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Indigenous arbuscular mycorrhiza and *Trichoderma* from systems with soybean predominance can improve tomato growth

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Abstract

In the last decades, there has been a tendency towards sustainable agriculture. Following this trend, edaphic fungi as Trichoderma and arbuscular mycorrhizal fungi (AMF) could increase plant growth contributing to diminish agricultural chemical supply. However, little information exists in current research regarding the possible effects of alternative practices to soybean monoculture systems on fungal groups, that could contribute to plant health and/or productivity. Thus, our objective was to assess changes in the abundance and diversity of indigenous AMF and Trichoderma from a long-term field experiment located in the Argentinean Pampas, in order to test the effect of alternative practices to soybean monoculture. The fungal ability to promote the growth of crops, such us tomato plant, was also tested. Soil samples were collected from a soybean monoculture system (Sb), a system including cover crop (Avena sativa) followed by soybean (CC/Sb) and a system including rotations (rot) of Soybean-Maize-Wheat crops CC/Sb-rot, M-rot and W-rot, respectively. Highest AMF and Trichoderma abundance was found at W-rot and M-rot systems, and highest AMF diversity was found at W-rot and CC systems. Furthermore, highest mycorrhizal colonization was found at CC/Sb and W-rot systems. Inoculated plants with single AMF consortium or with Trichoderma strains showed significant increases in comparison with the control. Dual inoculation increased tomato plant growth as compared to the control, and evidence of synergism was found by increases in shoot and root growth. Our results show that dual inoculation with indigenous AMF and Trichoderma from alternative crop systems to soybean monoculture could play an important role in tomato plant growth. This information could be useful to decrease production costs and environmental impacts.

Keywords: Inoculation, mycorrhizal colonization, Trichoderma, cover crops, crops rotation systems

Introduction

The global current need for food production has put pressure on increasing yields; consequently, agriculture has become more competitive and efficient (Cho *et al*., 2004). In Argentina, the main change in agricultural production has been the expansion of soybean cultivation as monoculture (Solbrig, 2005). This includes the permanent application of chemicals and little residue incorporation, which leads to a decrease in soil organic matter (SOM) content. This could affect the microbial biodiversity, increase soil degradation, and contribute to environmental deterioration (Marshall *et al*., 2011).

Abuscular mycorrhizal fungi (AMF), which belong to phylum Glomeromycota, form mutualistic associations with most terrestrial plants (Gupta *et al*., 2017). The AMF increase the growth of a wide variety of crops (Sharif *et al*. 2010) as well as the tolerance to biotic and abiotic stress (Sidhoum and Fortas, 2018); it also contributes to soil aggregates stability (Rillig *et al*., 2002). As result, in the last decades, the AMF have been highlighted as potential biofertilizers.

In soils, the plant-AMF system is in interaction with other microorganisms as *Trichoderma* fungi (Tiunov and Scheu, 2005). *Trichoderma* is widely distributed in different environments, especially in those with high SOM (Harman, 2006). Among other properties, *Trichoderma* can solubilize insoluble soil P compounds and has positive effects on plant growth and pest biocontrol. These properties could result from rapid growth and antagonistic action to plant root pathogens (Guigón-López *et al*., 2010). For this reason, *Trichoderma* is also considered a potential bioinoculant which enhances plant growth and health. Therefore, the combination of *Trichoderma* (P solubilizer) and AMF (P transporter)

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consortium could probably increase plant growth and also control fungal pests.

Positive interactions between *Trichoderma* harzianum and AMF were reported by Martínez-Medina *et al*. (2011) who registered a reduction of foliar disease produced by Fusarium fungus in melon (Cucumis melo). Due to *Trichoderma* antifungal properties, antagonism with AMF could be expected. Antagonistic effects of *Trichoderma* on AMF were found by Santander and Olave (2014) and were related to antifungal metabolite compounds released by *Trichoderma*. On the other hand, synergistic or neutral effects on potato plants were found by Ene and Alexandru (2008) with different *Trichoderma* strains (T. longibrachiatum, T. virens, and T. koningii) and AMF belonging to the Glomus and Gigaspora genus.

Currently, inoculation with fungi as biofertilizers is not used as a management practice in extensive crops. Cover crops (CC) have been considered as an alternative to increase SOM in soybean monoculture (Villamil *et al*., 2006). Avena sativa is a commonly used CC, because of the carbon residues it leaves in the soil (Duval, 2016); it also has numerous benefits, such as physical soil protection (Fernández *et al*., 2007). Another alternative to soybean monoculture that maximizes productivity per unit area by optimizing the use of resources and reducing pest are crop rotations including soybean with other crops (Kumar and Goh, 2000). However, little is known about how these practices could affect the soil microbial structure and or activity. In particular, it is interesting to know if some alternatives to soybean monoculture can affect microbial groups that could fulfill roles in plant health and/or productivity.

We hypothesize that alternative systems to soybean monoculture, such as CC or crop rotations modify soil microbial abundance and/or diversity. Therefore, there is a greater probability to find combinations of indigenous AMF-*Trichoderma* that could be considered as potential bioinoculants. The present study was performed to understand the effect of different soybean crop systems on the abundance and/or diversity of indigenous AMF and *Trichoderma* strains from the Argentinean Pampas and to select AMF-*Trichoderma* consortium with ability to increase plant growth. In addition, we aimed to advance our knowledge of the identity of selected strains of potential biotechnological interest.

Materials and Methods

Experimental site and soil samples collection

The study was carried out in a long-term field trial under no-tillage started in 2006 at the Instituto Nacional de Tecnología Agropecuaria (INTA) Research Station at Balcarce, Argentina (37° 45' S, 58° 18' W; 870 mm mean annual rainfall; 13.8 °C mean temperature; 138 m above sea level). The soil was a Typic Argiudoll (Mar del Plata series, USDA taxonomy), with less than 2% slope and loam texture at the surface layer (0-20 cm). The experimental design consisted of a randomized complete block design (RCBD) with three replications per treatment. Three treatments were evaluated: 1) soybean monoculture (Sb); 2) cover crop/soybean (CC/Sb); 3) crop rotation (rot) with the three phases of rotation in different plots (CC/Soybean-Maize-Wheat) with Wheat (W-rot), Maize (M-rot) and CC/Sb (CC/Sb-rot) in the year of study; that resulted in fifteen experimental units of 15 m x 5 m each.

