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In vitro selection of Strains of *Trichoderma* spp. with phosphate solubilizing and indole acetic acid producing capacities

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Abstract

Trichoderma spp., in addition to having biocontrolling effects on pathogens, exhibit phosphodissolvent and IAA production capacities; Our hypothesis suggests that the efficiency of some strains of *Trichoderma* in phosphodissolving and producing IAA is a function of the type of strain, the incubation time, and the concentration of specific precursors or inductors of hormones present in the system. The central aim of this investigation had as an objective the *in vitro* selection of strains of *Trichoderma* spp. isolated from the rhizosphere of beans and corn with phosphate solubilizing and indole acetic acid (IAA) producing capacities. Nine (9) different strains of *Trichoderma* were isolated and selected from the rhizosphere and rhizoplane of beans and corn. In order to evaluate their phosphodissolvent capacities, these strains were cultivated in three solid media and three liquid media with and without Phosphoric Rock (PR). The relative efficiency of solubilization (RSE), bioacidulation, and phosphodissolution was then determined. IAA production capacity was evaluated as a function of strain, time, and concentration of L-Tryptophan. For this, 9 strains of phosphodissolvent *Trichoderma* were tested, and 4 turned out to be IAA producers. There were highly significant differences ($P \leq 0.01$) in phosphodissolvent capacity and IAA production in relation to the variables studied and the strains evaluated. It is an important mechanism of adaptation to the ecological success of both plants and microorganisms which in turn represents a valuable genebank for agro-ecological and agrobiotechnological applications.

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Introduction

Trichoderma spp., has been reported to be a fungal promoter of vegetal growth (PGPF: Plant Growth Promoting Fungi) (Yedidia, 1999; Harman, 2000; Gravel *et al.,* 2007; Vinale *et al.,* 2008; Achá, 2008). This capacity is attributed to the fact that this fungus (i) produces amino acid (L-tryptophan) hormone inductors (ex: 3-indole acetic acid) (Gravel *et al.,* 2007; Altomare *et al.,* 1999), (ii) increases the velocity and percentage of seed germination, (iii) intervenes in the decomposition of organic material, liberating nutrients in available forms for the plant (Howell, 2003; Godes, 2007), (iv) for its phosphodissolvent capacity in ecosystems where phosphorous (P) is a limiting resource (Vera *et al.,* 2002; Valero, 2007; Valencia *et al.,* 2007; Moreno *et al.,* 2007; Hoyo-Carvajal *et al.,* 2009;), and (v) because it reduces effects caused by environmental stress in plants (Bjorkman *et al.,* 1998). These characteristics offer a microbial germplasm bank with important agrobiotechnological use perspectives (Richardson, 2001; Gyaneshwar *et al.,* 2002; Trolove *et al.,* 2003; Osorno and Osorio, 2014; Zúñiga and Becerra, 2014).

With respect to phosphodissolution capacity, several investigations report the existence of various mechanisms for the solubilization of inorganic phosphate (P) compounds. Among those that stand out are the production of lithic enzymes during the decomposition of organic matter (Iyamuremye and Dick, 1996; Bar-Yosef *et al.,* 1999; Marschner, 2008) and organic acids that generate competition between organic anions produced and phosphate ions at absorption sites on the surfaces of clayey soil minerals (Bolan *et al.,* 1994). Some organic acids commonly associated with microbial solubilization of P by fungi are: gluconic (Bar-Yosef *et al.,* 1999; Rodríguez *et al.,* 2006), oxalic, citric (Kucey and Leggett, 1989; Kim *et al.,* 1997; Osorio, 2008), lactic, tartaric, and aspartic (Venkateswardu *et al.,* 1984). These acids are products of microbial metabolism, in some cases of oxidative respiration, and in others the fermentation of carbonaceous substrates (ex: glucose) (Atlas and Barta, 1998; Mathews *et al.,* 2002; Prescott *et al.,* 2004). Other mechanisms proposed are the excretion of protons due to the assimilation of NH_4^+ by microorganisms (Kucey, 1983; Roos and Luckner, 1984; Abd-Alla, 1994; Whitelaw, 2000); desorption of P ions at absorption sites (He and Zhu ,1998; 1997); and chelation of Al^{3+} and Fe^{3+} (Iyamuremye and Dick, 1996; Bar-Yosef *et al.,* 1999; Marschner, 2008).

However, despite wide reports of the beneficial effects of microorganisms that solubilize P (MSP's), their results are inconsistent (Garbeva *et al.,* 2003; Gardener, 2004; Reva *et al.,* 2004; Chen *et al.,* 2007A). It is presumed that their inconsistency is because even simple inoculation of the soil-plant system with microorganisms alters rhizospheric ecosystem dynamics (de Freitas *et al.,* 1997; Habte and Osorio, 2001; Chigineva *et al.,* 2011). It is also known that response differs depending on the vegetal species (Flach *et al.,* 1987; Gregory, 2006), the mineralogical composition of the soil (Boul and Eswaran, 2000), and interactions between different microbial functional groups in the soil (Habte and Osorio, 2001; Vessey, 2003; Chigineva *et al.,* 2011; Zúñiga-Silgado, 2016). Reports made by Caipo *et al.,* (2002), also state that the type and quantity of inoculant affects the cellular physiology of both microorganisms and host plant as well as the availability of carbon substrate(s) as energy sources (Knox *et al.,* 2000; Caldeira *et al.,* 2008), the competitivity with other autochthonous microorganisms of the soil (Silvieira *et al.,* 2003; Collados, 2006) and environmental conditions (Johnson *et al.,* 1997), and plays a part in the differential response of crops to microbial inoculation.

In relation to the capacity of *Trichoderma* to produce specific hormone inductors, the presence of IAA of microbial origen has been found in the interior of plants (Libbert *et al.,* 1969 in: Arshad and Frankenberger, 1993). Thus, soil microorganisms that are IAA producers favor vegetal growth, as they make this substance available for the plant. These substances begin a chain of hormonal effects principally leading to the promotion of protein synthesis and cellular division and elongation; thus

producing macroscopic responses including germination and development, proportional plant growth, or the separate growth of different organs in different phenological stages. They also modify the apical dominance and therefore the architecture of the plant. A key effect for the nutrition of plants is given at the level of rhizogenesis and root lengthening (Valencia *et al.,* 2005), which may translate into better anchoring and the capacity to exploit a larger quantity of soil, favoring the uptake of nutrients. IAA acts as a catalyst or accelerator of primary meristematic tissues in young parts of the plant, accelerating their cellular reproduction and allowing them to develop more rapidly as compared to plants that have not been treated with this microorganism (Valencia *et al*., 2007). *T. harzianum* has also been reported to be a promoter of vegetal growth in cultivations of eggplant, vetch, beans, coffee, tomato, potato, and forest species, among others (Zambrano, 1989; Dandurand and Knudsen, 1993; Börkman *et al.,* 1998).

Given the relevance of this fungus, the adequate selection of strains at the laboratory level will contribute to the development of strategies for the integrated management of crops through the elaboration, evaluation, and application of bioproducts with multifuncional effects occasioned by the biocontrolling activity of pathogens summed with their phytostimulating and biofertilizing activities. Our hypothesis suggested that the efficiency of some strains of *Trichoderma* in phosphodissolvery and the production of IAA is a function of strain, incubation time, and concentration of precursors or specific inductors of hormones present in the system. The central aim of this investigation had as an objective the *in vitro* selection of strains of *Trichoderma* spp., with phosphate solubilizing and indole acetic acid (IAA) producing capacities in the rhizosphere of beans and corn.

