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Enhancing ^{15}N-uptake in maize (*Zea mays* L.) by native *Trichoderma* spp. strains in Central Mexico

Vera-Núñez, J. A.[1], Luna-Martínez, F.[1], Barcos-Arias, M. S.[2], Ávila-Miranda, M. E.[3], Grageda-Cabrera, O. A.[4] and Peña-Cabriales, J. J.[1*]

[1]Centro de Investigación y de Estudios Avanzados (Cinvestav)-IPN, Unidad Irapuato, km 9.6 Libramiento Norte, Carretera Irapuato-León, 36824 Irapuato, Guanajuato, México.
[2]Escuela Superior Politécnica del Litoral (ESPOL), Facultad de Ciencias de la Vida, Centro de Investigaciones Biotecnológicas del Ecuador, *Campus* Gustavo Galindo, km 30.5 Vía Perimetral, EC090112 Guayaquil, Ecuador.
[3]Instituto Tecnológico de Tlajomulco, km 10 Carretera Tlajomulco-San Miguel Cuyutlán, 46640 Tlajomulco de Zúñiga, Jalisco, México.
[4]Campo Experimental Bajío (CEBAJ), Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), km 6.5 Carretera Celaya-San Miguel de Allende, 38110 Celaya, Guanajuato, México.

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A study was undertaken to evaluate the role of *Trichoderma* strains on ^{15}N-uptake by maize under greenhouse and field conditions. The *Trichoderma* strains were isolated from different maize production systems in Guanajuato State, Central Mexico. A total of 39 *Trichoderma* isolates were obtained, 23 correspond to *Trichoderma harzianum* (Intra-Genic-Segment). Some colonized the endorhizosphere of maize better than others; however, *Trichoderma* diversity did not correlate with the maize production system (rainfall *vs.* irrigation conditions). In greenhouse conditions, biomass production and ^{15}N-uptake were equally variable; maize plants inoculated with selected *Trichoderma* species (high root colonization) and fertilized with 140 mg N kg^{-1} soil, showed similar increase in grain yield and ^{15}N-uptake *vs.* those fertilized with 280 mg N kg^{-1} soil. Biomass and ^{15}N-uptake directly correlated with the capacity of *Trichoderma* spp. to colonize the rhizosphere. Under field conditions, the N-fertilizer use efficiency was the highest when maize cv. P30G40 was inoculated with *T. harzianum* T35 at a N fertilization rate of 180 kg N ha^{-1} (78%). All measured parameters showed positive effects of inoculation under-scoring the feasibility of inoculants with these fungi based on *Trichoderma* to increase the N-fertilizer use efficiency applied to maize.

Key words: Biofertilizers, N-fertilizer use efficiency, ^{15}N isotope.

INTRODUCTION

Ever increasing populations present two important agricultural challenges; one is to enhance food security while ensuring sustainability, the other to conserve natural and biological resources. Sustainable

*Corresponding author. E-mail: jpena@ira.cinvestav.mx. Tel: +52 462 6239642. Fax: +52 462 6245896.

intensification of agricultural production thus demands an integrated approach to develop novel land, soil, water and plant resources. In view of the great genetic diversity and prime importance of maize in Mexico, a network of research institutions is developing and applying this integrated approach to maize agro-ecosystems (SAGARPA-CIMMYT, 2012). One of the present contributions has been to use *Trichoderma* species to enhance N uptake of local maize cultivars in Central Mexico.

Fungal species of the genus *Trichoderma* are ubiquitous inhabitants of the rhizosphere of plants. As some species of *Trichoderma* are important agents in the control of soil-borne pathogens of plants they have been extensively characterized (Mendoza-Mendoza et al., 2018).

Soils amended with *Trichoderma harzianum* displayed, not only a reduction in disease severity but also enhanced growth of the plants (Santiago et al., 2013). Several mechanisms by which *Trichoderma* spp. influence plant growth and development have been reported (Harman et al., 2004). Such mechanisms include the production of growth hormones (Contreras-Cornejo et al., 2009), solubilization of soil micronutrients (Ying-Tzu et al., 2018) as well as increased uptake and translocation of less-available nutrients (Zhao et al., 2014). *Trichoderma* enhanced uptake of P and N is of key importance in assessing agricultural production (Vera-Núñez et al., 2006). Akladious and Mohamed (2014) showed that inoculation of maize plants with *T. harzianum* T22 increased growth, chlorophyll content, starch content, nucleic acids content, total protein content and phytohormone content. Similarly, increases in Fe and P concentrations were observed in inoculated plants. Nevertheless, the mechanisms by which *T. harzianum* solubilize nutrients and stimulate their uptake are yet to be established. Therefore, the aim of this study was to confirm if colonization of the maize rhizosphere by *Trichoderma* spp. contributes to direct N-uptake. Thus, in order to evaluate the role of *Trichoderma* in maize cultivation, (1) native *Trichoderma* spp. were isolated and characterized from maize fields in the state of Guanajuato, Central Mexico, and (2) the effect of inoculation was assessed with selected isolates on biomass production and ^{15}N-uptake by maize.