The Sb system was maintained without weeds during the fallow period using glyphosate application at the beginning of spring. Soybean (Glycine max) was sown (0.35 m row spacing) using a plant density of 450,000 seeds ha^{-1} inoculated with *Bradyrhizobium japonicum*. At sowing, it was fertilized with 20 kg phosphorus ha^{-1} as triple superphosphate (0-46-0) and 15 kg sulfur (S) ha^{-1} as gypsum (16% S, 20% Ca (calcium), SO_4Ca $2H_2O$). The CC used in treatments CC/Sb and CC/Sb rot was oat (Avena sativa L.), using a density of 120 kg ha^{-1} (0.175 m row spacing). The CC was fertilized with N at tillering (90 kg N ha^{-1} as urea). Cover crop was killed just at flag leaf, with 3-4 L ha^{-1} of glyphosate (48% active principle). In the W-rot treatment, wheat (Triticum aestivum Buck Sy200) was sown using a density of 140 kg seeds ha^{-1} and fertilized with P (20 kg P ha^{-1} as triple superphosphate). N (150 kg N ha^{-1} as urea) and S (15 kg S ha^{-1} as gypsum) fertilization were applied at tillering. At wheat

Table 1: Chemical soil characteristics of different plots of field experiment. OM: Organic Matter; P-Bray: available soil phosphorus

Field plot	pH	OM (%)	P-Bray (mg kg^{-1})
Sb	5.69 (± 0.2)	5.12 (± 0.4)	33.37 (± 1.4)
CC/Sb	5.63 (± 0.8)	5.51 (± 0.8)	28.09 (± 1.2)
M-rot	5.25 (± 0.6)	5.54 (± 0.7)	26.01 (± 0.8)
W-rot	5.62 (± 0.5)	5.48 (± 0.9)	27.72 (± 1.1)
CC/Sb-rot	5.46 (± 0.3)	5.49 (± 0.5)	30.36 (± 0.9)

Values in parentheses corresponding to standard deviations (n = 3).

harvest, plots were sown with unfertilized soybean. In the M-rot treatment, maize crop (Zea mays, DK72-1-10 RR2) was sown using a density of 80,000 plants ha^{-1}(0,52 m row spacing) and fertilized with P (20 kg P ha^{-1} as ammonium phosphate (PO4 (NH4)3)). N fertilization was applied as urea at V6 stage (Ritchie and Hanway, 1982). Pests, weeds and diseases were traditionally controlled with chemical methods.

Fifteen composite soil samples, 10 sub-samples per plot, were separately collected in summer (January) by using a cylindrical sampler 5 cm in diameter, 0-15 cm depth. Subsamples were separately homogenized per treatment and replicates were stored in a refrigerator (4 ºC) until processed (two days later). Part of the soil samples were used for soil chemical parameters determination, as detailed in Table 1.

Genetic AMF-belonging glomerales diversity assessment

The genetic diversity of AMF was assessed with an emphasis on the most representative genera of AMF (*Glomus* and *Funneliformis,* belonging to the Glomerales order according to Redecker *et al*. (2013)) by a genotypic fingerprinting approach, using the polymerase chain reaction-Single Stranded Conformation Polymorphism (PCR-SSCP). Soil DNA extraction was performed separately for each field treatment by using the MoBio Power Soil DNA isolation kit, according to the manufacturer's instructions. A nested PCR was performed for amplifying a fragment of the large subunit of ribosomal DNA gene (28S) by a nested PCR. In the first PCR reaction, approximately 700 bp were amplified using the fungal specific primer pair LSU0061/LSU0599 (5'-AGCATATCAATAAGCGGAGGA-3′/ 5′-TGGTCCGTGTTTCAAGACG-3′). On the second PCR reaction, the products originating from the first PCR reaction (diluted 1:50) were used as template for amplifying an approximately 400 bp fragment, corresponding to the 28S rDNA gene of AMF belonging the *Glomus*/*Funneliformis* genera (*Funneliformis mosseae* =*Glomus mosseae*; *F. caledonium*= *Glomus caledonium* and *F. geosporum*= *Glomus geosporum*) (Van Tuinen *et al*., 1998). PCR products generated by LSUrk4f and LSUrk7r primer pairs were subsequently analyzed by SSCP using a Dcode Universal Mutation Detection System (Bio-Rad, USA). Five microliters of the PCR product were added to 3 µL of denaturing loading mixture (95% deionized formamide, 0,05% bromophenol blue, 0.05% xylene cyanol FF and EDTA 20 mM), denatured at 95ºC for 5 min, and immediately plunged into ice. Each PCR product was loaded into the 1 mm-thick gel 0.5X MDE® (Cambrex, Rockland ME, USA), 1X TBE (0.045 M Tris/borate, 1 mM EDTA) and run at 15°C, 8 W, 300 V for 4 h. PCR and SSCP conditions were performed as described by Kjøller and Rosendahl (2000) and by Rosendahl and Stukenbrock (2004). The SSCP gels were silver stained according to Benbouza *et al*. (2006), scanned and finally analyzed. The Shannon-Weaver (1949) diversity H' Index of Glomales was calculated (Van Elsas *et al*., 2012) based on the SSCP pattern as detailed in Eq. (1):

Eq (1) $$H' = -\sum_{i=1}^{S} Pi\ lnPi$$

Where Pi = Ni/N, Ni is the abundance of the ith ribotype, N is the total abundance of all ribotypes in the sample (lane of SSCP gels). The abundance of SSCP bands were indicated by the intensity of each SSCP band extracted by the Phoretix 1D PRO software (Nonlinear Dynamics, United Kingdom).

