Native mycorrhization of onion in response to the application of bioinoculants, inorganic fertilization, and water deficit

Micorrización nativa de la cebolla en respuesta a la aplicación de bioinoculantes, fertilización inorgánica y déficit hídrico

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ABSTRACT

Arbuscular mycorrhizal fungi are important components of the soil microbiota which interact with other beneficial microorganisms in the rhizosphere. The synergistic effect of this naturally occurring association between the soil and introduced microorganisms to improve growth and to cope with biotic and abiotic stresses plays a key role in crop productivity. In this research, the native mycorrhizal colonization of onion (*Allium cepa* L.) was evaluated in the presence of a plant growth promoting microbial consortium (MC), under inorganic fertilization and water deficit regimes, in a semiarid region of the Venezuelan Andes. The main objectives were to determine the colonization potential of arbuscular mycorrhizae and to quantify the presence of mycorrhizal spores in soil under field conditions. An onion crop was established with normal irrigation (NIr) (100% ETc) and water deficit (WD) (67% ETc), with different fertilization treatments (MC with 0%, 50%, and 100% NPK). Native mycorrhizal colonization was determined by counting vesicles and arbuscules in the roots of onion plants in all treatments, and mycorrhizal spores in the soil of the experimental plot were quantified before and after the trial in the rhizospheric soil for each treatment. The treatments showed no significant differences in native mycorrhizal colonization, but the soil spore count was higher in the MC 100% NPK NIr treatment. Additionally, the mechanization of the plot significantly reduced the presence of mycorrhizae in the soil, suggesting that greater implementation of non-conventional practices could improve preservation of biodiversity and increase soil health through agricultural management.

Key words: *Allium cepa*, mycorrhizae, plant-microorganism interactions, plant growth promoting microorganisms-PGPM, water stress.

RESUMEN

Los hongos micorrízicos arbusculares son componentes importantes de la microbiota del suelo e interactúan con otros microorganismos benéficos en la rizósfera. El efecto sinérgico de esta asociación que se produce de forma natural en el suelo y los microorganismos introducidos para mejorar el crecimiento, combatir el estrés biótico y abiótico desempeña un papel clave en la productividad de los cultivos. En esta investigación se evaluó la colonización micorrízica nativa de la cebolla (*Allium cepa* L.) en presencia de un consorcio microbiano (CM) promotor del crecimiento vegetal, bajo un régimen de fertilización inorgánica y déficit hídrico, en una zona de cultivo semiárida en los Andes venezolanos. Los objetivos principales fueron determinar la colonización de micorrizas arbusculares y cuantificar la presencia de esporas micorrízicas en el suelo en condiciones de campo. Se estableció un cultivo de cebolla con riego normal (IrN) (100% ETc) y déficit hídrico (DH) (67% ETc) y diferentes tratamientos de fertilización NPK con y sin el consorcio microbiano CM (CM con 0%, 50% y 100% NPK). Se determinó la colonización de las micorrizas nativas mediante el conteo de vesículas y arbúsculos en las raíces de las plantas de cebolla en todos los tratamientos, y se cuantificaron las esporas micorrízicas en el suelo de la parcela experimental antes de establecer el ensayo, y luego del ensayo en el suelo rizosférico para cada tratamiento. Los resultados no mostraron diferencias significativas entre los tratamientos en cuanto a la colonización micorrízica nativa, pero el conteo de esporas en suelo resultó mayor en el tratamiento 100% NPK CM IrN. Se determinó que la mecanización de la parcela redujo significativamente la presencia de micorrizas en el suelo, sugiriendo que una mayor implementación de prácticas no convencionales preserva la biodiversidad e incrementa la salud de los suelos con manejo agrícola.

Palabras clave: *Allium cepa,* micorrizas, interacción planta-microorganismo, microorganismos promotores del crecimiento vegetal-MPCV, estrés hídrico.

Received for publication: October 31, 2024. Accepted for publication: December 21, 2024. Doi: 10.15446/agron.colomb.v42n3.117329

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Introduction

Mycorrhizae are symbiotic associations generated between certain fungi and plant organs which are, in most cases, roots (Huey *et al*., 2020). This association increases the surface area of the roots, allowing the plants to absorb water and nutrients more efficiently from a large volume of soil (Sun *et al*., 2018).