MATERIALS AND METHODS

Isolation and characterization of *Trichoderma* strains

Three main sites were selected along a transect of approximately 250 km in the state of Guanajuato, Central Mexico. The sites in the following municipalities included: (1) Northern zone: Dolores and san Felipe; (2) Central zone: Irapuato; and (3) Southern zone: Penjamo. At each site, nine plants plus rhizosphere (30 cm diameter and 50 cm depth) at preflowering stage (approximately 60 days after sowing, DAS) from two to three maize fields (each 1 ha) were sampled using a spade sterilized with ethanol and flamed. Samples were taken from plots cultivated in the traditional manner (rainfed) as well as under intensive irrigation. The samples were stored at 4°C before use.

The colonization of maize roots by fungi was assessed using the methods of Phillips and Hayman (1970). In addition, 10 root pieces of 1 cm were placed equidistant in a Petri dish on *Trichoderma* selective media (TSM) [g L^{-1}]: 0.2 $MgSO_4 \cdot 7H_2O$, 0.9 K_2HPO_4, 0.15 KCl, 1.0 HN_4NO_3, 3.0 D (+) glucose, 0.25 chloramphenicol, 0.20 pentachloro nitrobenzene, 0.04 quintozene, 0.15 rose Bengal, and 15.0 agar at pH 6.0 (Elad and Chet, 1983). Three independent replicates were incubated for seven days at 28°C. Fungal colonies showing typical macroscopic *Trichoderma* characteristics were pre-selected and microscopic observations made to confirm the genus and the presence of typical phyalides (Barnett and Hunter, 1987). The proportion of root segments with which *Trichoderma* colonies was associated and recorded as the percentage of root colonization. Selected *Trichoderma* isolates were purified by culturing spores on TSM media giving isogenic strains that were used for mycelia compatibility and molecular characterization studies.

Compatibility among *Trichoderma* mycelia

The *Trichoderma* spp. isolates were evaluated for mycelia compatibility by direct confrontation in Petri dishes containing potato-dextrose-agar media (PDA). Five circular pieces of agar, 0.4 cm diameter, colonized with fresh mycelia of each *Trichoderma* spp. isolate were placed equidistant in Petri dishes with PDA media. All possible combinations were tested. Pairing was evaluated at 5 to 7 days after inoculation. Reactions in the contact area of mycelia were scored as follows: if two colonies grew together without inhibition, they were grouped in the same compatibility group. In contrast, if mycelia-free areas were found between colonies, this was recorded as incompatibility (Ávila-Miranda et al., 2006).

Molecular characterization of *Trichoderma* isolates

Purified isolates of *Trichoderma* spp. were cultured in potato-dextrose-broth media (PDB) at pH 6.0 to obtain mycelia for DNA extraction (Reader and Broda, 1985). Grouping of isolates by amplified fragment length polymorphism (AFLP) procedure (Vos et al., 1995) based on the IRDye Fluorescent AFLP Kit for Large Plant Genome Analysis (LI-COR Biosciences, 4647 Superior St., Lincoln, NE, USA) was followed with some modifications. A sample of 100 ng of genomic DNA was digested with *Eco*RI (5'-CTCGTAGACTGCGTACC/CTGACGCATGGTTAA-5') and *Mse*I (5'-GACGATGAGTCCTGAG/TACTCAGGACTCAT-5') and then adapters were ligated to the fragments following the manufacturer's instructions. Pre-amplification reactions (20 cycles: 94°C/30 s, 56°C/60 s and 72°C/60 s) used standard *Eco*RI+A and *Mse*I+G primers while selective reactions were amplified with the *Mse*I+GG/*Eco*RI+AT, *Mse*I+GG/*Eco*RI+AA, *Mse*I+GG/*Eco*RI+AG and *Mse*I+GG/*Eco*RI+AC primer combinations (12 cycles: 94°C/30 s, 65°C/30 s -minus 0.7°C per cycle- and 72°C/60 s). *Mse*I+GG primer was synthesized by SIGMA GENOSYS (Woodlands, TX, USA) and IRDyeTM-700 and IRDyeTM-800 labelled *Eco*RI+2 primers were purchased from LI-COR Biosciences. The AFLP products were separated on a LI-COR DNA analyser, Mod. 4200, viewed, scored and converted into numerical data files using SAGA^{MX} AFLP Quantar software. The XLS data were converted as present (1) or absent (0) and assembled into a raw data matrix. A square symmetric matrix of genetic distance was obtained using the Nei and Li (1979) coefficient. Dendograms were then generated by the UPGMA-un-weighted pair group method using arithmetic averages (Sneath and Sokal, 1973) and NTSYSpc 2 software

Table 1. Chemical characteristics of the experimental soil (Vertisoil).

Property	Content
Soil reaction	
pH (1:2 water)	8.2
Electrical conductivity (dS m^{-1})	0.6
Fertility (mg kg^{-1})	
N-inorganic	2
P-Bray	21
K	165
Ca	3163
Mg	238
Na	44
Fe	8
Zn	3
Mn	4
Cu	7
Organic matter (%)	0.6

(Applied Biostatics Inc.). Bootstrap analysis was based on 1,000 replications. A *T. harzianum* strain C4 donated by Dr. Alfredo Herrera from "Laboratorio de Expresión Génica de Hongos", Cinvestav-IPN, Langebio, was used as the reference.

Species identification was based on polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS1 and ITS2) region of the nuclear ribosomal rRNA gene cluster (White et al., 1990). The products obtained from each isolate were purified, sequenced and compared with *Trichoderma*-type sequences deposited in the NCBI Genbank databases.

