

Potato field-inoculation in Ecuador with *Rhizophagus irregularis*: no impact on growth performance and associated arbuscular mycorrhizal fungal communities

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Abstract A field trial was conducted in two localities of the Ecuadorian Andes to evaluate potato (*Solanum tuberosum* c.v. INIAP - Fripapa) response to inoculation with four commercial products containing the arbuscular mycorrhizal fungus (AMF) strain *Rhizophagus irregularis* DAOM 197198. In parallel, potato roots were analysed using 454 GS-FLX+ sequencing of c. 800 bp of the nuclear LSU rRNA gene to assess the associated AMF communities. To evaluate inoculation success, sequence reads of *R. irregularis* on the root samples were compared between inoculated and not inoculated plants by analysing the frequency of occurrence (FO) and relative read abundance (RA). None of the commercial products significantly increased potato yield. Instead, the AMF communities were dominated by an unknown *Acaulospora* sp. (Sp14) found at high FO and RA in both localities. *Rhizophagus*

irregularis was found in most of the roots of both inoculated and not inoculated plants at both localities. However, its abundance was unexpectedly low indicating poor inoculum establishment. Clearly, many factors have to be taken in consideration for the successful application of AMF-based inoculants. For the Ecuadorian field trials, several causes may explain the lack or poor establishment of *R. irregularis* such as inoculation technique, agricultural practices, biotic and abiotic conditions and competition with native AMF species.

Keywords Arbuscular mycorrhizal fungi · Commercial inoculum · LSU rRNA gene · *Rhizophagus irregularis* · *Solanum tuberosum*

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1 Introduction

Indigenous farmers in the Central Andean Highlands in South America have cultivated potatoes (*Solanum tuberosum*) for centuries (Hawkes 1990). In Ecuador, the demographic growth in the Andean region in the last decades and subsequent land pressure has forced farmers to cultivate potato at higher altitudes 2700–3800 m asl at slopes of up to 50% and with poor soil quality (Dercon et al. 2007). Novel policies implemented by Ecuadorian Government aimed to encourage agricultural production through the reduction of import tariffs and subsidies of agrochemicals (Pellegrini 2013) have resulted in large increases of potato productivity (SINAGAP 2014). For instance, potato yield increased from 4.1 t ha⁻¹ in 2001 to 12.8 t ha⁻¹ in 2014 (SINAGAP 2014). Emblematic of this situation is the province of Carchi in northern Ecuador with the highest national yields (22.52 t ha⁻¹). However, the number of pesticide poisonings in the province due to the misuse/overuse of pesticides is among the highest reported in the

world (Sherwood et al. 2002). Eco-efficient and economic alternatives to decrease the use of agrochemicals and their negative impacts on environment and farmers' health are needed.

AMF are soil fungi that belong to the phylum *Glomeromycota* (Schüßler et al. 2001). These below-ground microorganisms establish symbioses with the vast majority of plants including potato (Smith and Read 2008). They increase the nutrient absorbing surface of host roots enabling plants to efficiently exploit soil nutrient resources, increasing the utilization efficiency of fertilizers, especially the non-renewable phosphorus (P) (Tawaraya et al. 2012). They also have been extensively reported to increase plant resistance to biotic and abiotic stresses (Wehner et al. 2011). Inoculation of potato plants with efficient, easy to mass-produce AMF generalists, coupled with appropriate agricultural practices can help to decrease the use of agrochemicals in Andean agroecosystems. AMF inoculation can represent an attractive alternative to improve potato performance (Davies et al. 2005; Douds et al. 2007).

During the last years, several companies started to formulate inoculants containing single or mixed AMF isolates which are colonizers of a broad range of crops under diverse environmental conditions (Vosátka et al. 2012). *Rhizophagus irregularis* (formerly named *Glomus intraradices*, see Stockinger et al. 2009) is probably the more widely exploited AMF species, due to its wide distribution (Berruti et al. 2014), broad host compatibility (Sýkorová et al. 2007) and relatively easy mass-production using different techniques (Ijdo et al. 2011). A recent analysis of 231 field experiments in North America and Europe where potato was inoculated with *R. irregularis* strain DAOM 197198 showed a significant increase in the production of marketable potato tubers of inoculated plants (Hijri 2015), suggesting its potential interest for application in other bio-geographic areas and agro-ecosystems. The success of AMF inoculation in the field depends on several factors (Verbruggen et al. 2013), including compatibility with the target plant (Schüßler et al. 2016; Vályi et al. 2015) and the local soil properties (Herrera-Peraza et al. 2011). Hence, it is of prime importance to evaluate the establishment, infectivity and effectiveness of an introduced AMF strain (Fall et al. 2015; Lu et al. 2015; Schüßler et al. 2016) and its persistence in the field (Farmer et al. 2007; Pellegrino et al. 2012; Sýkorová et al. 2012).

Recently, the use of Next Generation Sequencing (NGS) technologies like 454 sequencing enabled us to explore in deep the plant-associated AMF. The analysis of potato roots in the Andes revealed a high number of unknown AMF species potentially novel to science (Senés-Guerrero and Schüßler 2016). The evaluation of the AMF community compositions associated with potato under particular environments is of crucial importance in order to unveil the role of the native AMF community when applying exotic AMF.

Additionally, the identification of possible preferential potato-AMF associations under Andean conditions such as altitude, soil and plant-genotype could help to develop context-adjusted and hence more efficient AMF inoculants.

In the present study, the effect of inoculating potato with four commercial formulations of *R. irregularis* strain DAOM 197198 on plant growth, marketable-tuber yield and on the associated AMF communities was evaluated at two experimental plots in the Andes of Ecuador. The AMF community structure of inoculated and not inoculated plants was evaluated by 454 GS-FLX+ sequencing by comparing the frequency of occurrence (FO) and relative read abundance (RA) of AMF species (Senés-Guerrero and Schüßler 2016). FO and RA of *R. irregularis* of inoculated and not inoculated plants were also used as criteria to evaluate the inoculation success of *R. irregularis* DAOM 197198 in potato roots.

