

## PREVENTION AND CONTROL OF *Fusarium oxysporum* Schltdl. USING BENEFICIAL ORGANISMS IN *Pinus oocarpa* Schiede ex Schltdl.

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### ABSTRACT

The most common disease in forest nurseries is damping-off, commonly caused by the fungus *Fusarium oxysporum* Schltdl. The objective of this study was to evaluate the effect of a commercial product based on microorganisms (Bactiva®) and a strain of *Trichoderma paratroviride* Jaklitsch and Voglmayr on the incidence of damping-off and the morphology of *Pinus oocarpa* Schiede ex Schltdl. plants inoculated with *F. oxysporum*. A completely randomized design was used, with treatments in an increased factorial arrangement, to evaluate two biological products (a commercial product and *T. paratroviride*) and three application forms (to the seed, to the substrate, and through irrigation) were evaluated, with an additional control inoculated only with *F. oxysporum*. The factors revealed that using the commercial product and applying it to the seed reduced the incidence rate to 25 and 23%, respectively. In terms of interactions, the application of the commercial product to the seed, to the substrate, and by irrigation, and with *T. paratroviride* to the seed and by irrigation, showed the lowest percentage of incidence (<30 %). At 195 days after sowing, *T. paratroviride* and substrate application improved plant morphology. The interactions showed that the highest root collar diameter (6.5 mm), plant height (23.2 cm), aerial dry weight (3.15 g), total dry weight (4.15 g), and Dickson quality index (0.61) were obtained with substrate-applied *T. paratroviride*. However, the lowest sturdiness quotient was obtained with the control (3.27), while for root dry weight and aerial dry weight over root dry weight, there were no differences among the treatments evaluated.

**Keywords:** Pinaceae, biological control, damping-off, *Trichoderma paratroviride*.

### INTRODUCTION

In Mexico, forest nurseries produce approximately 180 million plants per year, which are used in reforestation and ecosystem restoration to reduce soil degradation and mitigate the negative effects of climate change (Andivia *et al.*, 2021). The significance of nursery management practices lies in their impact on plant quality, specifically their

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morphological and physiological characteristics, which directly influence their ability to adapt to the conditions of the transplanting site (Rueda-Sánchez *et al.*, 2014; Aldrete *et al.*, 2023). On the other hand, according to Cibrián-Tovar *et al.* (2008), the decrease in forest mass is caused by land use change, overgrazing, and the presence of pests and diseases; the latter also cause the death of reforested trees and nursery plants, resulting in a significant economic loss for nurserymen.

Damping-off is a disease characterized by rotting at the base of the plant stem, resulting in wilting and death (Larios-Larios *et al.*, 2019). According to Mojica-Marín *et al.* (2009), it is caused by the genera *Fusarium*, *Rhizoctonia*, *Pythium*, and *Phytophthora*. In the Mexican state of Durango, Prieto *et al.* (2009) found this disease in all nurseries evaluated. *Fusarium* fungi, for example, are filamentous, cosmopolitan ascomycetes with well-developed, septate mycelium and distinctive conidiophores (Sumalan *et al.*, 2013). Their damage triggers a series of irreversible conditions in the host, which start in the roots or the above-ground parts of the plant (Ma *et al.*, 2013). To avoid the proliferation of this pathogen, appropriate management is required in the nursery. Among the management actions used, the application of chemicals stands out, which frequently endangers human health and may cause pathogen resistance (García-Díaz *et al.*, 2017). To prevent this disease, it is recommended to use certified and sanitized seeds and control irrigation after planting to avoid excess soil moisture (Gordon *et al.*, 2015).

Okorski *et al.* (2014) indicate that in the control of *Fusarium oxysporum*, natural enemies of the pathogen can be used as biological control agents, which contributes to environmental conservation and prevents the pathogen from developing resistance. Additionally, natural enemies feed on organic matter and have the ability to compete for the nutrients required by pathogens. Generally, they present several mechanisms of competition and inhibition in the presence of pathogens such as *F. oxysporum*, so they are an effective and direct alternative against these types of diseases (Okorski *et al.*, 2014).