Preparation of *Trichoderma* inoculum

Inoculants were prepared according to Ávila-Miranda et al. (2006). *T. harzianum* strains were plated out on PDA medium, incubated for 5 days at room temperature (24 ± 4°C) with a 12 h photoperiod. Conidia were harvested by pipetting 10 mL of sterile distilled water over the mycelial growth, which was rubbed with a sterile aluminium bar to separate the conidia. The suspensions were transferred into 100 mL sterile glass containers with 50 mL of sterile distilled water. Conidia concentrations were determined in 50 µL aliquots using a Neubauer chamber (Hausser Scientific, Horsham, Pasadena, CA, USA). The carrier for inoculum formulation consisted of a 1:1 v/v mixture of wheat bran and peat moss (Sunshines, Sun Gro Horticulture, Bellevue, WA, USA). The substrate was sterilized three times in an autoclave (1 h each time) over three consecutive days at 121°C, to eliminate those microorganisms that germinate during the period of cooling of the substrate. Approximately 2×10^4 conidia g^{-1} of substrate were applied and adjusted to 50% humidity on a dry weight basis. The substrate was incubated at room temperature (24 ± 3°C) for 10 days.

Inoculation with *Trichoderma* isolates under greenhouse conditions

Two seeds of maize cv. VS-486 were sown at 2 cm depth in pots containing 12 kg of an alkaline Vertisol from Irapuato, Guanajuato State, Central Mexico, which has been practised; the cereal-cereal rotation, wheat or barley is sown in autumn-winter and maize or sorghum in spring-summer, and usually deficient in N due, generally, to low levels of soil organic matter (Grageda-Cabrera et al., 2011). Selected characteristics of the experimental soil are shown in Table 1 (NOM-021-SEMARNAT-2000).

Six *Trichoderma* isolates from each site and crop production system with a high capacity to colonise roots and grouped according to genetic similarities were evaluated: (1) *Trichoderma asperellum* T7, (2) *T. asperellum* T12, (3) *T. harzianum* T16, (4) *T. harzianum* T28, (5) *T. harzianum* T35, and (6) *T. harzianum* T44. Each pot was inoculated with 6 g of inoculum containing approximately 2×10^4 conidia g^{-1}. The experiment was conducted at three levels of N-fertilizer: (1) 140, (2) 280 and (3) 336 mg N kg^{-1} soil. The applied N rates were equivalent to 50, 100 and 120% of the basal N-fertilization rate used for maize. Controls included: inoculated and non-fertilized (*Trichoderma* strains alone), uninoculated and fertilized and a zero control (uninoculated and non-fertilized). One week after germination, only the most vigorous maize seedling was kept.

A completely randomized experimental design with five replicates was used. Derived from other studies that showed a high N-fertilizer use efficiency (Grageda-Cabrera et al., 2011), ammonium sulphate (^{15}N-labelled fertilizer) was applied in two equal split application rates: (1) 50% N at sowing and (2) 50% N at 35 DAS. All pots were fertilized with 80 mg P_2O_5 kg^{-1} as $Ca(H_2PO_4)_2$ and 40 mg K_2O kg^{-1} as KCl at sowing. ^{15}N-ammonium sulphate containing 1% atom ^{15}N excess was applied to pots in the same way as the commercial fertilizer. Pots were kept clean by manual weeding once a week and irrigated approximately every 2 to 3 days with 450 mL distilled water pot^{-1}. At physiological maturity (about 120 DAS), 1 plant $replicate^{-1}$ was harvested, separated into straw (leaves + stems), roots and grain, which were weighed. Root colonization was assessed as described earlier.

Inoculation with "elite" *Trichoderma* strains under field conditions

A field experiment was laid down on a Vertisoil at INIFAP, Celaya, Mexico (20°44´ N, 101°19´ W). Using two main maize cultivars

sown in the zone: (1) P30G40 variety (Pioneer) and (2) H469C hybrid (INIFAP) were planted (1×10^5 plants ha^{-1} in six rows of 1 m wide and 6 m long $treatment^{-1}$). Two N-fertilizer rates were applied: (1) 180 and (2) 240 kg N ha^{-1} as ammonium sulfate enriched with 1% atoms ^{15}N excess was applied ^{15}N isotopic microplot (1 m wide and 1 m long) plus a supplemental fertilization of 80 kg P_2O_5 ha^{-1} as $Ca(H_2PO_4)_2$ and 40 kg K_2O ha^{-1} as KCl. Two "elite" *Trichoderma* strains were evaluated*:* (1) *T. harzianum* C4 and (2) *T. harzianum* T35 applied at a dose of 20 g inoculums m^{-2} (containing 2×10^4 conidia g^{-1} substrate) prepared according to the procedure described earlier. An experimental design of 2 maize cultivars × 2 N-fertilizer rates × 2 *Trichoderma* inoculants was used in randomized blocks with five replicates. A no-inoculated control treatment was included. The plants were irrigated using a drip system along a surface streak. At physiological maturity (about 120 DAS), five plants $treatment^{-1}$ from ^{15}N isotopic microplot were harvested to assess $^{15}N/^{14}N$ ratio and fresh straw (leaves + stems). In addition, grain yield was determined in four rows of 4 m long $treatment^{-1}$.