2 Materials and methods

2.1 Study location

The study was performed in two agricultural plots: (1) Zamora Huayco Research Station - Loja (04°0'1.59"S, 79°10'48.46" W; 2160 m asl; precipitation and mean temperature for the period, 768 mm and 15.4 °C, respectively) and (2) Santa Catalina Research Station - Quito (0°22'14.15"S, 78°33' 24.84"W; 3053 m asl, precipitation and mean temperature for the period, 618 mm and 12.9 °C, respectively), located in southern and northern Ecuador respectively. One plot (20 × 26 m) was selected in each locality. In Loja, the plot was left in fallow for one year before the establishment of the trial, while in Quito wheat was cultivated the preceding season. The inoculation trials were conducted from April to September 2012 in Loja and from March to August 2012 in Quito. Soil physico-chemical characteristics were determined from bulk soil of 10 samples randomly collected in each plot. The soil analyses were conducted at the *Laboratorio de Aguas y Suelos del Instituto Nacional de Investigaciones Agropecuarias* (INIAP), Quito-Ecuador. N-NH₄⁺ was determined by the phenate method (APHA 1996); P was extracted after Olsen (Olsen 1954); K, Ca, Mg and Fe by modifications of the Olsen extraction method (as stated by INIAP). Phosphorus was determined by photocolormetry (Fox et al. 1964) and the other elements by atomic absorption spectrophotometry (Hunter 1979). In Loja, the soil was silty with a pH = 4.91, organic matter (OM) content = 5.5%, N-NH₄ = 68 mg/kg, P = 30 mg/kg, K = 175.5 mg/kg, Ca = 300 mg/kg, Mg = 75.6 mg/kg and Fe = 350 mg/kg of soil. In Quito, the soil was sandy loamy with pH = 5.54, OM = 10.70%, N-NH₄ = 83 mg/kg, P = 51 mg/kg, K = 144.3 mg/kg, Ca = 1200 mg/kg, Mg = 62.4 mg/kg and Fe = 279 mg/kg of soil.

2.2 Plant material

Healthy potato tubers of *Solanum tuberosum* x *andigena* c.v. INIAP-Fripapa 99 were provided by the Experimental Station Santa Catalina - INIAP (Quito, Ecuador). The tubers were stored two weeks in mesh bags under dimmed light to promote sprouting.

2.3 AMF inoculum and inoculation techniques

Four commercial products were tested (number of spores per gram as described by the manufacturer): Mycorise® ASP (liquid, 400 spores/ml – ASP), Myke® Pro P-801 (powder, 800 propagules/g – P-801), Myke® Pro GR (granular, 110 propagules/g – GR) purchased from Premier Tech Biotechnologies (<http://www.premiertech.com/global/en/>) and Symplanta® (powder, 10.000 propagules/g – SYM) purchased from SYMPLANTA (<http://www.symplanta.com/>). According to manufacturers, inocula was produced in vitro and contained only *Rhizophagus irregularis* strain DAOM 197198 and inert carrier material. The products were stored at 4 °C until use. At planting, the solid-formulated inocula (Myke® Pro P-801, Myke® Pro GR and Symplanta®) were placed underneath the seed tuber (Douds et al. 2007). The liquid formulation (Mycorise® ASP) was applied manually to the sprouts of the tubers using a Pasteur pipette. The tubers were afterwards gently covered with soil.

2.4 AMF inoculum infectivity

Vitality of the products was tested prior to field application by measuring colonization of seedlings of *Plantago lanceolata* in 1 L pots containing a sterilized mixture of agricultural soil and mine sand (1:3 v/v). Each pot was placed in a sun bag (Sigma-Aldrich, St. Louis-Missouri, USA) to avoid contaminations by other AMF. The amount of applied product was adjusted to an approximate of 200 propagules per plant and three replicates were set up per product. A not inoculated control was also included. After 4 months, the plants were harvested and the roots stained with methyl blue. Percentage root length colonization (%RLC) was determined using the method of Trouvelot et al. (1986). The microscopical analysis revealed the presence of AMF structures (vesicles, hyphae and arbuscules) within the roots of inoculated plants. The average root length colonization percentages (%RLC) for each product were as follows: ASP (18.62% ± 2.4), P-801 (29.4% ± 3.5), GR (32.1% ± 2.1) and SYM (36.2% ± 4.6). No mycorrhizal structures were found in the roots of control plants.

2.5 Experimental design

In both locations, the experiment was established in a completely randomized design with 10 treatments. The

treatments refer to the commercial products (Mycorise® ASP, Myke® Pro-801, Pro GR and Symplanta) applied at 250 and 500 spores/tuber, except for Myke® Pro P-801 for which 100 and 250 spores/tuber were applied due to reduced availability of inoculum. Whatever the treatment, the spore concentration applied to potato tubers was higher than the 50 spores/tuber recommended for potato cropping by Premier Tech Biotechnologies (personal communication Ms. Dominique Le Quéré, Premier Tech). Two control treatments were also included: a chemical fertilized treatment (Fert) with a typical amount applied by farmers (50–120–100 Kg NPK per ha⁻¹) without AMF inoculation, which was applied evenly at planting along the furrow, and a not fertilized not inoculated (Control). Five replicates were considered per treatment. Each replicate consisted of a 4.2 m furrow containing 14 potato tubers spaced every 30 cm. The distance left among furrows was one meter. Guard furrows (not inoculated, not fertilized) were included to separate replicates.

2.6 Agricultural practices

In the two experimental sites, the plots were prepared mechanically with a disk plow. Prior to inoculation, organic amendment with chicken manure (5.83 tons ha⁻¹) was applied evenly in all the furrows regardless of the treatment at both sites, according to the local practices. Hilling and weeding were done manually at 30 and 60 days after planting (DAP). Three fungicides used routinely by farmers to control potato late blight (*Phytophthora infestans*): Revus® (Syngenta UK Limited, Cambridge, UK), Previcur® Energy 840 SL (Bayer CropScience AG, Monheim, Germany) and Soll® 76% WP (Agroquim C. Ltda., Quito, Ecuador) were applied 30 DAP and alternatively every 8–15 days, according to the climate conditions and following the recommendations of the manufacturers.