Arbuscular mycorrhizal fungi (AMF) aid plants to establish and survive in unfavorable environments by different means, inducing greater tolerance of the roots to pathogens by acting directly as protectors of the root system (Liu *et al*., 2018), improving plant mineral nutrition and increasing plant biomass, and by improving plant-water relations, since mycorrhizae enhance resistance to water stress (Eroğlu *et al*., 2020). For these reasons, AMF inoculation is being incorporated as a strategy applied to agroforestry and silvicultural practices to colonize the root-soil environment, improve plant growth, yield, nutrient content, and soil fertility (Jacott *et al*., 2017).

Plant growth-promoting microorganisms (PGPM) in the rhizosphere are beneficial bacteria and fungi of great importance in Agronomy, due to their prominent role in sustainable agricultural practices (Brauer *et al*., 2019). Plant growth-promoting rhizobacteria (PGPR) help to improve plant growth and health in various ways (Dheeman & Maheshwari, 2022). The direct mechanisms involve the availability of mineral nutrients and production of phytohormones that contribute to plant growth and adaptation (Shome *et al*., 2022). PGPR can also act indirectly by eliminating or reducing the load of phytopathogens through biocontrol activity, *i.e*., secreting various growth inhibitors such as lytic enzymes, bacteriocins, and antibiotics or by inducing natural resistance of the host plants (Ehinmitan *et al*., 2024).

The coexistence of PGPM and AMF in the rhizosphere is highly beneficial for growth and development of most plants (Hashem *et al*., 2016). This synergistic effect is the result of positive interactions between these microorganisms, playing an important role in mitigating a wide range of biotic and abiotic plant stresses such as drought, salinity, heavy metal exposure and pathogens, in addition to increasing plant growth and yield (Soussani *et al*., 2023). Therefore, the evaluation of the inoculation of these beneficial microorganisms represents a favorable option for the sustainable management of ecosystems (Chamkhi *et al*., 2022). Their greatest potential lies in directly influencing the reduction of the use of pesticides and chemical fertilizers that harm the environment (Gupta, 2020).

Onion cultivation may require a great investment due to the high cost of certified seeds, industrial fertilizers containing NPK and agrochemicals and other inputs to maintain its production cycle. This means that its marketing costs could be excessive. In turn, these practices have significantly modified the ecological balance of soils and have altered soil microbial populations, acidified or increased the salt content, and decreased organic matter content in soil (Wolińska *et al*., 2017). The indiscriminate application of inorganic products to soil could be reduced by using microorganisms with biofertilizing, biocontrolling and biostimulating characteristics, which would enable sustainable crop production with the least use of chemical compounds (Blanco & Castro, 2021). In onions, inoculation with *Azospirillum* and mycorrhizae in combination with inorganic NPK fertilizer increased bulb diameter, bulb dry weight, total dry weight per plant, and bulb yield, as well as improved the cost-benefit ratio (Singh *et al*., 2017). Similarly, inoculation with two mycorrhizal species (*Funneliformis mosseae* and *Claroideoglomus etunicatum*) under drought conditions increased the content of proline, soluble proteins, and total carotenoids in plants (Muhsen *et al*., 2019). Therefore, it is important to determine how other native beneficial microorganisms (mycorrhizae) influence these processes and determine the effects they exert on the soil flora to improve their growth.

For the above reasons, our hypotheses were: 1) the growth of native mycorrhizae in soil increases in the presence of a bioinoculant and with a lower dose of industrial fertilizers, as this growth would be stimulated by the synergistic action of the beneficial microbial consortium (MC), and the colonization process would not be inhibited by the presence of agrochemicals; 2) native mycorrhization improves under conditions of water deficit, due to enhanced root growth, which enables plants to explore a greater soil volume for water and mineral nutrients. To validate these hypotheses, the aim of our study was to determine the native mycorrhizal colonization of onion (*Allium cepa* L.) in the presence of a native plant growth-promoting MC of the Venezuelan Andes, under an inorganic fertilization regime and water deficit conditions in a semiarid region. Based on previous research, we aimed to determine whether there was a synergy between the PGPMs used and the native mycorrhizae of the experimental plot and whether this influenced the favorable effects of MC on the onion crop. This will allow us to stimulate the preservation of biodiversity and increase the quality of soils through agricultural management.

