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Efficiency of *Trichoderma viride* as a biocontrol agent for *Phytophthora capsici* in Pepper (*Capsicum annuum* L.)

Eficiencia de *Trichoderma viride como un agente biocontrolador para Phytophthora capsici en Pimiento* (*Capsicum annuum* L.)

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Abstract

Phytophthora capsici is one of the most devastating pathogens that limits the production of Paprika *(Capsicum annuum L.)* worldwide. Likewise, *Trichoderma viride* stands out as a biological agent due to its antagonistic effect, resistance inducer, growth stimulator, etc. The present work evaluated the effectiveness of *T. viride* as a biocontrol agent against *P. capsici* in Paprika using three growth methods (direct seeding, plantlet and bare root). Twelve treatments were developed under greenhouse conditions, including a control (without inoculum) and a completely randomized design with a factorial arrangement. *T. viride* inoculation was carried out 40 days after sowing at a concentration of 10^6 conidia ml^{-1} while *P. capsici* was inoculated 50 days after sowing using three colonized wheat grains per plant. The inoculation method of the controlling agent in the direct seeding and plantlet was given by drench, and in the bare root was carried out by immersing of the seedling for 5 minutes prior to the transplant. Then, the correlation between plant growth method and *P. capsici,* and the interaction between *T. viride* and the plant growth method were made. The results showed that the highest efficacy of *T. viride* as a *P. capsici* biocontrol agent was in the method of the plantlet and bare root. The correlation between the method of growing crop and root rot was lower in bare root (74 % severity). In the other two treatments (direct seedling and plantlet) 100 % of plants were dead; finally, the effect of *T. viride* as a growth inducer was not evidenced in any of the treatments. Regarding AUDPC, the direct seeding method showed a higher incidence. The bare root planting method obtained the lowest value of the *T. viride* and *P. capsici* interaction.

Keywords: *Biocontrol, Capsicum annuum L, Phytophthora capsici, Trichoderma viride, growth methods.*

Resumen

Phytophthora capsici es uno de los patógenos más devastadores que limita la producción de paprika (*Capsicum annuum* L.) en el Mundo. Asimismo, *Trichoderma* destaca como agente biocontrolador por su efecto antagonista, inductor de resistencia, estimulador de crecimiento, etc. El presente trabajo evaluó la eficacia de *T. viride* como controlador biológico para *P. capsici* en páprika bajo tres métodos de siembra (directa, plantín y raíz desnuda). Se instalaron doce tratamientos bajo condiciones de invernadero, incluyendo un testigo (sin inóculo) y se empleó un diseño completamente al azar con arreglo factorial. La inoculación de *T. viride* se

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llevó a cabo a los 40 días posterior a la siembra a una concentración de 10^6 conidias ml^{-1}, mientras que la inoculación de *P. capsici* se realizó a los 50 días posterior a la siembra empleándose tres granos de trigo colonizados por planta. El método de inoculación de *T. viride* en la siembra directa y plantín se hizo vía drench y en la siembra a raíz desnuda se realizó por inmersión de la plántula durante 5 minutos previo al transplante. Luego se realizó la correlación entre los métodos de siembra y *P. capsici*, y la interacción entre *T. viride* y los métodos de siembra. Los resultados mostraron que la mayor eficacia de *T. viride* como de *P. capsici* se registró en los métodos de siembra plantín y raíz desnuda; la correlación entre el método de siembra y la pudrición radicular fue menor en la siembra a raíz desnuda (74 % severidad). En los otros tratamientos (directa y plantín) el 100 % de plantas murieron; finalmente, el efecto de *T. viride* como inductor de crecimiento no se evidenció en ninguno de los tratamientos. Con respecto al ABCPE, el método de siembra directa mostró mayor incidencia; el menor valor de la interacción *T. viride* y *P. capsici* fue obtenido en el método de siembra raíz desnuda.

Palabras claves: *Biocontrolador, Capsicum annuum L, Phytophthora capsici, Trichoderma viride, growth methods.*

Introduction

Chili pepper (*Capsicum annuum* L.), one of the most widely grown vegetables worldwide, native from tropical and subtropical regions of America, cultivated approximately 3.8 million ha, and the harvested crops produce around of 41 million tons annually, representing 3.76 % of the global horticulture crop production and 6.51 % of global horticulture crop area (Food and Agriculture Organization [FAO], 2020b). By 2018, approximately 70 % of the global area of pepper crops was in Asia, 18 % in Africa, 7 % in America and 5 % in Mediterranean countries (FAO, 2020b).

