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Biocontrol agents against *Penicillium digitatum* in 'pera' orange

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Abstract

This work aimed to evaluate the efficiency of biocontrol agents in controlling green mold (*Penicillium digitatum*) in 'pera' orange fruits. *In vitro* experiments were carried out with a fungus isolate grown on BDA medium and tested against different concentrations of commercial products based on biocontrol agents: *Trichoderma harzianum* (0, 5, 10, 15, 20 and 25 mL L^{-1}), *Bacillus subtilis* (0, 2, 4, 6, 8 and 10 mL L^{-1}) and *B. licheniformis* + *B. subtilis* + *T. longibrachiatum* (0, 5, 10, 15, 20 and 25 g L^{-1}) and the fungicide imazalil as standard (2 mL L^{-1}). The experiment analyzed mycelial growth and spore germination. Based on the results, *in vivo* tests were carried out, evaluating the curative and preventive effect of applying biocontrol agents as inoculating with 10 µL of conidial suspension (10^8 conidia mL^{-1}). All biochemical agents displayed 100% control over mycelial growth and a linear effect to inhibit the germination of *P. digitatum*. The *in vivo* tests highlighted that all agents showed a linear effect, both in the curative and preventive effects, significantly reducing the development of green mold (AUDPC) in 'pera' orange fruits. The experiment concludes that the prophylactic application of 25 mL L^{-1} of *T. harzianum*, 10 mL L^{-1} of *B. subtilis,* and 25 g L^{-1} of *B. licheniformis* + *B. subtilis* + *T. longibrachiatum* in orange fruits 'pera' control *P. digitatum*.

Keywords: citrus farming, citrus, biological control, orange

Introduction

Brazilian citrus farming has great relevance in the world economy, being the largest producer of citrus and the largest exporter of concentrated orange juice (Chagas et al., 2018; IBGE, 2020). As a result of the COVID-19 pandemic, eating habits changed, increasing the *per capita* consumption of fresh citrus fruits and orange juice in 2020, with a tendency to continue increasing consumption by around 40% per month (CITRUS BR, 2020).

The post-harvest quality is the primary factor for the fruits' commercialization. Attributes such as appearance, flavor, aroma, texture, and nutritional value directly reflect consumer acceptance (Chagas et al., 2018). The green mold caused by the fungus *Penicillium digitatum* (Pers.: Fr) Sacc. is among the most critical post-harvest diseases (Dukare et al., 2018; Carmona-Hernandez et al., 2019).

The fungus *P. digitatum* is widely disseminated in all producing regions, being responsible for around 90% of total losses in citrus fruits (Benato et al., 2018; Pétriacq et al., 2018; Bazioli et al., 2019). Preventive and curative measures are required due to the aggressiveness of the fungus and its potential to cause losses, and to avoid or eliminate the incidence and severity of *P. digitatum* in fruits.

Its primary control method is still the application of systemic fungicides, such as imazalil (Costa et al., 2019). However, several studies have revealed the emergence of strains of *P. digitatum* resistant to the main fungicides, in addition to the severe implications that these products represent for the environment, animals, and humans (Ferraz et al., 2018; Pétriacq et al., 2018).

Among the most promising strategies is the use of biocontrol involving beneficial microorganisms in the control of diseases in the pre- and post-harvest of fruits (Dukare et al., 2018; Boffette et al., 2018; Carmona-

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Hernandez et al., 2019; Tian et al., 2020).

In citrus farming, the use of antagonistic microorganisms, such as those of the genus *Trichoderma* spp. and *Bacillus* spp., revealed positive results in the control of phytopathogens in citrus farming (Cunha & Kupper, 2018; Hussain, 2018; Cunha et al., 2018; Ahima et al., 2019; Tian et al., 2020). In general, research has emphasized the efficiency of *T. harzianum* and *B. subtilis* in the biocontrol of pre-harvest diseases, but little is known about the effectiveness of these agents in post-harvest rot, especially of *B .licheniformis + B. subtilis + T. longibrachiatum* in the biocontrol of *P. digitatum* in 'pera' orange fruits. Therefore, this work aimed to evaluate biocontrol agents' efficiency in controlling green mold (*Penicillium digitatum*) in 'pera' orange fruits.

Material and methods

Biocontrol agents and pathogen isolate

The experiment used three commercial products: Trichodermil® (2x10^9 viable *Trichoderma harzianum* spores mL $^{-1}$), Serenade® (1x10^9 UFC g^{-1} of the *Bacillus subtilis* strain QST 713 bacteria), and Nem Out™ (3.75 x 10^8 UFC g^{-1} of the mixture of *B. licheniformis, B. subtilis*, and *T. longibrachiatum*).

The biocontrol agents were incorporated into the melting BDA culture medium (50 °C) at concentrations of 0, 5, 10, 15, 20, and 25 mL L^{-1} for *Trichoderma harzianum,* 0, 2, 4, 6, 8, and 10 mL L^{-1} for *Bacillus subtilis*, and 0, 5, 10, 15, 20, and 25 g L^{-1} for *B. licheniformis + B. subtilis + T. longibrachiatum*. The fungicide imazalil (2 mL L^{-1}) was the positive control.

P. digitatum was used on 'pera' orange from the commercial orchard. The fungus was cultivated on BDA medium (potato dextrose agar) and incubated in a BOD-type growth chamber at 25 °C, alternating 12-hour photoperiod, for seven days to keep the isolated strain alive.

In vitro tests: mycelial growth and spore germination

The pairing tests were performed in duplicate through direct comparison according to Campanile et al. (2007), calculating the antagonism index. The biocontrol agents were poured into Petri dishes (90 mm), and after two hours, in the center of each Petri dish, a disc (6 mm) of the pathogen colony that had been growing for seven days was placed, leaving the fungal structures in contact with the culture medium. Then, the plates were placed in a BOD-type growth chamber at 25 °C, with a 12-hour photoperiod, for seven days.