Materials and methods

Study site and experiment design

The field experiment was carried out from February to mid-June 2017 at the IIAP-ULA Experimental Station in San Juan de Lagunillas, Mérida, Venezuela, at 1,100 m a.s.l. (UTM coordinates 8º30'75'' N; 71º20'28'' W). The soil of the plot corresponded to an Aridisol (USDA, 2020). The experimental site was characterized by an average rainfall of 509 mm, with two maximum peaks in April and October, average temperature of 23.7ºC, annual evaporation around 1,500 mm, vastly exceeding precipitation, so that crops require irrigation year-round (Blanco, Rada, Paolini *et al*., 2021). The soil characteristics (15-cm depth) were: sandy loam texture, water retention capacity 27%, organic matter 3.6%, pH 6.0, electrical conductivity 0.14 mS cm^{-1}, total N 0.12%, available P 30 mg kg^{-1}, K 568 mg kg^{-1}, Ca 2060 mg kg^{-1} and Mg 1872 mg kg^{-1} (Blanco, Rada, Paolini *et al*., 2021). The experimental plot was prepared by mechanization through disc plow and harrow passes, and the application of a pre-emergent and selective herbicide (ai: S-metolachlor) at a dose of 2 L ha^{-1} for weed control, one month before seedling transplant. Weeding during the trial was done manually, and irrigation was localized. An onion hybrid, F1 2000, was used, and seedbeds were prepared as described by Blanco, Rada, Paolini *et al*. (2021). These plants were transplanted 45 d after sowing (das) in six rows, for a total of 120 plants per bed and corresponding to a density of 360,000 plants ha^{-1}. Seedlings were planted in 2.4 m long x 1.4 m wide beds (3.36 m^2), spatially separated from each other by a trough to avoid contamination between treatments.

Microbial inoculum

The bioinoculant used corresponded to a microbial consortium (MC) of the strains ME01 (*Rhizobium tropici*) + Leu2A (*Bradyrhizobium japonicum*) selected for their plant growth-promoting effects on *A. cepa* seedlings (Blanco, Rada, Castro *et al*., 2021). The bioinoculated treatments received 2.5 ml of the MC ($1x10^8$ cells ml^{-1}) per seedling on day of transplant, and were reinoculated with 5 ml of the inoculum at the same concentration in the field, 15 d after transplant (dat). The applied MC belonged to the strain collection of the Laboratorio de Fitobiotecnología of the Universidad de Los Andes, Venezuela.

Experimental design

A 2×4 bifactorial design in completely randomized blocks with 3 replicates was used in the experiment. The irrigation factor consisted of two frequencies which corresponded to a given evapotranspiration value (ETc): NIr (daily irrigation; 100% ETc) and WD (irrigation every 3 d; 67% ETc) beginning 20 dat, this latter frequency corresponded to severe water stress (Blanco, Rada, Paolini *et al*., 2021). The fertilization factor consisted of four levels referenced to 100% of the nutrient requirement of NPK for onion yielding 40 t ha^{-1}. For this reference, net extraction per ha was 247 kg N, 240 kg P_2O_5 and 240 kg K_2O, which corresponded to 577 g of inorganic fertilizer with a commercial formula 15-15-15 NPK (ammoniacal N 10.3%, nitric N 4.7%; assimilable phosphorus 15%; potassium chloride 15%) plus 5.4 g of urea for each bed (3.36 m^2) (Blanco, Rada, Paolini *et al*., 2021). These fertilization rates were combined with the application of the MC biofertilizer, resulting in treatments as follows: MC+0% NPK, MC+50% NPK, MC+100% NPK, and 100% NPK (non-inoculated control). The fertilization schedule is given in Blanco, Rada, Paolini *et al*. (2021).

Native mycorrhizal colonization of onion roots

Root sampling of *A. cepa* plants was carried out during the experiment to evaluate the percentage of native colonization and the presence and number of mycorrhizal spores in the rhizospheric soil. For this, root sampling was carried out according to Sánchez de Prager (1999). Four plants were chosen at random, and root samples were collected from the first 10 cm, placed in polyethylene bags, and taken to the laboratory, where they were washed with distilled water to eliminate any rhizospheric soil residues.