AMF native spores abundance from soybean crops

AMF spores were extracted from 50 g of each field soil treatment by wet sieving, decantation of 60% sucrose gradient centrifugation and collection of the spores and soil particles in 53 µM sieves as described by Sieverding (1991). Isolated spores were surface cleaned by sonication (Quantrex S200 Sweep Zone (TM) Technology L & R kit), and surface sterilized with a solution of 5 mg/L streptomycin sulphate and neomycin sulphate at 25 µL/sample three times for 5 minutes (according to a modification of the protocols by Covacevich and Consolo, 2014).

The total number of AMF spores (clean and healthy spores, in which internal nuclei were evident) in each sample was quantified under a binocular magnifying glass (6 X magnification) and referred to 100 g of soil. The spores were transferred to 1.5 mL eppendorf® tubes with 100 µL of sterile distilled water and were multiplied.

AMF spores multiplication in trap plant cultures

Isolated spores of each treatment were multiplied during 9 weeks in a growth chamber, with a mix (50% V/V) of 10 g of ryegrass and clover as trap plants, which were seeded in 1 kg pots. Pots were filled with a mixture of soil (from the field experiment fumigated with formaldehyde following Covacevich and Echeverría (2003): perlite: sand (autoclaved) 1:1:1 in vol. After 9 weeks roots were separated from the substrate, washed and stained with a trypan blue (0.05%) in distilled water-acid lactic-glycerol (1:1:1 in vol) solution according to the modification of Phillips and Hayman (1970) method, in which the phenol reagent was omitted. Arbuscular mycorrhizal colonization (AMC), as well as the frequency of root infection,

colonization intensity and arbuscule content, were quantified (Trouvelot *et al*., 1986).

Trichoderma native abundance from soybean crops

Trichoderma strains were isolated from 10 g of soil separately for each field treatment through the plate decimal dilution method on Agar-TSM (*Trichoderma* Selective Medium; Elad *et al*., 1981) and abundance (Unit Forming Colony/100 g soil) was quantified after growth (28 ± 0.5 °C for 7 - 10 days). Individual colonies were then separately transferred to Petri dishes with PDA (potato dextrose agar medium) and kept for 14 days at 25 °C, which allowed for the multiplication of one isolated conidia (pure culture).

Trichoderma molecular identification

Genomic DNA from *Trichoderma* isolates was extracted from 100 mg of mycelium from pure cultures based on Raeder and Broda (1985); DNA quality was checked on 1.5 % agarose gel stained with GelRed®.

Polymerase chain reaction (PCR) that allowed for the amplification of internal transcribed spacers (ITS) of ribosomal DNA (rDNA) region from each *Trichoderma* isolate was performed with primers ITS1 (5-TCCGTAGGTGAACCTGCGG-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3) (White *et al*., 1990) which includes the ITS1, the 5.8S rDNA gene and the ITS2 regions. PCR was conducted in a mixture containing 10 ng of genomic DNA, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each of the four dNTPs, 0.5 mM of each primer, and 1 unit of Taq polymerase (Promega WI, USA). Thermal cycling conditions involved an initial denaturation step at 94 °C for 2.5 min, followed by 40 cycles of 94 °C for 15 s, 48°C for 1 min and 72 °C for 1.5 min, and a final extension at 72 °C for 10 min. PCR products were separated by electrophoresis in a 1% agarose gel mixing with GelRed® (Genbiotech, UK) during 1 h at 120 V. The expected size and the quality of the amplicons were checked on 1% agarose gel. PCR amplification products were purified and sequenced by Macrogen (Korea) using the ITS4 primer.

Trichoderma and AMF inoculation: Effect on tomato plant growth

In a tomato pot experiment, *Trichoderma* and AMF strains selected by their diversity and/or proliferation ability were inoculated, both alone and in combination, to test their ability to promote plant growth. The experimental design was randomized in blocks with 6 treatments and four replicates as detailed: 1) Uninoculated (Control); 2) Inoculated with AMF consortium from the field plot W-rot (AMF W-rot); 3) inoculated with AMF consortium from the field plot CC/Sb (AMF CC/Sb); 4) inoculated with *Trichoderma* from the field plot W rot (Tch W-rot); 5) Inoculated with *Trichoderma* from the field plot CC (Tch CC/Sb); 5) inoculated with consortium with AMF and *Trichoderma* from the field plot W-rot (AMF+Tch W-rot); 6) inoculated with consortium with AMF and *Trichoderma* from the field plot CC/Sb (AMF+Tch CC/Sb).

Certified tomato seeds (*Solanum lycopersicum,* "platense" cv.) were surface sterilized with 10% sodium hypochlorite for 1 min followed by 10% alcohol for 1 min, rinsed with sterile distilled water and kept in Petri dishes with distilled sterile water for 24 h, facilitating the hydration of seeds.

Tomato seed inoculation was separately done for each selected *Trichoderma* strain. Seeds were shaken for 30 min with a magnetic stirrer in an Erlenmeyer flask containing 90 mL of 5% Agar-water plus 10 mL of an aqueous suspension *Trichoderma* strains that was adjusted to $1x10^8$ conidia/mL and a drop of surfactant (Tween 20). For control treatment, the same procedure was performed in the absence of conidia (mock inocula). Treated seeds were dried on a sterile absorbent paper for 24 h at room temperature and then placed in a moist chamber for germination for 7 days at room temperature.

Two pre-germinated seeds were planted in 500 mL pots filled with a substrate composed of non-sterilized agricultural soil (0-20 cm) form the field experiment mixed with sterile sand and perlite (2:1:1 soil: sand: perlite w/v). Tomato plants were grown in a plant growth chamber (25 °C, 12 h light/12 h dark) for 45 days and watered every 72 h. After 15 days, one plant per pot was removed.

At sowing and at 10 days after sowing (DAS) tomato roots were inoculated with 2 mL/pot aqueous suspension containing 12 AMF spores of selected treatments (AMF W-rot and AMF CC/Sb); spores consisted of a homogeneous mixture of genus *Acaulospora, Rhizophagus, Funneliformis* and *Claroideoglomus* (according to the INVAM classification, http://invam.wvu.edu/). Non-inoculated soil pots received a 2 mL of mock inocula (Schroeder and Janos, 2004) which consisted of a filtrate of soil of selected treatments (40 g of soil shaken for 1 h in 400 mL of sterile deionized water filtered in Whatman No. 1).

