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ISOLATION OF NATIVE STRAINS OF *TRICHODERMA SPP*, FROM HORTICULTURAL SOILS OF THE VALLEY OF TOLUCA, FOR POTENTIAL BIOCONTROL OF *SCLEROTINIA*

[AISLAMIENTO DE CEPAS NATIVAS DE *TRICHODERMA SPP* DE SUELOS HORTICOLAS DEL VALLE DE TOLUCA, COMO BIOCONTROL POTENCIAL DE *SCLEROTINIA*]

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SUMMARY

The presence of *Trichoderma* mold strains was evaluated in seven localities in the southern part of the Valley of Toluca in the State of Mexico. This area has a high potential for growing vegetables. In the study, native strains of *Trichoderma* were isolated from soil samples, physiographic factors were identified, as well as the physicochemical properties of the soil which may affect *Trichoderma* occurrence. The potential of *Trichoderma* strains for control of *Sclerotinia spp.*, a pathogenic fungus which causes soft rot in lettuce, was evaluated. Eleven strains were isolated, most of them associated with the type of soil found in the San Francisco Putla and San Francisco Tetetla localities. Logistic regression analysis showed no relationship between the soil properties (organic matter content and pH) and the presence of *Trichoderma*. Tukey test ($p<0.05$) showed significant differences between the percentage of inhibition of *Sclerotinia* by the eleven native strains of *Trichoderma*. The TF10, TL4 y TX8 strains had a high biocontrol potential, with inhibition percentages of 80%, 86% y 88%, respectively. These strains are an ecological alternative for the control of S*clerotinia* spp.

Key words: *Trichoderma, Sclerotinia,* biological control

RESUMEN

Se evaluó la presencia de *Trichoderma* en siete localidades en la zona sur del Valle de Toluca, Estado de México. Esta es un área con un alto potencial en la producción de hortalizas. El estudio se dirigió al aislamiento de cepas nativas de *Trichoderma* a partir de muestras de suelo, identificación de factores fisiográficos, así como las propiedades físicas y químicas del suelo que determinan la ocurrencia de *Trichoderma*. Se evaluó el potencial de las cepas de *Trichoderma* para el control de *Sclerotinia* spp., hongo patógeno causante de la pudrición blanda en lechuga. Se aislaron once cepas, el mayor número de ellas asociadas al tipo de suelo se encontró en las localidades de San Francisco Putla y San Francisco Tetetla. El análisis de regresión logística mostró que no hay una relación entre las propiedades del suelo (materia orgánica y pH) y la presencia de *Trichoderma*. La prueba de Tukey ($p<0.05$) mostró diferencias significativas entre el porcentaje de inhibición de las once cepas nativas de *Trichoderma* sobre *Sclerotinia*. Las cepas TF10, TL4 y TX8 presentaron un alto potencial de biocontrol con porcentajes de inhibición del 80%, 86% y 88% respectivamente. Estas cepas representan una alternativa ecológica de control de S*clerotinia* spp.

Palabras clave: *Trichoderma, Sclerotinia,* control biológico

INTRODUCTION

Lettuce (*Lactuca sativa* L.*)* is one of the crops with more planted surface in the horticultural zone of the Valley of Toluca, in the State of Mexico. The production of this vegetable satisfies the country´s internal demand. Soft rot is the main diseases that affects this crop and can cause losses of up to 60% of the production (Hao and Subbarao, 2005; Wu and Subbarao, 2006).

Soft rot disease is caused by two species of fungus of the *Sclerotinia* genus, *S. minor* Jagger and *S. sclerotiorum* (Lib.) de Bary. Both species may be found in the same fields, being one of them normally predominant. Control of *Sclerotinia* infections is complicated since the sclerotia that are resistance structures and the primary inoculum for new infections may remain for long time in the soil. (Davis *et al.*, 2002). *Sclerotinia* species use different modes of infection in the plant, *S. minor* infects by eruptive germination of sclerotia while *S. sclerotiorum* infects by carpogenic germination (Hao and Subbarao, 2005). Soft rot disease is characterized by abundant growth of white and cottonlike mycelia, as well as by aqueous rotting of the plant's crown and root, and the pathogen can attack the crop in any phase of its development (Subbarao, 1998; Davis *et al.*, 2002; Rabeendran *et al*., 2006).

