Peruvian Journal of Agronomy 2 (3): 27- 34 (2018)
ISSN: 2616-4477 (Versión electrónica)
DOI: http://dx.doi.org/10.21704/pja.v2i3.1230

Received for publication: 15 November 2018
Accepted for publication: 10 December 2018

Cultural, biological and chemical control of the white rot fungus (*Sclerotium cepivorum*, Berk) in onions (*Allium cepa*) in Arequipa´s countryside

Control químico, biológico y cultural de la pudrición blanca (***Sclerotium*** *cepivorum*, Berk) de la cebolla (*Allium cepa*) en la campiña de Arequipa

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Abstract

This experiment was carried out at the province of Arequipa, to determine broccoli residual effects on the sclerotia population of *Sclerotium cepivorum* on the soil and determine the best fungicides and biocontrol agents on the biggest red onion production. In two field areas infected with *Sclerotium cepivorum*, soil samples were carried out to determine the number of sclerotia at the beginning and the end of the trial. One area received broccoli residues before treatment installation. The experiment had a laboratory and a field phase. The block design was used completely randomized (DBCA), using seven fungicide treatments (T_1) control, Iprodione (T_2), Thiabendazole (T_3), Boscalid (T_4), Carbendazim (T_5), *T harzianum* (T_6), *Bacillus subtilis* (T_7). Both areas received the same treatments. The density of sclerotia in the soil was not significant between these areas. However, the addition of broccoli had lower percentages of incidence and severity and higher yields in all treatments. At the area that received broccoli, the Boscalid (T4) and the Iprodine (T_2) fungicide were highlighted with 43.33 T/ha and 28.33 T/ha, respectively; the area without broccoli, the T4 with 38.33 T/ha and T2 with 25.56 T/ha. T5 (Carbendazim) had the lowest yield: 15.00 T/ha without broccoli and 19.58 T/ha with broccoli.

Keywords: Biocontrol agent, sclerotia, incidence, severity, pathogen.

Resumen

El experimento fue llevado a cabo en la provincia de Arequipa, con el objetivo de determinar el efecto de residuos de brócoli sobre la población de esclerotes de *Sclerotium cepivorum* en el suelo y determinar los mejores fungicidas y biocontroladores en la mayor producción de la cebolla roja. En dos áreas de un campo infestado con *Sclerotium cepivorum* se realizaron muestreos de suelo para determinar el número de esclerotes al inicio y al término del ensayo. Un área recibió residuos de brócoli antes de instalar los tratamientos. El experimento tuvo una fase de laboratorio, y una fase de campo. Se utilizó el diseño de bloques completamente al azar (DBCA), con 7 tratamientos fungicidas (T_1) control, Iprodione (T_2), Thiabendazole (T_3), Boscalid (T_4), Carbendazim (T_5), *T harzianum* (T_6), *Bacillus subtilis* (T_7). Ambas áreas recibieron los mismos tratamientos. La densidad de esclerotes presentes en el suelo no fue significativa entre las áreas; sin embargo, la incorporación de brócoli favoreció a menores porcentajes de incidencia y severidad, y mayores rendimientos en todos los tratamientos. En el área incorporada con brócoli, destacaron Boscalid (T4) e Iprodione (T2) con 43.33 T/ha y 28.33 T/ha respectivamente; el área sin brócoli el T4 con 38.33 T/ha y T2 con 25.56 T/ha. El rendimiento más bajo lo tuvo el T5 (Carbendazima): 15.00 T/ha sin brócoli y 19.58 T/ha con brócoli.

Palabras claves: Biocontrolares, esclerotes, incidencia, severidad, patógeno.

Introduction

Red onion is a vegetable of great importance and with the highest internal consumption nationwide, which is grown intensely in different areas. It also has a great social relevance since its production creates a permanent source of labor and favors, indirectly, the economic family income. National onion production is aimed to mainly cover the internal market, being red onion, the primary type of onions produced, which is focused, mostly, in Arequipa, department taking part and generating relevant amounts of production volumes, making more than the 70% of the national production (SIEA, 2012).

Production is influenced by many factors that determine the growing yields and final quality of harvests. It should be noted within these factors the negative effect diseases caused. Species from the *Allium* kind, are widely damaged by the *Sclerotium cepivorum* Berk fungus (Schwartz &Mohan, 2008). In Arequipa, onion growing is at high risk of being affected by the white rot, directly damaging the commercial product. This pathogen causes significant losses that might rise to the 100% since it is difficult to control and because control strategies used

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are poorly efficient, and so, many farmers have changed crops definitely or keep cultivating in completely infested grounds, which represents high production costs and considerable crop losses. Taking into consideration the great economic importance of this growing and the inefficiency of traditional control and preventive methods this research paper attempted to find feasible alternatives regarding the phytopathogen control that causes white rot, by means of biological and chemical controls and the addition of organic residues.

Objectives

- Determine the effect of broccoli residue addition on the sclerotia density found in the soil.
- Determine the best fungicides and biocontrol agents that allow better quality and higher production of red onions.

Materials and Methods

Experiment location

The experiment was carried out in two phases, one in the laboratory and the other at field conditions. The laboratory phase was developed at the Laboratorio del Departamento de Fitopatología (Department of Phytopathology´s laboratory) from the Universidad Nacional Agraria La Molina. And the field phase was carried out at the Chilina valley, district of Cayma, province and department of Arequipa, located at 16°28`south latitude, 71°27`west latitude and 2,300 m.a.s.l.

Sampling collection

Infested onion bulbs collected were those that showed symptoms (yellowish color on outer leaves, wilt) and signs (sclerotia and mycelium) of the *Sclerotium cepivorum* Berk pathogen. Collected samples were placed in paper bags and taken to the laboratory for pathogen isolation.

Isolation of Sclerotium cepivorum

Small portions of the infected tissue were cut, covering the disease growing area and survival structures. These pieces were disinfected using sodium hypochlorite at 1% (during 5´ for the affected tissue and 10´ for sclerotia); then they were grown in Petri dishes with potato dextrose agar oxytetracycline (PDAO), incubated at 20ºC. Once fungi colony was developed, subcultures were made until obtaining pure cultivation.

Laboratory phase

a) In vitro biological and chemical control test:

The potato dextrose agar (PDA) medium was prepared to carry out the poisoned food test. In an Erlenmeyer containing the selective medium (45ºC), a fungicide treatment was added along with the mixture homogenization and this was plated inside an aseptic chamber, using a 0.5 cm diameter punch, small areas of *Sclerotium cepivorum* mycelium were cut and placed in the middle of the surface of the medium contained in the plate. The procedure from the biological control test was the same used for the chemical control. Commercial and biological fungicide treatments used for this test are shown in table 1. The design was completely randomized (DCA, by its Spanish acronym), having four repetitions per treatment. The evaluation consisted of making daily entries of the radial growth of the fungi colony developed in the plate containing poisoned food, and these were compared to the reference samples. The experiment concluded when the reference sample showed fungi increase along the entire surface of the medium; the most efficient fungicides where those that did not allow the pathogen growth.

Table 1*: In vitro* treatments for the *Sclerotium cepivorum* control

Treatments	Active ingredients	Commercial name	Dose (g,mL/100L)	Type of product
T_1	Control		0.0 g	None
T_2	Iprodione	Rovral 50% PM	0.2 g	Contact
T_3	Thiabendazole	Mertect 500 SC	0.125mL	Systemic
T_4	Boscalid	Cantus 500 WG	0.2 g	Systemic
T_5	Carbendazim	Protexin 500FW	0.25 mL	Systemic
T_6	*T harzianum*	Tricho-D	0.15g	Biocontrol ag.
T_7	*Bacillus subtilis*	Serenade	0.5 mL	Biocontrol ag.

Field phase

a) Ground preparation and delimitation of experiment area:

Experiment was located at an agricultural land for commercial onion production having a white rot *(Sclerotium cepivorum Berk)* presence background.

b) Experiment field characteristics:

This study was carried out in a total area of 450 m^2, which was divided into two equal sections of 225 m^2 and located at each one blocks and patches of 6m^2. In one of the experiment area sections, broccoli residues were added in a quantity of 5 Kg/m^2, and it was covered with a transparent polyethylene fabric, and later, weekly irrigation was performed to make the residue decomposition process easier. After 30 days, furrow mapping and onion seedling plantation on the definite field at a 250,000 plant/ha density was carried out. The experiment was made under the completely randomized block design (DBCA) for the effect of fungicides and biocontrol agents in front of the pathogen, with 21 experimental units, three blocks with seven randomly distributed treatments per each block, both for the experiment area with broccoli addition and for the experiment area without broccoli addition. An individual analysis was carried out for each patch (with and without broccoli) and also, a homogeneity test of variances so to later proceed to make the combined analysis, where the brassica factor was included to analyze its effect. The statistical test F was performed for the different mean squares and the Turkey test at 0.05 of significance to compare averages of

treatments. Treatments (fungicides and biocontrol agents) used for this phase were the same used for the in vitro test, for both experimental field sections (Table 2). These treatments have been conducted in two Experiments:

- Experiment 1: Soil with no brassica addition.
- Experiment 2: Soil with brassicas.