Fungi of the genus *Trichoderma* are used as biological control agents against phytopathogenic fungi due to their mechanisms of action, which include antibiosis, mycoparasitism, production of secondary metabolites, and competition for space and nutrients (Hernández-Melchor *et al.*, 2019). Sánchez-Rangel *et al.* (2016) obtained similar incidence percentages when applying Captan, copper sulfate pentahydrate, and two *Trichoderma* strains (SP6 and SP12) on plants of *Carica papaya* L. Meanwhile, García-Díaz *et al.* (2019) demonstrated that *T. harzianum* decreased the incidence of *Fusarium circinatum* on seedlings of *Pinus greggii* Engelm. ex Parl. when using sawdust as substrate. Larios-Larios *et al.* (2019) reduced the incidence of damping-off in *Capsicum chinense* Jacq. seedlings by applying *Trichoderma* spp. to the foliage. Rodríguez-Pinto *et al.* (2021) reduced wilt caused by *Fusarium* spp. on *Solanum melongena* L. plants *in vitro* and in the field by using native *Trichoderma* strains isolated and used as biocontrol. Ortega-Cerón *et al.* (2024) reported that applying *T. harzianum* to the substrate on *Pinus devoniana* Lindl. plants decreased the pathogenicity of *F. circinatum*.

Among the species produced in forest nurseries, *Pinus oocarpa* Schiede ex Schltdl. has the potential to be used in reforestation programs, since it is capable of growing in both fertile and marginal soils; in addition, it presents adequate production characteristics due to its growth speed and the conservation status of its populations (Fabián-Plesníková *et al.*, 2020; CONAFOR, 2022). Considering the above, the objective of this research was to evaluate the effect of a commercial product based on microorganisms and a strain of *T. paratroviride*, using different forms of application, on the incidence of damping-off and the morphology of *P. oocarpa* plants inoculated with *F. oxysporum*, under the hypothesis that these products reduce the incidence of the pathogen and modify the morphology of the plants.

## MATERIALS AND METHODS

### Trial set-up

The experiment was established in a greenhouse of the Forestry Sciences Division of the Autonomous University of Chapingo in the municipality of Texcoco, State of Mexico, Mexico (19° 29' 34" N and 98° 53' 38" W, at an altitude of 2240 m). Seeds of *Pinus oocarpa* Schiede ex Schltdl. were collected from a natural population of the species in the municipality of San Simón de Guerrero, State of Mexico, by the National Institute of Forestry, Agriculture, and Livestock Research. Two *P. oocarpa* seeds, previously soaked in water (24 h) and disinfested in commercial chlorine (5 %), were sown in containers with a substrate of peat, perlite, and vermiculite 3:1:1 v/v, combined with a controlled-release fertilizer (7 g L<sup>-1</sup>). Since they are artificial substrates, they were considered to be innocuous to a certain extent, so no sterilization process was applied. At 35 days after sowing (dds), a thinning was carried out, where one plant per container was left. Irrigation was applied every third day with tap water without causing splashing between the trays.

### Experimental design and study factors

Two biological products were evaluated using a completely randomized design with treatments in an increased factorial arrangement. One of the products was a commercial product (Bactiva® TNI, Germany) based on fungi from the *Trichoderma* and *Gliocladium* genera, bacteria from the *Bacillus* and *Pseudomonas* genera, and a marine alga (*Ascophyllum nodosum*). The other was a strain of *Trichoderma paratroviride* that was collected from a plantation of *Eucalyptus* spp. in the state of Tabasco, Mexico. Both products were evaluated in three different application ways (to the seed, to the substrate, and through irrigation). Additionally, there was a control in which only *Fusarium oxysporum* was inoculated to the substrate at the base of the plant. Four replicates per treatment were carried out, which generated 28 experimental units, each one consisting of a tray with 42 plants in individual containers of 170 mL capacity.

### **Pathogen inoculation**

At 49 dds, 20 mL of distilled water with two drops of polysorbate 20 and a concentration of  $1 \times 10^7$  conidia per milliliter of *F. oxysporum* were applied to the substrate at the base of the plant. The inoculum was obtained from purified Petri dishes identified with the pathogen. At 90 dds, a pathogenicity test was performed by evaluating four plants with wilt symptoms per treatment (Soria *et al.*, 2012). In this understanding, where only the pathogen (control) was inoculated, only *F. oxysporum* was found, and in those where the commercial product and the *T. paratroviride* strain were applied, the presence of the pathogen and *Trichoderma* was confirmed.