Materials and methods

The present investigation was realized in the Biotechnology Laboratory the Mayor College of Antioquia University Institution and in the Laboratories of Ecology and Environmental Conservation and Vegetal Health National University of Colombia, Medellín (6°15´ N and 75°34´ W, 1450 m of altitude).

Composition of fungal community in soil and rhizospheres of beans and corn

Twenty samples of roots and rhizosphere soil of maize and beans cropswere collected 30, 60, and 90 days after being planted in the Municipality of Rionegro in the Antioquia Department. Samples with 250g of soil were stored in a refrigerated cooler for their transfer to the laboratory. They were then processed following the methodology reported by Calvo (2008).

Isolation of morphotypes of Trichoderma spp., that solubilize PR

Morphotype isolation of *Trichoderma* sp., was done from rhizosphere soil and root segments of beans and maize following the methodology reported by Hoyos-Carvajal (2008). Roots collected were submerged in 10mL of sterile 0.85% NaCl solution and centrifuged for 10 min at 200 rpm (Reyes *et al.,* 2002). Later, 1g of roots of each sample were transferred to 100 mL Erlenmeyer flasks containing 20mL of 10% peptone water and agitated at 100rpm for 5 minutes. Next, the roots were separated from the supernatant and dried on sterile absorbent napkins. Half of the roots were dissected in 1mm segments and planted in petri dishes with Rose Bengal Agar (RBA) with 300 mg L^1 of Streptomycin Sulfate and 100 mg L^{-1} of chloramphenicol (SIGMA) in complete darkness. The other half of the root samples were transferred into a mortar to be macerated. Aliquots of the macerated root samples were planted in petri dishes with Rose Bengal Agar (RBA), 300 mg L^{-1} Streptomycin Sulfate, and 100mg L^{-1} chloramphenicol (SIGMA) in complete darkness. Each petri dish was incubated for 24 hours at 25±1°C. A total of 30 petri dishes were planted.

Experiment 1: Solubilization of PR in solid media

The solid culture media utilized were a qualitative indicator of the solubilizing action of PR by strains of *Trichoderma* spp. In order to determine the efficiency of biosolubilization of PR, three different solid culture

media were tested:

(i) MMHA with hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) as a source of P: (MM-HA; $Ca10 (PO_4) 6(OH)_2$) for one liter of medium, which contained: 0.1g NaCl; 0.4g NH_4Cl; 0.78g KNO_3; 0.50g $MgSO_4 \cdot 7H_2O$; 0.1g $CaCl_2 \cdot 2H_2O$; 0.5mg $FeSO_4 \cdot 7H_2O$; 1.56mg $MnSO_4 \cdot H_2O$; 1.40mg $ZnSO_4 \cdot 7H_2O$; 10g glucose; and 2.7g MMHA (0.05% P) all filtered to 2µm; and 20g agar (Döbereiner *et al.,* 1999; Reyes *et al.,* 1999). The initial pH was adjusted to 7.0. Additionally, for the isolation of phosphodissolvent fungi, 30mg of streptomycin sulfate and 100mg chloramphenicol was added.

(ii) Modified Pikovskaya culture method (PVKM)-Agar with 0.5 g ferric phosphate ($FePO_4 \cdot H_2O$) per L instead of tricalcium phosphate (Nopparat *et al.,* 2009); the composition of which was: 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.004 g $MnSO_4 \cdot H_2O$, 0.2 g NaCl, D10 g -Glucose, 0.002 g $FeSO_4 \cdot 7H_2O$ (Sigma-Aldrich), 0.5 g yeast extract (Bioxon), 18 g agar (Bioxon), and distilled water (900 mL). For the isolation of phosphodissolvent fungi, 0.1 g chloramphenicol (Pfizer) was added. To this mixture, a solution of phosphate: 0.5 g gum arabic (Kremer), 0.5 g ferric phosphate ($FePO_4 \cdot H_2O$) (Química- Barquim), and 100 mL sterile distilled water was added. In this medium, dissolution was seen in the form of a clear halo around the colony or mycelium (Sundara and Sinha, 1963).

(iii) Osorio and Habte (2001) medium for PR solubilizers (MSPOH), the composition of which was: 1.0 g NaCl; 02 g $CaCl_2 \cdot 2H_2O$; 0.4 g $MgSO_4 \cdot 7H_2O$; 1.0 g NH_4NO_3; 10 g glucose L^{-1}; and 35 g unacidulated PR per liter of medium.

Additionally, PDA culture medium was used as a reference. The initial pH of the different media was adjusted to 7.0 with 1.0M NaOH. The three different media were sterilized in an autoclave for 15 minutes at 121°C. Ten (10) Petri dishes were used to plant each morphotype of *Trichoderma* for each culture medium.

Determination of phosphodissolvent capacity of Trichoderma spp.

In order to evaluate the effect of media on the phosphodissolvent capacity of the strains, the diameters of the colonies (DC) were measured as a function of time (3, 6, 9, and 12 days after inoculation). Additionally, the diameter of the solubilization halo (DSH) was measured and the relative efficiency of solubilization (RSE) of PR was calculated. The pH of the media was measured using pH indicator strips on their surfaces. The RSE was calculated using the method proposed by Vera *et al.* (2002):

$$RSE = 100 \times (DSH \div DC)$$

This value indicates the radius of action of the fungus on the substrate it solubilizes in relation to the size of the extended fungal mycelium. Data shows the average of the 4 petri dishes inoculated per strain.

Experiment 2: Kinetics of dissolution of PR in liquid media

Fungal biological PR dissolution efficiency was evaluated following the method developed by Osorio (2008). For this purpose, 75 mL of media containing a solution consisting of 1.0 g NaCl, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.4 g $MgSO_4 \cdot 7H_2O$, 1.0 g NH_4NO_3, 10 g glucose L^{-1}, and 3.5 g unacidulated PR per liter of medium was added to a 250 mL Erlenmeyer flask . The pH was adjusted to 7.0 with 0.1M NaOH. The Erlenmeyer flasks and their contents were sterilized in an autoclave at 120°C and 1.2 kg cm^{-2} for 20 minutes. For each morphotype of *Trichoderma* with PR solubilizing capacity, suspensions of spores in 1 mL $CaCl_2 \cdot 2H_2O$ 0.01*M*a, for a concentration of 7×10^9 spores per mL (Capuchino and Sherman, 1998) were used. Each Erlenmeyer flask was inoculated with one (1) mL of these suspensions. As a control, the uninoculated Erlenmeyer flasks received 1mL of $CaCl_2 \cdot 2H_2O$ 0.01*M* solution.

The broths were incubated at 25 ± 1°C, in an orbital agitator at 100 rpm for 12 days. Each treatment had 4

replicas. In order to establish dissolution kinetics, every 3 days, 1 mL from each Erlenmeyer flask was collected in order to measure the pH (Conductronic PC45, México) and the concentration of soluble P using the blue molybdate spectrophotometer method (890 nm) (Genesys 20, Thermo Spectronic, USA) (Murphy and Riley, 1962).