Plant growth and microbial determinations in a pot experiment

Tomato plants height and SPAD units (average 3 lectures per plant using a SPAD MINOLTA 502) were measured at 45 DAS. Shoots were cut and shoot fresh weight (SFW) was recorded. Leaf images were captured and

leaf area was calculated by using the ImageJ program (https://imagej.nih.gov/ij/). Shoot dry weight (SDW) was determined after drying shoots and the leaves (60 ºC for 72 h). Roots were separated from the substrate, fully recovered, washed, dried with absorbent paper, and root fresh weight (RFW) was measured.

Inoculation responsiveness (IR) was separately calculated for each inoculation treatment according to the following equation (Eq. 2):

$$\text{Eq (2)} \quad IR = \frac{SDW(I) - \text{mean SDW (NI)}}{\text{Mean SDW (NI)}} \times 100$$

where SDW is the individual SDW production of inoculated (I) plants and mean SDW of NI (control) plants. The IR was similarly calculated for SFW and for RFW.

After RFW quantification, roots were stained and AMC was quantified as detailed above (2.4 section). At the end of the pot experiment, abundance of *Trichoderma* in the pots substrate was assessed as described: 10 g of substrate of each pot was weighed and suspended in 90 mL of sterile distilled water; the solution was homogenized on a horizontal stirrer at 150 rpm for 25 min. and four serial decimal dilutions were made. Two hundred microliters of 10-1 and 10-2 dilutions were distributed in Petri dishes with Agar-TSM medium (Elad *et al*., 1981), which were incubated as described above for the isolation of species of the genus *Trichoderma* (2.5 section). Counts of Colony Forming Units (CFU) of *Trichoderma* were recorded (Covacevich and Consolo, 2014).

Statistical analysis

Tested variables were analyzed using an analysis of variance (ANOVA); means were separated by the Tukey's test (p<0.05) by using the SAS software (2005).

Results and Discussion

AMF and Trichoderma indigenous selection from soybean systems with potential to promote plants growth

AMF and *Trichoderma* indigenous strains selection from different soybean crop systems was based on their diversity and ability to proliferate in different crop systems. High genetic diversity (H') of AMF belonging to the genus *Glomus* and *Funneliformis* (Glomerales order) quantified through the PCR-SSCP strategy was obtained in W-rot system, followed by the CC/Sb system, whereas the lowest H' was obtained in the Sb monoculture system (Table 2). Highest abundance of AMF spores was found in the M-rot system, which almost doubled the abundance recorded in other treatments. Highest abundance of *Trichoderma* was recorded in the W-rot system. Our results clearly show that different soybean management systems modified the abundance of both studied fungi. Particularly, the inclusion of oat in rotations (CC/Sb-rot) increased the abundance of both fungal groups as well as the diversity of indigenous Glomerales of soybean crops.

The infective ability of native AMF propagules from different soybean crop systems was evaluated through Arbuscular Mycorrhizal Colonization (AMC) in trap plants. Highest infective capacity was found in trap plants with substrates from the CC/Sb and W-rot systems in which an AMC of 73% and 69% was reached, respectively. These degrees of colonization significantly exceeded those obtained in M-rot and CC/Sb-rot rotations (51% and 47%, respectively), while the lowest AMC was found in the trap plants from Sb monoculture system (29%).

Trichoderma isolates from all soybean cultivation systems showed abundant proliferation in culture medium. However, *Trichoderma* isolates from the CC/Sb and W-rot systems showed a fast conidia proliferation at 72 h of growth, as compared to isolates from the other systems.

Highest AMF diversity and infective capacity as well as the highest production of *Trichoderma* conidia found in the CC/Sb and W-rot systems suggest that native fungi of these systems would have better potential as promoters of plant growth, as compared to other evaluated systems.

Trichoderma species from treatments W-rot and CC/Sb were genetically identified as *Trichoderma* tomentosum and T. harzianum, respectively. Sequences of ITS-rDNA regions

Table 2: Genetic AMF diversity (PCR-SSCP) and AMF and *Trichoderma* abundance at field experiment

Field Experiment Treatments	AMF spores abundance (Nº spores/100 g soil)	Genetic AMF Diversity Index (H´)	*Trichoderma* abundance (CFU/100 g soil)
Sb	218 b	1.4	6.2 x10^3 b
CC/Sb	266 b	2.0	5.2 x10^3 b
M-rot	476 a	1.7	5.8 x10^3 b
W-rot	220 b	2.1	2.6 x10^4 a
CC/Sb-rot	254 b	1.7	4.0 x10^3 b

Values in columns with same letters show no significant differences among treatments (p< 0.05)

were deposited in the GenBank (https://www.ncbi.nlm.nih.gov/) under the accession numbers MG737695 and MG737696 (available online https://blast.ncbi.nlm.nih.gov/Blast.cgi). Mycorrhizal fungi were morphologically identified as belonging to the Acaulospora, Rhizophagus, Funneliformis and Claroideoglomus genera.

Tomato growth response to single and dual inoculation with selected AMF and *Trichoderma* indigenous from soybean crops

Significant increases in SDW, SFW, RFW and leaf area of tomato plants at 45 DAS were found as a result of single and/or dual inoculation with AMF-*Trichoderma* (Figure 1), as compared to the Control (Table 3). Similarly, Bader *et al*.