### **Application of the commercial product and *Trichoderma paratroviride***

The concentration used of the commercial product and *T. paratroviride* was  $1 \text{ g L}^{-1}$  and  $1 \times 10^7$  conidia  $\text{mL}^{-1}$  water, respectively. When applied to the seed, it was allowed to stand for 5 min in 200 mL of sterile distilled water with the corresponding treatment before sowing. The application to the substrate took place while the substrate was being prepared, and the established concentration was diluted in 200 mL of sterile distilled water. At 49 dds, the treatments were applied through irrigation, where the established concentration was combined in sterile distilled water (200 mL), and all the trays belonging to each treatment were uniformly irrigated.

### **Response variables**

#### **Incidence of *Fusarium oxysporum***

The incidence of this pathogen was evaluated weekly by recording the number of plants per experimental unit with typical symptoms of the disease (Soria *et al.*, 2012), i.e., color changes in needles and terminal bud wilting. In this sense, the accumulated percentage of dead plants in relation to the initial number of plants was obtained over 17 weeks from 49 dds. These values were transformed with the arcsine function prior to analysis.

#### **Morphological indices in *Pinus oocarpa* plants**

At the end of the experiment (195 dds), 12 plants per experimental unit were taken and removed from the container to eliminate all the root substrate. Root collar diameter was measured with a digital vernier in millimeters. Plant height was measured with a ruler graduated in centimeters, from the base of the stem to the apical bud. To measure root dry weight, aerial dry weight, and total dry weight, the root and aerial part of the plant were divided, placed separately in paper bags labeled with their experimental unit, and dried in an oven for 72 h at 70 °C. The ratio of aerial and root dry weight, sturdiness quotient, and Dickson's quality index were calculated according to Aldrete *et al.* (2023).

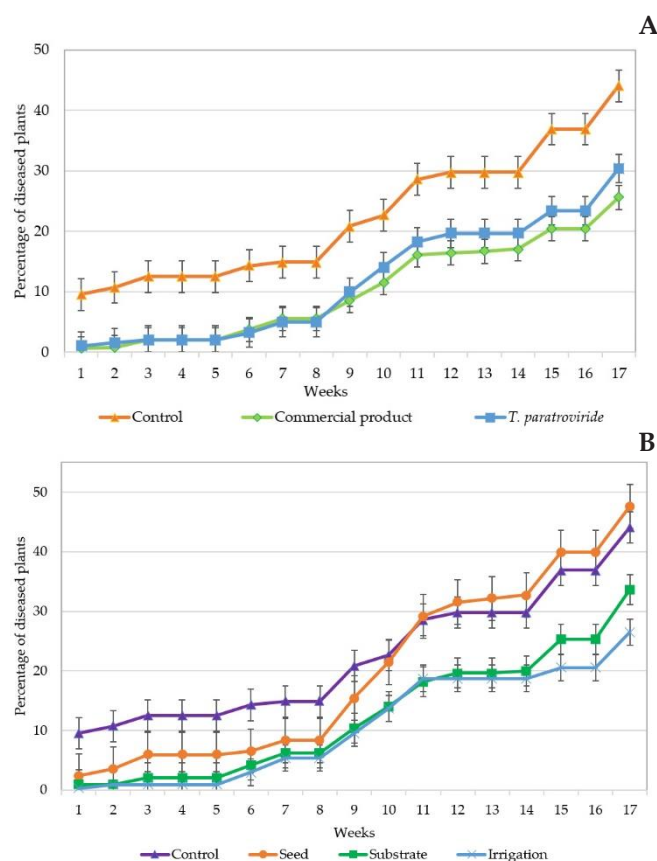
### Statistical analysis

Data were analyzed for normality (Shapiro-Wilk) ( $p \geq 0.05$ ), variance homogeneity (Bartlett) ( $p \geq 0.05$ ), and residue independence tests; likewise, an analysis of variance was performed by as proposed by Federer (1955), corresponding to a randomized design with treatments in an increased factorial arrangement, and a multiple comparison of means with Tukey's honest minimum significant difference test ( $p \leq 0.05$ ) using the R statistical program version 4.3.2.