Roots were stained using the procedure described by Phillips and Hayman (1970), which consisted of five steps to obtain an ideal staining and observe the mycorrhization within the roots: 1) thinning of the previously washed rootlets: very thin rootlets were placed in test tubes and 10% KOH was added to cover them and heated for 10 min in boiling water. Afterwards, the KOH was removed, and the rootlets were washed five consecutive times with distilled water; 2) bleaching of the roots: 10% H_2O_2 was added to cover the roots for 3 min, then removed, followed by seven consecutive washes with distilled water until the excess was removed; 3) acidification: the roots were covered with 10% HCl for 3 min at room temperature, and then washed five consecutive times until the acid was completely removed according to Sánchez de Prager (1999); 4) staining the roots: the roots were covered for 10 min with trypan blue (0.05%) and then heated in a boiling water bath for 15 min. The dye was then removed without rinsing; 5) decolorization of the roots: the roots were treated with lactoglycerol (lactic acid, glycerol, and water in a 1:1:1 ratio) and processed to determine the percentage of mycorrhizal colonization.

To determine the percentage of mycorrhizal colonization, stained roots were mounted on slides for evaluation under an optical microscope (McGonigle *et al*., 1990). The roots were placed in Petri dishes for better handling and eight segments approximately 1 cm long were taken using dissection needles and placed parallel to each other on a slide. Drops of clean lactoglycerol were added to keep the roots moist and preserve them for a longer time. A coverslip was placed on each slide, air bubbles were removed, and the edges were sealed with nail polish, allowing them to dry for 5 min. Finally, three parallel and equidistant lines were marked on the back of the slide, perpendicular to the rootlets to be examined under the microscope. At each intersection between the lines and the rootlets, the presence of vesicles and/or arbuscules was recorded, regardless of the intensity of mycorrhization. A value of one (1) was given for the presence and zero (0) for the absence of these structures. Colonization was determined with the following equations:

$$\text{Vesicular colonization (\%)} = \frac{\text{Nº of segments with vesicles}}{\text{Total Nº of segments}} \times 100 \quad (1)$$

$$\text{Arbuscular colonization (\%)} = \frac{\text{Nº of segments with arbuscles}}{\text{Total Nº of segments}} \times 100 \quad (2)$$

Quantification of mycorrhizal spores in the soil

For the isolation and quantification of mycorrhizal spores in the rhizosphere, soil from the chosen plants was used to determine native mycorrhizal colonization of the roots, following the method described by Sánchez de Prager (1999). The soil was obtained by inserting a gardening shovel vertically adjacent to the bulb, at a depth of approximately 4-8 cm. Using these subsamples for each treatment and replicate, a composite sample of more than 100 g was prepared and placed in previously labeled polyethylene bags, transported in ice containers to the laboratory and kept at a temperature of approximately 6ºC until processed.

For the separation of spores from the soil, a centrifugation in sucrose method was used (Sieverding, 1991). One hundred g of rhizospheric soil were introduced into a beaker with approximately 2,000 ml of distilled water. The sample was stirred on a stirring plate for 5 min and left to rest for 3 min to eliminate large particles by sedimentation. The suspension was passed through a series of sieves between 500 and 44 µm and washed with plenty of water. This process was repeated twice consecutively to collect the spores on a 44 µm sieve. The spores were transferred to a 50 ml centrifuge tube, and the volume was completed to 20 ml of distilled water. The sample was then centrifuged at 1800 rpm for 2 min to eliminate the supernatant. Twenty ml of distilled water and 20 ml of a 50% sucrose suspension were injected into the bottom of the centrifuge tube, taking care not to disturb the gradient, and again centrifuged at 3500 rpm for 2 min until a central ring formed within this gradient. Spores were extracted from this ring using 5 ml pipettes. They were placed on the 44 µm sieve, washed thoroughly with sterile distilled water and placed on a Doncaster plate to quantify them in a stereoscopic microscope (Thz75 Leica model 1446275). Additionally, spore counts were performed in the non-rhizospheric soil (before the test) and in non-rhizospheric soil without fertilizers or inoculate under the two irrigation regimes (after the test) to determine the effect of mechanization of the plot on the presence of mycorrhizae.

Statistical analysis

The results were analyzed by analyses of variance (ANOVA) and least significant difference test (LSD test) (P<0.05) using the statistical package Statgraphics Centurion XVI (2009). Correlations between the percentage of vesicle colonization and the number of spores were established with the statistical program PAST version 3.0 (Hammer *et al*., 2001).