Mycelial growth was assessed daily by measuring the colony's mycelial radius (mm) using a digital caliper (two orthogonal measurements), calculating the average values per plate. The values obtained were used to calculate the antagonism index (IA): IA% = [(RM - rm) x100] RM, where: IA= Antagonism index in percentage; RM= Mycelial radius without the presence of the antagonist (control); rm = Mycelial radius with the antagonist. The experimental design was completely randomized, with 17 treatments and 20 replications. Each experimental plot consisted of a Petri dish.

The potential of the biocontrol agents on spore germination was assessed by preparing spore suspensions of the pathogen obtained from pure colonies of the *P. digitatum* isolate. These suspensions were cultivated in BDA medium with distilled water + Tween ^{20}, scraping the Petri dish with a Drigalski loop and filtering through gauze. The suspension was calibrated to 10 ^{4} spores mL^{-1}, with Neubauer chamber in an optical microscope. Aliquots of 40 µL of the spore suspension were pipetted into individual wells of Elisa test plates, where 40 µL of each treatment and their respective concentrations were also placed.

Spore germination was stopped with 20 µL of lactophenol after an incubation period of 26 hours, at 25 °C, and evaluated by counting 50 spores per cavity with subsequent calculation of the germination percentage. Spores that presented germ tube emission were considered germinated, regardless of their size. Data were expressed as a percentage of spore germination in relation to total spores.

In vivo tests: Obtaining plant material and spore suspension

The *in vivo* tests used 'pera' orange fruits from a commercial orchard at a stage of ripeness suitable for consumption. The fruits were selected and standardized and then washed with neutral detergent (0.2%), sanitized with sodium hypochlorite (NaClO) 0.1% for 10 minutes, with subsequent rinsing in running water and then in distilled water, being allocated on the bench at room temperature until completely dry.

The suspension of pathogen spores was obtained as previously described in the *in vitro* tests but was calibrated to 10^8 spores mL^{-1} by a Neubauer chamber in an optical microscope.

Biocontrol agents' curative effect assessment

Fruits were inoculated with a suspension of *P. digitatum spores* (10^8 spores mL^{-1}) by applying an aliquot of 10 µL in a 2 mm deep wound in the previously demarcated fruit epidermis to evaluate the curative effect. After four hours of inoculation, the fruits were immersed in the treatments for 2 minutes at the same

concentrations as the *in vitro* test.

The fruits were immersed for 2 minutes in the treatments and then dried for four hours on a bench covered with Kraft® paper to evaluate the preventive effect. After drying, a 100 µL syringe performed a 2 mm deep wound in the previously demarcated epidermis, which was later inoculated with ten µL of *P. digitatum* spore suspension (10^8 spores mL^{-1}).

The fruits were stored at 25 °C (± 2 °C) and 80% (± 5%) RH, for seven days to evaluate the curative and preventive effects. During this period, the fruits from the control treatment remained wholly overtaken by green mold growth.

Five assessments of the diameter of the lesions on 'pera' orange fruits were carried out using a digital caliper to assess the severity. The data obtained were used to calculate the area under the disease progress curve (AUDPC) using the equation AUDPC = Σ $[(Y\ i_{+1} + Y_i)/2]$ $[T_i +1 - T_i]$ where Y_{i+1} = diameter of the lesion at time T_{i+1} and Y_i = diameter of the lesion at time T_i (Campbell & Adden, 1990).

The experimental design was completely randomized, with five replications consisting of four fruits as an experimental unit and carried out in duplicate. The data obtained were submitted to homogeneity, normality, and variance analysis (F test), and the means were compared by the Tukey test at 1% and 5% probability. The regression analyses were carried out at 1% and 5% probability using the Sisvar program.

Results and Discussion

In vitro tests

All biocontrol agents tested induced a 100% inhibition of *P. digitatum* mycelial growth at all concentrations evaluated (**Figure 1**). The concentrations of *T. harzianum, B. subtilis,* and *B. licheniformis + B. subtilis + T. longibrachiatum* were efficient and equal to the control observed for the fungicide, with all treatments differing from the control ($p<0.01$).

The spores' germination of *P. digitatum* displayed a linear effect ($p<0.01$) depending on the doses for all biocontrol agents (**Figure 2**), and there was a difference among the concentrations, the control, and imazalil treatments. The treatments with biocontrol agents significantly reduced the germination of *P. digitatum* spores. The fruits treated with *B. licheniformis + B. subtilis + T. longibrachiatum* (25 g L^{-1}) displayed the most significant controls, followed by *T. harzianum* (25 mL L^{-1}) and *B. subtilis* (10 mL^{-1}), showing germination inhibition of 94%, 88%, and 85% respectively, when compared to the control. The fungicide inhibited 100% spore germination.

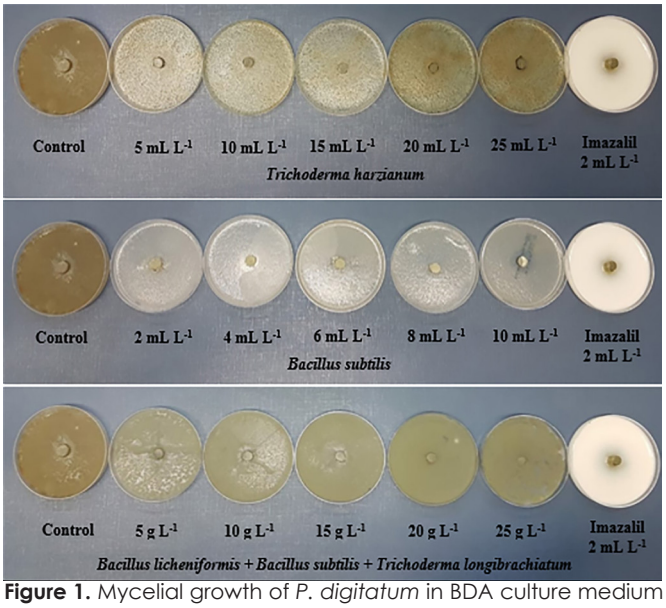

Figure 1. Mycelial growth of *P. digitatum* in BDA culture medium containing concentrations of *T. harzianum*, *B. subtilis*, and *B. licheniformis + B. subtilis + T. longibrachiatum*, and the active agent imazalil as a positive standard control.