For the above, it was necessary to pass the sample through a piece of filter paper and later centrifuge the filtrate at 4000 rpm for 10 min, after which it was then filtered in a duplicate vaccuum through Millipore 0.2 µm Ø and 0.45 µm Ø, filter paper, respectively. The procedure above was done in order to separate fungal cells from particulate matter.

Experiment 3: Selection of IAA producing strains of Trichoderma

Preliminary test for detection of indole acetic acid (IAA)

Tests for the capacity of individual strains of *Trichoderma* spp., to synthesize indole acetic acid (IAA) were conducted on an individual basis following the methodology reported by Rico (2009). Initially, a culture medium of potato broth (PDB: Potato-Dextrose-Broth) was prepared and autoclaved at 121°C for 15 minutes. Later, a previously filtered mixture of 0.153 g L-Trp was dissolved in 15 mL water. Next, an inoculation was done with each strain of *Trichoderma* spp., in 50 mL Erlenmeyer flasks with PDB culture media enriched with L-Trp (an amino acid precursor for the synthesis of IAA during the development of *Trichoderma* spp.,). The fungal growth media were constantly agitated at 100 rpm and 27 °C for 72 hours, after which they were centrifuged at 3000 rpm (Jouan MR 1812 centrifuge) for 15 minutes.

The supernatant was dispensed in MicroWell brand ELISA plastic plates with 96 250-microliter wells. For the reveal, Salkowski ($ClFe_3$=2% (w/v) 0.05M, H_2SO_4=35% H_2O=63%) solution was added, which detected indole derivatives (Gordon and Weber, 1951). As a negative or zero control, control wells with PDB and Salkowski solution were used. For positive control, two identified bacteria (*Bacillus subtilis* and *Bacillus amiloliquefasciens*) were used as test positives. The ELISA plate was placed in total darkness for 15 minutes, and for its later reveal, color change from yellow to diverse tonalities between the range of pink and hot fuchsia were observed, indicating the presence of IAA (Gordon and Weber, 1951).

in vitro quantification of indole acetic acid (IAA)

The intrinsic capacity of strains to produce IAA in the presence of different concentrations of tryptophan was evaluated taking note of methods reported by Ahmad *et al.* (2005) and Campos *et al.* (2007). For this, a calorimetric method measuring a Van UrkSalkowski reactive was prepared from ferric chloride and sulfuric acid (Mayer, 1958; Glickmann and Deessaux, 1995). This reactive is specific for the detection of compounds derived from indole.

For fungal growth, a liquid Burk's culture medium was used, which is free of nitrogen and is supplemented with glucose as a carbon source with mineral salts and distilled water (Park *et al.*, 2005). The pH of the media were adjusted to 6.8 with a potentiometer (Conductronic PC45, México) which were then autoclaved at 121°C for 15 minutes.

In order to evaluate fungal production of IAA, 50mL capacity screwcap test tubes were used. These contained 30mL Burk's broth supplemented with tryptone. Each treatment evaluated an increased dose of L-Tryptophan, at 1, 2, and 3 mg L^{-1}. Each treatment, with its respective control (nutritive broth without L-Tryptophan) was done in triplicate, with each of the tubes in the treatment being inoculated with 7.5mL of inoculate at a concentration of 4.6 x 10^6 spores mL^{-1}. The tubes were placed in a shaker with a constant agitation of 120 rpm at 27°C for 72 hours in total darkness. After this period, the fungal broths were centrifuged at 3000 rpm (Jouan MR 1812 centrifuge) for 15 minutes at 27±1°C, after which time 1 mL supernatant was transferred to a test tube and

mixed with 1mL of fungal culture supernatant and 2 mL Salkowski reactant at 2% (w/v) $FeCl_3$ 0.5*M* dissolved in 35% perchloric acid. This was agitated in an incubation vortex at 21°C for 30 min. After the reaction, an absorbancy test of each sample was done at 530nm in order to determine the quantity of auxins with a spectrophotometer (Genesys 20, ThermoSpectronic, USA). The control blank was prepared with sterile culture medium and the positive control of the reaction with the IAA solution pattern. The presence of IAA was determined by the development of a pink color characteristic of the presence of the acid. This test was interpolated using a calibration curve with an IAA (Sigma) pattern starting at a concentration pattern of 100 µg mL^{-1}.

Experimental design and statistical analysis

A completely random experimental design was used. For experiments 1 and 2, the treatments had a 4×4 factorial arrangement (4 incubation times, 4 culture media). The dependent variables were RSE (%), final medium, pH, and concentration of P in solution (mg L^{-1}). Each treatment had 4 replicates. In experiment 3, the treatments had a 4X4 factorial arrangement (4 incubation times, 4 L-Tryptophan doses). The dependent variable was indole acetic acid production. Each treatment had 4 replicates.

Results were analyzed using an analysis of variance (ANOVA), which determined assumptions and residuals. A Duncan test was used to separate media. Analyses were done at a $(P) \leq 0.05$ significance level. All analyses were done with Statgraphics statistical software, version Centurion XVI.

Results

Composition of fungal communities in soil and rhizospheres of beans and corn

Table 1 presents data from fungal populations present in two microhabitats: rhizospheric soil and the rhizosphere. The results demonstrated that significant differences exist $((P) \leq 0.05)$ between the populations studied. This may be explained by the fact that each environment presents favorable conditions for the growth of microorganisms. In this investigation, the total median values per gram of dry soil for the fungi are expressed in the order of 5.0 x 10^4 UFC g^{-1}. Highly significant differences were found between the number of fungal genera in the two microhabitats and the two cultivations studied $((P) \leq 0.01)$.

Table 1. Composition of fungal community in soil and rhizosphere of beans and corn.

Substrate	Total Microbiota UFC g^{-1}	Log UFC g^{-1}	Growth media	Fungal genera present in soil and bean rhizosphere (UFC g^{-1})					Fungal genera present in soil and rhizosphere of corn (UFC g^{-1})		
				Trichoderma	*Aspergillus*	*Penicillum*	*Paecillomices*	*Rhizopus*	*Trichoderma*	*Aspergillus*	*Penicillum*
Rhizosphere	1,6 x 10^4	4,01	PDA	6,5 x 10^4	5,4x 10^4	3,9 x 10^4	3,3x 10^4	2,4x10^4	2,5 x 10^4	1,4x 10^4	1,9 x 10^4
			MM-HA	7,8 x 10^4	6,0 x 10^4	3,4 x 10^4	3,5 x 10^4	2,0x 10^4	2,5 x 10^4	1,5 x 10^4	1,4 x 10^4
			PVKM	4,5 x 10^4	4,5 x 10^4	5,0 x 10^4	4,0 x 10^3	2,5x 10^4	6,0 x 10^4	1,5 x 10^4	1,5 x 10^4
			MSPOH	8,5 x 10^4	7,3 x 10^4	7,5 x 10^4	4,2 x 10^4	2,3x 10^4	5,5 x 10^4	1,3 x 10^4	1,5 x 10^4
Rhizoplane	1,3 x 10^4	2,5	PDA	1,8x 10^4	2,5 x 10^4	2,5 x 10^4	1,7 x 10^4	1,5x 10^4	2,8x 10^4	4,5 x 10^4	4,5 x 10^4
			MM-HA	1,0 x 10^4	2,0 x 10^4	2,9 x 10^4	1,3 x 10^4	1,0x 10^4	5,5 x 10^4	5,0 x 10^4	4,9 x 10^4
			PVKM	1,5 x 10^4	2,5 x 10^4	1,2 x 10^4	1,0 x 10^3	1,5x 10^4	5,0 x 10^4	4,5 x 10^4	4,0x 10^4
			MSPOH	1,8 x 10^4	1,5 x 10^4	1,5 x 10^4	1,7 x 10^3	1,5x 10^4	5,0 x 10^4	5,0 x 10^4	4,0 x 10^4

All values shown are the average of 4 replicas per treatment.