Table 2. Treatments for the control of *Sclerotium cepivorum on* field for both experimental field areas.

Treatments	Active ingredients	Commercial name	Dose (g,mL/200L)	Type of product
T_1	Control	None	0.0 g	None
T_2	Iprodione	Rovral 50% PM	400 g	Contact
T_3	Thiabendazole	Mertect 500 SC	250 mL	Systemic
T_4	Boscalid	Cantus 500 WG	400 g	Systemic
T_5	Carbendazim	Protexin 500FW	500 mL	Systemic
T_6	*T harzianum*	Tricho-D	300 g	Biocontrol ag.
T_7	*Bacillus subtilis*	Serenade	1000 mL	Biocontrol ag.

c) Sclerotia count of *Sclerotium cepivorum Berk.:*

The initial population (Pi, by its Spanish acronym) of sclerotia for both experimental field sections and the final population (Pf, by its Spanish acronym) was determined by the end of the experiment. Samples of 1kg of soil per patch were taken and as of treatment, considering each point from each experimental unit. Collected samples were placed in a paper bag and taken to a 60°C stove for five days. The sucrose flotation technique (Utkhede & Rahe, 1979) was to determine the sclerotia population, and they were put under the stereoscope to count the amount of sclerotia present. A homogeneity test of variances was made between brassica patches and without brassica patches, where it was determined that there is enough homogeneity to be able of performing a combined analysis.

d) Biological and chemical control test on field:

Applications of fungicides and biocontrol agents were made in two instances:

First application: after transplantation, a total of 150 plants were treated by root immersion for 15 minutes, in a fungicide solution or biocontrol agents according to the treatment and appointed doses.

Second application: After the appearance of first symptoms, which were presented upon 60 days after the transplantation (ddt). The application was made on the plant stem, using a 20-litre bag at a continuous stream.

Third application: During the crop´s critical time, which corresponds to the formation and thickening of the bulb. Application was done to the plant stem upon 105 (ddt), using a 20-liter bag at a continuous stream.

e) Variables to be evaluated:

Initial population (Pi) and final population (Pf) of sclerotia: The initial population (Pi) of sclerotia from both experimental field sections was determined by quantifying the final population (Pf) by the end of the campaign.

Disease incidence: The percentage of healthy and diseased plants was determined by the symptoms at the foliar part (wilt), every 15 days and by taking 30 plants as a sample, which was located at the middle of each patch. Epidemics were compared by estimating the area under the disease progress curve (*AUDPC*), calculations were made using the SAS statistical package, and a variance analysis to compare the *AUDPC* based on the Tukey test ($p \leq 0.05$).

Severity: By damage in harvested bulbs, examining a 30-plant sample, roots and bulbs with pathogen signs and according to the affected area percentage in degrees. The assessment scale used was a variation to the scale proposed by Arenas, 1997.

Yield: The production by kg from each treatment and each repetition was evaluated.

Results

In vitro test.

Isolation of *Sclerotium cepivorum:* Colonies of *Sclerotium cepivorum* isolated in PDAO showed the following characteristics: White colony, during the entire mycelial growth, very fine cottony mycelium adhered to the substrate, radial growth colony. Throughout the colony growth, small conglomerations of mycelium were formed or formation of sphere-shaped black sclerotia.

In vitro biological and chemical control of *Sclerotium cepivorum:* Chemical products tested "in vitro" inhibited the development of *Sclerotium cepivorum* as well as biological products. Chemical products that completely inhibited pathogen growth were Iprodione (T2) and Carbendazim (T5); therefore, they the most effective products; meanwhile, fungicides Thiabendazole (T3) and Boscalid (T4) allowed the development of the pathogen before completely inhibit it. On the other hand, biocontrol agents also effectively inhibited pathogen growth. *Bacillus subtilis* inhibits the development of *S. cepivorum* more rapidly than *T. harzianum*. Boscalid and *T. harzianum* products in the ANVA test and statistical significance turned out to be highly significant regarding the other treatments and at the Tukey multiple comparison test ($P<0.05$) it could be observed that in Thianendazol, Iprodione, *B. subtilis,* Carbendazim products there are not statistically significant differences among them. Finally, there is a highly significant difference of the reference in regards of treatments, since its growth was satisfactory and completed its growth upon four days. In Table 3, results from the variance analysis and the statistical significance from the *Sclerotium cepivorum* growth inhibition by fungicide and biocontrol agent inhibition are shown.

Table 3. Mycelial Growth (mm) of *Sclerotinia cepivorum* in *in vitro* test

Treatments	Evaluated products	Radial growth (mm)	Inhibition (%)	Tukey ∞0.05
T1	Control	43.44	0.00	a
T2	Iprodione	0.00	100.00	c
T3	Thiabendazole	1.50	96.50	c
T4	Boscalid	15.33	64.70	b
T5	Carbendazim	0.00	100.00	c
T6	*T.harzianum*	13.44	69.10	b
T7	*B. subtilis*	0.44	99.00	c

Field results

Experiment with no broccoli addition

Final and initial sclerotia population: The number of sclerotia in treatments and repetitions was variable; however, the variance analysis (ANVA) shows that there are no significant statistical differences between blocks and between treatments, with a coefficient of variation (CV) of 30.56%. The initial inoculum of *Sclerotium cepivorum* was increased in treatments; however, there are no significant statistical differences between blocks and between treatments for final sclerotia population, with a coefficient of variation (CV) of 28.38 %.

Incidence: In the variance analysis there are no statistical differences between blocks, but in fact, significant differences between treatments have been observed; there are differences between fungicides applied for controlling the *Sclerotium cepivorum*. When performing the comparison test as of Tukey, the T1 (reference), is statistically equal to treatments with fungicide: T5 (Carbendazim), and biocontrol agents: T7 (*B. subtilis*) and T6 (*T. harzianum),* recording the biggest incidences, and therefore, the pathogen behavior was not affected by the effect of fungicides and biocontrol agents. The T4 (Boscalid) showed a better fungicidal effect against the pathogen since it showed a lower incidence percentage of 17.78%, Table 4.

Table 4. Percentage of disease incidence, Area Under Disease Progress Curve (AUDPC), severity and control treatment percentage, on field without broccoli. (Alfa=0.05)

Treatment	Incidence	Sig.	% of disease	% of control	AUDPC	Sig.	Severity	Sig.
T1 (Control)	70.0	a	100.0	0.0	2941.7	a	54.6	a
T2 (Iprodione)	52.2	a b	58.4	41.6	1716.7	a	32.6	b
T3 (Thiabendazole)	48.9	a b	64.3	35.7	1891.7	a b	38.0	a b
T4 (Boscalid)	17.8	b	29.2	70.8	858.3	a b	11.7	c
T5 (Carbendazim)	74.4	a	100.0	0.0	2941.7	b	50.9	a b
T6 *(T.harzianum)*	56.7	a	68.8	31.2	2025.0	b c	43.9	a b
T7 *(B. subtilis)*	63.3	a	89.0	11.1	2616.7	c	39.5	a b

A comparison of epidemics was carried out using the area under the disease progress curve (AUDPC). In the comparison test of Tukey, the T1 and T5, there are no significant differences and the disease development was statistically higher in AUDPC values; while at treatment 4 (Boscalid), there are significant differences between treatments, since it shows a lower value of AUDPC. Table 4. The efficiency treatment percentage, with a higher rate of S. *cepivorum* control, was, T4 with 70.82%, followed by T2 with 41.64%.

Severity: The variance analysis, ANVA, determined that there are significant differences between blocks and treatments, with a C.V of 11.33 %, severity in the bulb was highly significant between treatments, and during treatments, it was shown that they had the highest incidence percentage. The T4 presented highly significant differences among them, and also shown the lowest severity levels, with a percentage of 11.67 %, which corresponds to degree 3, with the presence of pathogen´s plant structures in roots and the outer part of the bulb, which allow classifying these bulbs as commercial ones. The reference treatment presented the highest severity levels with 54.63 % of severity, which corresponds to a degree 5, with the presence of non-usable or non-commercial bulbs. Table 5 shows Tukey´s comparison test and the severity percentage in treatments.