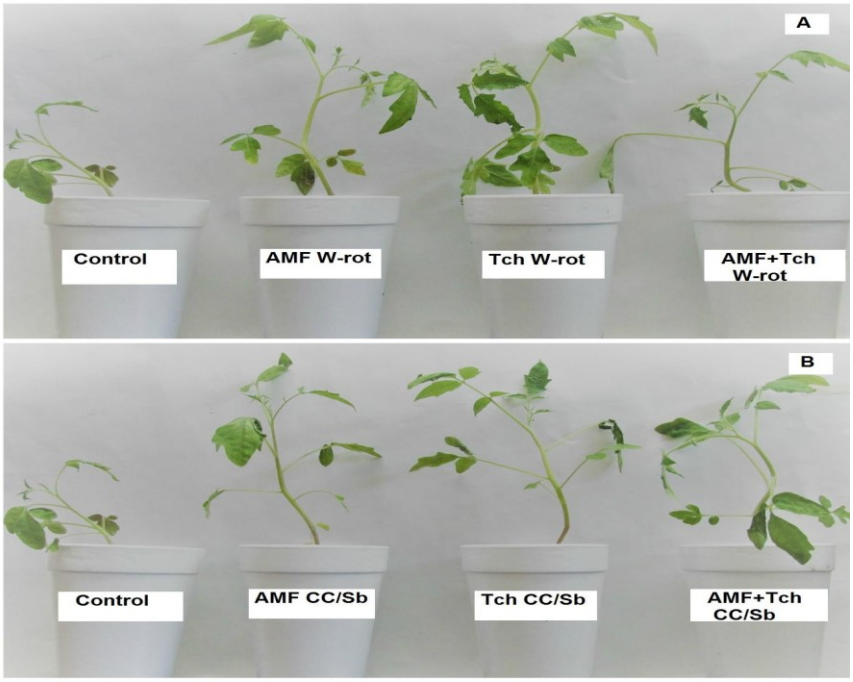

Figure 1: Tomato plants growth after 45 days. A: (from left to right) without inoculation (control), inoculated with AMF W-rot, Tch W-rot and inoculated with both fungi (AMF+Tch W-rot). B: (from left to right) without inoculation (control), inoculated with AMF CC/Sb, Tch CC/Sb and inoculated with both fungi (AMF+Tch CC/Sb)

Table 3: Growth parameters in tomato plants (45 DAS) as affected by single or dual inoculation with arbuscular mycorrhizal consortium (AMF) or *Trichoderma* (Tch) indigenous strains from soybean crops. CC/Sb: Soybean after oat as cover crop –CC- ; W-rot: systems including rotations (rot) of crops with the three phases of rotation in different plots (CC/Soybean-Maize-Wheat) with wheat (W-rot) in the year of study; SFW: shoot fresh weight; SDW: shoot dry weight; RFW: root fresh weight; CFU: colony forming units

Treatments	Shoot height (cm)	Greenness Index (SPAD)	SFW (g)	SDW (g)	RFW (g)	Leaf area (cm^2)	*Trichoderma* abundance (CFU g s.s)
Control	10.25 d	29 a	0.86 b	0.06 b	0.15 b	0.63 d	417.0 d
AMF W-rot	17.01 a	30 a	1.96 a	0.15 a	0.36 ab	1.45 b	1423.7 c
AMF CC/Sb	17.02 a	30 a	1.82 a	0.13 a	0.22 b	0.96 cd	1527.7 c
Tch W-rot	14.75 bc	30 a	1.55 ab	0.11 ab	0.50 a	1.09 c	3611.0 a
Tch CC/Sb	16.51 ab	31 a	1.55 ab	0.14 a	0.37 ab	2.16 a	4097.3 a
AMF+Tch W-rot	14.25 c	33 a	1.49 ab	0.10 ab	0.59 a	1.06 c	2569.3 b
AMF+Tch CC/Sb	14.25 c	31 a	1.66 a	0.11 ab	0.37 ab	1.25 bc	2465.0 b

Different letters in columns indicate significant differences among treatments (Tuckey, p <0.05).

(2019) showed increases up to 300% in tomato plants growth inoculated with T. harzianum indigenous from Argentina. Our results showed that inoculation responses (IR) were always positive, with a marked increase (above 60%) resulting from inoculation with *Trichoderma* strains. Higher IRSDW (above 140%) was found after single inoculation with T. harzianum indigenous from CC/Sb system of Argentina soybean fields, as compared to the Control (Figure 2). Bal and Altinatas (2008) reported that lettuce plants (Lactuca sativa) inoculation with T. harzianum increased only seedling fresh weight, without increasing roots weight. A higher IRRFW (above 244%) was obtained after single tomato inoculation with T. tomentosum indigenous from W-rot system, as compared to the Control. To our knowledge, few studies have reported the effect of T. tomentosum inoculation on plant growth. Rabeendra *et al*. (2000) and Ousley *et al*. (1993) reported the efficiency of T. tomentosum on the growth of cabbage and lettuce plants, respectively.

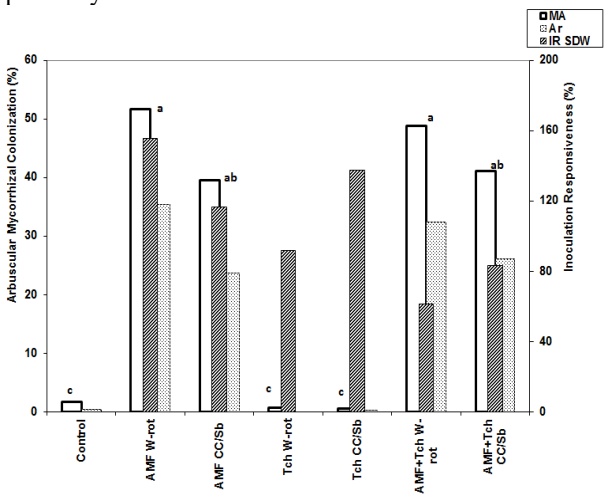

Figure 2: Arbuscular mycorrhizal colonization in tomato roots quantified as mycorrhizal intensity (MA) and Arbuscule content (Ar). Inoculation responsiveness in shoot dry weight (IR SDW) of inoculated plants relative to control

Control = not inoculated; AMF W-rot: Inoculated with AMF consortium from the field treatment W-rot; AMF CC/Sb: Inoculated with AMF consortium from the field CC/Sb treatment; Tch W-rot: Inoculated with *Trichoderma* from the field treatment W-rot; Tch CC/Sb: *Trichoderma* inoculated from the field CC/Sb treatment; AMF+Tch W-rot: Inoculated with consortium with AMF and *Trichoderma* from the treatment field W-rot; AMF+Tch CC/Sb: Inoculated with consortium with AMF and *Trichoderma* from the field CC/Sb treatment.