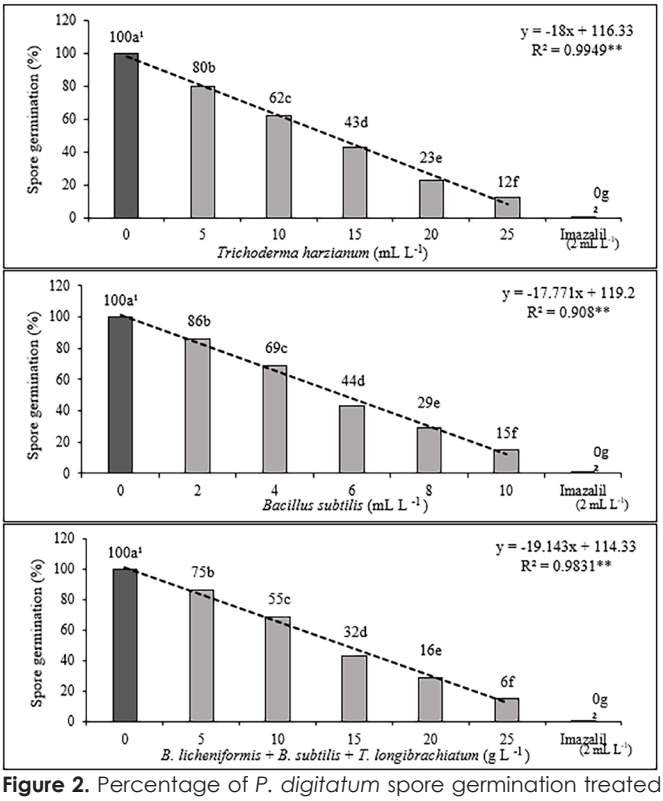

Figure 2. Percentage of *P. digitatum* spore germination treated with *T. harzianum* , *B. subtilis* and *B. licheniformis + B. subtilis + T. longibrachiatum* . Means followed by equal letters do not differ according to the Tukey test at the 1% probability level ($p<0.01$). ¹Control and ²Positive control.

In vivo tests: Curative and preventive control of P. digitatum in 'Pera' orange fruits

Regarding the incidence of *P. digitatum*, all fruits displayed disease symptoms except for the imazalil (fungicide) treatment. The symptoms started two days after inoculation. Regardless of the biocontrol agent

tested, the disease developed. However, when evaluating the severity (AUDPC), the presence of biocontrol agents linearly reduced the progress of the disease compared to the control in both the curative and preventive forms of treatment application (**Figure 3**).

3) highlighted that the preventive effect presented better performance to control *P. digitatum* in 'Pera' orange fruits. For all concentrations of *T. harzianum* there was a difference between curative and preventive effects. In both treatments (curative and preventive), the 25 mL L^{-1}

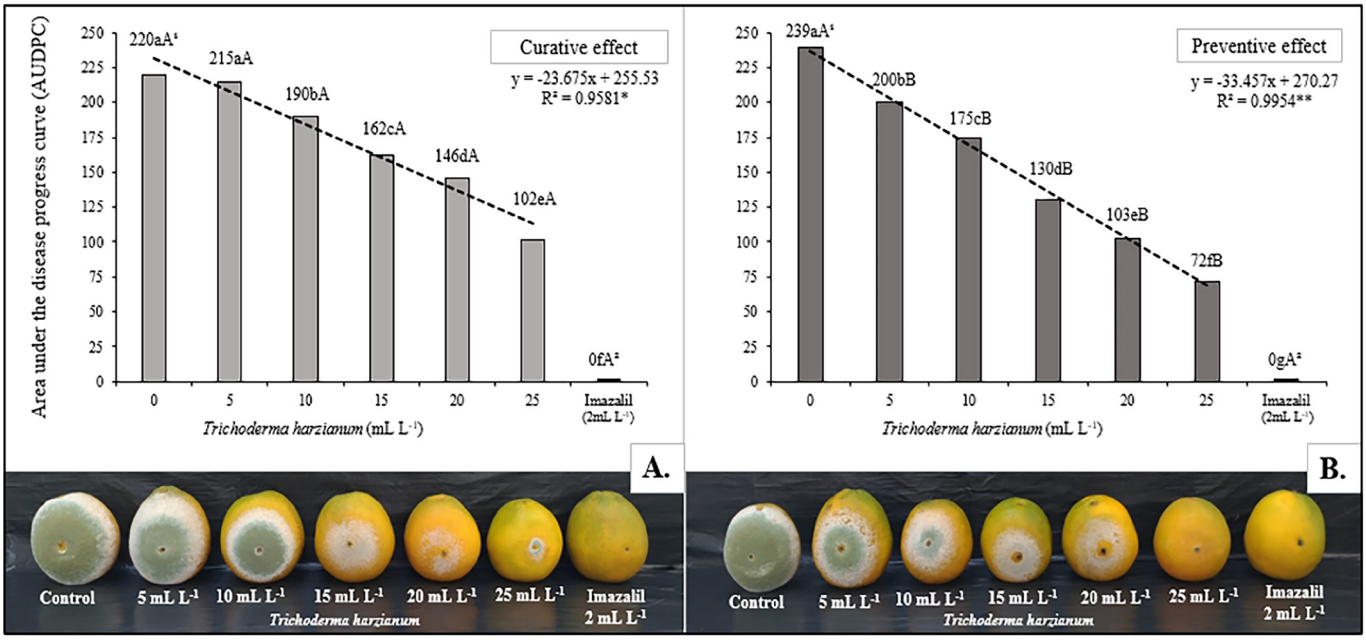

Figure 3. Area under the disease progress curve (AUDPC) for the severity of green mold in 'pera' orange fruits inoculated with *P. digitatum* treated with concentrations from *T. harzianum*, to analyze curative (A) and preventive (B) effects. Means followed by equal lowercase letters do not differ between treatments for the same effect, and equal capital letters do not differ between curative and preventive effects (Tukey : *p<0.05 and **p<0.01). ¹Control; ²Positive standard (fungicide).