Yields: The harvested commercial bulb yield was classified by first quality, second quality and total yield; variance analysis ANVA were carried out for each one, where for first yields highly significant differences were shown between blocks and treatments with a coefficient of variation of 22.03%. This shows that yields both from blocks and treatments were variable, presenting certain difference due to the high sclerotia density and high disease incidence, and as a consequence, different severity degrees were shown in the several phenological stages of the growing, impacting yields as a result.

The variance analysis in the second quality yield does not show significant differences between blocks and treatments, with a coefficient of variation of 24.80 %. As for the total yield, there are no differences between blocks, but they are between treatments. First quality yield, the T4 (Boscalid) showed the highest values with 28.06 T/ha, and it was significantly different in regards of the other treatments, lowest levels of yield were for the reference and T5 (Carbendazim) with 4.31 and 8.06 T/ha, respectively. As for the second quality yield, no significant differences between treatments are shown. Finally, as for the total yield, treatment 4 had the highest yield with 38.33 T/ha, and with a lower incidence percentage and lower degree of severity of bulbs, followed by treatment 2, (Iprodione) with 25.56 T/ha. The lowest yield was shown in treatment 5 (Carbendazim) with 15 T/ha. Table 5.

Table 5. Test for first and second yields and total yield with different fungicide treatments with no broccoli addition. (alfa=0.05).

Treatment	First yield T/ha		Second yield T/ha	Total yield T/ha	
	Mean	Sig.	Mean	Mean	Sig.
T1 (Control)	4.31	b	11.67	15	b
T2 (Iprodione)	11.06	b	13.89	25.56	ab
T3 (Thiabendazole)	9.72	b	15	24.72	ab
T4 (Boscalid)	28.06	a	10.28	38.33	a
T5 (Carbendazim)	8.06	b	6.94	15.97	b
T6 *(T.harzianum)*	8.94	b	13.89	22.83	ab
T7 *(B. subtilis)*	8.19	b	12.78	20.97	b

Experiment with broccoli addition

Initial and final sclerotia population: The number of sclerotia in treatments and blocks was variable, as a result, there several sources of initial inoculum. However, the variance analysis performed (ANVA) shows that there are no significant statistical differences between blocks and treatments, with a coefficient of variation (CV) of 29.42; the variance analysis performed (ANVA) showed that the final population of sclerotia in the experimental field does not present significant statistical differences between treatments and between blocks, with a coefficient of variation (CV) of 29.40 %.

Incidence: There are statistical differences between blocks and highly significant differences between treatments with a coefficient of variation of 9.11%. At the comparison test of Tukey, the T1 (reference) and T5 (Carbendazim) are statistically equal and presented the highest percentages of final incidence; while T3 (Thiabendazole), T6 (*T. harzianum)* and T7 (*B. subtilis*) presented an effect on the pathogen statistically equal, since there are no differences between these treatments. Lowest final incidence percentages and best fungicidal effect against the pathogen was presented in T4 (Boscalid). Table 6.

Table 6: Percentage test for disease incidence, AUDPC, severity and control percentage of treatments, on field with broccoli addition. (alfa=0.05).

Treatment	Incidence	Sig.	AUDPC	Sig.	% of disease	% of control	Severity	Sig.
T1 (Control)	68.9	a	2700.0	a	100.0	0.0	48.9	a
T2 (Iprodione)	45.6	b	1525.0	a b	56.5	43.5	34.7	a
T3 Thiabendazole)	53.3	a b	1650.0	b	61.1	38.9	35.7	a
T4 (Boscalid)	11.1	c	566.7	c	21.0	79.0	7.0	b
T5 (Carbendazim)	67.8	a	2366.7	c	87.7	12.4	39.9	a
T6 *(T.harzianum)*	55.6	a b	1766.7	c	65.4	34.6	40.9	a
T7 *(B. subtilis)*	54.4	a b	2183.3	d	80.9	19.1	37.9	a

At the area under the disease progress curve (AUDPC) there are no significant differences between blocks, but there are highly significant differences between treatments, and thus the epidemiological development in treatments had a different behavior among them. In the comparison test between T1 (reference) and 5 (Carbendazim), there are no significant differences. The T4 (Boscalid) shows a lower value of AUDPC, and therefore, there is a highly significant difference between treatments. As for the coefficient of variation, the highest percentage of control for S *cepivorum* with T4 is 79.01%, followed by T2 with 43.52%, the lowest percentage of control was obtained from the treatment 5 with a percentage of 12.35%. Table 6.

Severity: The variance analysis ANVA determined that there are no statistical differences between blocks but there are significant differences between treatments with a C.V of 9.40 %, the biggest damage in bulbs was presented in T1 (reference) with a 48.89 %, which corresponds to a degree 5, according to the assessment scale, where plant and conservation structures from the pathogen and non-profitable bulbs are shown. The T4 (Boscalid) with a severity percentage of 7.04 % corresponding to a degree 3 of severity, where harvested bulbs can be considered as profitable, or with low severity levels; therefore, T4 in regards of the other treatments, presented highly significant differences, likewise, incidence levels of the disease were lower in this treatment, increasing upon 105 days, bulb filling stage, and remaining constant throughout time. Table 6.

Yield: Variance analysis for first and second quality bulbs shows significant statistical differences between blocks and treatments, with a coefficient of variation of 23.43 % and 31.17 % respectively. The total yield shows that there are no differences between blocks; however, there were differences between treatments with a coefficient of variation of 15.72 %. In the comparison test for first quality yields, the T4 (Boscalid) showed the highest values with 38.33 T/ha, and it was significantly different regarding the other treatments, including the reference treatment, which showed the lowest levels of yield along with Treatment 5 (Carbendazim) with 5.19 and 8.28 T/ha, respectively. The biggest second quality yield is shown in the reference treatment with 20.83 T/ha, with a tendency of having a yield similar to the other treatments, excepting for the T4 (Boscalid). The highest total yield was presented by T4 with 43.33 T/ha, with significant differences between treatments using fungicides and biocontrol agents. Table 7.

Table 7: Test for first and second quality yields and total yield with different fungicide treatments with broccoli addition. (alfa=0.05).

Treatment	First yield T/ha		Second yield T/ha		Total yield T/ha	
	Media	Sig.	Media	Sig.	Media	Sig.
T1 (Control)	5.3	b	5.0	b	19.6	b
T2 (Iprodione)	12.8	b	18.1	ab	30.1	b
T3 (Thiabendazole)	12.1	b	15.8	ab	28.3	b
T4 (Boscalid)	38.3	a	20.8	a	43.3	a
T5 (Carbendazim)	8.2	b	11.4	ab	22.5	b
T6 *(T.harzianum)*	11.7	b	15.6	ab	27.5	b
T7 *(B. subtilis)*	9.7	b	12.8	ab	26.1	b

Combined analysis of the two experiments with broccoli and without broccoli

Final and initial sclerotia population: The combined analysis in its variation block source within brassicas; treatment brassicas and brassica treatment interaction does not show a significant statistical difference. The comparison test of brassicas factor in the two experimental fields (with and without broccoli) did not show significant differences in the final and initial sclerotia population.

Incidence: The combined analysis does not show statistical differences between the final incidence and AUDPC, so to determine the difference between these two experimental fields and final incidence treatments and AUDPC, a Tukey´s multiple comparison tests were performed. Where fields with an addition of broccoli and with no broccoli show no significant differences, thus, brassica effect did not impact on the pathogen behavior; however, AUDPC and final incidence averages for the field with addition of broccoli were lower in final incidence percentage with 50.95 % and AUDPC with 1822.6. The comparison test of the combined final incidence analysis shows statistical differences between chemical and biological treatments. The T4 showed highly significant differences with a lower final incidence percentage of 14.4 %.

T1 and T5 are the treatments with the highest values on the development of the disease progression curve AUDPC, there were no significant differences with treatment 7, but there were statistical differences regarding the other treatments. Treatment 4 showed significant differences between treatments using biocontrol agents and treatments using fungicides, with a lower level in AUDPC of 712.50. Fig.1.

The best treatment was Boscalid; treatments Thiabendazole and Carbendazim had a poor effect on the pathogen, which did not stop the disease development. Product efficiency decreases, and more applications will be needed to reach the same control percentage since control effect from fungicides is partial. As for the control effect from biocontrol agents, fighting capacity depends mainly on environmental conditions, soil type, formulation, pathogen and pathogen-host relation.