Peru is a great exporter of dry pepper, located in 2017 as the world's fourth largest exporter of dry pepper, totaling 32 million tons (Food and Agriculture Organization [FAO], 2020a). According to Ministerio de Agricultura y Riego (MINAGRI, 2019), the sowing projection for the 2019-2020 farming season adds up to 6.1 million ha, meaning a growth of 45.4 % with respect to the 2018-2019 season, reporting as main producing areas Barranca (32.4 %), Arequipa (22.2 %), Ancash (17.2 %), Piura (9.9 %), Ica (9.3 %) and Lambayeque (6 %).

This crop, grown in greenhouses and fields, is attacked for several diseases responsible for economic losses. One of the most destructive diseases is root rot, caused by the oomycete pathogen *P. capsici* (Tomah et al., 2020). This soil-borne pathogen is widespread throughout the world and can infect and cause severe losses in several crops, with a host range of over 15 plant families (Barchenger et al., 2018). Most of its host plants come from Solanaceae, Cucurbitaceae and Fabeaceae. Symptoms vary significantly according to the host, plant part infected and environmental conditions. The disease may manifest in a plant's underground parts, involving the root and crown rot of pepper (Lamour et al., 2012). During high disease pressure, pathogen dispersal causes aerial blight of leaves, fruit and stems (Callaghan et al., 2016).

For an integrated pest and disease management scheme, cultural and chemical methods, such as crop rotation, resistant varieties, and fungicides, are the most commonly used. Nevertheless, some chemicals employed to manage this pathogen usually fail due to the development of fungicide resistance (Silvar et al., 2006) or variable efficacy against the diverse propagules of the pathogen (Stasz & Martin, 1988). Likewise, highly resistant pepper cultivars showed susceptibility or moderate resistance when the plants were inoculated with *P. capsici* (Dunn & Smart, 2015) due to many pathogen physiological races (Monroy-Barbosa & Bosland, 2011). Hence, biological control employing fungal antagonists can be a robust and sustainable disease management strategy. In this sense, *the Trichoderma* genus is the most used for the biocontrol of soil fungus pathogens. These genera have also been shown to be particularly effective in managing *P. capsici* (Tomah et al., 2020). They inhabit diverse environments, undergo various interactions with other organisms, live in soil and saprophytically grow on wood, bark and many other substrates, and interact with animals and plants (Zeilinger, 2016). *Trichoderma* spp. possess many antagonistic mechanisms against pathogens of crops, such as lytic enzymes,

mycoparasitism, and competition for nutrients and space for their successful colonization (Harman, 2006; Mukherjee et al., 2012). Besides, the feature endophytic of this genus, which produces a broad spectrum of secondary metabolites, often results in improving assimilation of micro and macronutrients that, in some cases, they are not available to plants; increased root growth and development; enhanced productivity and, improved tolerance to various stresses including diseases, there are numerous reports of various *Trichoderma* spp. induced resistance against different plant pathogens (Siddaiah et al., 2017). *T. viride* is a mycoparasite that produces a lot of hydrolytic enzymes against the pathogenic crop. Moreover, in response to the high ability of biomass transformation is of great interest research fields and biological control industries. This fungus is considered one of the best biocontrol agents, because of its ability to produce many enzymes, such as chitinases, glucanases and proteases. Furthermore, *T. viride* produces glycosyl hydrolases, such as xylanases, cellulases and mannanases under suitable conditions (Elgorban, 2016).

The first goal of this study was to evaluate the efficiency of *Trichoderma viride* as a biocontroller for *P. capsici* under three methods of growing crops. The second aim was to determine the correlation between the methods of growing crops with *P. capsici*, and the last one was assessment the interaction between *T. viride* and the methods of growing crops. These results will provide a more effective biocontrol of *T. viride* against *P. capsici* in pepper. Also, it will contribute to a reduction in pesticide residues in the fruit and the environment.