Sampling and ^{15}N analytical methods

Straw and grain were dried at 70°C for 72 h and total-N (tN) was determined using the micro-Kjeldhal procedure and $^{15}N/^{14}N$ ratio following Rittenberg oxidation with sodium hypobromite (Mulvaney, 1993) and an optical emission spectrometer (Axmann et al., 1990). The amount of ^{15}N in plant derived from fertilizer (Ndff) was calculated using the following equations (Zapata, 2001):

$$\text{tN yield (kg N ha}^{-1}) = \text{Dry matter yield (kg ha}^{-1}) \times [\text{tN (\%)} / 100] \quad (1)$$

$$\text{Ndff (\%)} = {}^{15}\text{N in plant (\% atoms excess)} / {}^{15}\text{N in fertilizer (\% atoms excess)} \times 100 \quad (2)$$

where ^{15}N % atoms excess (a.e.) fertilizer applied = 1% ^{15}N a.e.

$$\text{N-fertilizer yield (kg N ha}^{-1}) = \text{tN yield (kg N ha}^{-1}) \times [\text{Nddf (\%)} / 100] \quad (3)$$

$$\text{N-fertilizer use efficiency (\%)} = [\text{N-fertilizer yield (kg N ha}^{-1}) / \text{N-rate (kg N ha}^{-1})] \times 100 \quad (4)$$

Statistical analyses

Analyses of variance and the differences between mean values were determined at $p \leq 0.01$ (greenhouse conditions) and $p \leq 0.05$ (field conditions) by the Honest Significant Difference Tukey's Test and Least Significant Difference Test, respectively using SAS system version 6.0 software (SAS Institute, 1996).

RESULTS AND DISCUSSION

Isolation and characterization of *Trichoderma*

All maize plants sampled were colonized by the *Trichoderma* spp. (Figure 1). Nevertheless, irrigated plants were more extensively colonized (46%) than rain-fed plants (32%). A total of 101 morphologically distinguishable *Trichoderma* isolates were obtained: 52% of the isolates from the irrigated systems and 48% rain-fed conditions (Table 2). Confrontation test confirmed that 65 of the isolates were equivalent to 39 monosporic lines which clustered in three groups (Figure 1). There was no correlation between AFLP-DNA marker groups and the origin of the isolates. Amplification of ITS enabled allocation of 59% of the isolates to *T. harzianum*, 35% to *T. asperellum*, 3% to *Trichoderma inhamatun* and 3% to *Trichoderma virens*. The Penjamo site furnished 93% of the *T. asperellum* isolates but no *T. harzianum.* All *T. harzianum* strains were isolated from the Irapuato and Dolores & san Felipe sites. Low numbers of *T. asperellum*, *T. inhamatum* and *T. virens* were present in Dolores & san Felipe (Figure 1 and Table 2). AFLP markers and ITS sequences gave similar groupings and clearly separated the *T. asperellum* isolates from the rest.

Responses to inoculation with *Trichoderma* isolates under greenhouse conditions

For reasons that are not known, inoculation with *T. harzianum* isolate T16 produced highly variable responses and for this reason, these data were not taken into consideration. Yet, significant statistical differences in root colonization due to inoculation were found (Table 3). Five *Trichoderma* strains more efficiently colonized the roots (77%) than the standard inoculum (60%). However, N fertilization had no significant effect on colonization. In other words, the present results show positive interactions between inoculations with *Trichoderma* without N or at low rate of N fertilizer, that is, 140 mg N kg^{-1} - (equivalent to 50% of N-rate used as base for maize).

Amelioration of straw and root dry weight was the greatest in treatments using the control inoculum without added fertilizer (Table 4) and produced a relatively high amount of biomass (23.8 g $plant^{-1}$). The highest grain yield production (25.8 g $plant^{-1}$) was found for the treatment maize inoculated with *T. harzianum* T35 at 140 mg N kg^{-1} soil. Total biomass production increased as a function of *Trichoderma* strain. *T. asperellum* T7 produced the highest biomass dry weight (103 and 111 g $plant^{-1}$) at 240 and 336 mg N kg^{-1} soil, respectively. Irrespective of inoculation, addition of 336 mg N kg^{-1} stimulated biomass production.

Significant differences ($p \leq 0.01$) were found in the total-N yield following treatment with *Trichoderma* spp. as compared to the controls (Table 5). Except for the treatments, *T. asperellum* T12 and *T. asperellum* T7 at 336 mg N kg^{-1}, total-N yield increased in direct proportion to higher rates of N-fertilization. Inoculation with *T. harzianum* T35 at 336 mg N kg^{-1} produced the highest total N-yields (620 mg N $plant^{-1}$), of which 85 mg N $plant^{-1}$ (14%) were derived from N-fertilizer equivalent to a N-fertilizer use efficiency of 25% (Table 5). In contrast and except for inoculation with *T. harzianum* T35, the N-fertilizer use efficiency decreased with increasing levels