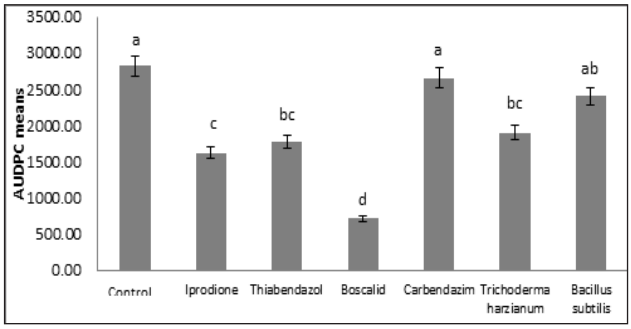

Figure 1: Combined analysis of AUDPC between study treatments

Severity: The variance homogeneity test between patches with broccoli and without broccoli showed that there is homogeneity to perform the combined analysis. There are significant differences too between brassica block factors and treatment factors. There are no significant differences in the brassica treatment factor because brassica effect was a determining factor for the disease development and in the manifestation of symptoms and signs. The Tukey´s comparison test 0.05, showed that the brassica factor did not present significant differences between them, the severity percentage for both fields was statistically the same in regards of its severity manifestation in harvested bulbs. T1 (reference) and T5 (Carbendazim) are statistically equal and with high severity percentages of 51.76% and 45.41% respectively. T4 (Boscalid) showed a lower severity percentage regarding the other treatments with 9.35%, is statistically different.

Yields: A variance homogeneity test was **performed** on the patches with broccoli and with no broccoli, which showed that there is homogeneity was done a combined analysis, the coefficient of variation was 44.32%. The brassica factor (with broccoli and no broccoli) in the Tukey´s comparison test, 0.05, presented significant differences between the addition of brassicas and with no addition of brassicas, therefore, this effect influences treatments and bulb production (T/ha). The First quality yield of treatments T5 (Carbendazim) and T1 (reference), showed the lowest values of yield, being statistically different regarding treatment T4 (Boscalid), which showed the highest value of yield with 33.20 T/ha. The second quality yield showed significant differences between treatments; thus, the brassica factor influences the yield. The total yield in the comparison tests showed significant differences and a higher yield in treatment 4 (Boscalid) with de 40.83 T/ha. Table 8.

Profitability analysis

Net profitability analysis reaches its highest value in treatment T4 (Boscalid) with 0.68 with no addition of broccoli, and profitability of 1.00 in the area treated with the addition of broccoli. Also, it should be noted that among

treatments being studied, in the area with brassica residues is seen three treatments showing positive profitability and unlike the untreated area, they contributed directly in obtaining a better production. The rest of treatments showed negative profitabilities in this trial. Therefore, from this point of view, the best treatment was treatment 4.

Table 8: Tukey´s comparison test for first and second quality yield and total yield in treatments.

Treatment	First yield T/ha		Second yield T/ha		Total yield T/ha	
	Media	Sig.	Media	Sig.	Media	Sig.
T1 (Control)	8.1	b	16.3	a	23.5	b c
T2 (Iprodione)	11.9	b	14.7	a	27.0	b
T3 (Thiabendazole)	10.9	b	16.5	a	27.4	b
T4 (Boscalid)	33.2	a	7.6	b	40.8	a
T5 (Carbendazim)	4.8	b	9.2	a b	17.3	c
T6 *(T.harzianum)*	10.3	b	14.9	a	25.2	b c
T7 *(B. subtilis)*	9.0	b	12.8	a b	19.2	b c

Discussion

In vitro biological and chemical control of *Sclerotium cepivorum:* Similar results are reported by Oliveira *et al* (1982), who performed fungicide tests with five isolations from different locations of *Sclerotium rolfsii* (fungus similar to *S. cepivorum),* where he found that the in vitro sensitivity effect from different fungicides on the radial mycelium growth and the growing tendency of the *Sclerotium rolfsii* fungus was effective. He also mentions that the Thiabendazole and Iprodione presented fungicidal effects, delaying the mycelial growth. It could be assumed that the same behavior would be present for another type of fungicide, such as the Boscalid, and that its fungicidal effect could vary according to the different fungus isolations. Contrasting results had already been noted by other researchers for this fungus, which previous works showed variation in reactions of the mycelial growth from different fungus isolation, using fungicides such as Utkhede and Rahe (1983), proved the effective control in vitro of *S cepivorum* with four isolations of the *B subtilis* bacteria. Regarding *T. harzianum,* a pathogen growth inhibition occurred, with a radial growth of 13,44 mm, which represents a 69.1%. These results also agree with reports from Oliveira *et al.* (1982), Arenas (1997), Obregón (2001); who obtained similar controls of *S. cepivorum,* by using *Trichoderma harzianum*.

Final and initial sclerotia population with no broccoli addition: According to what was reported and the number of initial sclerotia in treatments with densities of 0.45 to densities of 0.77 sclerotia per soil gram, are capable of producing very high incidences of disease on field, and their behavior regarding the disease development will depend on favorable conditions and control measures performed to inhibit or delay their infection. Rosas *et al.* (2010), point out that there is only enough one sclerotium per soil gran, this is equal to 1,000 sclerotia/kg of soil, to produce the death of 100% of onion plants.

Initial and final sclerotia population with broccoli addition: The initial population of *Sclerotium cepivorum* regarding the final population was increased in treatments in an aggregate way, Coley Smith *et al.* (1990), mentions that survival structures in S *cepivorum* are very resistant against adverse conditions and its survival is kept above the 92%, and feasibility can reach up to 96% between 5 and 10 years that remain in the ground.

Incidence with broccoli and without broccoli: The lowest control percentage was obtained from T5 and presented a behavior equal to the reference treatment. In the case of biocontrol agents, *T. harzianum* was more effective than *B. subtilis*. These results coincide with results obtained by Rosas *et al*. (2010), where T. *harzianum´*s efficiency was proved in a higher percentage than *Bacillus subtilis* for the control of *S cepivorum.* Table 4. According to these incidence results and disease progression, Coley Smith *et al*. (1990), mentions that the low efficiency of some fungicides (Thiabendazole, Carbendazim, Iprodione) is due to the microbial degradation, and also adds that, the control is more limited as sclerotia population increases in the soil.

Combined analysis of the two experiments with broccoli and without broccoli

Final and initial sclerotia population: Sclerotia density at the area treated with residues was reduced; however, there no significant differences between both experimental areas, Villar *et al*. (1990) mentions that when adding cabbage or broccoli residues on the soil infested with *S. cepivorum,* this significantly reduces the number of dead plants and the disease incidence.

Conclusions

Fungicides Iprodione and Carbendazim were the ones that completely inhibited the growth of the in vitro pathogen, which showed significant differences regarding the reference treatment. The best product for the in vitro biological control of *Sclerotium cepivorum* was *Bacillus subtilis*, which inhibited the fungus growth a lot faster than *Trichoderma harzianum* that also achieved a good control but with a slower inhibition.

Field conditions with broccoli residues and without broccoli residues, fungicides that had a better control effect were Boscalid with 79.01% and 70.82% and Iprodione with 43.52% and 41.64% against *Sclerotium cepivorum* and also having a lower incidence percentage and a lower severity level in bulbs. In field conditions with broccoli residues and without broccoli residues, *Trichoderma harzianum* had a better control effect with 34.57% and 31.16% against *Sclerotium cepivorum,* unlike *Bacillus subtilis* with 19.14% and 11.05%.

The effect of adding broccoli residues on the sclerotia density on the soil was not statistically significant, but with the addition of broccoli residues, there were significant statistical differences for incidence, severity and yields, both for patches with chemicals, biocontrol agents and on the reference one. Also, lower percentages of incidence, severity and higher yields were obtained, unlike the case with no addition of broccoli residues. Treatments that showed a higher yield in red onion production was treatment 4 (Boscalid) with yields of 43.33 T/ha for the area with the addition of broccoli, and 38.33 T/ha for the area with no addition of broccoli; followed by treatment 2 (Iprodione), where yield on the area with addition of broccoli was 28.33 T/ha and on the area with no addition of broccoli was 25.56 T/ha.