Isolation of phosphodissolvent morphotypes of Trichoderma spp.

The processing of samples of soil and roots permitted the recuperation of 68 primary isolates which were grouped into 18 morphotypes. Through micromorphological, physiological, biochemical, and metabolic tests; 9 strains of *Trichoderma* spp. were selected with phosphodissolvent capacities.

Experiment 1: PR solubilization in solid media

Determination of phosphodissolvent capacity of Trichoderma spp.

In MSPOH media, on the sixth (6) day of incubation, the diameter of the TRIC7 strain showed the

following characteristics: DC 42.6 cm Ø, DHS 7.2 cm Ø, RSE 276%, pH of medium 3.5 (Table 2), and concentration of soluble P ($H_2PO_4^-$) 64.3 mg L^{-1} (Table 3).

Similar results were obtained with the TRIC11 strain with the same culture medium and incubation time. This strain showed a DC of 3.4 cm Ø, a DHS of 7.5 cm Ø, an RSE of 221%, a pH of 3.5 (Table 2), and a concentration of soluble P ($H_2PO_4^-$) of 75.0 mg L^{-1} (Table 3). Similarly, the TRIC13 and TRIC48 strains showed a DC of 3.3 and 3.5 cm Ø, a DHS of 8.5 and 7.9 cm Ø, a maximum RSE of 257 and 226%, a pH of 3.5 (Table 2), and a concentration of soluble P ($H_2PO_4^-$) of 89.8 and 86.2 mg L^{-1}, in the same culture media and incubation time, respectively (Table 3).

On the sixth day of incubation, the RSE values of TRIC7, TRIC11, TRIC13, and TRIC48 oscillated between 221 and 276%. These results show a solubilization halo for these strains larger than the diameters of their mycelia (Table 2).

Table 2. Relative solubilization efficiency (RSE) of different strains in solid culture media. Diameter of mycelia and solubilization halo.

Strain	Time (d)	Diameter of the fungus colony DC (cm)				Halo diameter solubilization HDS (cm)				Relative Efficiency of solubilization P RES (%)				Final pH of the medium			
		PDA	MMHA	PVKM	MSPOH	PDA	MMHA	PVKM	MSPOH	PDA	MMHA	PVKM	MSPOH	PDA	MMHA	PVKM	MSPOH
*Trichoderma*sp. (TRIC7)	3	1.4	1.6	3.5	2.4	0.0	1.6	4.4	4.6	0.0	100	125	191	6.8	5.5	5.5	4.5
	6	3.8	2.8	3.6	2.6	0.0	4.8	5.4	7.2	0.0	171	150	276	6.8	4.5	5.0	3.5
	9	6.7	4.3	4.8	3.8	0.0	6.0	5.5	8.5	0.0	139	114	223	6.5	4.5	5.0	3.0
	12	10.0	5.1	5.8	4.2	0.0	6.6	6.6	8.8	0.0	129	113	209	6.0	4.0	5.1	3.0
*Trichoderma*sp. (TRIC11)	3	2.4	2.9	2.2	3.3	0.0	2.9	2.6	4.9	0.0	100	118	148	6.9	5.5	5.0	5.0
	6	4.3	3.3	2.6	3.4	0.0	6.5	3.5	7.5	0.0	196	134	221	6.5	4.5	5.0	3.5
	9	8.5	3.5	3.4	4.1	0.0	6.6	3.6	8.0	0.0	188	105	195	6.5	4.0	5.1	4.0
	12	10.0	4.2	4.6	5.8	0.0	6.7	4.6	8.2	0.0	159	100	141	6.0	4.0	5.0	4.5
*Trichoderma*sp. (TRIC13)	3	2.1	1.6	2.0	2.3	0.0	1.6	2.0	4.2	0.0	100	100	182	6.5	5.0	5.2	4.5
	6	4.6	2.1	2.3	3.3	0.0	4.6	3.6	8.5	0.0	219	156	257	6.5	4.5	5.0	3.5
	9	8.7	3.8	4.6	7.4	0.0	4.9	5.6	9.0	0.0	128	121	121	6.0	3.5	4.5	4.0
	12	10.0	4.4	4.9	8.5	0.0	4.9	5.9	9.5	0.0	111	120	111	6.0	3.5	4.5	4.5
*Trichoderma*sp. (TRIC33)	3	2.8	2.5	3.4	2.5	0.0	3.6	3.4	2.8	0.0	144	100	112	6.8	5.8	6.5	6.0
	6	4.9	3.4	3.5	2.7	0.0	5.4	4.5	4.3	0.0	158	129	159	6.5	5.5	6.5	5.5
	9	7.1	4.6	4.1	4.4	0.0	5.5	4.6	4.5	0.0	119	112	102	6.5	5.5	6.5	5.0
	12	10.0	4.9	4.3	4.5	0.0	5.9	4.7	4.6	0.0	120	109	102	6.5	5.5	6.5	5.0
*Trichoderma*sp. (TRIC36)	3	2.3	2.4	1.7	3.3	0.0	2.8	1.8	3.9	0.0	116	105	118	6.9	5.9	5.5	6.5
	6	4.7	2.9	2.7	4.1	0.0	4.5	3.4	6.5	0.0	155	125	158	6.5	5.5	4.5	6.5
	9	8.4	3.5	3.9	5.5	0.0	4.7	3.9	7.5	0.0	134	100	136	6.5	4.5	4.5	5.5
	12	10.0	4.8	5.3	6.8	0.0	4.9	5.3	8.0	0.0	102	100	117	6.5	4.0	4.0	4.0
*Trichoderma*sp. (TRIC48)	3	2.2	1.5	1.5	3.9	0.0	2.8	1.8	6.9	0.0	186	120	176	6.5	5.0	4.5	5.5
	6	5.9	3.5	2.5	3.5	0.0	6.8	3.9	7.9	0.0	194	156	226	6.5	4.5	4.0	3.5
	9	9.1	5.5	4.5	6.5	0.0	7.1	5.5	8.0	0.0	129	122	123	6.5	4.5	4.0	4.0
	12	10.0	6.5	4.5	7.2	0.0	7.5	5.6	8.6	0.0	115	124	119	6.5	4.0	4.0	4.5
*Trichoderma*sp. (TRIC53)	3	2.3	2.1	2.1	2.1	0.0	2.6	2.1	2.2	0.0	123	100	104	6.9	5.5	6.5	5.5
	6	4.0	2.2	2.3	2.8	0.0	3.4	2.7	4.2	0.0	154	117	150	6.5	4.5	5.0	5.5
	9	8.3	3.5	2.7	4.1	0.0	3.6	2.8	4.5	0.0	102	106	109	6.5	5.0	5.5	6.0
	12	10.0	3.7	3.3	6.3	0.0	3.7	3.4	6.9	0.0	100	103	109	6.0	5.0	6.5	6.0
*Trichoderma*sp. (TRI57)	3	3,5	4,8	3,5	4,2	0.0	4.8	4,2	4,4	0.0	100	120	105	6.5	602	6,2	6,8
	6	3,5	4,5	3,6	4,3	0.0	5.7	4,4	5,5	0.0	126	122	128	6.4	5,7	5,2	6,6
	9	3,9	5,2	3,9	4,5	0.0	5,8	4,5	5,6	0.0	111	115	124	6.0	5,0	4,1	6,5
	12	10.0	5.6	4,2	4,7	0.0	5,8	4,6	5,7	0.0	103	109	121	5.8	5,2	5,2	6,4
*Trichoderma*sp. (TRIC61)	3	2,0	3,3	2,0	1,9	0.0	3,3	2.4	2.4	0.0	100	120	126	6.8	5.8	6.5	6.8
	6	2,0	3,5	2,3	2,5	0.0	5,5	3.2	4.5	0.0	157	139	180	6.6	5.5	6.3	5.5
	9	3,0	7,5	3,0	3,7	0.0	7,8	3.3	4.9	0.0	104	111	132	6.7	5.3	6.1	5.3
	12	10.0	8,0	3,3	5,0	0.0	8.0	3.5	5.2	0.0	100	106	104	6.9	5.1	6.0	5.0