The curative effect of applying *T. harzianum* (Figure 3A) at a concentration of 5 mL L^{-1} was not efficient in controlling green mold on 'Pera' orange, not differing from the control. However, from a concentration of 10 mL L^{-1} there was a significant effect in inhibiting the *P. digitatum* development, showing a linear reduction in severity, reaching reduction percentages of 13.6%, 26.4%, 33.6%, and 53.6% at concentrations of 10, 15, 20, and 25 mL L^{-1}, respectively when compared to the control. Concentrations between 10 and 25 mL L^{-1} differed from each other and differed significantly also from the control and imazalil treatments.

The preventive effect of *T. harzianum* application (Figure 3B) displays a response similar to that presented by the curative effect, with a linear reduction depending on doses. Even though, in this case, the control was efficient at all concentrations, which displayed significant differences between them and from both control and imazalil treatments. The effect on green mold control caused a reduction in severity by 16.3%, 26.8%, 45.6%, 56.9% and 69.8% at concentrations of 5, 10, 15, 20, and 25 mL L^{-1}, respectively.

The comparison between the curative and preventive effects of *T. harzianum* concentrations (Figure concentration promoted the most significant reduction in the severity of *P. digitatum*. However, the reduction was even more significant in the preventive (69.8%) than in the curative effect (53.6%) when compared with their respective controls.

The curative effect applying *B. subtilis* (**Figure 4**A) showed a response similar to that observed for treatment with *T. harzianum*, with a linear reduction depending on doses. The concentration of 2 mL L^{-1} was inefficient in controlling green mold, not differing from the control. However, from a concentration of 4 mL L^{-1} there was a significant effect on inhibiting the development of *P. digitatum*, showing a reduction in severity of 13.8%, 22.4%, 31.9%, and 47.8% at concentrations of 4, 6, 8, and 10 mL L^{-1}, respectively, when compared to the control. Such concentrations differed from each other and both the control and imazalil treatments.

The preventive effect of treatment with different concentrations of *B. subtilis* (Figure 4B) displayed a response similar to that presented by the curative effect, with a linear reduction depending on the doses. Still, in this case, there was control efficiency for all concentrations evaluated, presenting significant differences between them and differing from the control and imazalil

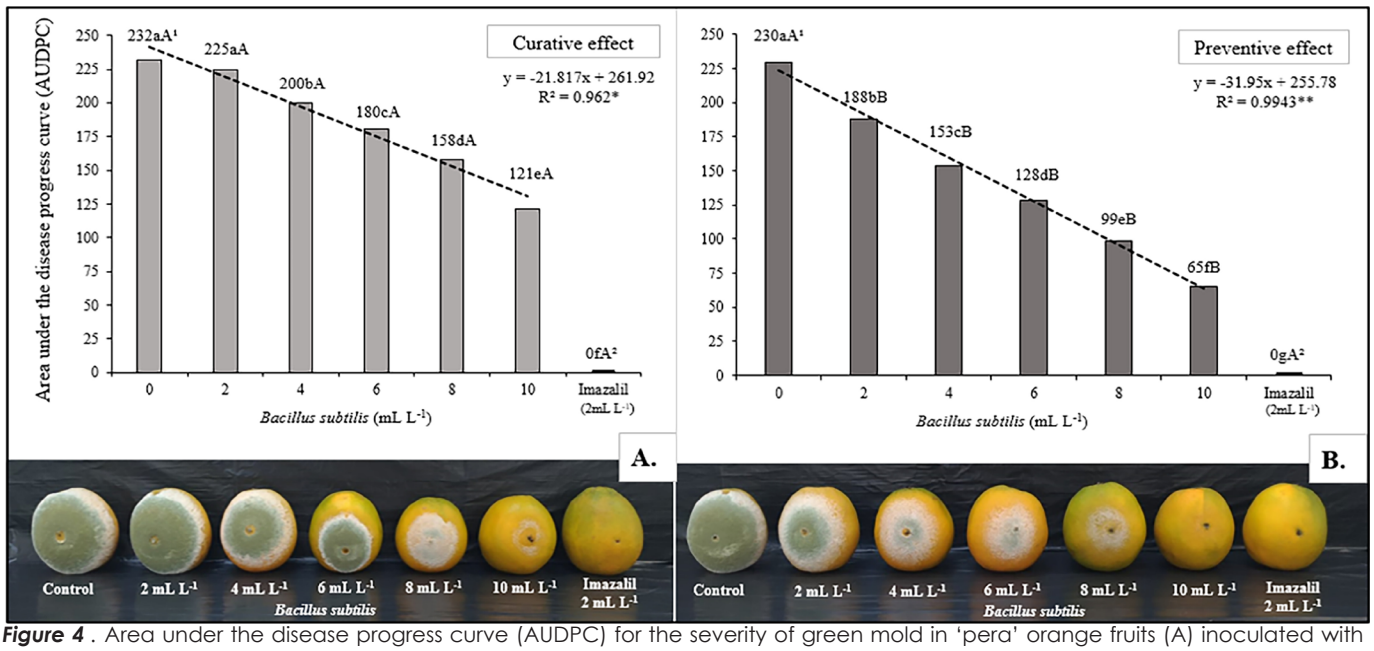

Figure 4 . Area under the disease progress curve (AUDPC) for the severity of green mold in 'pera' orange fruits (A) inoculated with *P. digitatum* treated with concentrations of *B. subtilis* to analyze curative (A) and preventive (B) effects. *Means followed by equal lowercase letters do not differ between treatments for the same effect, and equal capital letters do not differ between curative and preventive effects (Tukey : *p<0.05 and **p<0.01). ¹Witness; ²Positive standard (fungicide).*