## RESULTS AND DISCUSSION

### Incidence of *Fusarium oxysporum*

In both factors, the percentage of incidence increased similarly up to week eight between both biological products and the three forms of application (Figure 1).

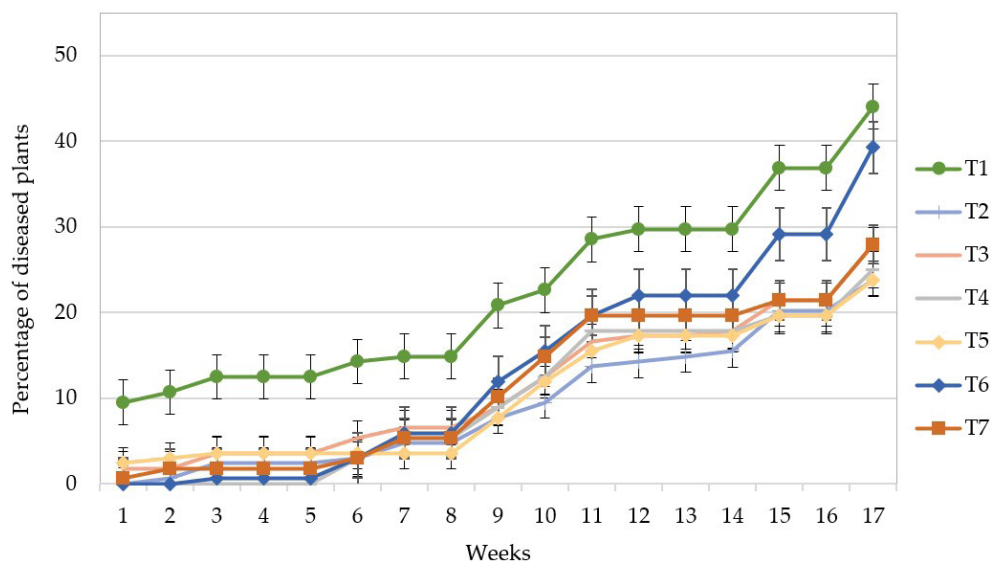


**Figure 1.** Percentage of cumulative incidence from inoculation of *Fusarium oxysporum* on *Pinus oocarpa* Schiede ex Schltdl. plants during 17 weeks of evaluation by the effect of the interaction of the product applied and the mode of application, and an additional control. A: biological product factor (Bactiva® and *Trichoderma paratroviride*); B: application form factor (seed, substrate, and irrigation). The standard deviation is indicated.

However, after this week, the percentages began to show different increases. In the biological product factor, *T. paratroviride* increased in relation to the commercial product until week 17, where the lowest percentage corresponded to the commercial product (25 %), followed by *T. paratroviride* (30 %) and the additional control (44 %) (Figure 1A). On the other hand, in the application method factor, application to the seed reported the lowest incidence percentages up to week 17 (23 %), in contrast to application through irrigation (26 %), to the substrate (33 %), and the control (44 %) (Figure 1B).

Regarding the interaction of factors and the additional control, the manifestation of disease symptoms in *P. oocarpa* plants started in the first week after inoculation (sdi) in the control ( $T_1$ ), in some plants of treatments  $T_3$ ,  $T_5$ , and  $T_7$ , and none of  $T_2$ ,  $T_4$ , and  $T_6$  (Figure 2). From the eighth sdi, there was a considerable increase in the percentage of incidence, where the application of *T. paratroviride* to the substrate and through irrigation presented a percentage close to the control. However, at 17 sdi, the control reported a 44 % incidence, a value close to that of *T. paratroviride* applied to the substrate (39 %) and higher than the rest of the treatments, which were found to be below 30 %.