Material and methods

***P. capsici* inoculum:** The isolate was obtained from *P. capsici* collection of the UNALM - U. TENNESSEE project; plants infected with inoculum were collected from pepper fields of Viru. The most colonized Petri plates with mycelium were chosen to be cultivated in PARB medium and incubated for seven days for the growth of the colony (Ocampo, 2003).

For mycelium multiplication, medium PARB fragments colonized were replicated to Petri plates containing Agar V8 medium (Ocampo, 2003).

Pathogen inoculum used in treatments was prepared over cooked wheat and passed through cooking (1 kilogram per 1.5 liter of water) over low heat for approximately ten minutes. Then, it was left to stand, drain and air. Subsequently, filled the bags with this wheat, leaving the upper third empty, were provisionally covered with cotton, tied with a garter without leaving air inside, and finally sterilized in the autoclave. After sterilization, the pure culture obtained in the V8 medium was seeded into the bags and incubated at 24 °C until the mycelium completely invaded the wheat.

***Trichoderma viride* Isolation:** *T. viride* strain from the Mycoteca of the Plant Pathology Department of the Universidad Nacional Agraria La Molina. The multiplication was carried out from the test tube containing the mycelium grown in PDA (potato dextrose agar) medium, from where they were transferred to Petri dishes containing PDA with oxytetracycline and then incubated at a 24 ºC until the growth of the colony covered the entire surface of the medium. For the treatment with *T. viride* on the plants the most colonized Petri plates were selected, 25 ml of deionized water was added, and rubbed with a triangle previously sterilized in order to homogenize each suspension. The suspensions were diluted to a concentration of 10^6 conidia ml^{-1}. The conidia count was performed with help of the Spencer Neubauer Striped hematocimeter. (French & Hebert, 1980).

Plant Production: The sowing of the Paprika cultivar was carried out on three different methods (direct seeding, plantlet and bare root) at the Phytopathology Greenhouse of UNALM. In direct seeding was used polypropylene bags containing 1000 g of sterile soil. For plantlet production was employed trays contained imported peat sunshine, and for bare root sowing, the seeds were sown in a row with a density of 50 seeds per 0.16 square meter (5 kg pot). They were kept under these conditions for 40 days until *T. viride* was inoculated. Afterward, plantlets placed on trays and plots were transplanted into polypropylene bags.

Controller inoculation (*T. viride*): It was performed at 40 days in the three seeding methods after the zero-day (sowing) at a concentration of 10^6 conidia ml^{-1}.

a. Direct sowing: 10 ml was applied by drenching at the base of the plant.
b. Plantlet: after five days of inoculation, 1 ml of controller solution per plant by drench. The plantlets were transplanted into a polypropylene bag containing 1 kg of sterile soil.
c. Bare root: the plants were immersed in the controller inoculum solution for five minutes, then transplanted into a polypropylene bag containing 1 kg sterile soil.

Pathogen inoculation (*P. capsici*): Inoculation was previously tested with five colonized wheat grains per plant. However, given that the disease symptoms shown per plant were very aggressive, the dose was reduced to three grains per plant. In this way the inoculation de *P. capsici* was carried out 50 days after sowing in each method, inoculating three colonized wheat grains per plant (Ocampo, 2003).

Evaluation of disease

In the greenhouse, disease severity was evaluated seven days after inoculation using a symptom scale: 0, healthy plant; 1, leaf epinasties; 2, pronounced epinasties; 3, epinasties plus leaf fall star; 4, severe loss of leaves; and 5, dead plant. The symptom scale data and disease incidence were recorded per plant on nine evaluations starting seven days after pathogen inoculation and ending with the death of the control inoculated with *P. capsici* at the 4-day interval in all treatments. The area under the disease progress curve (AUDPC) was calculated for each treatment according to Shaner & Finney (1977).

In addition, at the end of the experiment, the root length, root dry weight, foliage dry weight, and plant height of each plant were determined.

Experimental design: The experiment was organized in a completely randomized design with a 3x4 factorial arrangement (12 treatments, with ten repetitions), where firts factor (M) corresponded to sowing methods and second factor (P) to *P. capsici*. The variables studied were disease severity, root length, root dry weight, foliage dry weight and plant height. The recorded data were subjected to an analysis of variance (ANOVA) followed by Tukey's comparison of means, with a significance level of $\alpha = 0.05$ probability, the package statistical Statistical Analysis System (SAS) version 8.

