8a (treatments B and BPc) presented a greater accumulation of tannins in the cortex than non-inoculated plants (treatments C and Pc, respectively). The accumulation of phenolic compounds in plant tissues as a positive effect of the inoculation of biocontrol agents has been shown in other pathosystems. For example, Javed *et al.* (2021) described that inoculation with *Bacillus megaterium* and *Pseudomonas fluorescens* increased the deposition of

phenolic compounds in vascular sections of stems and roots in mung bean plants (*Vigna radiata*) infected by *Macrophomina phaseolina*, which prevented the colonization of the mycelium in the lumen of the xylem vessels, unlike the plants not treated with these bacteria. The observation of a higher density of tannins in the histological cortex cross-sections from the treatments inoculated with *Bacillus* sp. A8a suggests an action of the biocontrol agent on one of the first physical barriers of the plant's defense and its possible contribution in the tolerance of the avocado plants against wilting by *Phytophthora*. However, future research should focus on elucidating the mechanisms that regulate the biocontrol capacity of strain A8a.

Conclusions

This work confirms the biocontrol activity of *Bacillus* sp. A8a against wilting by *Phytophthora*, which was previously reported *in vitro,* and suggests that this activity may be partly due to the differential accumulation of tannins in the cortex of the plants inoculated with the bacteria. Future studies may confirm the biocontrol potential of *Bacillus* sp. A8a in field conditions. Finally, to maximize its efficiency in the field, it is critical to corroborate the successful colonization of the *P. americana* rhizosphere by strain A8a and to improve our understanding of its interactions with the native microbiota in the avocado rhizosphere, in order to contribute to a more sustainable integrated management of wilting by *Phytophthora*.

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