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Peruvian Journal of Agronomy 2 (3): 35-43 (2018)
ISSN: 2616-4477 (Versión electrónica)
DOI: http://dx.doi.org/10.21704/pja.v2i3.1231

Received for publication: 30 November 2018
Accepted for publication: 28 December 2018

Pseudomonas of the rhizosphere of avocado (*Persea americana* Mill.) with biocontrol activity of *Phytophthora cinnamomi* Rands isolated in the central coast of Peru

Pseudomonas de la rizosfera del Palto (*Persea americana* Mill.) con actividad de biocontrol de *Phytophthora cinnamomi* Rands aisladas en la costa central del Perú

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Abstract

In the rhizosphere, as the zone of biological activity, diversity of microorganisms can be found like bacteria of the genus *Pseudomonas*. They are characterized for controlling pathogens like *Phytophthora cinnamomi*, also to be promoters of growth. For this reason, in the present study, bacteria of the genus *Pseudomonas* were isolated from the avocado rhizosphere of the provinces of Casma, Huaral, and Lima. A total of six strains (R2, R5, R7, R10, S10 and S6) were selected for evaluating their biocontrol capacity against *P. cinnamomi* under in vitro and greenhouse conditions. In the in vitro test, strains S6 and S10 controlled 30.3 and 44 %, respectively. Under greenhouse conditions, *Pseudomonas* strains were inoculated on 4-month-old avocado cv. "Zutano" plants. Five months later, we evaluated the following variables: severity in roots, height increase, fresh root and leaf weight, and percentage of root and leaf dry matter. In greenhouse, the best strains in the control of *P. cinnamomi* were S6, R2, R7 and R10, controlling 55.2, 39.5, 33.7 and 31.0 %, respectively. In the increase of height, the strains S6, R2, R7 and R10 reached 11.4, 9.3, 7.6 and 5.1 cm, respectively. The percentage of dry matter of roots, strains S10, R10 and R7 obtained 29.6, 27.5 and 27.9 %, respectively. In this study, it was observed that although the application of *Pseudomonas* controls *P. cinnamomi*, it also induces the root and apical growth of avocado.

Keywords: avocado, promoter of growth, *Pseudomonas*, rhizosphere.

Resumen

En la rizósfera como zona de actividad biológica, se pueden encontrar diversidad de microorganismos como bacterias del género *Pseudomonas* que se caracterizan por controlar patógenos como *Phytophthora cinnamomi*, también por ser promotores de crecimiento (PGPR). Por esta razón en la presente investigación se aislaron bacterias del género *Pseudomonas* de la rizósfera de palto de las provincias de Lima, Huaral y Casma. Se seleccionaron 6 cepas (R2, R5, R7, R10, S10 y S6) con las que se realizaron pruebas para evaluar su capacidad biocontroladora frente a *P. cinnamomi* in vitro e invernadero. En la prueba *in vitro* las cepas S6 y S10 controlaron un 30.3 y 44 %; respectivamente. En condiciones de invernadero, se inocularon cepas de *Pseudomonas* en plantones de palto cv. Zutano de 4 meses de edad; transcurridos cinco meses, se evaluó variables como severidad en raíces, incremento de altura, peso fresco radicular y foliar y porcentaje de materia seca radicular y foliar. En invernadero, las mejores cepas en el control de *P. cinnamomi* fueron S6, R2, R7 y R10 que controlaron un 55.2, 39.5, 33.7 y 31.0 %; respectivamente. En el incremento de altura, las cepas S6, R2, R7 y R10 alcanzaron 11.4, 9.3, 7.6 y 5.1cm; respectivamente. El porcentaje de materia seca de raíces, las cepas S10, R10 y R7 obtuvieron 29.6, 27.5 y 27.9 %; respectivamente. En este estudio se observó que si bien la aplicación de *Pseudomonas* ejerce un control sobre *P. cinnamomi* también induce el crecimiento radicular y apical de la planta.

Palabras Claves: palto, PGPR, *Pseudomonas*, rizósfera.

Introduction

Peru has an avocado producing area of around 30,320 hectares with a total production of 349,317 tons. The coastal departments of Ancash, Ica, La Libertad, and Lima have the biggest cultivated area. (MINAGRI, 2014).

Peru is the second exporter of avocado worldwide, with 175.6 million kilos exported (Arteaga, 2016). Therefore, avocado production in Peru will continue to be an attractive business due to the opening of new markets and new consumers who value their nutritional properties.

The production of the avocado depends on climatic, edaphic, nutritional and sanitary factors. Within the sanitary aspect, diseases are one of the factors that augment the production costs of the fruit. Among the most important, is "root rot" caused by *Phytophthora cinnamomi* (Chromista, Heterokontophyta). This pathogen limits tree development, reduces fruit production and quality, directly affecting profitability.

Currently, this oomycete is controlled by chemical products, such as fosetil-Al and metalaxyl (Mora, 2007),

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but the constant use of chemical products diminishes its effectiveness over time due to the resistance that this phytopathogen develops towards the fungicide. For these reasons, biological control is considered to take advantage of soil microorganisms with antifungal activity, including bacteria of the genus *Pseudomonas*, responsible for the suppression of some soil pathogens (Raaijmakers *et al.,* 2002).

Bacteria of the genus *Pseudomonas* have been widely studied as biological controllers for their ability to colonize the root, compete aggressively with other microorganisms, adapt to different situations of environmental stress, synthesize antibiotics and enzymes, and activate systemic resistance in plants (Weller *et al*., 2006).

For this reason, in the present work, we evaluated under in vitro conditions the antagonistic effect of strains of *Pseudomonas* spp. isolated from the rhizosphere of avocado on *P. cinnamomi* by scoring the progress of the pathogen in centimeters. In addition, in the greenhouse; the severity of the infection of *P. cinnamomi* in avocado seedlings previously inoculated with the strains of *Pseudomonas* spp. was measured by the percentage of lesions in roots, and the effect of these strains on the growth of the avocado seedlings was measured by the increase of height of the stem in centimeters.

Materials and methods

Field work. The sampling was carried out in commercial avocado fields that reported root rot problems in Casma, Huaral and Lima. In each field, 10 avocado trees were randomly selected. Roots were collected from each tree with a volume of rhizospheric soi from four cardinal points, with a total equivalent of 250g of soil. These samples were transported and kept in cold conditions by using gel packs to maintain their temperature and humidity.

Laboratory work. The laboratory work (in vitro isolation) was carried out in the facilities of the Laboratory of the Department of Phytopathology of the National Agrarian University of La Molina (UNALM for its acronym in Spanish), located in the district of La Molina, Province and Department of Lima, at 12° 05' 06" S of latitude, 76° 57' 07" WG of longitude and 243.7 m.a.s.l.

Sample Treatment. The microflora of both the rhizosphere and the soil was analyzed separately. Under aseptic conditions, 3 g of root of each sample was placed in test tubes with 9 mL of peptonated water in continuous agitation, performing 7 serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}), adapted from Andres (1991). For the soil sample near the root, 10 g were placed in 90 mL of peptone water (ratio 1: 9), following the same previous procedure.

Count of *Pseudomonas* spp. In regard to the quantification of *Pseudomonas* spp., 1 mL of the dilution (10^{-2} to 10^{-5}) was seeded in triplicate in tubes containing 9 mL of asparagine broth. These tubes were incubated for four days at 37°C (Burges, 1960). This procedure was performed for both root and soil.

Isolation and identification of *Pseudomonas* spp. The tubes that were positive (fluorescent) to the asparagine broth culture were striated in the King-B culture medium and incubated for 48 hours, and fluorescence was evaluated in ultraviolet light at 366 nm (Schaad, 2001) together with the morphology of the colonies (Palleroni, 2005). The colonies that were positive for fluorescence were then selected.

From each isolate, a colony was selected, which was striated in the Tryptone Soy Agar (TSA) culture medium to ensure the purity of each isolate and to be stored in TSA agar wedges at 4°C for the next tests (Martínez, 2010).

Pathogen isolation (*Phytophthora cinnamomi* Rands). Rootlets of 1-3 millimeters in diameter that presented the typical symptomatology corresponding to a black and firm rot that originated from the zone of elongation were selected. They were washed with distilled water and segments of about 1 cm long that presented the area of pathogen progress were cut. Then, they were immersed for a few seconds in a 70% alcohol solution in order to prevent possible contamination (Zentmyer, 1980).

These pieces of 1 to 2 mm^2 of diseased and asymptomatic tissue were seeded in selective culture medium CMA (Corn Meal Agar) with PARB (Pimaricin-Ampicillin-Rifampicin-Benomyl) (Erwin & Ribeiro, 1996). They were incubated at 22°C for seven days, and the growth rate of the colony and its characteristics were observed (Alvarado-Rosales *et al.*, 2007).

Each pure colony in CMA was transferred in V8 juice agar medium for four days at 21°C. From slices of grown mycelium in V8 juice agar that was introduced in Petri dishes containing 1% soil solution, structures of the pathogen were visualized as sporangia.