All values shown are the average of 4 replicas per treatment.

In contrast to these strains, in the TRIC33, TRIC36, TRIC53, TRIC57, and TRIC61 strains, the formation of halos were smaller, showing low RSE values.

The diametral growth of the fungal strains was larger in the control treatments than in the phosphate media ($p \leq 0.05$) (Table 2). In this treatment, the average diameter of mycelia on the twelfth (12) day of incubation was 10.0 Ø and the maximum decrease in the pH of growth media was 5.8 (Table 2). These values (DC) disagree with the concentration of soluble P ($H_2PO_4^-$) obtained with respect to different growth

media (Table 3).

Experiment 2: Quantification of soluble phosphate in liquid media

In MSPOH media, on the sixth (6) day of incubation, the TRIC7, TRIC11, TRIC13, and TRIC48 strains showed concentrations of soluble P ($H_2PO_4^-$) of 66.3, 75.9, 89.8, and 86.2 mg L^{-1}, respectively. In the same order, they exhibited a decrease in pH from 7.0 to 3.64, 3.63, 3.64, and 3.80. Considering that these strains solubilized an average of 12.5 times more P than the control treatment and the other strains evaluated, they attained the highest in vitro solubilization efficiency of PR ($P \leq 0.01$) (Table 3).

Table 3. Concentration of soluble P ($H_2PO_4^-$) and pH in liquid media with and without inoculation of fungal strains.

Treatment	Incubation time (d)	Variables evaluated in the middle MM-HA		Variables evaluated in the middle PVKM		Variables evaluated in the middle MSPOH	
		Medium pH	Soluble P ($H_2PO_4^-$)	Medium pH	Soluble P ($H_2PO_4^-$)	Medium pH	Soluble P ($H_2PO_4^-$)
Absolute control (uninoculated)	3	6.55	1.5	6.71	1.3	6.69	1.3
	6	4.60	1.1	6.64	1.8	6.67	1.7
	9	5.45	1.9	6.35	1.8	6.46	1.7
	12	6.50	1.9	6.13	1.7	6.41	1.6
Trichoderma sp. (TRIC7)	3	6.00	10.8	5.98	6.3	5.65	61.0
	6	5.58	37.7	5.68	6.9	3.64	66.3
	9	5.48	28.3	5.56	6.7	3.03	53.0
	12	4.33	37.7	5.30	6.6	4.41	48.5
Trichoderma sp. (TRIC11)	3	5.69	25.8	6.53	5.8	5.75	68.9
	6	5.48	39.0	5.97	6.9	3.63	75.9
	9	5.38	25.3	5.86	6.2	4.01	65.3
	12	4.21	21.3	5.71	6.5	4.52	59.5
Trichoderma sp. (TRIC13)	3	4.98	33.7	6.02	6.4	5.67	68.9
	6	4.75	68.0	5.86	6.9	3.64	89.8
	9	4.83	48.8	5.54	6.7	4.02	79.8
	12	4.38	55.7	5.53	6.5	4.91	69.8
Trichoderma sp. (TRIC33)	3	5.73	2.7	6.96	1.2	6.00	33.2
	6	5.74	2.9	6.70	2.5	5.76	34.0
	9	5.76	2.6	6.77	2.7	5.30	34.2
	12	5.70	2.5	6.47	1.9	5.19	34.2
Trichoderma sp. (TRIC 36)	3	5.96	5.8	5.91	3.2	6.79	36.0
	6	5.67	6.5	4.90	4.4	6.68	36.0
	9	5.58	5.8	4.73	3.9	5.23	36.5
	12	5.45	6.1	4.42	3.5	6.31	36.5
Trichoderma sp. (TRIC48)	3	5.87	7.3	5.59	4.2	5.53	55.0
	6	5.57	7.9	5.57	5.4	3.80	86.2
	9	5.37	6.8	5.46	4.9	3.99	66.0
	12	5.18	6.5	5.45	4.3	3.45	52.0
Trichoderma sp. (TRIC53)	3	5.82	2.1	3.97	1.4	6.82	29.3
	6	5.68	2.8	2.96	1.7	6.72	29.9
	9	5.62	2.5	3.03	1.8	6.55	25.5
	12	5.55	2.3	3.04	1.0	6.34	25.0
Trichoderma sp. (TRI57)	3	5.92	1.3	3.47	1.4	6.62	19.3
	6	5.68	1.5	2.96	1.7	6.62	19.9
	9	5.72	1.4	3.83	1.8	6.05	15.5
	12	5.55	1.0	3.74	1.0	6.44	15.0
Trichoderma sp. (TRIC61)	3	5.83	3.6	6.86	2.2	6.02	43.2
	6	5.74	4.9	6.60	2.5	5.72	44.0
	9	5.76	3.7	6.57	1.7	5.00	44.2
	12	5.60	3.3	6.47	1.7	5.12	44.2

Soluble P ($H_2PO_4^-$) ($LSD_{0.05}$= 8.0) and final pH of culture media ($LSD_{0.05}$= 0.2) are data that show the behavior of fungal colonies in MM-HA, PVKM, and MSPOH liquid media. All values shown are the average of 4 replicas per treatment.

At the end of twelve days of solubilization kinetics, the soluble concentration of P ($H_2PO_4^-$) decreased to 48.5 mg L^{-1} in TRIC7, to 59.5 mg L^{-1} in TRIC11, to 69.8 mg L^{-1} in TRIC13, and to 52.0 mg L^{-1}in TRIUN48 (Table 3). This behavior confirms that at higher fungal biomasses there are lower concentrations of P in solution, allowing the selection of day six (6) as the optimal time in which the highest concentration of liberated P is found in solution ($P \leq 0.05$). The concentration of soluble P ($H_2PO_4^-$) was lower in the other strains evaluated (Table 3).

Experiment 3: Selection of IAA producing strains of Trichoderma spp.