Results and discussion

The parameters were evaluated by comparing trials with their controls respectively inside each method of growing crops. The result of treatments on morphological parameters is shown in Table 1.

Interaction between *P. capsici*, *T. viride* and method growing crop: Treatments inoculated with *T. viride* (T3, T7, T11) resulted in increases of most growth parameters assessed in comparison to the experimental control (T2, T6, T10), which correspond a treatment inoculated only with *P. capsici*. Greater values for the parameters as root length and foliage dry weight were registered on the plantlet method; and root dry weight on the bare root method. All treatments showed significant statistical differences in favor of the effect of *T. viride* ($\alpha = 0.05$) (Table 1, Figures 1 and 2). The values achieved in the plantlet and bare root method respond to its greater absorption capacity of controlling inoculum, directly influenced by the inoculum method. In both cases, the contact of the roots with the controlling inoculum is significatively compared to the direct seeding method, where the application was by drench. Then, due to having a more compact soil surface, only some of the solution is absorbed by the roots due to losses that can occur for percolation and/or leaching. According to Pineda-Insuausti et al. (2017), the plantlet method is favored by the positive interaction that occurs between *T. viride* and plantlet substrate, based on the high enzymatic capacity that *T. viride* possesses to degrade substrates, and thus increase the efficiency of assimilation of nutrients by the plant concerning the bare root planting method. The result achieved is also favored by an increase in the absorption capacity of the root due to the effect of root breakage at the time of transplanting (Harman, 2004).

Table 1. Average of evaluated parameters for the efficiency test of *T. viride* as a bio controller for *P. capsici* under three sowing methods in Pepper.

TREAT.	COD	ROOT LENGTH (mm)			ROOT DRY WEIGHT (g)			FOLIAGE DRY WEIGHT (g)			PLANT HEIGHT (cm)			SEVERITY (SCALE)		
1	DS(T)	242.8	a	b	0.43	a	b	0.95		b	22.6	a	b	0	a	b
2	DS (Pc)	95,2		b c	0,16		b c	1,04		b c	28,89	a	c	5,0		b
3	DS (Pc + Tv)	117,3		b	0,19		b	1,12		b	30,17	a	c	5,0		b
4	DS (Tv)	347,4	a	b	0,62	a	b	1,93	a	b	37,80	a		0-	a	b
5	P (T)	285,3	a	b	0,52	a	b	1,16		b	34,10	a	b	0-	a	b
6	P (Pc)	23,4		b c	0,10		b c	0,37		b c	24,80	a	c	5,0		b
7	P (Pc + Tv)	177,2		b	0,32		b	1,07		b	24,70	a	c	4,5		b
8	P (Tv)	371,2	a	b	0,67	a	b	2,14	a	b	31,80	a		0-	a	b
9	BR (T)	497,1	a		0,90	a		2,05	a	b	33,90	a	b	0-	a	
10	BR (Pc)	122,0	a	c	0,22	a	c	1,15	a	c	21,70	a	c	3,7	a	b
11	BR (Pc + Tv)	250,4	a	b	0,45	a	b	1,83	a	b	26,50	a	c	2,5	a	b
12	BR (Tv)	360,8	a		0,65	a		2,03			31,80	a		0-	a	
	CV %	36,27%			37,14%			44,48%			17,91%			109,89%		

Data is average of ten repetition. Different letters indicate significance for p <0.05. DS.: direct seeding, P: plantlet, BR: bare root.