treatments. The effect on the control of green mold on 'Pera' orange reduced the severity by 18.3%, 33.8%, 44.3%, 57.0%, and 71.7% at concentrations of 2, 4, 6, 8, and 10 mL L^{-1}, respectively.

The comparison between the curative and preventive effects of *B. subtilis concentrations* (Figure 4) highlighted that the preventive effect also presented the best performance for controlling *P. digitatum* in 'pera' orange fruits. For all concentrations of *B. subtilis* there was a difference between curative and preventive effects.

In both application effects (curative and preventive) (Figure 4), the 10 mL L^{-1} *B. subtilis* concentration significantly reduced the *P. digitatum* severity. However, in the preventive effect, the reduction was more significant (71.7%) than in the curative effect (47.8%) when compared with their respective controls.

Both curative and preventive effects of applying *B. licheniformis* + *B. subtilis* + *T. longibrachiatum* (Figures 5A and 5B) presented a response similar to that observed for the *T. harzianum* and *B. subtilis* treatments. Besides, the 5 g L -1 concentration was also inefficient in curing green mold on 'Pera' orange, not differing from the control. However, from a concentration of 10 g L^{-1}, there was a significant effect on inhibiting the *P. digitatum* development, showing a severity reduction of 13.0%, 22.2%, 31.3%, and 47.4% at concentrations of 10, 15, 20, and 25 g L^{-1}, respectively, when compared to the control. Such concentrations differed from each other, the control, and imazalil treatments.

The preventive effect of treatment with *B. licheniformis* + *B. subtilis* + *T. longibrachiatum* also had control efficiency for all concentrations evaluated, presenting significant differences between them and differing from the control and imazalil treatments. Again, there was a significant effect on the control of green mold on 'Pera' orange, with a reduction in severity of 17.4%, 31.5%, 52.3%, 61.8%, and 75.5% at concentrations of 5, 10, 15, 20 and 25 g L^{-1}, respectively.

The comparison between the curative and preventive effects of *B. licheniformis* + *B. subtilis* + *T. longibrachiatum* (**Figure 5**)highlighted that the preventive effect presented the best performance for controlling *P. digitatum* . In both treatments (curative and preventive), the concentration of 25 g L^{-1} of *B. licheniformis* + *B. subtilis* + *T. longibrachiatum* promoted the most significant reduction in the severity of *P. digitatum*. However, the decline was more significant in the preventive effect (75.5%) than in the curative effect (47.4%) compared to their respective controls.

Analyzing the curative and preventive effect of *T. harzianum*, *B. subtilis*, and *B. licheniformis* + *B. subtilis* + *T. longibrachiatum*, verified that the preventive effect promoted the most significant green mold severity reduction on fruits in the tests carried out, and determined that the best concentration of each treatment was higher: 25 mL L^{-1}, 10 mL L^{-1}, and 25 g L^{-1}, respectively. The analyses also depicted a significant interaction ($p<0.05$) between the two sources of variation: treatment and application effect (**Table 1**).