With the dependent variable production of IAA in mind, the analysis of variance permitted the determination of whether strain, day, and concentration of L-Tryptophan influenced, respectively, the results. The results showed that these variables were representative in the study with a high level of significance ($P \leq 0.01$). For the variable strain, the value ($P \leq 0.01$) was found, indicating significant differences between the strains evaluated. The variable time had a value of ($P \leq 0.01$), indicating significant differences between the strains evaluated. The variable concentration of L-Tryptophan had a value of ($P \leq 0.01$), indicating significant differences between the strains evaluated. Two high accuracy tests the Duncan and Fisher LDS were used to determine the differences between these variables; with both clustering the average IAA values generated with respect to the variables tested.

Preliminary test for the detection of IAA

The processing of the soil and root samples permitted the recovery of 68 primary isolates which were then grouped into 18 morphotypes. Through micromorphological, physiological, biochemical, and metabolic tests, 9 strains of *Trichoderma* spp., with phosphodissolvent capacities and 14 strains with the capacity to detect compounds derived from indole were selected. Of the 9 phosphodissolvent strains of *Trichoderma* spp., the TRIC7, TRIC11, TRIC13, and TRIC48 strains also tested positive for the presence of IAA (Table 4).

Table 4. Colorimetric response to the production of IAA in the presence of L-Tryptophan.

Strain	Time (d)	L-triptofano mg L^{-1}			
		0	1	2	3
TRIC7	3	-	+	++	+++
	6	-	++	+++	+++
	9	-	+++	++++	++++
	12	-	+++	++++	++++
TRIC11	3	-	+	++	+++
	6	-	++	+++	+++
	9	-	+++	++++	++++
	12	+	+++	++++	++++
TRIC13	3	-	++	++	++
	6	-	++	++	++
	9	-	+++	+++	++++
	12	+	+++	+++	++++
TRIC48	3	-	++	++	++
	6	-	++	++	+++
	9	-	++	+++	++++
	12	+	+++	++++	++++

- = Intensity of null color + = Intensity of incipient color ++ = Intensity of moderate color +++ = Intensity of high color.

Due to the fact that IAA is synthesized through various metabolic pathways that may or may not depend on L-Tryptophan as a precursor the presence of indole detected can be considered to be directly proportional to the intensity of the colors fuchsia or red during the time of reaction. Thus, as reaction time increases, so does the concentration of IAA that is generated (Table 4). Colorimetric tests show that

the TRIC11, TRIC13, and TRIC48 strains, in the absence of L-Tryptophan, generated a change in color over time (Table 4).

In vitro quantification of indole acetic acid (IAA)

The capacity for the production of IAA in the presence of L-Tryptophan on the part of the *Trichoderma* spp., strains selected was quantified through the addition of Van Urk Salkowski reagent. This generated a calibration curve which was made from patterns of IAA (Sigma) diluted in distilled water at known concentrations. Using absorbancy data (530 nm) as a function of IAA mg $^{L-1}$ concentration, a linear regression was made in order to determine the equation of the line y = 1.675x, with the value r^2 = 0.999. An adequate dispersion over the line was considered as a reference for the calculation of IAA (mg L^{-1}) produced by the four phosphodissolvent IAA producing strains.

Table 5. Concentration of IAA (mg L^{-1}) in the presence of L-Tryptophan generated by *Trichoderma* spp.

Strain	Time (d)	L- Triptófano mg L^{-1}			
		0	1	2	3
TRIC7	3	0	0.945	0.992	1.576
	6	0	1.841	2.281	2.517
	9	0	2.245	2.517	3.674
	12	0	3.534	3.790	4.258
TRIC11	3	0	1.742	2.881	3.517
	6	0	3.792	4.916	7.3.13
	9	0	4.218	7.143	11.788
	12	0	7.314	9.986	15.972
TRIC13	3	0	2.235	2.417	3.974
	6	0	5.437	7.776	9.987
	9	0	7.143	8.005	10.928
	12	0	10.743	11.472	13.152
TRIC48	3	0	3.534	3.798	4.273
	6	0	4.057	5.283	6.007
	9	0	7.346	9.845	10.773
	12	0	9.953	11.716	12.874

In completing the statistical analysis, data obtained in the negative control was not taken into account in making groups for the comparison of media. According to the determinations made, the TRIC7 (P≤0.05) strain had the lowest production of IAA in relation to the TRIC11, TRIC13, and TRIC48 strains. The variable day was highly significant (P≤0.01) in relation to the TRIC11, TRIC13, and TRIC48 strains. The variable concentration of L-Tryptophan was highly significant (P≤0.01) in relation to the TRIC11, TRIC13, and TRIC48 strains. The results corroborate the capacity of some strains of *Trichoderma* spp., to dissolve PR and produce IAA efficiently.

Discussion

The results of this investigation confirm the hypothesis that success in the *in vitro* selection of strains of *Trichoderma* with phosphodissolvent and IAA producing capacities is a function of the strain evaluated, the growth medium, the incubation time, and the concentration of specific hormonal inductors.

Composition of fungal community in soil and rhizosphere of beans and corn

One of the most important aspects that determines the composition of the microbial community in the rhizosphere is the type of vegetal species, due to the

fact that for different plants, according to the genotype, root morphology, and physical and structural transformations that these make in the adjacent soil, there is variation in the structure of microbial communities (Marschner *et al.,* 2001). The bean is a plant, the foliar architecture of which favors the accumulation of necromass in the foot, which encourages a higher colonization of fungi due to its decomposing activity for the litter accumulated around the stem (Sabogal, 2002). Due to the above, a large quantity of organic matter is obtained in the soil that favors the establishment of fungi that respond to the rhizospheric effect exercised by the exudates of the bean rhizosphere.

In gramines, the metabolism generates a larger quantity of root exudates in which organic acids predominate. The offering of gramine exudates is deposited in the soil near the rhizosphere. In beans, the offering of exudates occurs in the foot; however, a larger quantity of decomposing fungi associated with the rhizosphere of corn may explain the results obtained. In general terms, the amount of fungi found were within the same range (quantity), although there was a larger number of fungi in the rhizospheres of corn than there was in beans. Solubilizing fungi were found to predominate in both cultures, but they showed a higher proportion in corn.

On the other hand, it is possible that the presence of a large quantity of phosphate solubilizing fungi found in the corn rhizosphere and the high micorrhization characteristic of the gramines (Sánchez, 1996; Cepeda and Gamboa, 2001) plays an important role in their uptake of phosphate as compared to that obtained in beans. Cepeda and Gamboa (2001) state that the quantity of phosphorous available in the soil determined by the Bray II method of 4.2 ppm in soils in eastern Antioquia is considered to be very low. These soils also show high quantities of Fe and Al and an acidic pH of 4.9. These factors are related to the high percentage of phosphoric retention (98.9%), and make it so that phosphate solubilizers are one of the most important microorganism functional groups associated with the plants that predominate in this ecosystem.

The presence and vegetal growth promoting activity of phosphate solubilizing fungi is summarily important, as it offers plants the possibility of achieving better adaptation to ecosystems that are limited in phosphorous due to their simultaneous utilization of assimilable forms of phosphorous that favor efficiency in the uptake of nutrients and the achievement of greater length and proliferation of roots. Fungal species have been found that produce enzymes such as acid phosphatases and phytases that increase soluble phosphorous in the soil and stimulate root lengthening and the proliferation of buds in different plants (Martinez and Martinez, 2000; Valero, 2003; Villarroel, 2009; Ocampo *et al.,* 2012).