2.7 Data collection and harvest

Plant emergence was evaluated at 30 DAP and plant height of all the plants was measured at 60 DAP using a metric tape by taking the length from soil surface to the apices of the tallest stem. At 120 DAP, two randomly selected plants per replicate and treatment were harvested and pooled. Above and below-ground parts were processed separately. Roots and tubers were cleaned under tap water. Dry weight (DW) was estimated on above (shoot and leaves) and below-ground (roots and tubers) by weighing the dried samples in an oven at 70 °C until a constant weight was reached (Davies et al. 2005). Total N (Kjeldahl method); P (Vanadate-Molybdate method) and K (atomic absorption spectrometry) contents of above and below-ground plant parts were determined by *Laboratorio de Aguas y Suelos* (INIAP). However, in the Fert treatment in Loja only three replicates from the five could be analysed

due to mortality. Remaining plants in the experimental furrows were harvested manually at 150 DAP. Marketable tubers (>30 g) were collected and their number and fresh weight recorded for each plant.

2.8 AMF root colonization

A subsample of two grams of fine roots was collected and homogenized from each of the two harvested plants per replicate before drying. These root samples were divided in two plastic tubes, each containing approximately one gram of root material, and preserved in 90% EtOH. One tube was used for the assessment of %RLC, while the other was preserved at -20°C for DNA extraction and 454 GS-FLX+ sequencing.

Roots for staining were cut in 1 cm length fragments, cleared with 10% KOH at 60°C for 30 min, rinsed three times with tap water, acidified in 10% HCl for 1 min and stained with 0.05% methyl blue dissolved in 90% lactic acid for 30 min at 60°C . Root pieces were then destained in 50% lactic acid overnight (modified after Urgiles et al. 2009) to remove excess methyl blue. Thirty randomly selected root pieces per replicate (150 per treatment) were mounted on slides and assessed for AMF colonization under a bright-field microscope (Leitz Wetzlar, Germany) at $100\times$ and $400\times$ magnifications. %RLC was estimated using colonization classes where: $0 = 0\%$, $1 < 1\%$, $2 = 2\text{--}10\%$, $3 = 11\text{--}50\%$, $4 = 51\text{--}90\%$, $5 > 90\%$ assigned to each root fragment depending on the percentage of the root length colonized by AMF (Trouvelot et al. 1986). Average colonization of the 30 root pieces was calculated using the formula: $\%M = 95n^5 + 70n^4 + 30n^3 + 5n^2 + n^1/N$. Where n^5 , n^4 , n^3 , n^2 , n^1 are respectively the number of fragments in the classes as 5, 4, 3, 2, 1 and N is the total number of fragments analysed.

2.9 DNA extraction and 454 GS-FLX+ pyrosequencing

Roots collected at 120 DAP and preserved at -20°C were used for DNA extraction and 454 GS-FLX+ sequencing. In both locations, four replicates per treatment were chosen for DNA sequencing analyses. Only the treatments with the higher level of inoculum (ASP, P-801, GR, SYM) and the two not inoculated controls (Fert, Control) were selected for AMF diversity estimation. However, only 47 out of the possible 48 root composite samples were analysed due to mortality of one repetition in the Fert treatment in Loja. DNA extraction and PCR were performed as described in Senés-Guerrero et al. (2014) using the FastDNA Spin Kit for soil (MP Biomedicals, Heidelberg, Germany) following the instructions of the manufacturer but using Lysing Matrix A tubes with an extra big ceramic bead instead of Lysing Matrix E. The first PCR was performed as described by Krüger et al. (2009) with the AMF specific primers SSUMAf-LSUMAr and 35 cycles of $0.5\text{ }\mu\text{l}$ of DNA template.

The primers target a region covering approx. 200 bp of the nuclear small subunit (SSU) ribosomal RNA (rRNA) gene, the complete internal transcribed spacer (ITS) region and approx. 900 bp of the large subunit (LSU) rRNA gene. The product of the first PCR served as template for a nested PCR by which a fragment of approx. 760 bp targeting the LSU rRNA gene was re-amplified, using fusion-primers (Senés-Guerrero and Schüßler 2016). Amplicons were 454 sequenced using the GS-FLX+ Titanium Sequencing Kit (Roche, Basel, Switzerland) at the company IMGM Laboratories (Martinsried, Germany).

2.10 Data analyses

Bioinformatic analysis, species delimitation and annotation were done as described in Senés-Guerrero and Schüßler (2016). Briefly, low quality and ambiguous reads along with sequences shorter than 500 bp were discarded in the QIIME pipeline (Caporaso et al. 2010). Sequences were divided into clusters using UCLUST (Edgar 2010) at a similarity threshold of 98% and the representative sequences (RS) of the cluster were used for further analyses. A maximum-likelihood phylogenetic tree was calculated with RAxML at the CIPRES Science Gateway (<http://www.phylo.org/portal2/>) with 1000 bootstraps and the GTRGAMMA model, using reference sequences from defined AMF species (Krüger et al. 2012), further reliable sequences from public databases, and from AMF sequences obtained from clone libraries derived from Peruvian (Senés-Guerrero et al. 2014), Bolivian and Ecuadorian (Senés-Guerrero and Schüßler 2016) potato roots. The reference sequences alignment and the phylogenetic tree served as a “backbone” for the individual phylogenetic placement of sequences by using the RAxML Evolutionary Placement Algorithm (EPA) with the GTRCAT model (Berger et al. 2011; Berger and Stamatakis 2011). In this way, species delimitation consisted of two steps: in the first, a reference phylogenetic tree based on long, 1.5 kb sequences was computed and in the second, each RS of a 98% similarity cluster was individually placed by EPA into this reference tree and annotated to species. To allow comparisons, species were annotated with the same species numbers as used in previous studies (Senés-Guerrero et al. 2014; Senés-Guerrero and Schüßler 2016).

2.11 Diversity analyses

Rarefaction curves were obtained by using the function described by Jenna Jacobs (<http://www.jennajacobs.org/R/rarefaction.html>) using a subsample size of 5 and the Shannon-Weaver diversity index (H) was calculated using the vegan package (Oksanen et al. 2013) of R 2.15.1 (R Development Core Team 2011). The 454-read frequency of occurrence (FO) and relative abundance (RA) were used as

criteria to compare AMF communities among treatments. Frequency of occurrence refers to the presence/absence of a species in a sample. The relative read abundance (as percentage) represents the proportion of reads of each AMF species (in each treatment) from the total amount of reads for that treatment. To identify changes in the AMF community composition related to the sites (Loja and Quito) and the treatments, a principal component analysis (PCA) was performed using the vegan package of R with the function scale = TRUE, which adjusts the total inertia to the number of variables.