Cultural, chemical and biological methods have been used to deal with soft rot disease with variable success. Application of chemical products, such as fludioxonil, fluazinam and iprodion, has been effective to reduce *Sclerotinia* infections (Hubbard *et al*., 1997: Matheron and Porchas, 2004). However some of those products can produce damage the crops by phytotoxic effects (Hao *et al*., 2003) eliminate beneficial organisms along with the pathogens as they are non-specific (Rey *et al.*, 2000), may cause health problems in the individuals who apply them and have a negative impact on the environment by accumulation due to persistence. All theses reasons have generated doubts about the convenience of their use.

Biological control (BC) is an ecological alternative for the management of plant diseases that are important in agriculture (Heredia and Delgadillo, 2000). It is known that some fungi are antagonistic to pathogenic organisms and thanks to this effect can decrease the damage caused by diseases in agroecosystems (Infante *et al.*, 2009) and it has been stated that BC must be done taking advantage of the diversity of native microorganisms in soil (Altieri, 1999).

Trichoderma genus has been widely studied and used for biological control. *Trichoderma* shows ecological plasticity, a high enzymatic ability to degrade substrates, are easily isolated, are rapidly cultivated and are very efficient to control a broad range of phytopathogens such as *Fusarium, Pythium, Rhizoctonia* and *Sclerotinia* (Quiroz-Sarmiento *et al.*, 2008). Products including *Trichoderma harzianum* have been used successfully in the suppression of damping-off in carrot caused by *Rhizoctonia solani* (Adams, 1990*)*. However, in other cases, formulas have not been effective, since they contain species that are not compatible with the environment or with the characteristics of the region where they are applied (Rabeendran *et al.*, 2006) for this reason, it is very important to use native strains in biocontrol. The objectives of this study were to identify the physical and chemical properties of the soil and the physiographic factors determining the establishment of *Trichoderma* in seven localities of the horticultural zone of the Valley of Toluca, as well as the isolation of native strains with the potential to act as biological control of *Sclerotinia.*

MATERIALS AND METHODS

Study site localization

The study zone is located at the south of the City of Toluca, between 19º 05´ and 19º 10´N and between 99º 30´ and 99º 40´W. In this area 50 *Sclerotinia* spp. infected lettuce plots were located (Table 1). Infected plots were distributed across 7 towns in the municipalities of Tenango del Valle, Rayón, Joquicingo and Texcalyacac.

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Table 1. Plots found in each locality, for isolation of native strains of *Trichoderma* spp.

Municipality	Town	Plots
Tenango del Valle	Santa María Jajalpa	9
	Tenango de Arista	7
	San Francisco Putla	10
	San Francisco Tetetla	7
Texcalyacac	San Mateo Texcalyacac	5
Joquicingo	San Pedro Techuchulco	5
Santa María Rayón	San Juan la Isla	7

In order to draw a map including the edaphic and physiographic factors of the study zone and to relate these factors to the presence or absence of *Trichoderma* all plots were georeferenced using the Global Positioning System (GPS),

Soil Sampling

Soil sampling included winter-spring (irrigation) and summer (rainy season) culture cycles. Four soil samples of 250 g were obtained from each analyzed plot, at a depth of 15 cm. Two of these samples where obtained from nearby healthy plants and the other two from nearby plants with soft rot symptoms. Collected samples were kept at -72ºC, until tests were performed. Sclerotia found on lettuce plants were isolated and propagated in the laboratory for further use in *Trichoderma – Sclerotinia* antagonism tests.

Determination of Physicochemical properties of soil

Soil samples were removed from the ultra-low temperature freezer, dried at room temperature (RT) and processed according to the applicable protocol. pH determination was performed to 1:2 soil-water suspensions according to AS-02 method of the NOM-021-RECNAT-2000. Organic matter quantification (OM) was performed using Walkley and Black (AS-07) method, texture was estimated by the Bouyoucos (AS-09) method.