The identification of *P. cinnamomi* was conducted by following the Dichotomous Key to Taiwan Species of *Phytophthora* (Ho, 1992).

Antagonism test against *Phytophthora* cinnamomi. From each previous isolate, the strains that controlled *Phytophthora cinnamomi* were identified. These strains were used in the challenge test in Nutrient Agar culture medium. It was necessary to prepare culture medium in which the corresponding reagents were included because the results of this test were not separately obtained. In each plate containing medium PDA + King-B + Nutrient Agar a controller was included (*Pseudomonas* spp. strain) and a pathogen disk.

This methodology was carried out with the objective of identifying strains of *Pseudomonas* spp. with greater control over *P. cinnamomi*, demonstrating a higher percentage of inhibition of the radial mycelial growth of the pathogen (% I).

Greenhouse phase. The greenhouse phase was carried out in the Greenhouse Research Station of the Department of Phytopathology of UNALM.

Vegetal material. We used for four-month old plants of avocado var. 'Zutano' which were treated before to the germination (immersed in Homai fungicide solution in 5 g/l dose of product), according to the disinfection protocol of the nursery of the Fruit Trees Research Program of National Agrarian University - La Molina. The transplant to bags was made in January 2016 to be taken to the greenhouse.

Preparation of the Substrate. The substrate mixture consisted of 50% soil, 25% river sand and 25% organic matter (Herrera & Narrea, 2011). Then, it was sterilized at 121°C for 15 minutes. The substrate was used in black polyethylene bags measuring 8x16x2.2 cm^3 (5.5 l), where the sterile soil was placed. Then, we proceeded to transplant the avocado.

Preparation, density and inoculation of the controller. Selected strains of *Pseudomonas* were seeded in peptone water at 27°C and 150 rpm (Revolutions per Minute) until reaching a population of $6x10^8$ (Colony Forming Units) CFU/mL (Martínez, 2010).

Pathogen inoculation. According to Drenth & Sendall (2001), it is recommended to use mycelium developed in wheat previously sterilized at 121°C and a pressure of 1.1 bar (15 lb/in^2 or 15 psi) for 30 minutes for two consecutive times.

The inoculation was done in five-month-old avocado plants. The application was made around the root system of the avocado at a depth of 5 cm (the dose of inoculum was 2.5 g of wheat with mycelial development/kg of soil) (Zentmyer & Richards, 1952). The humidity of the substrate was maintained with periodic irrigation to favor the development of the pathogen.

The mycelium of *Phytophthora cinnamomi* was inoculated ten days after the inoculation of the controller, according to Martínez (2010).

Evaluated parameters.

Disease severity and root length. The severity and root length were measured by processing digital photographs of avocado roots using ASSESS software (Lamari, 2002).

Plant Height. We used a millimeter ruler. Measurement (length of the main stem) was taken from the neck of the plant to the terminal bud at the time of inoculation of the biocontroller and at the end of the experiment. The results were expressed in centimeters.

Fresh weight, and dry root and foliar matter. To obtain the percentage of dry matter, firstable the roots and leaves of the plant were placed in paper bags to be weighed; then they were taken to the stove at a temperature of 70°C for 48 hours to obtain the dry weight. The weights were expressed in grams using electronic balance.

Biochemical characterization of *Pseudomonas* spp. The following biochemical tests were carried out, according to Palleroni (2005): (i) production of fluorescent pigments, (ii) resistance to high and low temperatures, (iii) and gelatin liquefaction, in order to characterize strains of non-pathogenic fluorescent *Pseudomonas* from the rest of species

Fluorescence in King-B culture medium. The culture medium of King-B is used for the detection of fluorescein, a green or blue fluorescent soluble in water. After growth of 24-48 hours at 27°C, the colonies, previously striated in King-B, were observed with an ultraviolet lamp (366 nm).

Growth at 4 and 41°C. Strains of *Pseudomonas* spp. were planted in tubes containing TSA culture medium, which were incubated at 4 and 41°C.

Jellied liquefaction. This test is used to determine the capacity of a microorganism to produce enzymes of the proteolytic type (gelatinases) that liquefy the gelatin. It is positive for *Pseudomonas aeruginosa* (pathogenic) (Mac Faddin, 1980).

The tubes containing nutrient gelatin were prepared considering three replications per treatment. The strains of *Pseudomonas* spp. were then inoculated by puncture and incubated at 37°C for 48 hours. Finally, they were placed in the refrigerator at 4°C for 2 hours. The test was positive if the inoculated medium became liquid, and the test was negative if the inoculated medium maintained its characteristics (Mac Faddin, 1980).

Experimental design. The statistical design used in the antagonism test against *Phytophthora cinnamomi* was a completely randomized statistical design (CRD), with ten treatments of strains isolated from *Pseudomonas* spp., four dishes per treatment and control treatment of *P. cinnamomi* pathogen (Table 1). The parameter that was evaluated in this test was the percentage of inhibition of grown radial mycelial, and the differences between the average of cultures analyzed out through the Tukey test (P = 0.05).

In greenhouse, the statistical analysis of variance (ANOVA) and Tukey test with a level of significance at 5% were conducted to identify whether or not there are significant differences between the results obtained with each evaluated strain. All statistical analyses tools were applied with minimum confidence of 95% and SAS 9.2 software (SAS, 2009).

Table 1. Treatments used in the present study.

Treatment	Description
T1	*P. cinnamomi* + strain of *Pseudomonas* spp. (R2)
T2	*P. cinnamomi* + strain of *Pseudomonas* spp. (R5)
T3	*P. cinnamomi* + strain of *Pseudomonas* spp. (R7)
T4	*P. cinnamomi* + strain of *Pseudomonas* spp. (R10)
T5	*P. cinnamomi* + strain of *Pseudomonas* spp. (S10)
T6	*P. cinnamomi* + strain of *Pseudomonas* spp. (S6)
T7	Control treatment: *P. cinnamomi* (Phy)
T8	Absolute control treatment (T)

Results

Isolation, purification and identification of *Pseudomonas* spp. A bacterial density of *Pseudomonas* spp. was obtained from the sampled areas which varied between 10^5 and 10^8 NMP/g of roots and dry soil. In Table 2, it can be observed that the Casma area presented the highest levels of bacterial density because the sampled farm promotes the microbial flora with applications of organic matter. We worked with Casma strains because they had a higher inoculum potential compared to the other areas sampled.

Table 2. Count of *Pseudomonas* spp. (NMP/g) isolated from the central coast of Peru.

Location		NMP/g
Lima	Soil	$2.3x10^6$ organisms /g of soil
	Root	2.3x106 organisms/g of root
Huaral	Soil	$9.3x10^5$ organisms /g of soil
	Root	$4.3x10^5$ organisms/g of root
Casma	Soil	$>1.1x10^8$ organisms /g of soil
	Root	$>1.1x10^8$ organisms/g of root

The isolates that grew and emitted fluorescence in asparagine broth were striated in King-B culture medium (also called *Pseudomonas* agar F) for the isolation of all *Pseudomonas* spp. (Schaad, 2001), thus differentiating them from other gram-negative microorganisms that do not emit fluorescence. A total of 14 positive isolates were obtained for this test.

Antagonic effect in vitro test. Figure 1 shows that the isolates from soil S7, S10, S2, S9, S4, S1, S6 and S3 inhibit the advance of *P. cinnamomi*; being constant in time, while in isolations from roots (R7, R10, R4 and R5) there is a strong inhibition at 4 DAS (days after sowing), but it is lost quickly over time.

All the evaluated strains reported some control varying the percentages of inhibition between 28.3 to 47% (Figure 2).

The isolates R2, R5, R7, R10, S6 and S10 showed growths above the mycelium of *P. cinnamomi*, being reduced to a yellow gelatinous mass. From there, samples were taken to be observed in a microscope. In isolates from the confrontation *P. cinnamomi* and *Pseudomonas* spp. fluorescence observed in a microscope (40X), the disintegration of the cytoplasmic content of the mycelium of *P. cinnamomi* was observed.

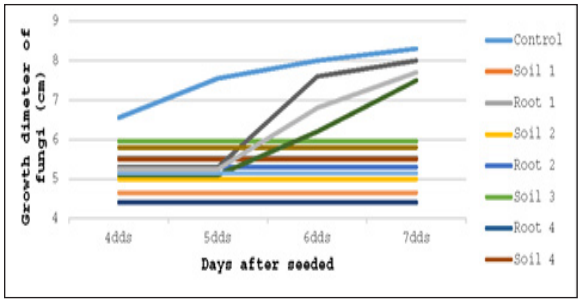

Fig. 1. Advance of *P. cinnamomi* (in centimeters) against soil and root isolates of *Pseudomonas* spp. for 7 days after seed in vitro test.