The data of the sequencing run was deposited in the Sequence Read Archive at NCBI under accession number PRJNA247467.

2.12 Statistical analyses

Statistical analyses were carried out for each site (Loja and Quito) separately. Mycorrhizal colonization and plant growth parameters were analysed by one-way analysis of variance (ANOVA) for the factor inoculation. Mycorrhizal colonization was Log_e- or arcsin-transformed to fulfil the assumptions for ANOVA. Mean comparisons were made using orthogonal contrasts for the crop performance parameters (plant emergence, plant height, yield, etc.). All the analyses were carried out using the software SPSS Statistics 20.

3 Results

3.1 Plant growth and tuber yield

Regardless of the treatment and experimental site (i.e. Loja and Quito), no differences were observed in the emergence of potato plants estimated at 30 DAP (data not shown, $P > 0.05$). Plant height at 60 DAP was significantly higher in the Fert treatment, compared to the inoculated treatments in Loja ($P < 0.001$). No differences were observed in the above and below-ground dry weights (DW) of the plants at 120 DAP ($P > 0.05$), among treatments and experimental sites (Online Resource 1). Finally, the concentration of N, P, K in above and below-ground tissues did neither differ significantly between not inoculated and inoculated plants ($P > 0.05$) nor between plants inoculated with the two doses of inoculum ($P > 0.05$ – Online Resources 2, 3).

Whatever the location, the number of tubers and their fresh weight, estimated at 150 DAP, did not differ significantly ($P > 0.05$) between the inoculated treatments and their respective controls. However, the Fert treatment in Loja and Quito showed significantly higher ($P < 0.01$) values compared with the inoculated treatments (Table 1).

3.2 AMF root colonization

Regardless of whether inoculated or not, all potato plants were colonized by AMF showing hyphae, vesicles and arbuscules

Table 1 Potatoes yield evaluation 150 days after planting (DAP). Four commercial inoculants of *R. irregularis* strain DAOM197198 (ASP, P-801, GR and SYM) were applied at two dosages. Controls: Fert (chemically fertilized, not inoculated) and Control (not inoculated and not fertilized)

	Loja		Quito	
	Number of tubers	Yield (g plant ⁻¹)	Number of tubers	Yield (g plant ⁻¹)
ASP ²⁵⁰ a	7.2 ± 0.5 ^b	243 ± 33.3	4.5 ± 0.5	391.3 ± 32.6
ASP ⁵⁰⁰	8.7 ± 0.8	344.8 ± 48.3	4.8 ± 0.6	345.5 ± 70.5
P-801 ²⁵⁰	9.3 ± 1.5	292.6 ± 85.1	4.6 ± 0.1	382.4 ± 24
P-801 ⁵⁰⁰	10.6 ± 1.4	398.4 ± 84.6	4.8 ± 0.6	375.2 ± 54.9
GR ¹⁰⁰	8.1 ± 0.8	294.5 ± 51.8	5.2 ± 0.9	326.3 ± 63.1
GR ²⁵⁰	10.4 ± 2.2	415.9 ± 93.4	6.0 ± 0.9	465.5 ± 78.8
SYM ²⁵⁰	10.7 ± 1.8	378.4 ± 41.7	5.8 ± 0.5	384.0 ± 29
SYM ⁵⁰⁰	7.1 ± 1.3	248.0 ± 48.9	4.6 ± 0.4	385.5 ± 54
Fert [†]	19.3 ± 2.6	424.5 ± 93.7	6.6 ± 0.7	575.6 ± 63.2
Control	9.1 ± 1.4	425.7 ± 123.7	4.9 ± 0.6	364.0 ± 55.5
Linear Orthogonal contrasts				
Inoc. vs Control	ns	ns	ns	ns
Fert vs Inoc	**	ns	**	**
Low vs High	ns	ns	ns	ns

Inoc, inoculated; Not-Inoc., not inoculated

^a Number of applied propagules per tuber. Low stands for the lower and High for the higher number of propagules

^b Values are means ± SE, $n = 5$. (Note: each replicate comprised twelve plants). Statistical significance *, $P < 0.05$; **, $P < 0.01$; ns = not significant difference

[†] Only three replicates were available due to mortality

within their roots. However, the overall %RLC level was low; it was significantly higher in Quito ($6.22\% \pm 0.39$) as compared to Loja ($4.27\% \pm 0.34\%$) at 120 DAP ($P < 0.001$, data not shown). In Loja, no significant difference in %RLC was observed between the Control treatment and the inoculated treatments ($P > 0.05$). However, Fert treatment displayed lower %RLC values compared to the inoculated treatments ($P < 0.01$). Treatments with higher doses of inoculum applied showed higher %RLC values than plants with lower doses ($P < 0.01$). In Quito, no differences were observed in %RLC between the not inoculated control treatment and the inoculated treatments, between the Fert treatment and the inoculated treatments and between the two doses of inoculum application ($P > 0.05$) (Table 2).

3.3 AMF community composition and molecular tracing of *Rhizophagus irregularis*

The number of recovered and quality-filtered sequences longer than 500 bp was 172,750. After singleton and non-AMF sequence removal, 164,756 reads of an average read length of 723 bp were left for analysis. These were clustered at 98% nucleotide identity, a threshold ensuring that no interspecific sequence variants are clustered (Senés-Guerrero and Schüßler

2016) to obtain 2912 representative sequences (RS). The RS were affiliated to the reference sequences phylogenetic tree (Online Resource 4) by EPA to forty AMF clades interpreted as species. From these, 30 species were found in Loja and 31 in Quito. Twenty-one species were shared between both localities. The overall 40 identified species belonged to 10 genera: *Acaulospora* (10 species), *Rhizophagus* (7 species), *Claroideoglomus* (5 species), *Scutellospora* (4 species), *Funneliformis* (4 species), *Gigaspora* (3 species), *Paraglomus* (2 species), *Archaeospora* (2 species), *Diversispora* (2 species) and *Pacispora* (1 species), distributed in 8 of the 11 families of the phylum *Glomeromycota*.