Columns with different letters indicate significant differences in mycorrhizal colonization among inoculation treatments (Tuckey, $p<0.05$). Statistical analysis results were similar for MA and Ar.

Tomato growth increase due to AMF inoculation is well-known (Cavagnaro *et al*., 2007; Mujica Pérez and Fuentes Martínez (2012). Thougnon Islas *et al*., (2014) detected slight (not statistically significant) evidences on tomato plant growth promotion (IR up to 40%) associated with an incipient colonization (23 DAS) by AMF indigenous from soybean monoculture systems of Argentinean Pampa. In our study, AMF indigenous from W-rot and CC/Sb increased SDW above 110%, as compared to the Control. Our results suggest a growth promoting potential by *Trichoderma* and AMF from rotation systems and/or CC/Sb. We also obtained higher IRSDW (above 60%) after AMF and *Trichoderma* indigenous dual inoculation from rotation and/or with CC/Sb systems, with respect to the control (Figure 2). We found tomato plant root growth (RFW) and shoot growth (SFW) increases when plants were inoculated with AMF+Tch W-rot (inoculum T. tomentosum consortium with AMF) and AMF+Tch CC/Sb (inoculum T. harzianum consortium with AMF), respectively (Table 3). The positive IR after dual inoculations found in our experiment is in agreement with some reports. Colla *et al*. (2015), for instance, reported that tomato plants inoculated with G. intraradices and T. atroviride, showed enhanced growth, as compared to control treatments. The increase in root and shoot weight was associated with an increased in nutrient uptake (e.g. P, Mg, Fe, Zn and B). Martínez-Medina *et al*. (2011) reported growth increases after AMF and *Trichoderma* dual inoculation in melon crop (Cucumis melo). Similarly, Parkash *et al*. (2011) found that both AMF and *Trichoderma* viride single and dual inoculation had a positive effect on plant growth and root mycorrhizal colonization in Dendrocalamus strictus plants. Moreover, Dubsky *et al*. (2002) reported that AMF and T. harzianum dual inoculation increased growth of Cyclamen persicum and decreased plant mortality caused by spontaneous infection by Cryptocline cyclaminis fungal pathogen.

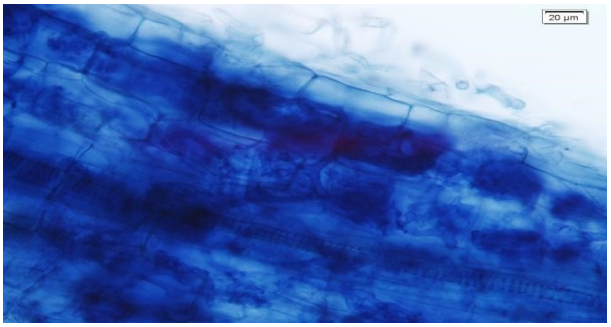

Figure 3: Mycorrhizal colonization of tomato roots after inoculation with arbuscular mycorrhizal fungi (40x magnification)

In this study, we found that highest *Trichoderma* abundance was recorded after single inoculation with Tch W-rot and also with Tch CC/Sb (Table 3). *Trichoderma* abundance decreased in pot substrates that received dual inoculation (about 30-40%, Table 3). In contrast, we found that AMC of tomato plants was not inhibited by *Trichoderma* since mycorrhizal colonization was higher than 40% in all AMF (single and also AMF+*Trichoderma*) inoculated plants (Figure 2); no differences in AMC were found between single and dual inoculation from the same crop system. Characteristic AMF arbuscules were found in inoculated tomato plant roots (Figure 3). Some studies showed positive (Datnoff *et al*., 1995) and neutral (Fracchia *et al*., 1998) interactions between *T. harzianum* and AMF species as *Glomus intraradices* and *G. mosseae*, respectively. However, detrimental effects of *Trichoderma* on mycorrhizal colonization were reported by Santander and Olave (2014), who found that *Trichoderma* depressed root mycorrhizal colonization of melon plants by *G. intrarradices*. In our study, a decrease in the population development of *T. harzianum* around the roots was found in the presence of AMF consortium, in agreement with prior findings (Sosa Rodríguez *et al*., 2006; Chandanie *et al*., 2009). It is unknown if the release of certain metabolites by the AMF can depress the development of *Trichoderma*. Future studies should deepen the knowledge of the mechanisms involved in the relations between both fungi in co-inoculation.

It is worth mentioning that some basal mycorrhizal colonization and low *Trichoderma* abundance were found even in non-inoculated treatments. We highlight that our results were promising since data were obtained from non-sterile soil conditions. Although the mechanisms and interactions of soil microorganisms are not well understood, it was suggested that soil microbiota could suppress the activity of introduced microorganisms by competition (Cook, 1991) and thus could depress inoculation responses. Results from our experiment have shown plant growth increases, AMF root colonization and *Trichoderma* abundance after inoculation, regardless of native microbiota. A possible advantage from non-sterile soil used in this experiment, would be that our results resemble field conditions.

In conclusion, we detected highest AMF abundance, *Trichoderma* and AMF diversity in alternative systems to soybean monoculture (such as the inclusion of oat as CC or rotation systems with wheat and maize). It is likely that the greater SOM provided either by the CC or by the crop rotation, has favored greater diversity of soil microbiota in general and AMF and *Trichoderma* in particular. Future studies should deepen the understanding of the factors of crop management practices that specifically favors the abundance and diversity of plant growth promoters microorganisms. Furthermore, both single and dual inoculation with native fungi from alternative systems to soybean monoculture would be important for horticultural crops because high inoculation responses were obtained. We suggest that root colonization prior to transplanting could provide root protection to pest and dryness, and improve tomato crop establishment under field conditions, in addition to decreasing production costs and environmental impacts.

Conflicts of interest

The authors declare no conflict of interest.