In the biochemical characterization tests, according to the Bergey Manual (Palleroni, 2005), carried out on strains of *Pseudomonas* spp. selected (R2, R5, R7, R10, S6 and S10) the results were obtained (Table 3) in which it is determined that the strains evaluated do not correspond to *Pseudomonas aeruginosa* (pathogenic to man).

Table 3. Reaction of different strains of *Pseudomonas* spp. to the biochemical tests.

	Fluorescent pigments	Grows at 4°C	Grows at 41°C	Gelatin liquefaction
P. fluorescens	+	+	-	+/ -
P. putida	+	+	-	-
P. aeruginosa	+	-	+	+
Strain R2	+	+	-	-
Strain R5	+	+	-	-
Strain R7	+	+	-	-
Strain R10	+	+	-	-
Strain S10	+	+	-	-
Strain S6	+	+	-	-

Greenhouse evaluation.

Evaluation of the severity of avocado roots. Treatments R5, S10, R10, R7 and R2 (60.4, 66.2, 69.0, 77.5, 78.4 and 90.7%, respectively) show percentage of severity that is not significantly different from the treatment inoculated with the pathogen. On the other hand, the S6 treatment has a significant difference with the control treatment and the treatment inoculated with *P. cinnamomi* with only 44.8% of severity in avocado roots (Figs. 2 and 3, Table 4).

Figure 2, strains R5 and S10 showed severity values (90.7 and 78.4%, respectively) above the control inoculated with *P. cinnamomi* (77.4%), this loss of antagonistic capacity could be due to population decline of these strains (Van *et al.*, 1997).

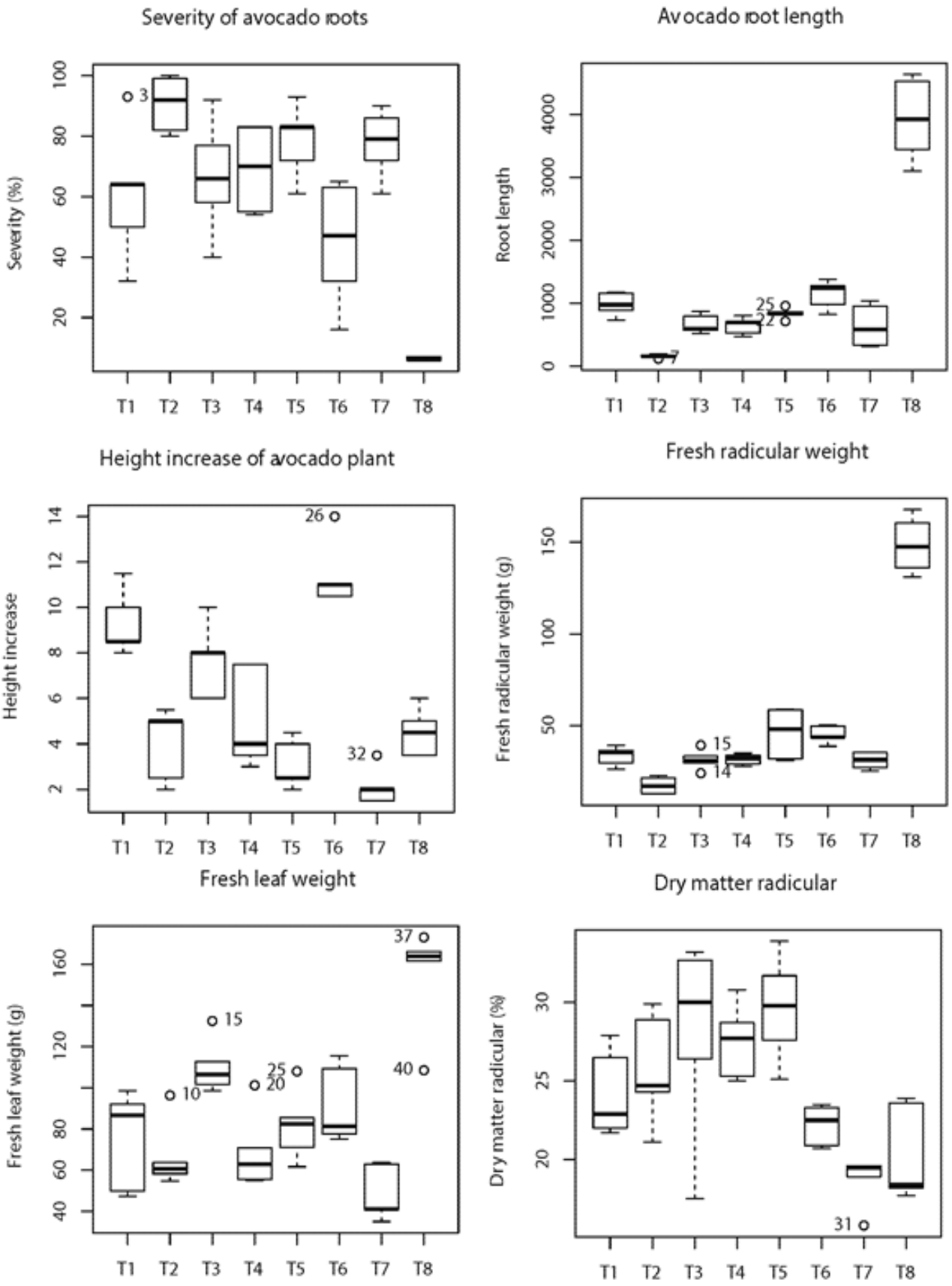

Fig. 2. Measurement of severity of avocado roots (%), avocado root length, height increase of avocado plant (cm), fresh radicular weight (g), fresh leaf weight (g) and dry matter radicular (%) of treatments inoculated with strains *Pseudomonas* spp. against *P. cinnamomi,* under greenhouse conditions.

It is also observed that the best treatment in the control of *P. cinnamomi* in greenhouse was T6 (inoculated with the S6 strain of *Pseudomonas* spp.) which showed 59.0% of control.

Table 4. Measurement of avocado variables with the comparison test of means (Tukey) of treatments inoculated with strains *Pseudomonas* spp. against *P. cinnamomi,* under greenhouse conditions.

Treatments	Strains	Severitya (%)	Increase in heightb (cm)	Foliar biomassc (g)	Root lengthd (cm)	Biomass of rootse (g)	Root dry matterf (%)
T1	R2	60.462 AB	9.3 AB	74.86 BCD	989.1 B	33.320 BC	24.200 ABCD
T2	R5	90.720 A	4.0 D	66.70 CD	160.6 C	17.340 C	25.780 ABC
T3	R7	66.274 AB	7.6 BC	110.38 B	672.2 BC	31.500 BC	27.960 AB
T4	R10	69.034 AB	5.1 CD	69.04 CD	640.5 BC	31.500 BC	27.500 AB
T5	S10	78.456 A	3.1 D	81.68 BCD	841.1 B	45.680 B	29.620 A
T6	S6	44.848 B	11.4 A	91.76 BC	1142.2 B	45.220 B	22.180 BCD
T7	Phy	77.466 A	2.1 D	48.68 D	648.1 BC	30.880 BC	18.680 D
T8	T	6.578 C	4.5 D	154.68 A	3924.6 A	148.640 A	20.360 CD

Coefficient of variation (alpha 0.05): 24.86[a], 25.17[b], 22.07[c], 26.17[d], 17.55[e], 13.98[f].

Avocado root length evaluation. The effect of *Pseudomonas* sp. on the length of avocado roots showed a significant difference between treatments. In all treatments, shorter root length was observed compared to the control treatment (Figs. 2 and 3, Table 4). Therefore, inoculated *Pseudomonas* strains influenced root growth but did not achieve significant difference with respect to the control.

Treatments S6, R2 and S10 presented longer root lengths (1142.2, 841.1 and 989.1, respectively) than the control inoculated with the pathogen (648.1).

Evaluation of the height increase of avocado plant. Inoculation of strains S6 and R2 showed a greater increase in height, 11.4 and 9.3 cm respectively, compared to the control treatment (Fig. 2). There is no significant difference between treatments S6 and R2, according to the Tukey test at 95% (Table 4). Figures 2 and 3 show all treatments with inoculation of *Pseudomonas* spp. have values that are above the control inoculated with *P. cinnnamomi* (2.1 cm). The treatments inoculated with strains of *Pseudomonas* spp. present greater amplitude of the interval of variance of their repetitions.