Curves of species accumulation showed plateau levels for almost all the treatments in Loja except the Control, indicating that sequencing depth was sufficient to represent the AMF species diversity, whereas this was not the case for Quito (Fig. 1). In Loja, the Shannon diversity indices of the not inoculated treatments (Fert and Control) displayed higher values $H = 1.57$ and $H = 1.61$, respectively compared with the inoculated treatments that ranged from $H = 0.90$ – 1.37 . In Quito, the Shannon indices were highly similar among treatments (either if the plants were inoculated or not) ranging from $H = 0.79$ – 1.11 (data not shown).

Based on FO, the most frequent species-rich genera in Loja were *Acaulospora*, *Rhizophagus*, *Claroideoglomus* and *Scutellospora*. The five most frequent species in decreasing order were an unknown *Acaulospora* sp. (Sp14), *Scutellospora* sp. (Sp67), *R. irregularis* (Sp09), *Acaulospora laevis* (Sp25) and *Scutellospora* sp. (Sp44) (Table 3). In Quito, the most frequent and species in decreasing order were

Table 2 Root length colonization (%RLC) of potato 120 days after planting (DAP). Four inoculants of *R. irregularis* strain DAOM197198 (ASP, P-801, GR and SYM) were applied at two dosages. Controls: Fert (chemically fertilized, not inoculated) and Control (not inoculated and not fertilized)

	Loja	Quito
ASP ²⁵⁰ a	3.2 ± 0.4^b	7.4 ± 1.8
ASP ⁵⁰⁰	6.1 ± 1.0	9.0 ± 1.4
P-801 ²⁵⁰	3.5 ± 0.8	7.9 ± 0.8
P-801 ⁵⁰⁰	6.7 ± 0.8	5.9 ± 0.6
GR ¹⁰⁰	2.6 ± 0.9	4.3 ± 0.6
GR ²⁵⁰	7.1 ± 1.2	7.7 ± 0.5
SYM ²⁵⁰	3.3 ± 0.9	4.2 ± 1.7
SYM ⁵⁰⁰	3.9 ± 0.8	3.6 ± 0.8
Fert [†]	1.9 ± 0.2	5.6 ± 0.9
Control	4.4 ± 0.7	6.8 ± 0.8
Linear orthogonal contrasts		
Inoc. vs Not-Inoc.	ns	ns
Fert. vs Inoc.	**	ns
Low vs High	**	ns

Inoc, inoculated; Not-Inoc., not inoculated

^a Number of applied propagules per tuber. Low stands for the lower and H for the higher number of propagules

^b Values are means $\pm$ SE, $n = 5$. (Note: Each replicate comprised twelve plants). Statistical significance *, $P < 0.05$; **, $P < 0.01$; ns = not significant difference

[†] Only three replicates were available due to mortality

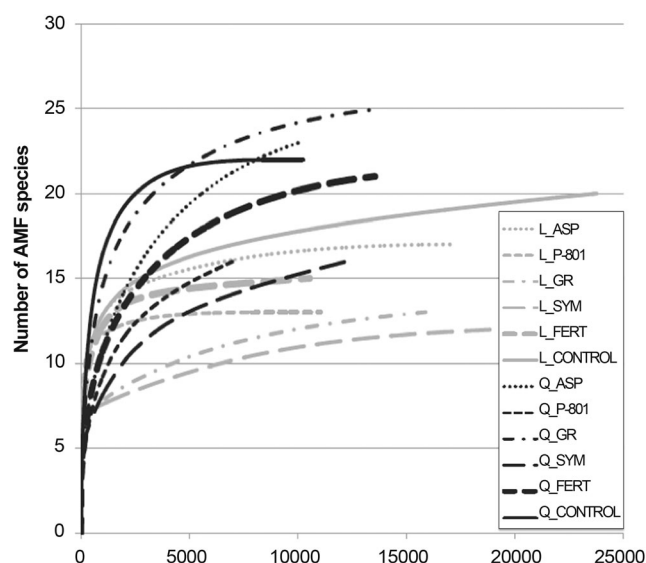


Fig. 1 Rarefaction curves of the number of arbuscular mycorrhizal fungal (AMF) species per treatment and location. L (Loja) and Q (Quito). ASP = Mycorise ASP (liquid); P-801 = Myke Pro P801 (powder); GR = Myke Pro GR (granular); SYM = Symplanta (powder); Fert = chemical fertilizer and Control

Table 3 Frequency of occurrence (FO) of AMF species in Loja and Quito in the inoculated (ASP, P-801, GR and SYM) and not inoculated (Fert and Control) treatments