Results

Percentage of vesicle colonization in roots

The results of mycorrhizal colonization showed only the presence of endomycorrhizal vesicles; no arbuscules were present in any of the samples. No significant differences were found between treatments for the presence of vesicles (P>0.05). However, the LSD test showed a slight tendency for a higher percentage of colonization in the roots in the 50% NPK + MC treatment under both irrigation conditions compared to the other treatments. The highest percentage (89.9%) corresponded to the 50% NPK + MC (WD) treatment, while the lowest (73.6%) corresponded to the 100% NPK + MC (WD) treatment (Fig. 1).

Spore quantification

The number of spores found after screening the soils is shown in Figure 2. The highest spore count (53.07%) was obtained in the 100% NPK + MC treatment under normal irrigation, while the lowest value (10%) was observed in the 0% NPK + MC and 100% non-inoculated NPK treatments (production control) under the same irrigation conditions. Significant differences were found between the eight treatments analyzed, with a greater number of spores in the soil

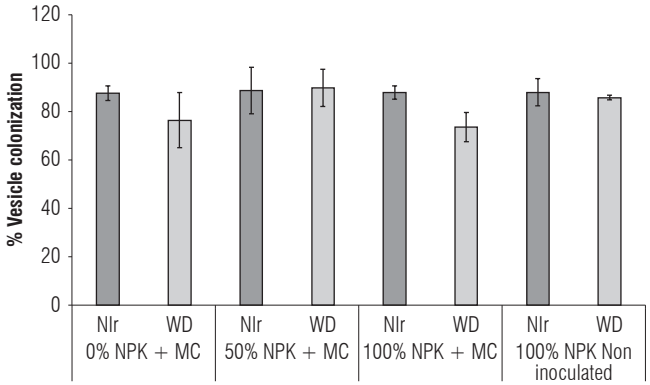

FIGURE 1. Percentage of vesicle colonization in *A. cepa* roots subjected to different treatments of inorganic NPK fertilization + MC (ME01+Leu2A (*Rhizobium tropici* + *Bradyrhizobium japonicum*) under water deficit conditions. NIr = normal irrigation 100% ETc, WD = water deficit 67% ETc. MC = microbial consortium. Error bars indicate the standard deviation.

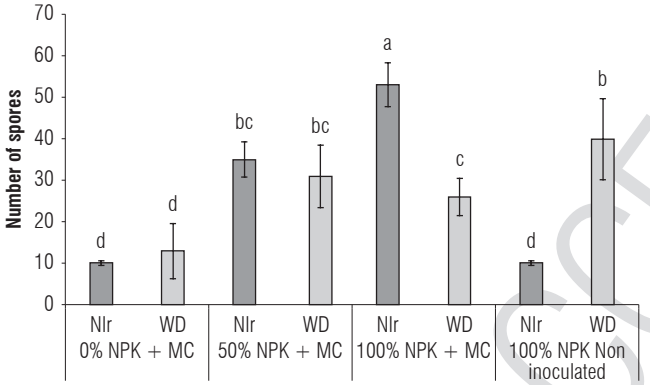

FIGURE 2. Number of spores per 100 g of rhizospheric soil of *A. cepa* for the different treatments of inorganic NPK fertilization and inoculation with biofertilizers under conditions of water deficit. MC = microbial consortium, NIr = normal irrigation, WD = water deficit. The percentage represents the applied dose of NPK fertilizer. Mean values (n=3). Different letters indicate statistically significant differences between treatments and conditions at a confidence level of 95% according to the LSD test. Error bars indicate the standard deviation.

under conditions of complete fertilization, inoculation with MC and normal irrigation.

No correlation was found between the percentages of root colonization and the number of mycorrhizal spores (P>0.05). Additionally, Figure 3 shows the spores isolated from the soil, as observed under the stereoscopic microscope (Figs. 3A-B), along with the vesicles observed on the onion roots (Fig. 3C). No arbuscules were observed in the root tissue.