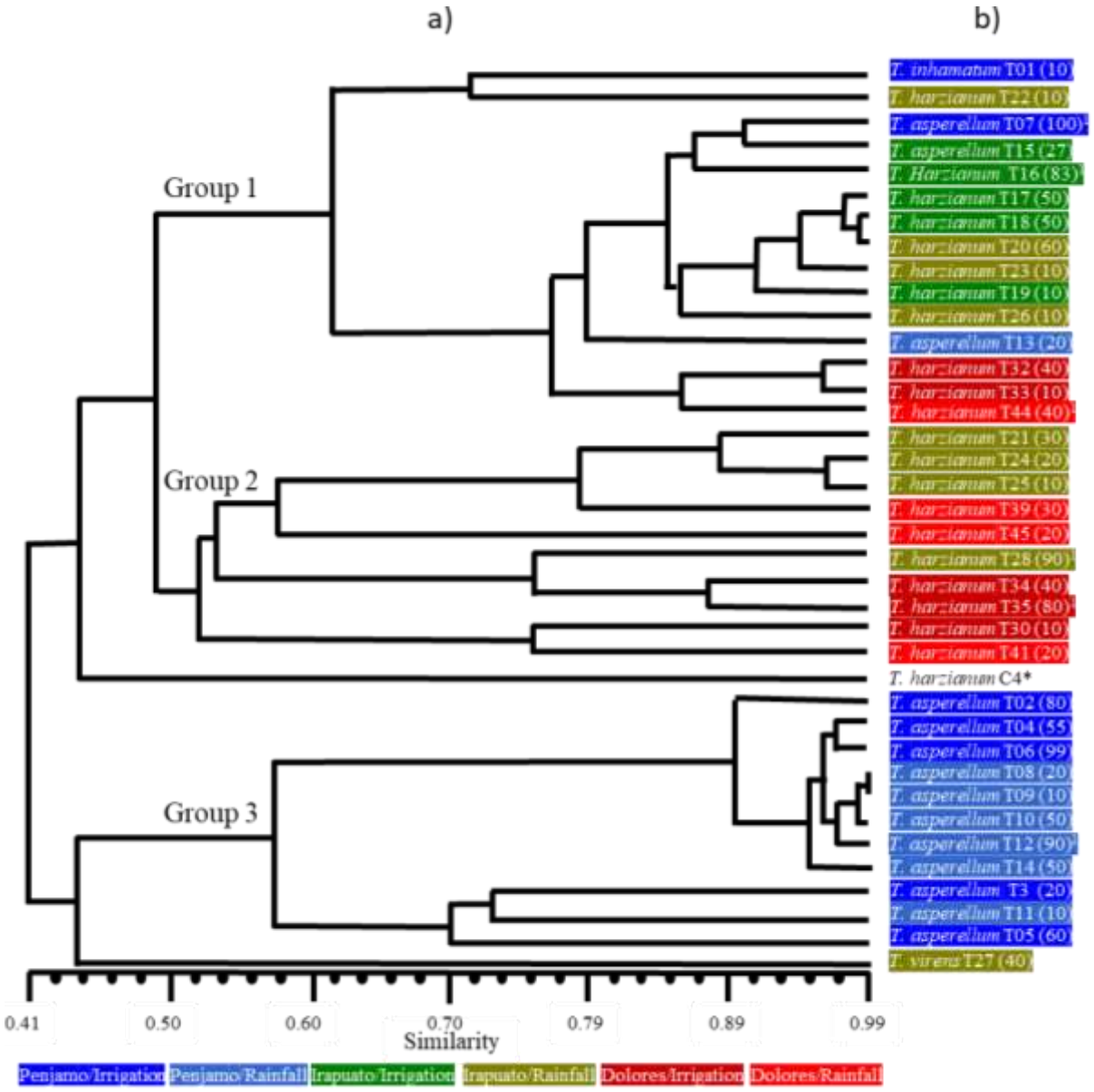

Figure 1. Dendogram of *Trichoderma* spp. isolates obtained from maize crop production systems in Guanajuato State, Central Mexico. (a) Similarity of *Trichoderma* isolates using 416 AFLP markers. Bootstrap values of 50% and more are indicated above nodes, (b) phylogenetic of *Trichoderma* spp. strains using 18s rRNA technique. Values in brackets represent percentage of colonization and, [1]selected isolates.

of N-fertilization. In all cases, N-fertilizer use efficiency of the inoculated and fertilized treatments was higher than that of the only fertilized treatment.

Responses to inoculation with "elite" *Trichoderma* strains under field conditions

Both maize cultivars studied showed positive responses in grain yield to higher rates of N-fertilizer (Table 6) and many of the differences were statistically significant ($p \leq 0.05$). The N-fertilizer use efficiency ranged from 38 to 78%. The highest N-fertilizer use efficiencies were observed when *T. harzianum* strain T35 was inoculated onto maize cultivar P30G40 at a N-rate fertilizer application of 180 kg N ha^{-1}. Surprisingly, the lowest N-fertilizer use efficiencies were obtained when the same isolate was inoculated on cultivar H468C and a higher N-rate 240 kg N ha^{-1} applied (50%). This implies that *T. harzianum* isolates are more efficient at gathering nutrients for the plants when soil N-levels were lower.

Plant rhizosphere is an important soil environment for

Table 2. Isolates of *Trichoderma* spp. obtained from maize production systems in Guanajuato state, Central Mexico.

System		Isolates[1]		
		Penjamo	Irapuato	Dolores & san Felipe
Irrigation	Total	23	16	13
	Confrontations	16	7	10
	Monosporics	7	5	6
Rainfall	Total	13	17	19
	Confrontations	8	10	14
	Monosporics	7	9	5

[1]At preflowering stage (approximately 60 DAS).