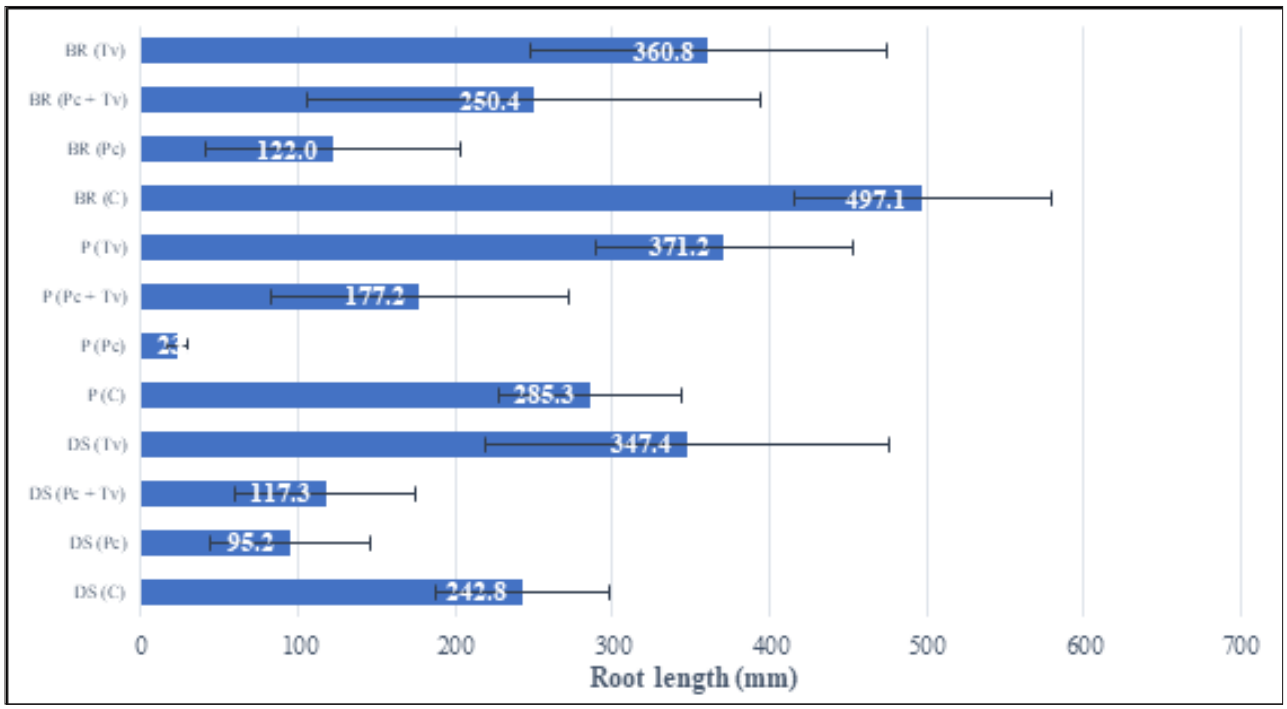

Figure 1: Root length (mm), according to control treatments of *P. capsici* on *T. viride* under three sowing methods (α = 0.05).

In concern to plant height, treatments did not show significant statistical differences with their controls (Figure 3).

Interaction between the method of growing crop and root rot (*P. capsici*): The treatments inoculated with *P. capsici* (T2, T6, T10) showed vast differences in values with their respective controls (T1, T5, T9 / without inoculum). Table 1 shows the significant difference in values in the parameters root length, root dry weight and foliate dry weight obtained in the bare root method, indicating that they were the most affected plants. However, the plants most affected by their control were confirmed in direct seeding; conversely, the least affected plants to their control were reported in the bare root method. Likewise, all treatments showed significant statistical differences in favor of the control without inoculum (Figures 1 and 2). This result would respond to the volume of radical mass developed by the plant at the time of pathogen inoculation. However, it was inoculated on the 50th day after sowing in the three growing methods. Growing a crop that develops the lowest root mass is the bare root, resulting from the high density shown in the plot. Unlike the plantlet and direct seeding method, under this same criterion, the result observed