Effects of mechanization on soil mycorrhizal populations

Figure 4 shows the number of mycorrhizal spores one month before the trial, when no mechanization had been

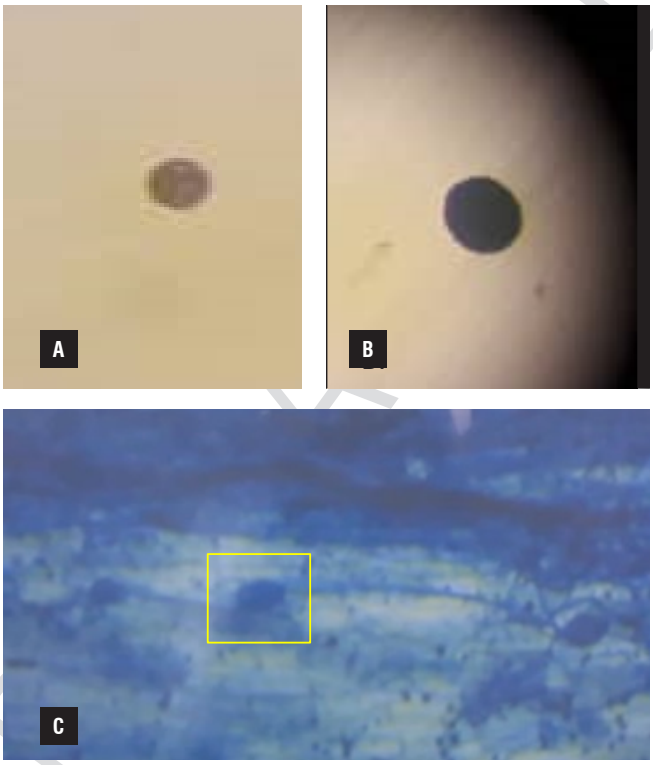

FIGURE 3. Images of mycorrhizal structures observed in the soil and roots of *A. cepa*. A and B) soil spores observed under a 500X stereoscopic microscope, C) vesicles in root tissue observed under a 400X microscope.

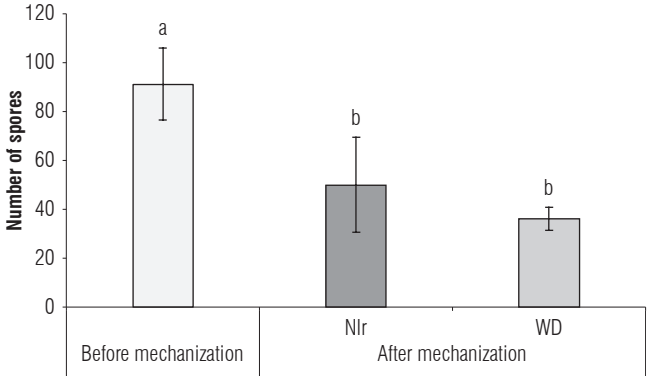

FIGURE 4. Number of spores per 100 g of non rhizospheric soil prior to the mechanization (reference values) and after mechanization (0% NPK Non inoculated) under irrigation conditions: Nir = normal irrigation, WD = water deficit. Mean values (n=3). Different letters indicate statistically significant differences at a 95% confidence level according to the LSD test. Error bars indicate the standard deviation.

applied to the plot, and after the trial (65 dat), at the end of the *A. cepa* bulbing stage. A decrease of 41.94% in the number of spores under NIr and 56.07% under WD conditions was detected after mechanization of the land, which implies an almost 50% reduction in the population of mycorrhizae in the soil due to the plowing prior to the trial.

Discussion

Tolerance to water stress is an important issue for crops, especially in arid environments. Onion cultivation is severely affected by periods of drought, which can reduce growth, yield and quality parameters (Bolandnazar *et al*., 2007). In this study, the WD condition used did not show a significant negative effect between treatments or on the presence of endomycorrhizal vesicles in onion plants. The absence of significant differences in the colonization percentage suggests that the positive effect on onion growth and development in the 50% NPK + CM treatments with respect to yield, CO_2 assimilation rate, leaf N content, etc., as shown by Blanco, Rada, Paolini *et al*. (2021) and Blanco, Rada, and Paolini (2023), was due solely to the presence of MC and not to a symbiosis between the beneficial bacteria in the MC and native mycorrhizae. According to Álvarez and Reyes (2018), effective mycorrhization during crop establishment depends on greater mycotrophy by AMF, which in our study did not occur for the F1 2000 hybrid onion. In another study with the pink Creole variety, mycorrhizal colonization with the applied mycorrhizal inoculum increased by more than 400% over the non-inoculated treatments, even though these latter treatments also showed smaller quantities of mycorrhizal hyphae. This suggests that mycorrhizal populations are naturally in the soil (Arandia *et al*., 2020) and can colonize this species, as shown in our study.