The time in which symptoms appeared was similar to that reported by García-Díaz *et al.* (2019), who inoculated the substrate with *F. circinatum* on *P. greggii* plants and



**Figure 2.** Percentage of cumulative incidence from inoculation of *Fusarium oxysporum* in *Pinus oocarpa* Schiede ex Schltdl. plants during 17 weeks of evaluation by the effect of the interaction of the product applied and the mode of application for the control of the pathogen, and an additional control.  $T_1$ : control;  $T_2$ : commercial product (Bactiva®) applied to the seed;  $T_3$ : commercial product applied to the substrate;  $T_4$ : commercial product applied by irrigation;  $T_5$ : *Trichoderma paratroviride* applied to the seed;  $T_6$ : *T. paratroviride* applied to the substrate;  $T_7$ : *T. paratroviride* applied by irrigation. The standard deviation is indicated.

found that the manifestation of the first symptoms occurred 20 days after inoculation. These findings were different from those of Robles-Carrión *et al.* (2014), where inoculating *F. oxysporum* to the substrate in plants of *Vasconcellea × heilbornii* (V.M. Badillo) V.M. Badillo, reported that symptomatology began 30 days after inoculation. Contrary to Soria *et al.* (2012), who suggest that a plant must have a wound in order for the pathogen to enter the tissue, the effects demonstrated in this study concur with García-Díaz *et al.* (2019), who assert that *Fusarium* spp. can directly infect the tissue even in the absence of a wound.

On the other hand, the commercial product and the application to the seed attenuated the incidence of the pathogen. Likewise, *T. paratroviride* applied to the seed and by irrigation reduced the incidence in a similar way to that of the commercial product in all forms of application. Bactiva® has a mixture of *T. harzianum*, *T. reesei*, *T. viride*, *Gliocladium virens*, *Bacillus subtilis*, *B. megaterium*, *B. polymyxa*, *Pseudomonas fluorescens*, and *Ascophyllum nodosum*. The findings demonstrated a better efficacy of the commercial product against the *T. paratroviride* strain, attributed to the mixture of strains present in the product, of which several authors have demonstrated the positive effects of the *Trichoderma* (Amaral *et al.*, 2019; Rodríguez-Pinto *et al.*, 2021; Ortega-Cerón *et al.*, 2024) and *Bacillus* genus (Mejía-Bautista *et al.*, 2016) in the control of *Fusarium*.

Similarly, when biological products were applied to the seed, a lower percentage of incidence was observed. Kthiri *et al.* (2020) indicate that when applying *Trichoderma* spp. to the seed there is an early relationship between the fungus and the roots of the seedling, which allows a better colonization in the rhizosphere and presents a positive effect on the development of the plant by having a greater tolerance to stress and a better absorption of nutrients.

The incidence reduction caused by *Trichoderma* application coincides with García-Díaz *et al.* (2019), Rodríguez-Pinto *et al.* (2021), and Ortega-Cerón *et al.* (2024), who were able to reduce the incidence of *Fusarium* spp. by applying *Trichoderma* spp. on *P. greggii*, *Solanum melongena*, and *P. devoniana*, respectively. According to Navaneetha *et al.* (2015), the positive effect of *Trichoderma* as a biocontrol agent against different pathogens is due to its ability to synthesize proteins, enzymes, antibiotics (antagonistic compounds), and substances that promote plant growth, such as vitamins and hormones. Joo and Hussein (2022) reported that the volatile organic compounds produced by *Trichoderma* spp. are a key factor in suppressing harmful rhizosphere microorganisms and enhancing defense. Amaral *et al.* (2019) found that *T. viride* shows good root colonization and has a prominent impact on defense mechanisms in pine plants.

### Morphological indexes of plants

According to the analysis of variance, for the biological product factor, there were only significant differences in plant height (PLH), aerial dry weight (ADW), and total dry weight (TDW); the highest values were obtained with the *T. paratroviride* strain, with 19.81 cm and 2.43 and 3.31 g, respectively (Table 1). Regarding the application method,

significant differences only occurred in root collar diameter (RCD), ADW, TDW, and sturdiness quotient (SQ). Substrate application produced higher values in RCD, ADW, and TDW, but lower values in SQ, whereas seed application produced intermediate values (Tables 1 and 2).