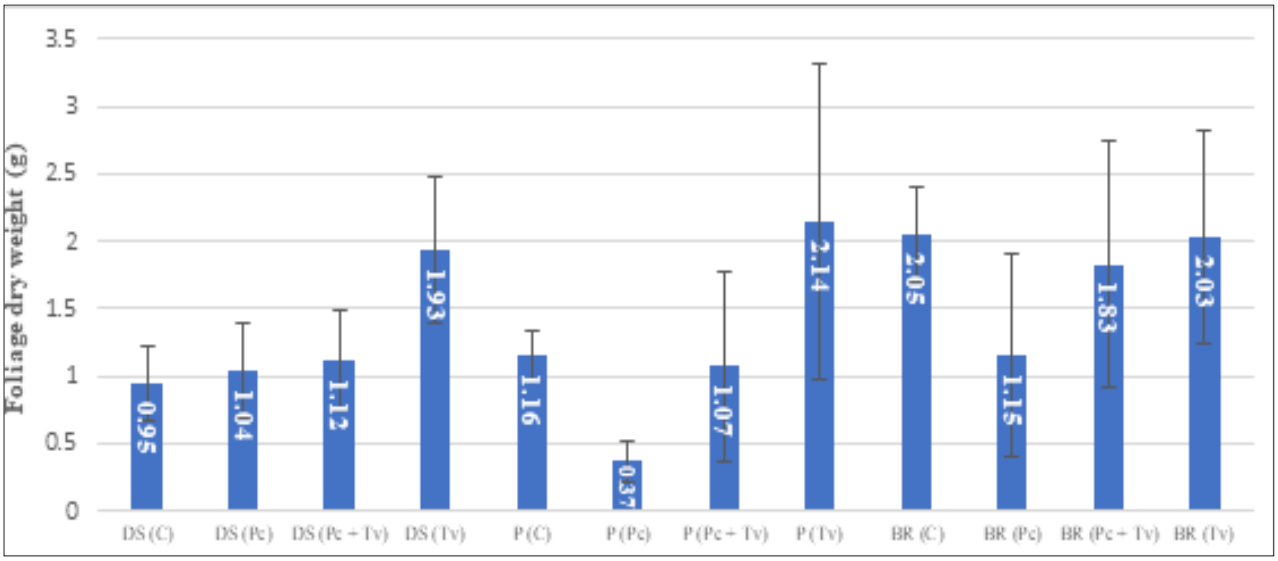

Figure 2. Foliage dry weight (g), according control treatments of *P. capsici* on *T. viride* under three sowing methods (α = 0.05).

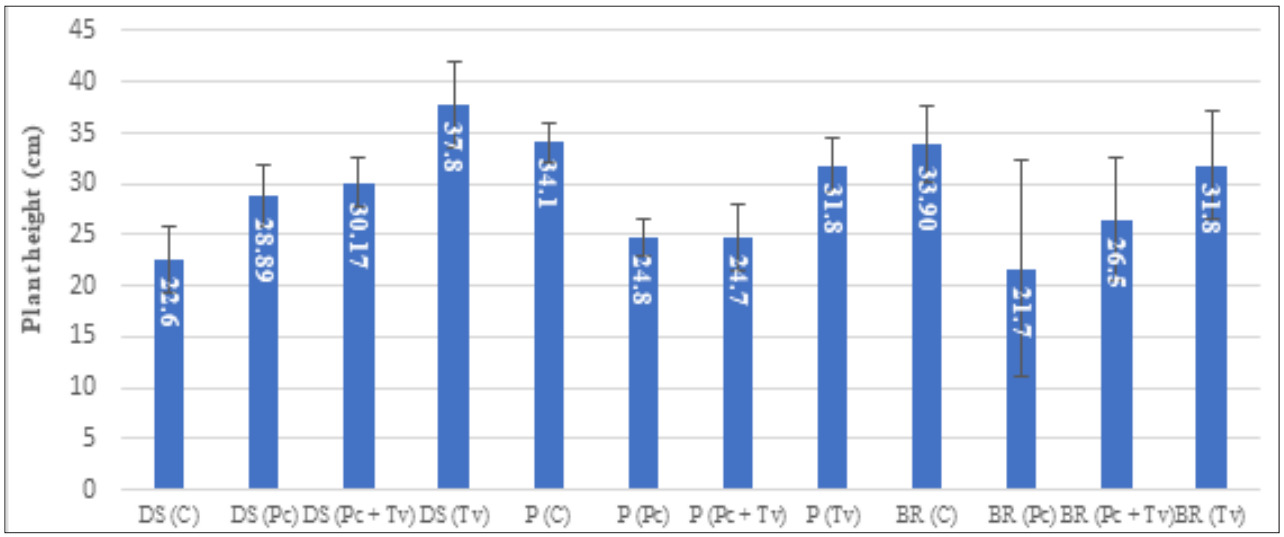

Figure 3: Plant height (cm), according to control treatments of *P. capsici* on *T. viride* under three methods (α = 0.05).