Table 3. Root colonization of maize plants inoculated with *Trichoderma* spp. strains at different N-fertilization rates under greenhouse conditions.

Strain	N-rate (mg kg^{-1})[1]				Average
	0	140	280	336	
	Colonization (%)				
T. asperellum T7	88±6	90±5	77±13	73±7	82
T. asperellum T12	85±12	81±18	71±18	75±8	79
T. harzianum T28	86±13	90±16	76±15	71±8	81
T. harzianum T35	76±13	78±16	67±15	67±8	72
T. harzianum T44	67±13	84±12	69±14	59±4	70
Control	46±9	54±8	70±17	70±7	60
Average	80	85	72	69	-
CV (%)[2]			17		
Tukey ($p \leq 0.01$)[3]:					
Strain (S)			12		
N-rate (N)			NS[4]		
S×N			NS		

[1]Average values of five replicates ± standard deviation; [2]Coefficient variation; [3]Honest Significative Difference Test Tukey's ($p \leq 0.01$); [4]No significant difference ($p \leq 0.01$).

plant-microorganism interactions (Albareda et al., 2006) and affect plant nutrient status and plant resistance to biotic or abiotic stress (Neubauer et al., 2013) as well as promote nutrients solubilization increasing its bioavailability for plants (Oliveira et al., 2009).

Trichoderma spp. are well-studied filamentous fungi commonly found in soil communities. *Trichoderma* life is based on three major nutritional modes: saprotrophy, mycotrophy, and dependence upon plant-derived sugars (Druzhinina *et al*., 2011). *Trichoderma* spp. and similar saprophytic fungi are well adapted to N-deficient environments (Frey et al., 2000). Adaptations include re-assimilation of N from degenerated hyphae, targeted hyphal growth towards locally enriched nutrient sites (Johansen, 1999) and translocation of cytoplasm from mycelium to hyphal apices in N-depleted regions (Frey et al., 2000). Fungal hyphae may also translocate mineral N to N-poor substrata when the absolute amount of N in a decomposing substrate increases during the early stages of decomposition. Furthermore, lateral and upward movement of ^{15}N-labeled inorganic-N from mineral soil to the decomposing litter has been demonstrated (Frey et al., 2000).

Another niche occupied by *Trichoderma* spp. is the rhizosphere, which attracts them due to the presence of root-derived sugar and exudates. *Trichoderma* strains able to colonize and grow within the root systems of plants are called “rhizosphere competent strains” and they are particularly suitable as inoculants due to their long persistence on the roots and beneficial properties such as growth promotion and biocontrol of plant pathogens (Vargas et al., 2009; Vieira et al., 2018).

Table 4. Biomass production by maize plants inoculated with *Trichoderma* spp. strains at different N-fertilization rates under greenhouse conditions.

Strain	N-rate (mg kg^{-1})	Dry weight[1]			
		Root	Straw	Grain	Total[2]
		(g plant^{-1})			
T. asperellum T7	0	9.3±2.3	7.7±1.4	1.2±0.0	24.1±3.3
	140	22.4±0.0	14.6±0.5	21.2±3.3	71.6±3.8
	280	35.5±8.0	31.6±8.2	19.5±7.9	102.9±7.4
	336	42.8±2.7	22.7±2.5	20.0±1.6	110.5±4.9
T. asperellum T12	0	10.8±4.2	13.6±4.8	0.5±0.0	38.1±13.3
	140	15.4±7.1	14.9±4.9	19.0±0.2	64.8±19.0
	280	19.2±6.4	17.0±2.0	17.5±2.7	65.3±12.7
	336	40.8±1.3	18.3±0.6	14.2±5.8	89.8±0.5
T. harzianum T28	0	9.0±2.0	7.2±1.4	6.9±4.6	25.7±7.3
	140	33.0±9.7	18.6±6.0	21.3±3.8	90.0±17.8
	280	31.4±2.9	15.7±2.5	13.0±7.4	76.3±7.0
	336	37.2±1.0	22.7±1.8	7.4±3.1	82.8±13.3
T. harzianum T35	0	11.4±1.6	11.4±2.4	4.4±0.0	32.6±13.8
	140	29.0±6.4	14.4±2.1	25.8±0.0	76.1±18.4
	280	18.5±4.7	14.8±2.2	22.2±0.1	67.3±8.1
	336	24.6±3.7	16.4±1.1	21.7±1.8	77.8±4.0
T. harzianum T44	0	6.1±3.1	6.6±2.8	8.7±0.0	21.9±2.3
	140	38.8±8.3	21.9±8.5	16.7±6.8	93.7±21.4
	280	41.2±6.4	24.1±6.4	20.7±2.6	104.7±18.0
	336	35.3±1.4	25.2±6.2	13.1±0.4	91.5±9.2
Control	0	23.8±0.0	25.6±0.1	5.4±0.0	61.9±0.1
	140	28.6±7.1	15.6±1.3	21.6±2.7	75.6±4.7
	280	40.9±4.1	22.6±2.4	11.9±0.0	84.9±6.4
	336	38.4±6.9	22.2±0.5	0.0±0.0	81.5±3.3
CV (%)[3]	-	18.2	20.0	16.1	19.3
Tukey ($p \leq 0.01$)[4]:					
Strain (S)	-	1.1	4.3	2.9	10.7
N-rate (N)	-	0.9	3.5	2.4	9.6
S×N	-	2.2	8.7	5.9	23.5

[1]Average values of five replicates ± standard deviation; [2]Including bract dry weigh; [3]Coefficient variation; [4]Honest Significant Difference Test Tukey´s ($p \leq 0.01$).