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References

Bader, A., F. Covacevich, G. Salerno and V.F. Consolo. 2019. Native *Trichoderma harzianum* strains from Argentina, produce indole-3 acetic acid and phosphorus solubilization, promote growth and control wilt disease on tomato (Lycopersicum sculentum Mill.) *Journal of King Saud University - Science* DOI: https://doi.org/10.1016/j.jksus.2019.04.002

Bal, U. and S. Altintas. 2008. Effects of T. *harzianum* on lettuce in protected cultivation. *Central European Agriculture Journal* 9: 63-70.

Benbouza, H., J.M. Jacquemin, J.P. Baudoin and G. Mergeai. 2006. Optimization of a reliable, fast, cheap and sensitive silver staining method to detect SSR markers in polyacrylamide Gels. *Biotechnologie, agronomie, société et environment* 10: 77-81.

Cavagnaro, T.R., S.K. Sokolow and L.E. Jackson. 2007. Mycorrhizal effects on growth and nutrition of tomato under elevated atmospheric carbon dioxide *Functional Plant Biology* 34: 730-736.

Chandanie, W.A., M. Kubota and M. Hyakumachi. 2009. Interactions between the arbuscular mycorrhizal fungus

Glomus mosseae and plant growth-promoting fungi and their significance for enhancing plant growth and suppressing damping-off of cucumber (*Cucumis sativus* L.). *Applied Soil Ecology* 41: 336-341.

Cho, G.D., M. Kim and W.W. Koo. 2004. The relative impact of national monetary policies and international exchange rate on long-term variations in relative agricultural prices. Center for Agricultural Policy and Trade Studies, Department of Agribusiness and Applied Economics, North Dakota State University, 1187: 2016-93683.

Colla, G., Y. Rouphael, E. Di Mattia, C. El-Nakhel and M. Cardarelli. 2015. Co-inoculation of *Glomus intraradices* and *Trichoderma atroviride* acts as a biostimulant to promote growth, yield and nutrient uptake of vegetable crops. *Journal of the Science of Food and Agriculture* 95: 1706-1715.

CooK, J. 1991. A Customized approach to biological control of wheat root diseases. p. 461. In: Biological Control of Plant Diseases: Progress and Challenges for the Future. Proceeding of a NATO Advanced Research Workshop on Biological Control of Plant Diseases. E.C. Tjamos, G.C. Papavizas and R.J. Cook (eds.). May, Greece Springer.

Covacevich, F. and H.E. Echeverría. 2003. Utilización de formaldehído para la erradicación de hongos micorríticos arbusculares de muestras de suelo. *Ciencia del suelo.* 21: 9-17.

Covacevich, F., H.E. Echeverría and M.C. Pagano. 2012. Arbuscular mycorrhizal fungi: Essential belowground organisms for earth life but sensitive to a changing environment. *African Journal of Microbiology Research* 6: 5523-5535.

Covacevich, F. and V.F. Consolo. 2014. Herramientas para el estudio y manipulación de Hongos Micorrícicos Arbusculares y Trichoderma. Universidad Nacional de Mar del Plata. 115 p.

Datnoff, L.E., S. Nemec and K. Pernezny. 1995. Biological control of Fusarium crown and root rot of tomato in Florida using *Trichoderma harzianum* and *Glomus intraradices*. *Biological Control* 5: 427-431.

Dubsky, M., F. Sramek and M. Vosatka. 2002. Inoculation of cyclamen (*Cyclamen persicum*) and poinsettia (*Euphorbia pulcherrima*) with arbuscular mycorrhizal fungi and *Trichoderma harzianum*. *Rostlinna vyroba* 48: 63-68.

Duval, M.E., J.A. Galantini, J.E. Capurro and J.M. Martinez. 2016. Winter cover crops in soybean monoculture: Effects on soil organic carbon and its fractions. *Soil & Tillage Research* 161: 95-105.

Elad, Y., I. Chet and Y. Henis. 1981. A selective medium for improving qualitative isolation of *Trichoderma* spp. from soil. *Phytoparasitica* 9: 59-67.

Ene, M. and M. Alexandru. 2008. Microscopical examination of plant reaction in case of infection with *Trichoderma* and Mycorrhizal fungi. *Roumanian Society of Biological Sciences* 13: 13-19.

Fernández, R., A. Quiroga, F. Arena, C. Antonini and M. Saks. 2007. Contribución de los cultivos de cobertura y las napas freáticas a la conservación del agua, uso consuntivo y nutrición de los cultivos. *Manual de Fertilidad y Evaluación de Suelos.* EEA INTA Anguil, Argentina. 51: 59.

Fracchia, S., M.T. Mujica, I. García-Romera, J.M. Garcia-Garrido, J. Martin, J.A. Ocampo and A. Godeas. 1998. Interactions between *Glomus mosseae* and arbuscular mycorrhizal sporocarp-associated saprophytic fungi. *Plant and Soil* 200: 131-137.

Guigón-López, C., V. Guerrero-Prieto, F. Vargas-Albores, E. Carvajal-Millán, G.D. Ávila-Quezada, L. Bravo-Luna and M. Lorito. 2010. Identificación molecular de cepas nativas de *Trichoderma* spp. su tasa de crecimiento in vitro y antagonismo contra hongos fitopatógenos. *Revista mexicana de fitopatología* 28: 87-96.

Gupta, M., N. Naqvi and P. Kumar. 2017. AMF-Centralized database of arbuscular mycorrhizal distribution, phylogeny and taxonomy. *Journal of Recent Advances in Applied Sciences* 30: 1-5.

Harman, G.E. 2006. Overview of Mechanisms and Uses of *Trichoderma* spp. *Phytopathology* 96: 190-194.

Kjøller, R. and S. Rosendahl. 2000. Detection of arbuscular mycorrhizal fungi (Glomales) in roots by nested PCR and SSCP (single stranded conformation polymorphism). *Plant and Soil* 226: 189-196.

Kumar, K. and K.M. Goh. 2000. Biological nitrogen fixation, accumulation of soil nitrogen and nitrogen balance for white clover (*Trifolium repens* L.) and field pea (*Pisum sativum* L.) grown for seed. *Field Crops Research* 68: 49-59.