The analysis of each effect separately revealed

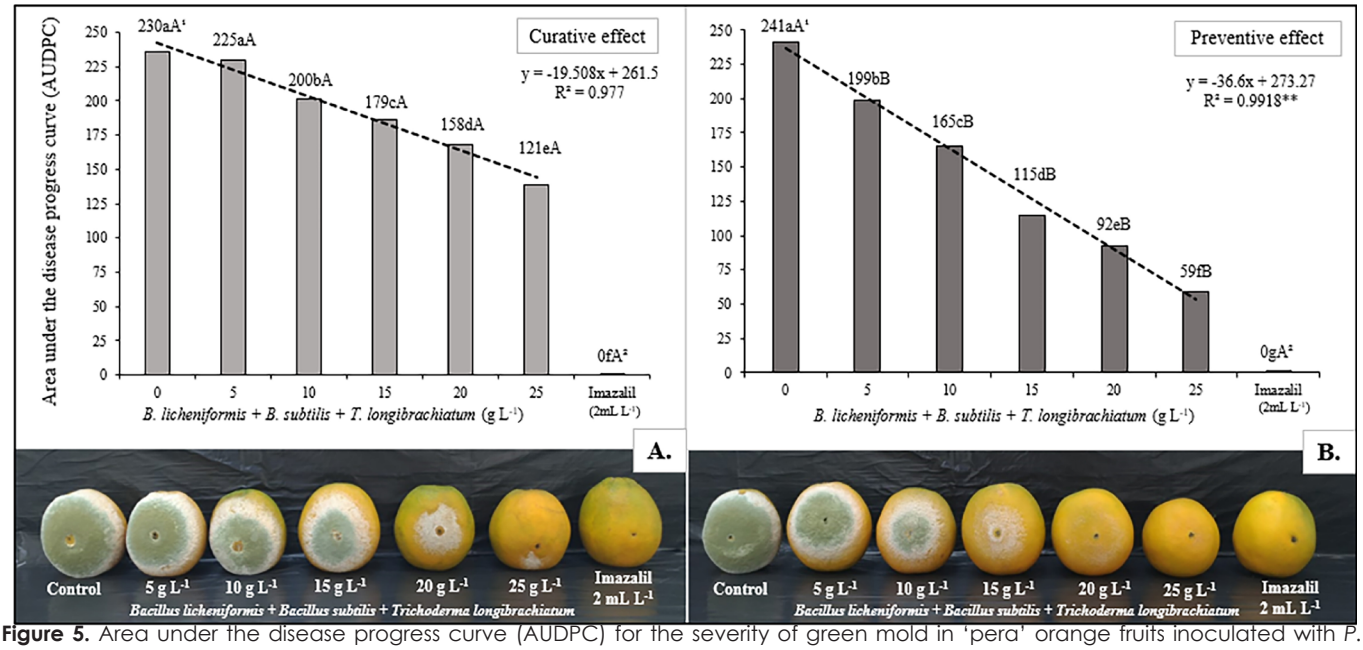

Figure 5. Area under the disease progress curve (AUDPC) for the severity of green mold in 'pera' orange fruits inoculated with *P. digitatum* for analysis of curative effect (A) and preventive effect (B) treated with concentrations of *B .licheniformis* + *B. subtilis* _ + *T. longibrachiatum*. Means followed by equal lowercase letters do not differ between treatments for the same effect, and equal capital letters do not differ between curative and preventive effects (Tukey : *p<0.05 and **p<0.01). ¹Witness; ²Positive standard (fungicide).

that the significant curative effect of the biocontrol agents. Applying *T. harzianum* on the fruits differed from other biocontrol agents, as it presented the lowest AUDPC. Treatments with *B. licheniformis* + *B. subtilis* + *T. longibrachiatum*, and *B. subtilis* , did not differ from each other and were less efficient in reducing AUDPC than *T. harzianum*. For the preventive effect, the treatments with *B. licheniformis* + *B. subtilis* + *T. longibrachiatum* and *B. subtilis* displayed the lowest AUDPC.

The comparison among the forms of treatment application stressed a significant difference between the curative and preventive application of treatments. The preventive effect of applying *T. harzianum, B. subtilis*, and *B. licheniformis* + *B. subtilis* + *T. longibrachiatum* reduced disease severity significantly compared to the curative effect. Prophylactic application of 25 mL L^{-1} of *T. harzianum,* 10 mL L^{-1} of *B. subtilis,* or 25 g L^{-1} of *B. licheniformis* + *B. subtilis* + *T. longibrachiatum* reduced AUDPC by 29%, 46% and 51%, respectively, when compared to the curative application (Table 1).

Biocontrol agents showed different control responses depending on the application form, with the highest AUDPC in fruits treated curatively. The joint use of *B. licheniformis* + *B. subtilis* + *T. longibrachiatum* provided the best control of *P. digitatum,* followed by treatments with *T. harzianum* and *B. subtilis* , when applied preventively. Given these findings, it was possible to verify that the behavior of biocontrol agents on *P. digitatum* depends on the application form (curative or preventive) on the fruits.

The use of antagonistic microorganisms in citrus farming has emerged as a potential alternative to fungicides. Several studies show positive effects with the application of biocontrol agents in the suppression of phytopathogens, especially those related to the post-harvest stage, such as *P. digitatum* and *P. italicum* (Kupper et al., 2012; Ferraz et al., 2018; Hussain, 2018; Cunha et al., 2018; Ahima et al., 2019; Tian et al., 2020). To date, such research has emphasized the efficiency of *T. harzianum* and *B. subtilis* in the biocontrol of post-harvest diseases. However, little is known about their effectiveness post-harvest, especially *B. licheniformis* + *B. subtilis* + *T. longibrachiatum* in the biocontrol of *P. digitatum* in 'Pera' orange fruits.

Table 1. Comparison of the average severity (AUDPC) of green mold in 'pera' orange fruits treated post-harvest with different biocontrol agents to evaluate the curative and preventive effects

Treatments	'Pera' orange fruit	
	Curative effect	Preventive Effect
T. harzianum (25 mL L^{-1})	102.0bA	72.0aB
B. subtilis (10 mL L^{-1})	121.0aA	65.0bB
B. licheniformis + *B. subtilis* + *T. longibrachiatum* (25 g L^{-1})	121.0aA	59.0cB
CV (%)	9.6	8.4

Means (n=40) followed by equal lowercase letters in the columns do not differ between treatments for the same effect, equal capital letters in the lines do not differ between curative and preventive effects for the same treatment, using the Tukey test at 5% probability.

According to the results obtained in the *in vitro tests,* biocontrol agents *T. harzianum, B. subtilis*, and *B. licheniformis* + *B. subtilis* + *T. longibrachiatum,* completely inhibited *P. digitatum*, with all concentrations evaluated being as efficient as the fungicide imazalil. Studies indicate that this finding relates to the main mechanisms of action of the genera Trichoderma and Bacillus (Silva et al., 2019; Tian et al., 2020).

As seen for *P. digitatum,* species of *Trichoderma* spp., such as *T. harzianum* and *T. longibrachiatum*, offer potential control over phytopathogens, as they present different modes of action on the aggressor, such as competition for nutrients and space, antibiosis and mycoparasitism (Gajera et al., 2013; Di Francesco et al., 2016; Dukare et al., 2018; Hussain, 2018). Isolates of *Trichoderma* spp. were also effective against the fungus P. digitatum under in vitro conditions. In all results, the isolates proved their antagonistic capacity in double culture techniques and culture filtrate assays (Mishra et al., 2011; Hussain, 2018).

Antagonistic bacteria of the genus *Bacillus*, such as the species *B. subtilis* and *B. licheniformis,* are efficient in competing for nutrients and space, synthesizing antibiotics and other compounds (antibiosis), acting through mycoparasitism, among other potential antagonistic mechanisms, such as the formation of biofilms, quorum sensing, and siderophores (Carmona-Hernandez et al., 2019). Therefore, these species effectively control *P. digitatum* and other pathogens.