Here, it was shown that the interaction between native *Trichoderma* and rhizosphere in commercial maize fields is efficient under both irrigated and rain-fed conditions. The present data also show that the fungal diversity associated with maize roots is restricted to four *Trichoderma* spp.: *T. asperellum*, *T. harzianum*, *T. inhamatum* and *T. virens.* In the region surrounding Penjamo site, only T. asperellum was found whereas T. *harzianum* predominated in fields of other locations.

Fungal species of the genus *Trichoderma*, being prevalent in soil, have been extensively used as biological control agents (Olson and Benson, 2007).

Table 5. Total N yield and N derived from fertilizer in maize plants inoculated with *Trichoderma* spp. strains at different N-fertilization rates under greenhouse conditions.

Strain	N-rate	N-yield[1]		
		Total	Fertilizer	Efficiency[2]
	mg kg^{-1}	mg plant^{-1}		%
T. asperellum T7	140	421.8±43.0	61.8±8.30	44.1
	280	485.7±77.8	72.0±23.8	25.8
	336	445.6±99.5	86.6±20.5	25.8
T. asperellum T12	140	418.4±52.8	66.0±10.4	47.1
	280	480.1±33.7	64.3±6.90	23.0
	336	362.8±98.1	65.0±20.7	19.3
T. harzianum T28	140	402.1±99.1	49.1±18.8	35.1
	280	511.0±95.4	95.2±19.8	34.0
	336	455.8±95.9	73.1±18.3	21.8
T. harzianum T35	140	576.5±12.8	96.6±3.00	69.0
	280	500.4±75.9	97.0±7.50	34.6
	336	620.1±59.7	84.6±13.0	25.2
T. harzianum T44	140	401.4±96.9	72.3±17.1	51.6
	280	532.6±26.9	97.0±28.4	34.6
	336	465.0±12.9	94.3±9.70	28.1
Control	140	402.9±59.6	55.8±15.9	39.9
	280	320.0±11.2	75.2±2.70	26.9
	336	132.5±26.4	26.5±7.10	7.9
CV (%)[3]	-	16.6	18.7	24.3
Tukey ($p \leq 0.01$)[4]:				
Strain (S)	-	70.3	6.6	6.5
N-rate (N)	-	55.6	3.9	5.3
SxN	-	141.1	7.8	12.9

[1]Average values of five replicates ± standard deviation; [2]N-15 isotopic method; [3]Coefficient variation; [4]Honest Significant Difference Test Tukey´s ($p \leq 0.01$).

Some strains also showed several plant growth-promoting traits including the capacity to solubilize nutrients, and to produce cellulases, chitinases, proteases, indol acetic acid, and siderophores (Ying-Tzu et al., 2018). However, subsequent events like oxidative, the synthesis of salicylic acid by the plants, and the secretion of elicitor-like proteins by *Trichoderma* spp. differentiate this fungus from pathogens (Mendoza-Mendoza et al., 2018). The results of tomato biomass and yield implied that *T. harzianum* strain SQR-T037 had the capacity to recognize and adhere to the plant roots (Chen et al., 2011). The benefits of *T. asperellum* strain T6 on plant growth under salt stress may be related to its capacity to chelate or solubilize and reduce Fe (Zhao et al., 2014). *T. asperellum* T34 is a recently commercialized strain which has been demonstrated to be an effective biocontrol agent able to increase the uptake of micronutrients by plants (Santiago et al., 2013). The mechanism of phytostimulation by *Trichoderma* spp. involves multilevel communication with root and shoot systems, as it releases into the rhizosphere auxins, small peptides, volatiles and other active metabolites, which promote root branching and nutrient uptake capacity, thereby boosting plant growth and yield (López-Bucio et al., 2015).

It is well known that crop productivity relies heavily on nitrogen (N) fertilization (Xu et al., 2012). *T. virens* GV41 increased N-use efficiency, and favored the uptake of native N present in soil. The positive effect of

Table 6. Grain yield and ^{15}N-fertilizer uptake by maize cultivars inoculated with *Trichoderma harzianum* "elite" strains at different N-fertilization rates under field conditions.

Treatment			Yield[1]			Efficiency[2]
Cultivar	N-rate	Strain	Grain	Total-N	N-Fertilizer	
	kg ha^{-1}		Mg ha^{-1}	kg ha^{-1}		(%)
P30G40	180	C4	14.2±0.5	272±14	109±7	61±4
		T35	15.2±0.8	311±18	140±15	78±8
		Control	12.8±0.9	239±23	103±6	57±3
	240	C4	13.6±0.7	246±15	105±3	44±1
		T35	13.5±1.4	239±11	98±5	41±2
		Control	14.6±1.6	256±13	152±8	64±3
H468C	180	C4	12.6±0.9	216±10	121±7	67±4
		T35	14.7±0.8	254±8	108±6	60±3
		Control	11.1±0.7	225±11	92±5	51±3
	240	C4	17.6±0.8	297±14	130±7	54±3
		T35	11.7±0.6	195±11	90±5	38±2
		Control	14.5±0.9	180±7	136±7	57±3
CV (%)[3]	-	-	6.2	4.7	3.8	4.3
LSD ($p \leq 0.05$)[4]:						
Cultivar (C)	-	-	8.19	163.88	24.30	13.29
N-rate (N)	-	-	4.01	85.36	30.95	65.22
Strain (S)	-	-	3.75	99.06	33.83	8.68
C×N	-	-	4.92	48.39	26.38	15.37
C×S	-	-	3.76	0.000	109.99	28.84
N×S	-	-	8.07	134.06	0.000	51.58
C×N×S	-	-	4.86	120.89	21.36	10.88