The highest number of spores occurred in the 100% NPK + MC treatment under the NIr condition, a treatment that had neither water nor nutrient deficit and was enriched with the inoculation of MC. The higher number of spores present in this treatment show that their germination could have been inhibited due to the full dose of fertilizers plus MC, which caused toxicity on the microbial metabolism, as demonstrated by Blanco (2021) during the determination of microbial activity variables in the soil in this same field trial.

According to our results, the native mycorrhizal population in the soil was considerably affected by field mechanization. Bowles *et al*. (2017) showed that no-plowing enhanced AMF activity and diversity by improving soil fertility, water storage and conservation, while reducing soil erosion. In contrast, long-term conventional plowing decreased AMF richness and induced a marked alteration in community composition by changing the functional quality of AMF, spore density, vesicles, or hyphal networks (Schalamuk *et al*., 2004; Zhang *et al*., 2015). The decrease in the number of spores in soil after field mechanization suggests, as reported in other studies, that soil mechanization negatively affects the native mycorrhizal population. Furthermore, the agronomic management of the plot between mechanization and the establishment of the trial could have inhibited the reestablishment of the native mycorrhizal population, since the soil was kept bare and herbicides were applied (Blanco, 2021). However, spores may be more resistant than other propagules and could remain in the soil longer throughout the crop cycle, compared to vesicles, whose main function is storage (Zangaro *et al*. 2012). According to Cuenca (2015), identifying a mycorrhizal species solely based on the morphology of the spores is insufficient; molecular information is needed for correct classification. Therefore, we choose not to suggest any genus for the spores observed in our study.

Several studies have reported the ability of AMF to enhance plant growth and yield under stress, promoting tolerance to adverse conditions through mechanisms such as improving water use efficiency and nutrient acquisition through the production of hormones and plant growth regulators, enhancing photosynthetic rate, regulating ionic balance, and producing antioxidants (Campanelli *et al*., 2013; Huey *et al*., 2020; Nasslahsen *et al*., 2022; Wahab *et al*., 2023). However, in our study, the interaction between native mycorrhizae and *A. cepa* was not significant in influencing production, as evidenced by the similarities found between the treatments with respect to the colonization. This indicates that there was no synergy between MC and native mycorrhizae, which also depends on the characteristics of the soil, especially its nutritional conditions. In our case, the soil was fertile, with high native biological activity (Blanco, 2021). The main effect of the synergism between mycorrhizae and PGPB is to enhance a generalized stimulation of the host's nutrition, although more local effects may occur at the root level, especially during the pre-colonization stage, when the two microorganisms interact as rhizospheric residents, or during the development of the tripartite symbiosis. The host genotype also influences this interaction (Spagnoletti *et al*., 2013).

Conclusions

The study showed that the effect of native mycorrhization of onion plants was not significant for the variables evaluated, but it confirmed that the positive effect of microorganisms on onion growth shown previously in the same field trial was due only to the action of the inoculated MC (*Rhizobium tropici* + *Bradyrhizobium japonicum*) and not to the additional action of native mycorrhizae. Soil mechanization significantly reduced the number of spores present in the soil, so it is recommended carrying out other land

conditioning procedures that are more environmentally friendly for the native microbiota.

Acknowledgments

We would like to thank the Decanato de Investigación at UNET (Project CODEIN 02-001-2020) and the MinCyT (Project FONACIT 2024PG146) for their financial support. We are grateful to Auxiliadora Olivo from the Laboratorio de Fitobiotecnología-ULA for her collaboration in the field, including the selection of roots for our experiments and preservation of the samples. We would also like to thank Tibisay Ramírez from UNET for providing us with some essential materials to determine mycorrhization. Finally, we acknowledge the collaboration of the following UNET laboratories: Biofertilizantes, Bioambiental, and Investigación en Biotecnología y Química de Polímeros.

Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author´s contributions

Conceptualization: ELB, FR, JP. Research: ELB, LA, YC. Methodology: ELB, LA, YC, MEP. Field data collection: ELB, YC. Laboratory analysis: ELB, LA, MEP. Data curation: ELB, LA, MEP. Data analysis: ELB. Writing – original draft: ELB, LA, YC. Writing – reviewing and editing: all authors. All authors have read and approved the final version of the manuscript.

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