It can be inferred from the above that the presence of phosphate solubilizing strains of *Trichoderma* spp., associated with plants may initiate similar effects and these could thus be utilized as inoculants in agricultural systems in cold climates just as the other strains evaluated (Cepeda and Gamboa, 2001; Rodríguez and Rubiano, 2002; Useche, 2003) have been. A value added from the utilization of these microorganisms in agriculture is the potential that they have to also act as biological control agents, which has been reported in different studies (Kloepper, 1993; Altamore *et al.,* 1999; Rodríguez and Fraga, 1999; Torres-Rubio *et al.,* 2000).

The results obtained in this study support the additive hypothesis (Bashan *et al.,* 1993), according to which, microorganisms that promote vegetal growth act through the sum of different mechanisms that operate simultaneously or in succession and the sum of these different activities when they are induced under advantageous environmental conditions result in the stimulation of plant growth. The association of plants with microorganisms that act through various mechanisms in an integrated manner, such as phosphate solubilizers, may be an important mechanism of adaptation for the ecological success of both the plants and the microorganisms, and at the

same time represents a valuable germplasm bank for agroecological and biotechnological applications.

Experiment 1: Solubilization of PR in solid media

The RSE of the fungal strains TRIC7, TRIC11, TRIC13, and TRIC48, with solubilization halos three (3) times larger than the diameter of their mycelia (Table 2) turned out to be larger than the strains of *Trichoderma* spp., *Paecilomyces* spp., and *Aspergillus* spp. evaluated by Vera *et al.* (2002) and Hernández *et al.* (2011) in comparable culture media. However, other strains evaluated in this study (TRIC33, TRIC36, TRIC53, TRIC57, and TRIC61) showed the formation of incipient halos; their RSE values were lower. Even so, several authors (Jones *et al.,* 1998; Whitelaw, 1999 and Hernández *et al.,* 2011) mention that the absence of solublilization in solid culture media does not necessarily indicate that certain microorganisms lack solubilizing ability, but rather that solid media are not sensitive enough to detect the activity of some of them. Therefore, this underscores the need to evaluate them in liquid media in order to obtain adequate results.

Nahas (1996) reported that phosphodissolvent bacteria and fungi obtained in the soils of Sao Paulo (Brazil) generated concentrations of soluble P ($H_2PO_4^-$) between 13.3 and 33.1 mg L^{-1}, respectively. Sridevi and Mallaiah (2009) also reported phosphodissolvent strains of *Rhizobium* spp., that exhibited RSE values between 60 and 150%, accompanied by decreases in the pH of the media used to between 7.02 and 4.04, as well as concentrations of soluble P between 15.6 and 62.0 mg L^{-1}. To this end, the strains of *Trichoderma* evaluated in this investigation showed RSE values between 128-176%, decreases in pH from 7.0 to 3.0, and soluble P between 15.0 and 89.8mg L^{-1} (Tables 2 and 3).

With respect to the diametral growth of fungal strains, this was larger in the control treatments than in media with PR ($P \leq 0.05$) (Table 2). This data suggests that the production of organic acids for the dilution of PR has a high energetic cost that decreases the production of fungal biomass.

The composition of the culture medium has a notable influence on the growth and solubilization of fungi. In this regard, Nahas (2007) and Hernández *et al.* (2011), emphasize that the production of substances implicated in solubilization require the absorption of nutrients, especially carbonaceous compounds, important in the accumulation of biomass.

The results of the solubilization kinetics of PR show a cause and effect relationship between the acidification of the pH of the media and the concentration of solubilized P ($P \leq 0.05$) (Tables 2 and 3). This inverse relationship suggests that it is the production of organic acids that is the principal mechanism of dissolution of PR; however, the fact that at the same pH the same concentration of P is not obtained suggests that other mechanisms of dissolution of PR may exist, such as those suggested by Thomas (1985) and llmer and Schinner (1992). Two examples include, the type of organic acid, and the complexing of Ca^{2+} by organic anions. These authors confirm that under buffered conditions, microbial metabolism changes and this effect allows the liberation of distinct organic acids to those that microorganisms commonly liberate when the initial pH of the culture medium is not adjusted.

In this way, the process of microbial solubilization of PR may be more affected by the type of organic acid secreted than by the quantity of acid (Halder *et al.,* 1990; Gyaneshwar *et al.,* 1998). For example, in a study done by Osorno and Osorio (2014) with the fungi *Mortierella* sp. (M) and *Aspergillus niger* (A), which produce oxalic acid, (Osorio, 2008) and citric acid (Velásquez *et al.,* 2010), respectively, significant differences were found with respect to P concentration in solution. Consonantly, the *Trichoderma* strains evaluated in this study also diminish the pH of the medium (presumably through organic acids), with some strains generating very low values, but do not cause the same dissolution of PR. In the same way, the mechanism of bioacidulation and biosolubilization of PR, as, for example, the type of organic acid that the fungus generates, causes a

higher concentration of phosphorous in solution, which affects the efficiency of phosphodissolution and permits a pattern of selection secured by PSM.

It follows that the efficiency of *in vitro* solubilization of PR shown by the strains of *Trichoderma* evaluated can be compared with other fungi of known effectiveness in dissolving PR and other phosphatic compounds such as *Penicillium janthinellum* (Ñustez and Acevedo, 2005), *Paecilomyces lilacinus* (Thom) Samson (Hernández *et al.,* 2011), *Trichoderma harzianum* (Zhang *et al.,* 2013; Zúñiga-Silgado, 2016), *Mortierella* sp., and *Aspergillus* sp., (Osorio, 2011; Osorno and Osorio, 2014). They are also comparable to the solubilization efficiency of bacterial genera such as *Bacillus, Azospirillum, Pseudomonas* and *Rhizobium* (Rodríguez *et al.,* 2004; Sridevi and Mallaiah, 2009; Ahmad *et al.,* 2011), among others.

Experiment 2: Kinetics of dissolution of PR

On the sixth day of incubation, the TRIC7, TRIC11, TRIC13, and TRIC48 strains showed the highest concentrations of soluble P ($H_2PO_4^-$) and the highest bioacidulation of pH. If we consider that these strains solubilized an average of 12.5 times more P than the control treatment and the other strains evaluated, they achieved the highest *in vitro* solubilization efficiency of PR ($P≤0.01$) (Table 2). At the end of twelve days of solubilization kinetics, the concentration of soluble P decreased in the culture media with these strains. This behavior confirms that at higher fungal biomass, there are lower concentrations of P in solution, allowing the selection of day six (6) as the optimal time with the highest amount of liberated P ($P≤0.05$) (Table 2).

In this respect, Hernández *et al.* (2011) mention that it is probable that the quantity of solubilized phosphorous diminishes over time as it is incorporated into fungal metabolism. Reyes *et al.* (1999a), in their investigations with *Penicillum rugolosum* Thom, attributed the decrease in liberated P to the process of immobilization of P during mycelial growth. As phosphorous is indispensable in the cellular functions of all living things, in processes such as the production of ATP, DNA, and RNA; fungi may store this element as a reserve substance in the form of polyphosphates within cellular vacuoles (Carlile *et al.,* 2001; Hernández *et al.,* 2011). Although in the control treatments the quantity of P in solution was 12.5 times smaller with respect to the inoculated treatments, the results indicate that there was dissolution of PR, although these results were statistically insignificant with respect to experiment time.