On the other hand, for the interactions and the additional control, the analysis of variance only found significant differences in the RCD, PLH, ADW, TDW, SQ, and Dickson quality index (DQI) (Tables 1 and 2), where the application of *T. paratroviride* to the substrate had the highest RCD (6.5 mm), PLH (23.2 cm), ADW (3.15 g), TDW (4.15 g), and DQI (0.61). However, the best SQ was obtained with the control (3.27). As this variable shows the balance between the aerial and root parts, the lower the index, the better the balance in the plant. In this sense, sturdiness allows estimating the physical resistance of the plants during planting operations and their resistance to the mechanical effect of the wind (Gomes *et al.*, 2002).

**Table 1.** Multiple comparison of means of the variables root collar diameter, plant height, and dry weight by the effect of the factors, their interaction, and the additional control in *Pinus oocarpa* Schiede ex Schltdl. plants inoculated with *Fusarium oxysporum* at 195 days after planting.

T	Biological product	Application form	RCD (mm)	PLH (cm)	PSR	ADW (g)	TDW
	PC		4.61 a	17.45 b	0.76 a	1.94 b	2.71 b
	TP		5.02 a	19.81 a	0.87 a	2.43 a	3.31 a
		Seed	4.71 ab	18.5 a	0.78 a	2.10 ab	2.88 ab
		Substrate	5.65 a	20.00 a	0.85 a	2.56 a	3.42 a
		Irrigation	4.08 b	17.3 a	0.82 a	1.90 b	2.72 b
T <sub>1</sub>		Control	4.61 ab	15.04 b	0.71 a	1.76 b	2.47 b
T <sub>2</sub>	PC	Seed	5.04 ab	18.37 b	0.81 a	2.08 b	2.90 b
T <sub>3</sub>	PC	Substrate	4.80 ab	16.80 b	0.71 a	1.97 b	2.69 b
T <sub>4</sub>	PC	Irrigation	3.98 b	17.18 b	0.76 a	1.77 b	2.54 b
T <sub>5</sub>	TP	Seed	4.37 b	18.75 ab	0.74 a	2.13 b	2.87 b
T <sub>6</sub>	TP	Substrate	6.50 a	23.20 a	0.99 a	3.15 a	4.15 a
T <sub>7</sub>	TP	Irrigation	4.17 b	17.76 b	0.87 a	2.02 b	2.90 b
	General mean factors		4.81	18.63	0.81	2.19	3.01
	General mean treatments		4.75	17.94	0.80	2.10	2.91
	CV (%)		17.39	10.62	20.85	18.27	16.37
	MSD <sub>0.05</sub> biological product		0.78	1.79	0.14	0.36	0.44
	MSD <sub>0.05</sub> form of application		1.17	2.67	0.21	0.54	0.65
	MSD <sub>0.05</sub> treatments		1.93	4.46	0.37	0.90	1.11

T: treatment; RCD: root collar diameter; PLH: plant height; PSR: root dry weight; ADW: aerial dry weight; TDW: total dry weight; PC: commercial product (Bactiva®); TP: *Trichoderma paratroviride*; CV: coefficient of variation; MSD<sub>0.05</sub>: minimum significant difference with a significance of 0.05. Values with the same letter are statistically equal according to Tukey's honest minimum significant difference test ( $p \leq 0.05$ ).

**Table 2.** Multiple comparison of means of the variables evaluated for the effect of the factors, their interaction, and additional control in *Pinus oocarpa* Schiede ex Schltdl. plants inoculated with *Fusarium oxysporum* 195 days after sowing.