Evaluation of fresh radicular weight. Regarding fresh radicular weight, treatments S10 and S6 (45.6 and 45.2 g) show a significant difference compared to the treatment inoculated with *P. cinnamomi* (30.8 g), but lower than the absolute control treatment (148.6 g), as observed in Figs. 2 and 3. For this variable, the repetitions of each treatment were close values, thus a shorter interval of variance.

Evaluation of the dry matter radicular. On the percentage of dry matter of roots, there is a significant difference between the treatments. Treatment S10 with 29.0% of dry matter of roots is the best compared to the control inoculated with *P. cinnamomi* (18.6%).

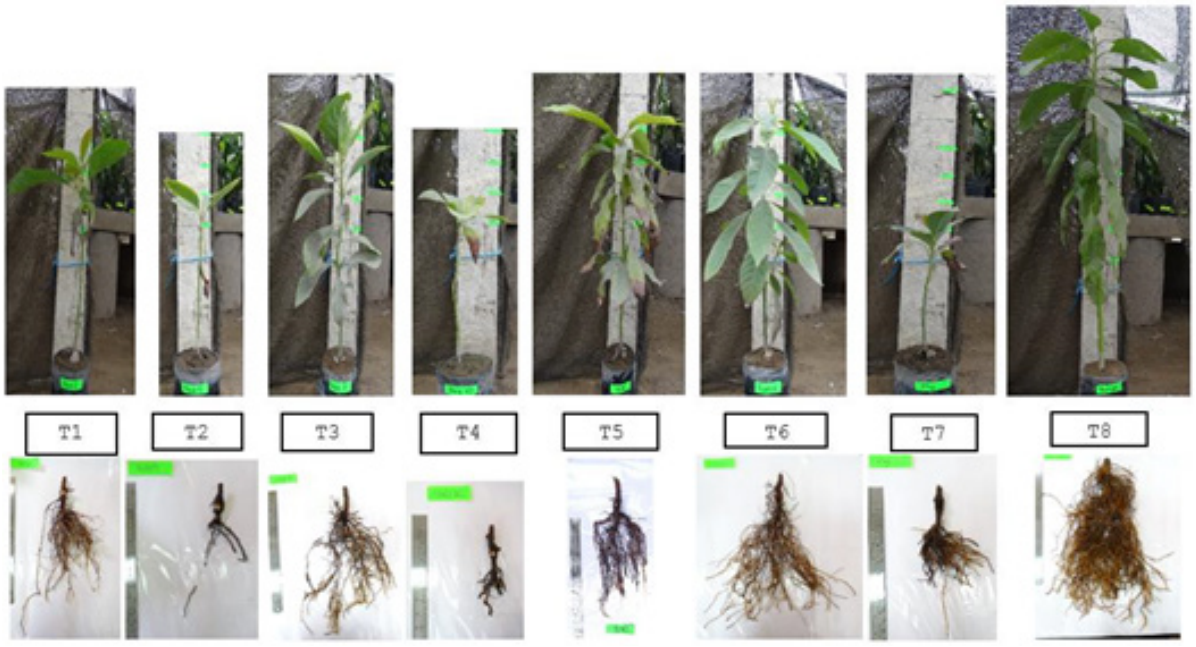

Fig. 3. Photograph of foliar and root area of avocado cv. 'Zutano', six months after inoculated with soil and root isolates of *Pseudomonas* sp. and *P. cinnamomi*, under greenhouse conditions.

Evaluation of fresh leaf weight. Treatments R7 and S6 (110.4 and 91.8 g) show a significant difference compared to the treatment inoculated with *P. cinnamomi* (48.7 g), but lower than the absolute control treatment (156.6 g), as observed in Figs. 2 and 3.

Dry leaf matter. Figure 2 shows there is no significant difference among treatments, and that the repetitions of treatments T2 (R5), T3 (R7) and T5 (S10) present higher values than the absolute control (T).

Discussion

This study shows the inhibition of *Phytophthora cinnamomi* against *Pseudomonas* in vitro and greenhouse. Villa *et al.* (2005) found that strains of *Pseudomonas* sp. inhibited the growth of *Sclerotium rolfsii* between 60 and 90%. Other studies also report that *Pseudomonas cepacia* and *P. fluorescens* were significant for suppressing *P. cinnnamomi* that grew in vitro and in vivo (Yang *et al.*, 2001).

The coagulation of the cytoplasm was observed; this same partial degradation or coagulation of the cytoplasmic content was observed in cultures of *Phytophthora capsici* with *Pseudomonas fluorescens* in which Diby *et al.* (2005) attributes it to hydrolytic enzymes. Broadbent & Baker (1974) also demonstrated that *P. putida* and *P. fluorescens* isolated from avocado soils caused massive mycelia lysis of *P. cinnamomi* in vitro.

The strains of isolated *Pseudomonas* were not pathogenic for humans according to Rodríguez *et al.* (2005) who demonstrated that to isolate *Pseudomonas aeruginosa* (opportunistic pathogen in humans) the bacteria should be cultured in a gelatin culture medium, thus demonstrating the presence of gelatinases. The promising strains of *Pseudomonas* gave a negative result to this test. Therefore, they are not pathogenic to man. *Pseudomonas putida* and *Pseudomonas* fluorescens are unable to grow at 42°C and do not produce pyocyanin unlike *P. aeruginosa*, corroborated by the UK Standards for Microbiology Investigations (2015).

Several possible mechanisms have been described by which soil *Pseudomonas* suppresses conditions related to pathogenicity. De la Fuente *et al.* (2000) identified three native strains of fluorescent *Pseudomonas* (UP61, UP143 and UP148) producing HCN, fluorescent siderophores such as pyoverdine proteases and antibiotics [2, 4-diacetylchloroglucinol (DAPG), PLT (piolterine), PRN (pyrrolnitrine) and phenazine derivatives] with antifungal activity (Julisch *et al.*, 2001). Thomashow and Weller (1996) also described the production of antibiotics by fluorescent *Pseudomonas*.

Therefore, the control effect of treatment T6 (S6) could be explained by mechanisms such as competition for iron, competition for colonization sites and nutrients exuded from the root, as well as the induction of plant defense mechanisms (Van Weels *et al.* 1997). Another mechanism of control is the production of extracellular enzymes, such as chitinases, laminases and glucanases that can degrade the walls of fungal cells. Van Weels *et al*. (1997) isolated a strain of *Pseudomonas stutzeri* that produced extracellular chitinase and laminase, and found that these enzymes digest and lyse the mycelium of *Fusarium solani*.

Marques *et al.* (2010) who mentioned that the production of IAA by PGPR generally causes the elongation and accumulation of P and N in the tissues of the plant would explain the increase in root length. In the root system, it has been seen that high levels of IAA increase the formation of lateral and adventitious roots, but inhibits the growth of the primary root.

This improvement in growth was also observed in the 1970s when some fluorescent *Pseudomonas* strains improved the growth of potatoes and sugarcane when applied to the seeds (Schroth & Hancock, 1982).

The increase in height is explained because *Pseudomonas* can act as promoters of plant growth in two ways: directly by suppression of pathogens or indirectly through the secretion of phytohormones and vitamins, or by increasing the absorption of minerals per plant.

Glick (1995) proposes that *Pseudomonas* can manifest its growth by promoting effects indirectly, stimulating the beneficial actions of other microorganisms associated with the roots, such as mycorrhizae. When the stimulation of plant growth occurs in the absence of other microorganisms, it has been attributed to the increase in the availability of mineral nutrients, such as phosphate or nitrogen, due to the production of phytohormones stimulating plant growth or to the degradation of ethylene precursors in the root by these bacteria.

Faggioli *et al.* (2007) also observed that the inoculation of corn plants with PGPR does not significantly influence the height but the percentage of dry matter (Fig. 2).

Conclusions

The soil sampled from Casma area had a higher quantity of *Pseudomonas* spp. organisms. The *Pseudomonas* spp. isolated from soil showed greater controlling effect on *Phytophthora cinnamomi* compared to isolates from roots, evaluated in vitro. The strain of *Pseudomonas* spp. S6 (T6) showed greater control of *P. cinnamomi* in the greenhouse and the strain of *Pseudomonas* spp. S10 (T5) showed greater root system development in the greenhouse. It was ruled out that all *Pseudomonas* spp. inoculated were pathogenic to humans (*Pseudomonas aeruginosa*).

Acknowledgments

This research was funded by Sociedad Agrícola Virú S.A. (Virú) company which grant me the first place in the contest “Premio Virú 20 años”. We thank Dr. C. Arbizu Berrocal for assistance.

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