The pH in the media decreased during the time of evaluation of P solubilization kinetics (Tables 2 and 3). This decrease in treatments in which TRIC7, TRIC11, TRIC13, and TRIC48 were evaluated, was an effect of the phosphodissolvent activity of the fungi (Gómez-Guiñan and Zabala, 2001; Pradham and Sukla, 2005; Chun-Chao *et al.,* 2007). Hernández *et al. (*2011) also reported that one of the principal mechanisms employed by fungi to dissolve phosphatic complexes is the production of organic acids. There were significant differences between control treatments and inoculates with respect to bioacidulation and phosphate concentration in solution.

The above coincides with other studies in which it was reported that when pH was decreased (5.5 to 2.4), the concentration of soluble P was augmented (0.32 to 110 mg L^{-1}) (Thomas *et al.,* 1985; Pandey *et al.,* 2008; Xiao *et al.,* 2009; Hernández *et al.,* 2011; Zúñiga-Silgado, 2016).

The favorable interaction between bioacidulation and biosolubilization depends on diverse factors, among those that stand out are the solubilization capacity of the organism, the type of organic acid produced which forms organic mineral complexes and reduces the pH of the medium as a result of metabolic activity (Wakelin *et al.,* 2004; Scervino *et al.,* 2010; Osorno, 2013; Osorno and Osorio, 2014), as well as the type of phosphatic compound, the labile carbon source, and the nitrogen source applied to the system (Barroso and Nahas, 2005; Pradham and Sukla, 2005; Souchie *et al.,* 2006; Hernández *et al.,* 2011).

The higher the capacity of strains to sporulate, form

UFC, and generate biomass in MM-HA medium (with $Ca_{10}(PO_4)_6(OH)_2$)) with respect to modified PVK medium (with $FePO_4 \cdot H_2O$) may be related to the degree of solubility of both chemical compounds (Table 4). The first has a higher solubility (2.21 x 10^{-4} g L^{-1}) than $FePO_4 \cdot H_2O$ (1.78 x 10^{-9} g L^{-1}), so it solubilizes more easily, generating higher concentrations of soluble P, which may have permitted a better morphophysiological response (Barroso and Nahas, 2005; Hernández *et al.*, 2011). Similarly, the higher sporulation, formation of UFC, and production of biomass obtained in MBSP may have been due to the higher presence of free ions of Ca^{+2} in the medium, which agrees with that described by Mosley *et al.* (1989), Shaw and Hoch (2007), Kozlova *et al.* (2010), and Hernández *et al.* (2011).

Experiment 3: Selection of IAA producing Trichoderma strains

In the IAA detection test, fourteen individual *Trichoderma* spp., strains showed a change to a red-pink color, indicating the presence of IAA. According to results reported by Salkowski (1889), Bric *et al.* (1991), Glickmann and Dessaux (1995), and Anwar (2000), its detection in the medium permits the establishment of whether the microorganism of interest has the ability to synthesize IAA (a vegetal growth hormone) through a determined precursor (L-Trp), and thus, to evaluate the specificity found in the Salkowski calorimetric reaction for the detection of indole compounds. In the calorimetric test, it was shown that theTRIC11, TRIC13, and TRIC48 strains generated a change in coloration over time in the absence of L- Tryptophan.

This color change resulted as a consequence of an oxidative reaction of sulfuric acid in which, through a transamination, an amine group was substituted for a chloride ion that came from ferric chloride (Bric *et al.,* 1991; Ahmad *et al.,* 2005). Based on the above, the TRIC7, TRIC11, TRIC13, and TRIC 48 strains showed a minimal production of IAA compared to the increase in the hormone obtained as a function of time and the concentration of L-Tryptophan. This behavior agrees with that reported by Celis and Gallardo (2007).

Just as Gravel *et al.* (2007), in an aseptic environment, demonstrated that a strain of *T. vatroviride* had the ability to synthesize IAA *in vitro* through the induction of different precursors such as L-Trp, tryptamide, and tryptofol (200 μg mL^{-1}) in culture media, showing that seedling roots of tomato grew in the presence of variable concentrations (0-10 μg mL^{-1}); it is presumed that the concentrations of IAA obtained in some strains of *Trichoderma* spp., evaluated in this investigation may be sufficient to have a direct influence on phytostimulation if it is kept in mind that regulators of vegetal growth act in small concentrations for this function. Based on the above, it is pertinent to advance in the development and field evaluation of a multifunctional bioproduct as a biocontrol and promoter of growth and vegetal development. The favorable attributes of strains of the antagonist *Trichoderma* have made the elaboration of biological products that have environmentally friendly characteristics possible. However, it is important to note that their success as products is assisted by a precise selection of strains as much from a physiological point of view as from a use object attribute and for a strict system of quality for their production.

The versatility, the range of biological mechanisms, and the ecological plasticity that the strains of *Trichoderma* evaluated in this study possess make them promising as bioinputs in agrobiotechnology.

The presence and promotive activity of vegetal growth in phosphate solubilizing fungi is summarily important, as it offers plants the possibility to achieve better adaptation in ecosystems in which phosphorous is a limiting resource. In this way, phosphodissolvent strains will simultaneously mediate the contribution of assimilable forms of phosphorous that favor efficiency in nutrient uptake and the achievement of longer length and proliferation of roots as a result of the action of IAA produced by these microorganisms. Various phytohormones, among them IAA, have important

roles as signal molecules that are mediators in the establishment of symbioses such as micorrhization and the association between diazotrophs and legumes (Hirsch *et al.,* 1997) which may also be considered to be a beneficial indirect effect of the production of IAA by the microorganisms isolated in this study.

Due to the above, it can be inferred that the presence of strains of *Trichoderm*a spp., that produce IAA and solubilize phosphates associated with plants may trigger similar effects and be utilized as fungal inoculants in agricultural systems (Whitelaw, 1999; Cepeda and Gamboa, 2001; Vera, 1999; Rodríguez and Rubiano, 2002; Useche, 2003). A value added to the use of these microorganisms in agriculture is their potential to also act as biological controls, which has been reported in several different studies (Torres-Rubio *et al*., 2000; Altamore *et al.,* 1999; Rodríguez and Fraga, 1999; Kloepper, 1993).

Conclusion

This study permitted the standardization of three distinct techniques for the selection of fungal morphotypes with phosphodissolvent and IAA production capacities from the rhizoshpere and rhizoplane of beans and corn. Appropriate isolation permits the selection of phosphodissolvent and IAA production fungi *in vitro,* and later, an evaluation that includes plant response to inoculation with these microorganisms (*e.g. in situ* and *ex situ).*

The microorganism promoters of vegetal growth act through the summation of different mechanisms that operate simultaneously or in succession, and the sum of the different activities when they are induced under favorable environmental conditions result in the stimulation of plant growth. The association of plants with microorganisms that act through various mechanisms in an integrated manner, such as phosphate solubilizers and producers of bioactive substances such as IAA. The above will permit advancement in the study of these organisms as potential inoculation tools in soils as important mechanism of adaptation to the ecological success of both plants and microorganisms which in turn represents a valuable genebank for agro-ecological and agrobiotechnological applications.

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