Marshall, C.B., J.R. McLaren and R. Turkington. 2011. Soil microbial communities resistant to changes in plant functional group composition. *Soil Biology and Biochemistry* 43: 78-85.

Martínez-Medina, A., A. Roldán and J.A. Pascual. 2011. Interaction between arbuscular mycorrhizal fungi and *Trichoderma harzianum* under conventional and low input fertilization field condition in melon crops: growth response and *Fusarium* wilt biocontrol. *Applied Soil Ecology* 47: 98-105.

Mujica Pérez, Y. and A.G. Fuentes Martínez. 2012. Efecto a la biofertilización con hongos micorrízicos arbusculares (HMA) en el cultivo del tomate en condiciones de estrés abiótico. *Cultivos tropicales* 33: 40-46.

Ousley, M.A., J.M. Lynch and J.M. Whipps. 1993. Effect of *Trichoderma* on plant growth: a balance between

inhibition and growth promotion. *Microbial Ecology* 26: 277-285.

Parkash, V., S. Sharma and A. Aggarwal. 2011. Symbiotic and synergistic efficacy of endomycorrhizae with *Dendrocalamus strictus*. L. *Plant Soil Environ* 57: 447-52.

Phillips, J. and D. Hayman. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society* 55: 158-161.

Raeder, U. and P. Broda. 1985. Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* 1: 17-20.

Rabeendran, N., D.J. Moot, E.E. Jones and A. Stewart. 2000. Inconsistent growth promotion of cabbage and lettuce from *Trichoderma* isolates. *New Zealand Plant Protection* 53: 143-146.

Redecker, D., A. Schüßler, H. Stockinger, S. L. Stürmer, J. B. Morton and C. Walker. 2013. An evidence-based consensus for the classification of arbuscular mycorrhizal fungi (Glomeromycota). *Mycorrhiza* 23: 515-531.

Ritchie, S.W. and J.J. Hanway. 1982. How a corn plant develops. Iowa State University of Science and Technology. Cooperative Extension Service Ames, Iowa. Special Report No. 48.

Rillig, M.C., S.F. Wright and V.T. Eviner. 2002. The role of arbuscular mycorrhizal fungi and glomalin in soil aggregation: comparing effects of five plant species. *Plant and Soil* 238: 325-33.

Rosendahl, S. and E. Stukenbrock. 2004. Community structure of arbuscular mycorrhizal fungi in undisturbed vegetation revealed by analyses of LSU rDNA sequences. *Molecular Ecology* 13: 3179-3186.

Santander, C. and J. Olave. 2014. Efecto de la interacción del hongo micorrícico arbuscular (AMF) *Glomus intraradices* y *Trichoderma harzianum* sobre la producción de plantines de melón en zonas áridas. *Idesia (Arica)* 32: 21-28.

SAS Institute. 2005. SAS User´s guide. Statistics. SAS Institute, Inc., Cary.

Schroeder, M.S. and D.P. Janos. 2004. Phosphorus and intraspecific density alter plant responses to arbuscular mycorrhizas. *Plant and Soil* 264: 335-348.

Shannon, C.E. and W. Weaver. 1949. The mathematical theory of information. University of Illinois Press, Chicago USA.

Sharif, M., E. Ahmad, M.S. Sarir and S. Perveen. 2010. Response of different crops to arbuscular mycorrhizal inoculation in phosphorus deficient soil. *Soil and Environment* 29(2): 192-198.

Sidhoum, W. and Z. Fortas. 2018. Growth and mycorrhizal responses to cadmium stress in some halophytic plants. *Soil and Environment* 37(2): 169-177.

Sieverding, E. 1991. Vesicular arbuscular mycorrhizal management in tropical agroecosystems. *Deutsche Gesellschsft fur Technische Zusammenarbeit (GTZ) tropics*, Federal Republic of Germany. 371 p.

Solbrig, O.T. 2005. The dilemma of biodiversity conservation. Agricultural expansion in the Argentine Pampas. *Harvard Review of Latin America, Flora and Fauna* 3: 34-35.

Sosa Rodríguez, T., J. Sánchez Nieves, E. Morales Gutiérrez and F. Cruz Cortés. 2006. Interacción micorrizas arbusculares-*Trichoderma harzianum* (Moniliaceae) y efectos sobre el crecimiento de *Brachiaria decumbens* (Poaceae). *Acta biológica colombiana* 11: 43-54.

Tiunov, A.V. and S. Scheu. 2005. Arbuscular mycorrhiza and Collembola interact in affecting community composition of saprotrophic microfungi. *Oecologia* 142: 636-642.

Thougnon Islas, A.J., M. Eyherabide, H.E. Echeverría, H.R. Sainz Rozas and F. Covacevich. 2014. Capacidad micotrófica y eficiencia de consorcios con hongos micorrízicos nativos de suelos de la provincia de Buenos Aires con manejo contrastante. *Revista Argentina de Microbiología* 46: 133-143.

Trouvelot, A. 1986. Mesure du taux de mycorhization VA d'un systeme radiculaire. Recherche de methodes d'estimation ayant une significantion fonctionnelle. *Mycorrhizae: physiology and genetics* 217-221.

Van Elsas, J.D., M. Chiurazzi, C.A. Mallon, D. Elhottovā, V. Krištůfek and J. Falcão Salles. 2012. Microbial diversity determines the invasion of soil by a bacterial pathogen. *Proceedings of the National Academy of Sciences* 109: 1159-1164.

Van Tuinen, D., B. Zhao and V. Gianinazzi-Pearson. 1998. PCR in studies of AM fungi: From primers to application. p. 387-400. In Mycorrhiza manual. Springer Berlin Heidelberg.

Villamil, M.B., G.A. Bollero, R.G. Darmody, F.W. Simmons and D.G. Bullock. 2006. No-till corn/soybean systems including winter cover crops. *Soil Science Society of America Journal* 70: 1936-1944.

White, T.J., T. Bruns, S.J.W.T. Lee and J.L. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications* 18: 315-322.