Several studies have revealed positive results from the use of *Bacillus* spp. isolates in citrus farming, such as the studies by Chen et al. (2018) and Tian et al. (2020), who verified that the application of cell-free supernatant (CFS) obtained from *B. subtilis* isolate ET-1 and *B. amyloliquefaciens* Isolated DH-4 effectively inhibited *P. digitatum* in *in-vitro* and *in-vivo* tests, demonstrating that it can be used as part of the integrated management of green mold in citrus. Furthermore, in the study carried out by Tian et al. (2020), the authors detected that the intense antifungal activity of the CFS of *B. subtilis* isolated ET-1, against *P. digitatum,* occurred due to the synthesis of five types of antimicrobial substances: macrolactin, bacillaene, iturins, fengycin, and surfactin.

Mohammadi et al. (2017) studied the effect of ten antagonistic bacteria (4 strains of *B. subtilis*, 2 strains of *B. pumilus*, 2 strains of *B. cereus*, 1 strain of *B. megaterium*, and 1 strain of *Agrobacterium radiobacterium*) *in vitro* against *P. digitatum*. All bacteria inhibited mycelial growth and spore germination of the fungus (except *A. radiobacter*). These results emphasize the antagonistic potential of *Bacillus* spp. species as important biocontrol agents for *P. digitatum.* (Mohammadi et al., 2017).

The suppression of mycelial growth and germination of *P. digitatum spores* by antagonistic bacterial species, such as those of the genus *Bacillus*, acts mainly through antibiosis. These bacteria suppress the growth and development of the pathogen by secreting chemical substances and other antibiotic compounds, inhibiting cell wall synthesis, destroying and altering cell membrane structures, and damaging protein synthesis. That is why they are so efficient as biocontrol agents (DUKARE et al., 2018).

Furthermore, according to Di Francesco et al. (2016) and Dukare et al. (2018), the biological control mechanism of *Trichoderma* spp. and *Bacillus* spp. also correlates with the ability to produce volatile antifungal metabolites. Volatile organic compounds are active at low concentrations and belong to various chemical groups, such as alcohols, aldehydes, ketones, esters, lactones, terpenes, and sulfur compounds. Due to their volatility, these compounds can travel great distances in structurally heterogeneous environments, as well as in solid, liquid, or gaseous compounds, being an excellent advantage for the success of antagonistic microorganisms (Carmona-Hernandez et al., 2019).

Most fungal pathogens, such as *P. digitatum*, according to Wang et al. (2018), infect fruits through wounds, stomata, and lenticels, starting the process with the germination of spores and subsequent formation of the germ tube. Biocontrol agents *T. harzianum, B. subtilis,* and *B. licheniformis* + *B. subtilis* + *T. longibrachiatum* also inhibited the germination of *P. digitatum* spores, showing a linear effect depending on the doses. However, the most outstanding germination control was observed for the highest treatment concentration with *B. licheniformis* + *B. subtilis* + *T. longibrachiatum*, indicating that combining species from the two genera in the same product presented greater efficiency of action.

The presence of two species of the genus *Bacillus* (*B. licheniformis* and *B. subtilis*) in the studied formulation possibly expanded the pathogen spectrum of action since the mode of action of this genus involves the synthesis of antibacterial and antifungal metabolites, such as surfactin, bacillomycin, and fengycin (DUKARE et al., 2018; TIAN et al., 2020). The isolated application of biocontrol agents, according to Dukare et al. (2018), is typically not sufficient to achieve a consistently high level (>95%) of disease control. Therefore, combining biocontrol agents is an advantageous approach, exploring the synergistic effects of the combination of microorganisms

and, thus, improving the performance and effectiveness of the biocontrol method.

The *in-vivo* trials, both curative and preventive applications of *T. harzianum, B. subtilis*, and *B. licheniformis + B. subtilis + T. longibrachiatum* in 'Pera' orange fruits, proved to be effective in controlling green mold in citrus, by significantly reducing AUDPC, especially at the highest concentrations of each biocontrol agent. These results indicate that biocontrol strategies involving these beneficial microorganisms represent a promising alternative to reduce or even replace fungicides to control post-harvest diseases.

The reality of many orchards shows that chemical control in pre- and post-harvest is still the most used method for controlling *P. digitatum*, as well as other phytopathogens (Erasmus et al., 2015; Ferraz et al., 2018; Costa et al., 2019; Fenta et al., 2019; Tian et al., 2020). However, the prolonged and uncontrolled use of fungicides has triggered a series of problems in agriculture in general. There are currently several cases of pathogen strains resistant to the modes of action of many active ingredients, in addition to the increasing detection of fruit residues, putting consumer health at risk (Fischer et al., 2013; Ferraz et al., 2018).

The continuous use of fungicides in citrus post-harvest has led to the development of many cases of resistance of strains of *P. digitatum* and *P. italicum* to synthetic fungicides, from the benzimidazole, sodium octaphenylphenate, and imidazole groups (Erasmus et al., 2015). Evaluating the sensitivity of 75 strains of *P. digitatum* to seven fungicides, Sánchez-Torres & Tuset (2011) found that among the strains assessed, 84% were resistant to thiabendazole and 77% to imazalil, which are the main fungicides used in post-harvest of citrus.

Worldwide, systemic fungicides, such as imazalil, are the most used post-harvest treatments to control green mold in citrus fruits (Costa et al., 2019). In Brazil, control of *P. digitatum* in *packing houses* has been carried out for more than 25 years with imazalil and thiabendazole (Fischer et al., 2013). Furthermore, studies reveal that the repeated use of some active ingredients allows the emergence of resistant *P. digitatum populations*, besides the severe implications that these products represent for the environment, animals, and humans (FERRAZ et al ., 2018).