in direct seeding responds to a higher root mass at the time of inoculation of the pathogen. This effect is supported by what has been verified by Hickman (1970) about the accumulation of *P. capsici* zoospores around the apices of the pepper roots claiming the theory of an attraction of the same by chemotaxis mechanism from root exudates after being managing to reproduce the phenomenon using capillaries that incorporated exudates or root extracts. Likewise, specific chemoreceptors are pointed out on the surface of zoospores that even motivate the orientation of the germ tube during their germination (Apaza et al., 1996).

Comparing in treatments the interaction between planting methods and root rot (T2, T6, T10 inoculated only with *P. capsici*). Table 1 shows that the significant value in the parameters root length, root dry weight and foliage dry weight was reached by the bare root method (T10: 122 mm, 0.22 g, 1.55 g, respectively); which showed significant statistical differences with the other treatments (Figure 1 and 2). The results obtained in the bare root method could be supported in that after starting the plants from an area of high density to another of much lower density, it positively influences the morphological development of the plant (Ritchie & Dunlap, 1980).

Interaction method of growing crop and *T. viride*: Treatments inoculated only with *T. viride* (T4, T8, T12) showed light differences with their respective controls (T1, T5, T9 without inoculum). Therefore, the direct seeding method obtained the most significant difference in values in the parameters root length, root dry weight and foliage dry weight. Likewise, all results showed significant statistical differences in favor of the control without inoculum (Table 1, Figures 1 and 2). The result would show that the radical-inducing effect of *T. viride* was not observed, and the highest value obtained in direct seeding would fall more to an effect given by the method of growing crop in which the development of the root is not interrupted and has more space for its development.

The interaction between *T. viride* and the different growing methods (T4, T8, T12 inoculated only with *T. viride*) were compared. It is observed in Table 1 that the highest value for the parameters root length, root dry weight and foliage dry weight was reached by the plantlet method (T8: 371.2 mm, 0.66 g., 2.14 g, respectively). Despite this, the statistic did not show significant differences between the plantlet and direct seeding, while the bare root showed a significant statistical difference. None of the three methods showed significant statistical differences with their control ($\alpha = 0.05$). The significant interaction between the *T. viride* and plantlet method would be influenced by the substrate that supports the seedling, which is based on the Trichoderma mechanism to solubilize nutritional elements that are unavailable in their original form to plants (Harman, 2003). It is also attributed to the high enzymatic capacity of *T. viride* to degrade substrates (Infante et al., 2009). In this way, it increases the efficiency of the assimilation of nutrients by the plant.

Disease development

Disease severity: When the treatment inoculated only with *P. capsici* (T2, T6 y T10) were compared, the lowest severity was observed in the bare root method (T10: scale 3.7), which shows a significant statistical difference with other sowing methods. The severity observed in

Figure 4. Paprika plants 76 days after sowing: A. Direct seeding (T1, T2, T3, T4); B. Plantlet (T5, T6, T7, T8) y C. Bare root (T9, T10, T11, T12).

direct seeding and plantlet methods were scale 5 (100% dead plants) (Table 1, Figure 4). This result could respond to the positive changes already mentioned in the root morphology due to root-break and transplant given in this sowing method.

On the other way, the best effectiveness of *T. viride* over *P. capsici* was obtained in the bare root sowing method (T11: scale 2.5) (Table 1), which showed a significant statistical difference with the other sowing methods. Both the sowing methods as *T. viride* inoculation method, influence this result. In such a way, the break of roots in the extraction increases the absorption capacity by the root and the immersion given corresponds to the inoculation method more efficiently than the drench used to inoculate the plantlet and direct sowing method. From this perspective, the result is supported by the *T. viride* mechanism of inducing the plant's physiological and biochemical defense mechanisms, such as the activation of resistance-related compounds (resistance induction) (Harman, 2004).