Isolation of *Trichoderma* strains

Isolation of *Trichoderma* strains from soil samples was carried out by the serial dilution method of Guigón-López and González-González, (2004). Each soil sample was homogenized and a 10 g sub-sample was taken, placed in a test tube containing 90 mL of a saline isotonic solution (0.85% sterile sodium chloride) and shaken for 20 minutes. 1 mL of that mixture was diluted with 9 ml of isotonic solution in a test tube, and mixed by 2 minutes; this procedure was repeated until 5 dilutions were obtained. 1 mL from the last three dilutions was plated in phytone yeast extract agar plates and incubated at 25°C for 7 days. *Trichoderma* resembling colonies were selected and isolated to pure strains by consecutive culture in order to be further analyzed.

Microscopic identification of *Trichoderma* strains.

In order to confirm the identity of those strains suspected to be *Trichoderma* a sample of mycelium was taken from each one of the pure strains, placed on a slide, and stained with methylene blue was added. Microscopic structures as size and shape of conidia and phyalides were observed and determined with the dichotomous keys (Samuels *et al.*, 2008) and identified using an optical microscope coupled to the Motic Images Plus 2.0 program, just those strains confirmed by this method were kept and labeled with a code, for its handling in further analyses.

***Sclerotinia* strains isolation.**

The sclerotia obtained from the lettuce plants were disinfected by immersion in 5% sodium hypochlorite for three minutes, washed three times with sterile distilled water, placed on sterile filter paper and allowed to dry. Later on, each sclerotium was placed in the center of a Petri dish, with Phytone Yeast Extract Agar and incubated at 25°C for 7 days (Mónaco *et al*, 1998). *Sclerotinia* pure strains were isolated from initial cultures by consecutive mycelium transfers.

***In vitro* confrontation tests**

In vitro confrontation tests were carried out by the dual cultivation method using pure strains of *Trichoderma* (antagonist) and *Sclerotinia* (pathogen) (Martínez and Solano, 1994). A sclerotium was placed at one end of a Phytone Yeast Extract Agar plate and one cm^2 of active mycelium of *Trichoderma* was placed in the other end; plates were incubated at 25º C for seven days. This assay was done in triplicate for each isolated *Trichoderma* strain. Percentage of inhibition of radial growth (PIRG) was recorded every 24 h, using the following formula:

$$PIRG=[(R1-R2)/R1] \times 100$$

Where, R1 is the radial growth of the control non confronted *Sclerotinia* strain and R2 is the radial growth of the *Trichoderma* confronted *Sclerotinia* strain (Samaniego *et al*., 1998, cited by Martínez *et al*., 2008).

Experimental design.

The confrontation tests were performed according to a completely random design, using three repetitions for each one of the eleven isolated native strains of *Trichoderma* that were confronted against *Sclerotinia spp*.

Statistical analysis.

Statistical differences in pH and OM values between the localities were determined by variance analysis. The possible relationship between physical properties of the soil (pH and organic matter content) and the presence of *Trichoderma* strains was determined using logistic regression. The potential of each

Trichoderma strain as bio control agent for *Sclerotinia* infection was assessed by variance analysis and Tukey means comparison test ($p<0.05$), using the Statgraphics plus statistical package, version 4.1.

RESULTS AND DISCUSSION

Isolated native strains of *Trichoderma*

Eleven native strains belonging to the *Trichoderma* genus were found and identified by macroscopic and microscopic characteristics of the colonies, according to Infante (2009), strains were distributed along five of the seven sampled localities (Table 2). *Trichoderma* strains were expected to be found in more than 50% of the sampled plots because of its cosmopolitan character and the fact that it is a natural inhabitant of soils, but *Trichoderma* strains were found in just 22% of plots; these results differ to those reported by Michel-Aceves *et al.*, 2001, in that work native strains of *Trichoderma* were found in 88% of the sampled sites. Michel-Aceves *et al.* mention the collection season is the main factor that may affect the presence of the fungus in soils and reported spring and summer as the best seasons to find a high number of isolates; however, even when the present study cover both seasons the number of isolates was lower.