	Loja							Quito						
	ASP	P-801	GR	SYM	Fert	Control	Total	ASP	P-801	GR	SYM	Fert	Control	Total
<i>Acaulospora</i> sp. (Sp14)	++++	+++	++++	++++	+++	+++	21	++++	++++	++++	++++	++++	++++	24
<i>Acaulospora</i> sp. (Sp15)							0	+			+			2
<i>Acaulospora</i> sp. (Sp21)				+			1	++		+	+	+++	+	8
<i>Acaulospora</i> sp. (Sp23)	++		+		+	++++	8							0
<i>Acaulospora laevis</i> (Sp25)	++		+++	+	+++	++	11	+++	++++	++++	++++	++	++	19
<i>Acaulospora</i> sp. (Sp27)							0	+		+	+	+	+	5
<i>Acaulospora scrobiculata</i> (Sp48)			+				1							0
<i>Acaulospora sieverdingii</i> -like (Sp49)	+++	++		++	+	++	10	+++	+++	+++	+++	+++	+++	18
<i>Acaulospora</i> sp. (Sp50)						++	2							0
<i>Acaulospora</i> sp. (Sp51)							0		+	++	+	+		5
<i>Archaeospora schenkii</i> (Sp52)		++	++				4							0
<i>Archaeospora</i> sp. (Sp53)			+				1							0
<i>Claroideoglossus</i> sp. (Sp07)	+				+		2	+	+	+		+	++	6
<i>Claroideoglossus claroideum</i> (Sp39)		+			+	+++	5	+	+	+	+			4
<i>Claroideoglossus etunicatum</i> (Sp41)	+	+			+	+	4			+				1
<i>Claroideoglossus etunicatum</i> -like (Sp54)	++++	+	+		+	+	8							0
<i>Claroideoglossus</i> sp. (Sp55)	++	+	+		+	+	6							0
<i>Diversispora</i> sp. (Sp56)							0	+					+	2
<i>Diversisporales</i> (Sp57)	+						1	+	++	++	+	+++	++++	13
<i>Funneliformis caledonius</i> (Sp11)						++	2	+	+	+		+	+++	7
<i>Funneliformis mosseae</i> (Sp12)			+			++	3	+	+	+			++	5
<i>Funneliformis</i> sp. (Sp58)					+		1						+	1
<i>Funneliformis</i> sp. (Sp59)					+		1						+	1
<i>Gigaspora margarita</i> (Sp60)							0	+		++			++	5
<i>Gigaspora rosea</i> (Sp61)							0			++		+	+	4
Gigasporaceae (Sp62)							0	+	++	++	+	++	++++	12
<i>Pascispora scintillans</i> (Sp63)					+	++	3				+			1
<i>Paraglossus laccatum</i> -like (Sp20)	+	++		+++	+	+	8	++	+++	++	++	+	++++	14
<i>Paraglossus occultum</i> (Sp64)							0		++	+		+		4
<i>Rhizophagus</i> sp. (Sp08)							0	+		+				2
<i>Rhizophagus irregularis</i> (Sp09)	+++	+	+	++++	+	++	12	++++	++++	++++	++	++++	++++	22
<i>Rhizophagus</i> sp. (Sp29)	+			+		+	3	++		++		++	++	8
<i>Rhizophagus intraradices</i> (Sp32)	++			+			3							0
<i>Rhizophagus invermaium</i> (Sp35)		+	+				2	++		+++		+	+	7
<i>Rhizophagus proliferus</i> (Sp65)	+	+					2							0
<i>Rhizophagus</i> sp. (Sp66)	++			+	+	+	5	++		++	+	+++	+++	11
<i>Scutellospora spinosissima</i> (Sp36)				+			1	++++	+++	++++	+++	++++	++++	22
<i>Scutellospora gilmorei</i> (Sp37)							0	+++	+	++	+++	+++	+++	15
<i>Scutellospora</i> sp. (Sp44)	++	+	+++	+	+	+++	11	+	+	+		+		4
<i>Scutellospora</i> sp. (Sp67)	++	+	+++	++	++	++++	14					+		1

The “+” represents the occurrence of the AMF species in each of the four replicate, except Fert treatment were only 3 replicates were analysed due to plant mortality

Acaulospora sp. (Sp14), followed by *R. irregularis* (Sp09) and *Scutellospora spinosissima* (Sp36) and *A. laevis* (Sp25) (Table 3). The FO of AMF species did not differ markedly

among inoculated and not inoculated treatments. However, the genus *Funneliformis* was found more frequently in the Control treatment in both localities (Table 3).

Based on the RA, the most abundant genera in Loja were in decreasing order *Acaulospora*, *Rhizophagus* and *Scutellospora* (Fig. 2a). Among these, *Acaulospora* sp. (Sp14) was the most abundant species representing 44% of the overall AMF sequences. *Rhizophagus irregularis* accounted for 13% of the overall AMF sequences. The number of AMF species among treatments ranged from 12 to 25 with the highest number of species in the Control (see Fig. 2a, b). In Quito the most abundant genera were *Acaulospora* and *Scutellospora*. The most abundant species was also *Acaulospora* sp. (Sp14), representing 61% of the overall AMF sequences, *Rhizophagus irregularis* represented only 0.3% of the total sequences. The number of AMF species in the treatments ranged between 16 and 28 (see Fig. 2c, d). The RA in inoculated and not inoculated treatments in Quito displayed a similar pattern, suggesting an even distribution of the AMF species in this site. In Loja the different patterns in the RA suggests an uneven distribution of the AMF species in the plot (Fig. 2a). Considering both the FO and RA, the most abundant AMF colonizers in Loja were *Acaulospora* sp. (Sp14), *R. irregularis* (Sp09) and *Scutellospora* sp. (Sp67). Sequences of *R. irregularis* were found in three (ASP, P-801 and SYM) out of the four inoculated treatments. However, a considerable number of sequences were also found in the Control treatment indicating that native *R. irregularis* strains were present in the plot. In Quito the most abundant colonizers were *Acaulospora* sp. (Sp14), *A. laevis* (Sp25) and *S. spinosissima* (Sp36). Sequences of *R. irregularis* were also found in all the treatments (either inoculated or not) but at very low rates. Contrary to what we expected after inoculation of

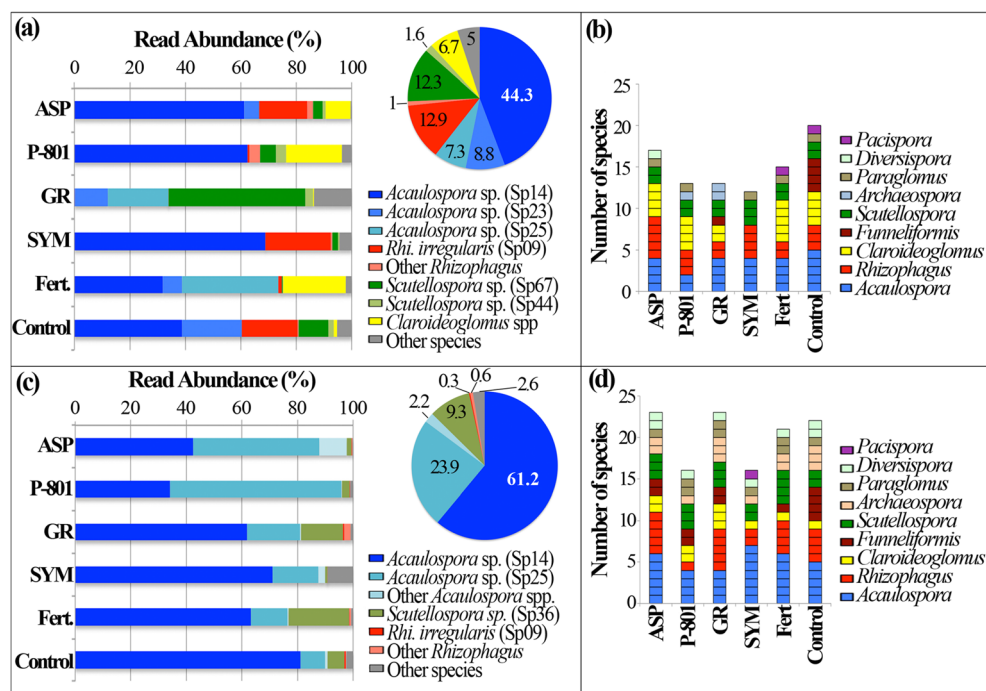
potato with *R. irregularis* DAOM 197198, the overall RA of this species showed no differences among inoculated and not inoculated treatments, neither in Loja nor in Quito. The PCA shows that the AMF community composition is different in Loja and Quito but no strong differences related to the controls and treatments are observed (Fig. 3).