AUDPC

Figure 5 shows the AUDPC values for the different treatments evaluated, where it is observed that, in the direct sowing method, the treatment inoculated with both *P. capsici* and *T. viride* (T3: 29) and the treatment inoculated only with *P. capsici* (T2: 30.55) show similar values. In the plantlet sowing method, the lowest value was observed in the treatment inoculated only with *P. capsici* (T6: 12). Concerning the treatment inoculated with both *P. capsici* and *T. viride* (T7: 18.15), this result would be influenced by the fact that, although it had a higher value in the treatment 7, it showed a lower degree of severity than the T6 treatment. In the bare root sowing method, the major value was reached by the treatment inoculated only with *P. capsici* (T10: 14.4) with respect to the treatment inoculated with both *P. capsici* and *T. viride* (T11: 9.75). Likewise, less degree of severity was observed in the T11 treatment.

CONCLUSIONS

The highest efficiency of *T. viride* as a bio controller of *P. capsici* was obtained in the bore root sowing method (32 % effectiveness), followed by plantlet (10 % effectiveness) and in sowing direct were obtained 100 % of died plants.

The best relation between the sowing method and *P. capsici* was obtained in the bore root

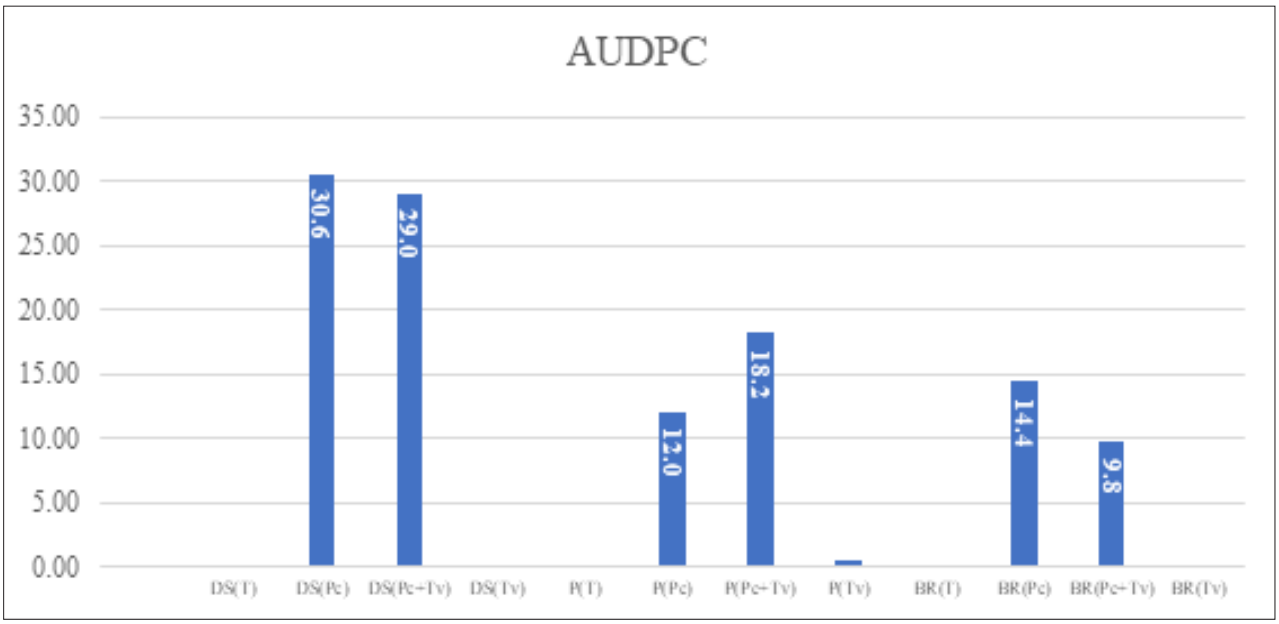

Figure 5. Area averages under the disease progress curve (AUDPC) for the nine evaluations according to treatment in the efficiency test of *T. viride* as a bio controller for *P. capsici* under three sowing methods in the pepper crop (*Capsicum annuum*)

sowing method (74 % severity). The other two sowing methods show 100 % severity (100 % died plants).

The best interaction between the sowing method and *T. viride* was obtained in the plantlet sowing method. The parameters assessment that best show the effect of *T. viride* are: root length, root dry weight, foliage dry weigh and disease severity.

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