Edaphic and physiographic factors in the *Trichoderma* distribution zone

Figure 1 illustrates the presence or absence of *Trichoderma* (red and green points respectively). It can be seen that even when the sampling zone is near to water bodies in 78% of the plots *Trichoderma* was not found; this make it clear that at least in this zone the proximity to rivers is not a determining factor for the establishment of *Trichoderma.* On the other hand, the profile of the sampling area is slightly wavy and the mold was found at different altitudes, which confirms its ecological plasticity, as pointed out by Samuels, 2006 and Infante *et al.*, 2009. Although *Trichoderma* as genus can be found in all latitudes and in all types of soils, geographic distribution of species of *Trichoderma* are quite different, while some species are broadly spread, as is the case of *T. pseudokoningii*, others have a limited geographic distribution, as is the case of *T. viride* that is not commonly found in the colder northern regions and in even more so for *T. aureoviride*, whose distribution is limited to the United Kingdom and northern Europe (Samuels, 2006).

Table 2. Isolated *Trichoderma* strains, by locality

Town	Strains	Code
San Francisco Tetetla	4	TL2,TL4.TL5,TL6
San Francisco Putla	3	TF6,TF8,TF10
Santa María Jajalpa	2	TX7,TX8
San Juan la Isla	1	TJ6
Tenango de Arista	1	TT6
San Pedro Techuchulco	0	-
San Mateo Texcalyacac	0	-

With respect to the type of soil where *Trichoderma* was found, the greater number of *Trichoderma* isolates (8) was obtained from phaeozem, and only two isolates were found in leptosol soil, although 58% of the sampling sites are found in leptosol (Table 3). This opens the possibility that the type of soil may be an important factor in the establishment of this *Trichoderma*. However, it has been reported that the presence or absence of *Trichoderma* depends on many factors, mainly the species involved, environmental conditions and pathosystem (Duffy *et al.*, 1997).

Determining physicochemical properties of soil

pH values of soil at sampling sites went from 4.1 to 8, that is, from strongly acidic to slightly alkaline (Table 3). pH value was found to be significantly different among sampling localities (Tukey $p<0.05$). Although this property depends on many factors, it is worth mentioning that for this study, those localities that have a pH values similar are placed near each other while when geographic distance increase so does the difference in pH values.

Native *Trichoderma* strains were found in acid soils (Table 3). This can be explained by the fact that acidity is a factor which affects presence, density and longevity of this fungal genus (Michel-Aceves *et al.*, 2001); pH also has an influence on the production of enzymes involved in the degradation process of fungi attacks (Kredics *et al.*, 2003; Samaniego, 2008) and some species of this genus produce organic acids (gluconic, citric or fumaric acid) that decrease the pH of soils, allowing phosphates, micronutrients and minerals to become soluble, and which are necessary for plant metabolism (Vinale *et al.*, 2008). The results of the present study coincide with what was reported by Okoth *et al.*, (2007), who reported that *Trichoderma* is abundant in acid soils.