4 Discussion

It is well known that potato plants need a high supply of N, P, K, Ca, Mg and S for a good development, which are often applied as inorganic fertilizers (White et al. 2007). However, the efficiency of potato plants to recover these nutrients from the soil is relatively low (Struik 2007). Therefore, inoculation with appropriate AMF is seen as an eco-efficient alternative to decrease high-inputs of chemical fertilizers (Cheng et al. 2008; Douds et al. 2007; Hijri 2015).

In the present study, no significant increase of plant growth or yield was detected with any of the inoculants or their concentrations. However, the analyses of roots revealed low levels of %RLC of inoculated plants in both localities, not significantly different from the not inoculated controls. Overall %RLC levels were comparable with those reported in other field studies (e.g. Cesaro et al. 2008; Douds et al. 2007; Tambascio et al. 2014). Hundreds of studies indubitably demonstrate the benefits of AMF on mycorrhizal plants compared with non-mycorrhizal plants under sterile soil conditions. However, at field level AMF inoculation has displayed contrasting results (Rodríguez and Sanders 2015). Indeed,

Fig. 2 Bars in figures (a) and c represent the relative read abundance of the most abundant AMF species found in the six treatments (ASP, P-801, GR, SYM, Fert and Control) in Loja and Quito, respectively. Each treatment consisted of four replicates, except Fert treatment in Loja were only 3 replicates were available. In the inserted pie graph the overall relative read abundance in percentage of each of these species is represented. Figures (b) and d represent the number of AMF genera and species found per treatment in Loja and Quito, respectively



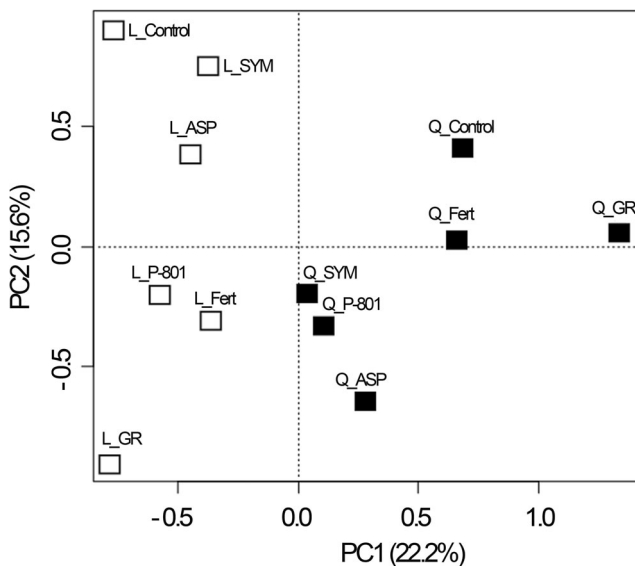


Fig. 3 Principal component analysis based on Euclidean distances of read abundance of the AMF community in Loja (open squares) and Quito (filled squares) under the six different treatments. ASP = Mycorise ASP (liquid); P-801 = Myke Pro P-801 (powder); GR = Myke Pro GR (granular); SYM = Symplanta (powder); Fert = chemical fertilizer

potato responses to inoculation in the field ranged from neutral (e.g. Cuenca et al. 2008; Velivelli et al. 2015) to positive (e.g. Cheng et al. 2008; Douds et al. 2007; Hijri 2015). The different experimental conditions make comparisons difficult and impossible to draw a single conclusion. A recent meta-analysis of 231 trials in Canada and Europe where potato was inoculated with *R. irregularis* strain DAOM 197198 under field conditions showed a significant increase of marketable potato yield in most of the trials (Hijri 2015). We can speculate that certain experimental conditions such as inoculation technique, nutrient-rich soils with high organic content, interaction between inoculum and local AMF communities or agricultural practices (i.e. frequent fungicides application), could have negatively impacted the establishment of the inocula.

4.1 Inoculation technique

One expectation for AMF inoculation is an increase of root colonization in inoculated plants (e.g. Farmer et al. 2007; White et al. 2008). We hypothesized that the apparent lack of effect of inoculation on %RLC could have several reasons. For instance, it is possible that %RLC increases were most evident in the first weeks/months after inoculation and decreased afterwards. It is also possible that placing the *R. irregularis* propagules in the planting hole underneath the tuber could be not an appropriate inoculation method for the developing roots to come in contact with the germinating hyphae as spores can be easily runoff with watering or rain events. A recent paper showed that spraying the AM inoculum

on potato tubers before planting was effective to promote marketable tuber yields. However, no information on the percentage of the AMF root colonization of inoculated plants or soil-nutrient contents were reported (Hijri 2015). In those experiments a liquid formulation of *R. irregularis* strain DAOM 197198, Myke® Pro Potato-L (Premier Tech Biotechnologies, Rivière-du-Loup, QC) was sprayed directly onto the tubers before planting at a rate of 70 spores per tuber. Although in our experiment we did not use Myke® Pro Potato-L (because it was not available at that time) we used 4 different formulations of the same AMF strain including a liquid formulation similar to Myke® Pro Potato-L.