T	Biological product	Application form	ADWR	SQ	DQI
	PC		2.55 a	3.83 a	0.42 a
	TP		2.84 a	4.10 a	0.48 a
		Seed	2.72 a	3.97 ab	0.43 a
		Substrate	2.96 a	3.62 b	0.52 a
		Irrigation	2.41 a	4.32 a	0.40 a
T <sub>1</sub>	Control		2.47 a	3.27 b	0.43 ab
T <sub>2</sub>	PC	Seed	2.57 a	3.66 a	0.46 ab
T <sub>3</sub>	PC	Substrate	2.78 a	3.52 a	0.42 ab
T <sub>4</sub>	PC	Irrigation	2.32 a	4.32 a	0.38 b
T <sub>5</sub>	TP	Seed	2.88 a	4.29 a	0.40 b
T <sub>6</sub>	TP	Substrate	3.14 a	3.71 a	0.61 a
T <sub>7</sub>	TP	Irrigation	2.50 a	4.32 a	0.43 ab
	General mean factors		2.70	3.97	0.45
	General mean treatments		2.64	3.84	0.45
	CV (%)		16.90	10.87	19.10
	MSD <sub>0.05</sub> biological product		0.41	0.39	0.07
	MSD <sub>0.05</sub> form of application		0.61	0.59	0.11
	MSD <sub>0.05</sub> treatments		1.04	0.97	0.20

T: treatment; ADWR: aerial dry weight over root dry weight; SQ: sturdiness quotient; DQI: Dickson quality index; PC: commercial product (Bactiva®); TP: *Trichoderma paratroviride*; CV: coefficient of variation; MSD<sub>0.05</sub>: minimum significant difference with a significance of 0.05. Values with the same letter are statistically equal according to Tukey's honest minimum significant difference test ( $p \leq 0.05$ ).

Among the biological products, the *T. paratroviride* strain showed a positive effect on plant morphology; likewise, among the application methods, higher morphology values were observed when the products were applied to the substrate and to the seed. Ferreira *et al.* (2024) mention that the use of *Trichoderma* strains in the production of forest seedlings promotes the solubilization and availability of nutrients for these, with numerous benefits for their development. Specifically, Gutiérrez-Flores *et al.* (2022) indicate that the increase in plant growth caused by *Trichoderma atroviride* is due to a process called anastomosis, where thin hyphae combine with other hyphae to form a network, which facilitates the exchange of liquids and nutrients. In the same way, the *Trichoderma* symbiosis, when applied to the substrate and the seed, increases the ability to uptake a greater amount of nutrients in plants and, in turn, provides a balance between the aerial and root parts, which favors greater tolerance to water stress (Navarro *et al.*, 2006; Kthiri *et al.*, 2020). It should be noted that the biological products were applied to the substrate and seed prior to *Fusarium* inoculation so that the strains of the two biological products were already established

at the time of pathogen inoculation, as opposed to irrigation, which was applied at the same time as the pathogen.

Most of the diameter values obtained in this research were within the range proposed by Prieto and Sáenz (2011), who indicate that they should be greater than 4 mm to be considered high-quality plants. The results obtained are similar to those of García-Díaz *et al.* (2019), who applied *T. harzianum* and obtained diameters up to 3.11 mm in *P. greggii* inoculated with *F. circinatum*. For their part, Regliński *et al.* (2012) reported better diameter, height, and total dry weight in *Pinus radiata* D. Don plants when using *T. atroviride*. On the other hand, other studies reported an increase in plant height when applying *Trichoderma* spp. on forest plants (Nunes *et al.*, 2021) and *T. harzianum* on *Pinus sylvestris* L. (Saleh *et al.*, 2023). In terms of DQI, Prieto and Sáenz (2011) establish that plants with values higher than 0.5 present a high quality, with only *T. paratroviride* applied to the substrate having an index higher than indicated. This agrees with Romero *et al.* (2008), who indicate that inoculating *Trichoderma* to the substrate promotes the growth and development of the seedling, as there is better contact and colonization between the substrate and the root.

## CONCLUSIONS

The commercial product and *Trichoderma paratroviride* reduced the incidence of damping-off and modified the morphology of *Pinus oocarpa* plants inoculated with *Fusarium oxysporum*. The lowest percentage of incidence was obtained with the commercial product and the form of application to the seed; similarly, the interactions revealed that applying the commercial microorganism-based product to the seed, substrate, and irrigation, as well as *T. paratroviride* to the seed and through irrigation, resulted in the lowest percentage of incidence. Better results in plant morphology were obtained when *T. paratroviride* was applied to the substrate. The interactions revealed that applying *T. paratroviride* to the substrate resulted in better growth; however, this treatment did not reduce the percentage of incidence, so it is recommended to use *T. paratroviride* on the seed, as it demonstrated good growth and lower pathogen incidence.

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