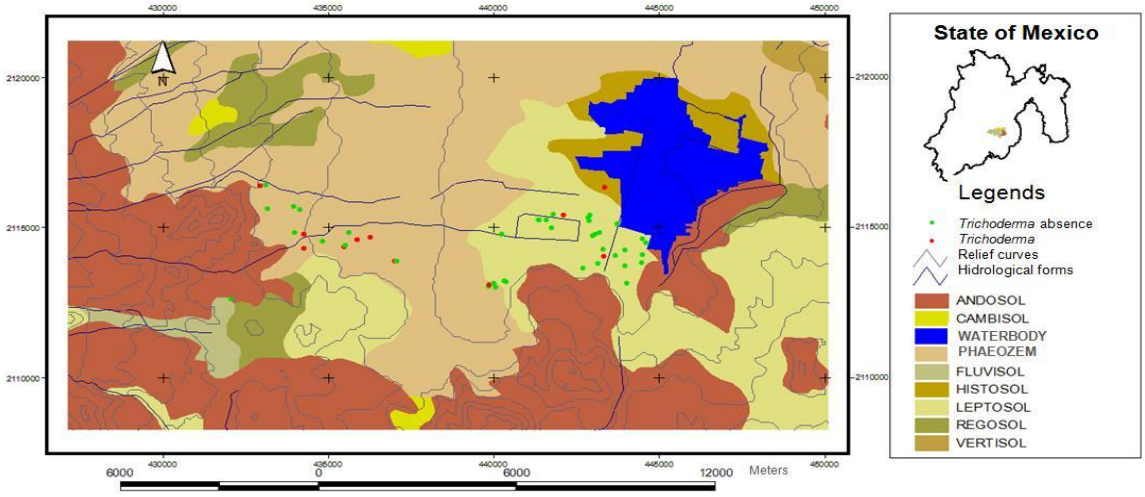

Figure 1. *Trichoderma* spp. distribution in the horticultural zone of the Valley of Toluca. Figure by: Ángel Rolando Endará Agramot

An organic matter (OM) content between 2.7 to 21.9% were found in the sampled horticultural soils (Table 3). Tukey test showed a significant difference ($p<0.05$) among sampling localities. These results differ to those reported by Reyes *et al*., (2002), in that work an OM content of 8.5 % in the cultivated soils of the Nevado de Toluca National Park (NTNP) was reported. The higher values that were found for OM can be attributed to the fact that certain producers add organic compounds to their plots to improve the soil, such as chicken, sheep or cow manure; records show a higher use of chicken manure, with an OM content of 20 – 40% (Estrada, 2005). Also, two sampled plots were planted for the first time; this may explains the high percentage of OM in the soil at least for those two cases. Low values of OM content found in some plots are possibly due to the fact that the constant agricultural use of the sampling sites has caused a decrease in OM content of those plots. These results agree with those of Michel-Aceves *et al*., (2001), as in that work low values of up to 2.3% of organic matter were reported for the same zone.

Trichoderma native strains were found mainly in soils with low OM content (Table 3), this does not match up with the results of Osorio-Nila *et al*., (2005), where high organic matter content increase the establishment of *T. lignorum* in lettuce cultivation soils of the Valley of Tenango Municipality, in the State of Mexico.

Textures found in the evaluated localities were, sandy crumb, limey crumb and clayey crumb, with the greater number of isolates coming from sandy crumb. No significant relationship was found for the presence of *Trichoderma* and soil properties (pH and OM) by logistic regression analysis (Table 4). These results agree with the study by Michel-Aceves *et al*, (2001), where no significant correlation was found between the presence of *Trichoderma* and soil properties.

Table 3. Physicochemical properties of soil in the sampled localities

Town	Number of strains[v]	Type of soil	Mean pH[x]	Mean OM[y]	Texture
San Francisco Putla	3	Phaeozem	4.9a	2.7a	Sandy crumb
San Francisco Tetetla	4	Phaeozem	5.0b	5.3b	Sandy crumb
Tenango de Arista	1	Phaeozem	5.4b	8.9d	Clayey crumb
San Juan la Isla	1	Leptosol	5.7c	11.8e	Limey crumb
San Mateo Texcalyacac	0	Leptosol	6.1d	21.9g	Sandy crumb
Santa María Jajalpa	2	Histosol	6.5e	6.0c	Clayey crumb
San Pedro Techuchulco	0	Leptosol	6.6e	13.0f	Sandy crumb

The analyses correspond to 50 plots distributed among seven towns.
[v] Number of *Trichoderma* strains found by locality.
[x, y] Mean value obtained from pH, OM parameters (N=150).
The values in the same column, marked with different letters, show statistical differences ($P<0.05$).

Table 4. Logistic regression between the presence of *Trichoderma* and soil properties

Soil property	Regression coefficient	Standard error	Chi squared	Probability level	R^{2z}
pH	-0.1458606	0.2868994	0.26	0.611170	0.005469
OM	-0.1786044	0.1005832	3.15	0.075784	0.058225

[z] Refers to r^2 values obtained through logistic regression between the presence of *Trichoderma* and soil properties.