4.2 Soil fertility/soil nutrient content

Soil analyses of the experimental plots revealed high nutrient contents and organic matter. Nutrient-rich soils have been reported to inhibit root colonization and hence the effects of AMF inoculation (Smith and Read 2008). The addition of chicken manure at planting could have also impacted negatively the establishment of the inocula by increasing the nutrient availability and decreasing the soil pH (e.g. Carreón-Abud et al. 2015).

4.3 Tracing of inoculated strains/interaction between inoculum and local AMF communities

Despite the low levels of AMF root colonization a diverse community of AMF species was found with a clear dominance of the genus *Acaulospora*. Early studies, mostly from temperate regions, reported members of the *Glomeraceae* as the most prevalent in AMF communities from agricultural fields (Borriello et al. 2012; Sasvári et al. 2011; Wang et al. 2008). However, this information should be taken with caution as differences in AMF communities and methodological biases towards PCR-amplification of members of *Glomeraceae* existed in some earlier studies (Kohout et al. 2014). In another study on potato fields from Italy, *R. irregularis* (as *Glomus intraradices*) was the most frequently detected species (Cesaro et al. 2008). In a recent survey in the Andes of Ecuador, Bolivia and Peru, the community composition of AMF associated with potato was assessed through 454 GS FLX+ sequencing, resulting in 41 AMF species identified (Senés-Guerrero and Schüssler 2016). A conserved core of species belonging to *Acaulospora*, *Cetranspora*, *Claroideoglomus* and *Rhizophagus* were found to simultaneously colonize 67% of all studied potato plants, representing the dominant AMF taxa under those environments. We found some clear similarities with the above-mentioned study. For instance, the same unknown *Acaulospora* sp. (Sp14) was found as the major colonizer of potato occurring in most of the root samples at very high frequencies (FO) and relative read abundances (RA). This

could indicate a possible host preference of this species under Andean edapho-climatic conditions. However, the genus *Cetraspora* reported by Senés-Guerrero and Schüßler (2016) were low or absent in our samples. Instead, the species *Scutellospora* sp. (Sp67) and *A. laevis* (Sp25) in Loja and *A. laevis* (Sp25) and *S. spinosissima* (Sp36) in Quito were found at high FO and relative RA rates. These differences could not be ascribed to a bias in the sampling strategy or sampling processing as the same procedures were used in both studies. Instead, differences may be related to soil characteristics, geography, and geographical distance, which have been shown to affect AMF communities (Jansa et al. 2014) even at small-scale distances (Mummey and Rillig 2008; Wolfe et al. 2007). The genus *Acaulospora* appears to be very widespread in this agro-ecosystem (Senés-Guerrero and Schüßler 2016).

In Loja, the figures representing the FO and RA of the AMF species showed dissimilar patterns of the composition of the AMF communities among treatments. These differences could be partially explained by the history of the plot that was left in fallow for one year before the establishment of the experiment allowing the growth of a diverse type of weeds. In Quito, the patterns of FO and RA of each AMF species were more evenly distributed among treatments. This plot was cultivated with wheat the preceding season. The lack of differences in the %RLC of plants among inoculated and not inoculated treatments in Quito indicates that the establishment of *R. irregularis* DAOM 197198 was rare or even absent.

The widespread occurrence of *R. irregularis* (Berruti et al. 2014; Kivlin et al. 2011) makes it difficult to separate the effects of native strains from those of the inoculated ones or to distinguish increases in frequency and abundance of certain AMF strains following inoculation. The tracing of inoculated strains and their potential impact on the native AMF communities has been rarely studied because it can be difficult to discriminate strains using a single molecular marker. Microsatellite simple sequence repeats (SSR) have been used to identify *R. irregularis* strains under in vitro culture conditions but not in natural habitats (Mathimaran et al. 2008), whereas under field conditions they could be identified by using the *mtLSU rRNA* gene (Börstler et al. 2008, 2010; Peyret-Guzzon et al. 2016; Sýkorová et al. 2012). However only *R. irregularis* isolates can currently be discriminated by *mtLSU* markers (Formey et al. 2012) and there is a limited amount of published sequence data for comparison (Thiéry 2010). For applied studies, tracing AMF introduced as inocula in field experiments is crucial and analysing such data with high throughput methods would be ideal to obtain vast quantities of information (Schlaeppli et al. 2016).

In this study DNA-based monitoring showed that *R. irregularis* must have been part of the native AMF

community in Loja and Quito, since it occurred independently of whether it was inoculated or not. Although, using 454 GS-FLX+ sequencing does not allow to distinguish the inoculated *R. irregularis* strain from the native ones, this approach has the advantage of including the analysis of the native AMF community composition at the species level, which provides information on whether the inoculated AMF species was already part of the native AMF and it allows to trace strains in an indirect manner if the introduced fungus can be qualitatively (species not present in native community) or quantitatively (species abundance increased by inoculation) distinguished from the native population. Farmer et al. (2007) reported that the frequency of occurrence of inoculated AMF species can be increased in roots by inoculation, yet, in the present study there was no increase neither of FO nor RA of *R. irregularis* in the inoculated compared to the not inoculated plants. By using mitochondrial large subunit ribosomal DNA (*mtLSU*) marker Sýkorová et al. (2012) could detect an introduced *R. irregularis* haplotype in the roots of *Phalaris arundinacea* several months after inoculation. This can suggest a slow establishment of the AMF to adapt to the local conditions. For that reason, lacking effects after inoculation could also be due to a slow establishment of the strain.

In conclusion, despite the lack of effect on potato performance by *R. irregularis* inoculation we found a diverse AMF community associated with potato plants with some similarities between both experimental plots and also earlier studies, such as the clear dominance of the species *Acaulospora* sp. (Sp.14). Although, *R. irregularis* was frequently found also in roots of not inoculated controls, its relative read abundance was low and not different from the not inoculated plants. This indicates that the inoculated AMF strain was rare or absent in the roots. Competition with the native AMF community, poor adaptation to the biotic or abiotic conditions or inappropriate placement of the inocula could have led to the observed outcomes.

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