In many citrus-producing regions, the application of imazalil is still considered safe post-harvest. However, agricultural systems need to reformulate the practices adopted concerning the use of pesticides and the maximum residue limits (MRL) allowed in a product. Therefore, it is likely that the use of imazalil in post-harvest applications will begin to have restrictions due to the continued reduction of MRLs permitted in many countries (Pétriacq et al., 2018).

Therefore, research that explores the use of alternative products, as is the focus of this work, offers strategies that offer new dimensions and flexibility in the choice of disease management in the production of healthier foods and reduce pathogen resistance to products. traditional (Fischer et al., 2013; Dukare et al., 2018; Boffette et al., 2018; Tian et al., 2020).

As seen, citrus fruits are very susceptible to infection by *P. digitatum*, especially in the post-harvest phase, that is, during storage that precedes fruit consumption (Tian et al., 2020). According to Boffette et al. (2018), when the fungus is in ideal conditions, with a temperature in the range of 25 °C and relative air humidity above 80% (factors monitored in in-vivo tests), added to the availability of nutrients to stimulate *spore* germination, the initiation and success of the infection are favored (COSTA et al., 2019). According to Nicoli et al. (2009), under ideal temperature and humidity conditions, in less than two days, the lesion reaches approximately 50 mm in diameter, reaching the juice vesicles (pulp). In fact, in 'Pera' orange fruits, the first symptoms appeared 48 hours after inoculation with the pathogen.

Studying the infection process of *P. digitatum*, Costa et al. (2019) described that the fungus produces enzymes capable of dissolving the middle lamella of fruit tissues, which is why the first symptom observed is the appearance of soft rot at the site of infection (a watery spot with slight discoloration of the tissue). Subsequently, the presence of white mycelia and the production of olive-green spores appear in the initial lesion region. As the days go by, the disease progresses until it takes over the entire fruit (Cunha et al., 2018).

In *in-vivo* tests, the fungus took around 168 hours after inoculation to completely take over the fruits of the control treatment, that is, five days after the first symptoms. However, treatments with *T. harzianum , B. subtilis*, and *B. licheniformis + B. subtilis + T. longibrachiatum*, especially at the highest doses, were efficient in containing the evolution of infection by *P. digitatum,* and it can be observed that the diameters of the lesions were smaller, with absence or reduction of sporulation.

Biocontrol agents displayed differences between curative and preventive effects, as fruits treated preventively had lower disease severity. In their study, Moretto et al. (2014) state that biocontrol agents' application after harvest may be too late to compete

effectively against the pathogen since it may have established itself in the fruit while still in the field (latent infection). In cases like this, the antagonistic microorganism should present a curative action, controlling these pre-existing infections and preventing subsequent infections by inhibiting the fungus sporulation. The highest AUDPCs in this study were in fruits treated curatively, demonstrating that the biocontrol agents displayed a higher antagonistic than preventive efficiency.

Biocontrol agents promoted a significant reduction in the disease severity when applied preventively, and the longer the fruit storage after treatments with *T. harzianum* , *B. subtilis*, and *B. licheniformis* + *B. subtilis* + *T. longibrachiatum* , the lower the disease severity, indicating the effective action of these treatments in protecting the fruits from future infections. A similar study also found that the preventive application of biocontrol agents to fruits was more effective in containing *P. digitatum* infection. (Wang et al., 2018).

In commercial situations, the preventive effect of applying biocontrol agents is a promising alternative. Reinfection of the same fruit or healthy fruits can occur during handling and processing within the packaging (Moretto et al., 2014). A single spore of *P . digitatum* can infect a fruit and produce millions of spores after seven days under optimal environmental conditions (Benato et al., 2018).

The preventive effect of applying *T. harzianum, B. subtilis*, and *B. licheniformis* + *B. subtilis* + *T. longibrachiatum* in 'Pera' orange fruits demonstrated that they showed an efficient capacity to grow and quickly colonize the fruit surface for a more extended period, regardless of environmental conditions. In addition to rapidly using available nutrients, they reduce their availability to pathogens (Dukare et al., 2018).

This effect is significant, as in citrus farming, the incidence of green mold on fruits is the main post-harvest rot, as alone, is responsible for around 90% of total losses in the citrus chain (Bazioli et al., 2019), reducing fruit quality, marketing period and shelf life (Fischer et al., 2008; Fischer et al., 2011). Faced with such aggression, biocontrol agents that act quickly to suppress *P. digitatum* are interesting.

Biocontrol agents employ direct and indirect inhibitors as mechanisms to suppress fungal growth by synthesizing antifungal compounds and specific metabolites that act on the pathogen, inhibiting its metabolism and development, besides damaging or killing pathogen propagules on fruits (Dukare et al., 2018). The synthesis of antibiotics is another primary strategy to control the possible deterioration of fruits during storage, as they reduce the growth of pathogens, even at very low concentrations (Wang et al., 2018; Wang et al., 2020).

Biocontrol agents have been successfully implemented in biological control strategies in the fruits' post-harvest, as they are faster in consuming the nutrients available on the surface of the fruits, colonizing with greater agility and thus reducing the chances of the pathogen spreading. install on site (Dukare et al., 2018).

Conclusion

All evaluated concentrations of *T. harzianum, B. subtilis*, and *B. licheniformis* + *B. subtilis* + *T. longibrachiatum* were 100% efficient in inhibiting the mycelial growth of *P. digitatum*.

The application of *B. licheniformis* + *B. subtilis* + *T. longibrachiatum* inhibited the germination of *P. digitatum* spores by 94%, followed by *T. harzianum* (88%) and *B. subtilis* (85%).

The preventive application of *B. licheniformis* + *B. subtilis* + *T. longibrachiatum* promoted the best control of *P. digitatum* in 'pera' orange fruits, followed by treatments with *T. harzianum*, and *B. subtilis*.

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