***In vitro* confrontation tests**

The eleven native *Trichoderma* strains isolated in this work showed a higher growth rate than *Sclerotinia* (Table 5). These results may be related to the fact that *Trichoderma* has the capability to colonize rapidly almost any substrate (Infante *et al*, 2009). On the other hand, while *Sclerotinia* normally is a very aggressive mold, in this case it showed a growth rate that was lower than *Trichoderma* strains; this growth rate is similar to the one reported by Sanogo and Puppala, 2007, when evaluating the *in vitro* growth of *S. sclerotiorum.*

In the confrontation tests, the eleven native strains of *Trichoderma* differ statistically ($p<0.005$) in their potential to inhibit *Sclerotinia spp.* growth (Table 6). The TL6 strain get the lowest level of inhibition of *Sclerotinia* spp (0.08%) while TX8 (88%), TL4 (86%) and TF10 (80%) were the strains with greater inhibitory capacity of all. The differences obtained for inhibition capability among native *Trichoderma* strains may be related to the fact that the isolated strains could be different species, since they had different color pattern, as well as differences in growth patterns and inhibition mechanisms. It has also been reported that each mold may have different antagonistic potential (Bowen *et al*., 1996; Hermosa *et al*., 2000; Herman *et al*., 2004; Schubert *et al*., 2008; Komón-Zelazowska *et al*, 2007).The results obtained in the present study are similar to those reported by Michel-Aceves *et al*. (2004), where inhibition percentages were found going from 5.35 up to 42.02% in the biocontrol of *Fusarium subglutinans* by *Trichoderma;* also to those of Arzate *et al*. (2006), in the antagonistic action of *Trichoderma spp*. over *Mycospharella fijiensis,* where inhibition percentages of 14.41 to 73.48 were found in banana plants. Thus, it will be interesting to carry out further studies, including identification of native strains at molecular level, as well as the analysis of the inhibitory mechanisms involved and its bioregulatory action over *Sclerotinia spp*.

Table 5. Radial growth rate of native strains of *Trichoderma* and *Sclerotinia spp*.

Strain[w]	Radial growth $(mm)_x$	Growth rate (mm/day)[y]
TX7	90.0a	12.857
TX8	90.0a	12.857
TJ6	89.76[a]	12.824
TT6	90.0a	12.857
TL2	89.99[a]	12.852
TL4	$87.57b_z$	12.511
TL5	90.0a	12.857
TL6	90.0a	12.857
TF6	90.0a	12.857
TF8	90.0a	12.857
TF10	90.0a	12.857
Sclerotinia spp.	$78.94c_z$	12.00

[x] Radial growth (mm) of each strain, for 7 days
[y] Growth rate (mm/day) of the eleven isolated strains of *Trichoderma* and *Sclerotinia* spp, during seven days of evaluation.
[z] Values with different letters are statistically different (Tukey, $p\leq0.005$).

Table 6. Comparison of means of *in vitro* inhibition percentages in dual cultures of native *Trichoderma* strains and *Sclerotinia spp*.

Native strain[x]	Inhibition (%)
TL6	0.08[a]
TL2	14.93b
TT6	17.81c
TL5	21.82d
TJ6	23.66e
TF6	34.35f
TX7	52.65g
TF8	59.38h
TF10	80.07i
TL4	86.18j
TX8	88.73k

Values with different letters are statistically different (Tukey, $p\leq0.005$)

CONCLUSIONS

The number of *Trichoderma* isolates that can be found in the Valley of Toluca horticultural zone may vary. One of the important factors for the establishment of Trichoderma is the type of soil. In this study, the phaeozem favored the presence of *Trichoderma* strains. Another factor increasing the occurrence of this genus is soil acidity.

Trichoderma proved to be a successful organism that colonize the substrate rapidly and get hold of space over *Sclerotinia* spp. Confrontation tests allowed us to identify three strains with a high potential for biocontrol of *Sclerotinia*; these strains are a natural resource and an alternative to reduce the use of chemicals which have been applied in these study sites and have not shown efficient results in the control of soft rot due to this pathogen. However, it is important to carry out other studies that will allow us to show the effectiveness of these strains at the greenhouse level and on the field.

On the other hand, the molecular identification at the level of species, of the native strains that were found, will allow us to manage their potential, in an optimal and correct manner, in further studies.

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