[1]Average values of five replicates ± standard deviation; [2]N-15 isotopic method; [3]Coefficient variation; [4]Least Significant Difference (LSD) Test ($p \leq 0.05$).

biostimulants on nutrient uptake and crop growth was species-dependent (Fiorentino et al., 2018). The results suggest that induction of increased or suppressed plant growth occurs through the direct effect of *T. harzianum* on root development, in combination with indirect mechanisms, such as mineral solubilization, including solubilization via acidification, redox, chelation and hydrolysis (Rui-Xia et al., 2015). The best plant growth responses (height, root length, leaf area, and root, stem and total dry weight) were achieved by inoculating conidia of either *T. tomentosum* (EMMFRS1C2) or *T. harzianum* (MichV6S3C2) in combination with tryptophan (Herrera-Jiménez et al., 2018). Expression and activity of H^+-ATP_{ase} suggesting that the proton-motive force generated by H^+-ATP_{ase} may be an accelerator of nutrient uptake and metabolism (Simkovic et al., 2015). Downregulation of defense genes and upregulation of C and N metabolism genes observed in the microarrays were accompanied by enhanced growth, and increased C and N (Domínguez et al., 2016).

Furthermore, *Trichoderma* spp., in association with plant roots, can trigger systemic resistance and improve plant nutrient uptake (Contreras-Cornejo et al., 2016). Nutrient deprivation or different nutrient sources are universal signals for conidiation, and light is a determinant for the utilization and/or uptake of specific C sources by *Trichoderma* spp. (Tisch and Schmoll, 2010).

N and P can directly act as signals that alter post-embryonic root development, modifying primary and lateral root growth and root hair formation and may be also involved in the success of plant-microbe associations (Giehl and Von Wirén, 2014). Modifications in root architecture in cucumber plants induced by *Trichoderma* strains overexpressing WT or qid74 gene increased plant biomass through an efficient use of N, P, K and micronutrients (Samolski et al., 2012). The root tips are able to sense the local and internal concentrations of nutrients to adjust growth and developmental processes, and to increase or decrease the exploratory capacity of the root system (Ruiz-Herrera et al., 2015).

Consequently, it would be expected that the root capacity to acquire macro and micronutrients boost in the presence of microbes via increased nutrient solubilization and/or transport (Krouk et al., 2010). The best biostimulation effects from the *Trichoderma* treatments were observed when crops are grown under low N availability (Fiorentino et al., 2018).

Similarly, *Trichoderma* as well as arbuscular mycorrhizal (AM) fungi can affect nutrient accumulation and alter nutrient ratios in plant tissues. Maize-*Trichoderma* interactions somehow stimulate root of the host plant and additional N-supplies increase root-development under sub-optimal conditions and would thus superficially resemble those of many plants with AM (Bowen and Rovira, 1999) and like the AM-plant interaction is of benefit to both partners. Larger root systems are better able to explore the surrounding soil for water and nutrients. Increased hyphal growth probably serves a similar function since it has been shown in AM that many compounds including those containing N are readily transferred from hyphal to plant cells (Bowen and Rovira, 1999). In cereals the contribution of associative and endo-phytic nitrogen fixation (BNF) is known and it appears to be highly variable, depending on the bacterial strain, the plant genotype, growth stage, and environmental conditions. In plant growth promoting rhizobacteria (PGPR), in particular *Azospirillum*, the production of phytohormones rather than BNF is considered to be a major factor for plant growth promotion. In maize, BNF expressed as nitrogen derived from air ranged from 12 to 33% (Montañez et al., 2009). However, the impact of associative BNF and plant-growth promotion is more marked in soils with poor fertility and it is inhibited by high N fertilization dose.

Evidence of maize-*Trichoderma* interaction were provided: (1) five of the 38 *Trichoderma* strains tested showed high root colonization capacities (≥80%); (2) an inverse relationship between root colonization and rate of N-fertilizer application was observed; (3) grain production was the highest when *Trichoderma* inoculants and N (140 mg N kg^{-1} soil) were added simultaneously; (4) total biomass production was increased by inoculation with *Trichoderma* spp.; (5) higher N-fertilizer uptake was obtained as a result of inoculation with *T. harzianum* T35 and *T. harzianum* T44 combined with the addition of external N (280 mg N kg^{-1}) and the recovery of ^{15}N was always higher in this combination than with fertilizer addition by itself and; (6) the capacity of *Trichoderma* isolates to colonize maize roots is directly correlated with the uptake of ^{15}N-fertilizer and therefore ^{15}N-fertilizer use efficiency.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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