BIOFERTILIZERS IN MEXICAN LIME (*Citrus aurantifolia* (Christm.) Swingle): ARBUSCULAR MYCORRHIZAL FUNGI AND *Azospirillum brasilense* IN GREENHOUSE †

[BIOFERTILIZANTES EN LIMÓN MEXICANO (*Citrus aurantifolia* (Christm.) Swingle): HONGOS MICORRÍZICOS ARBUSCULARES Y *Azospirillum brasilense* EN INVERNADERO]

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SUMMARY

Background: The majority of terrestrial plants have evolved in symbiosis with beneficial microorganisms, which help them acquire minerals that are scarce in soil, such as phosphorus and in some cases nitrogen. Thus, the development and use of biofertilizers based on microorganisms is important for partial or total replacement of chemical fertilizers. The use of arbuscular mycorrhizal fungi (AMF) and *Azospirillum brasilense* helps to boost Mexican lime (*Citrus aurantifolia*) plant growth, making them more vigorous and productive. **Objective:** To evaluate the effect of beneficial microorganisms in Mexican lime plant growth in a greenhouse. **Methodology:** An experiment with Mexican lime was established under greenhouse conditions and a completely randomized bifactorial design: (A) beneficial microorganism with four levels: consortium HMA Cerro del Metate; *Rhizophagus intraradices* (Ri); *Azospirillum brasilense* (Ab); and without microbial inoculum (WI); (B) chemical fertilization N-P-K (nitrogen-phosphorus-potassium) with four levels: high (180-180-180 kg ha^{-1}); intermediate (90-90-90 kg ha^{-1}); low (45-45-45 kg ha^{-1}) and without fertilization. In total, 16 treatments with seven replicates were performed; plant growth and microbiological response variables were evaluated with an analysis of variance (ANOVA) and Tukey's multiple comparison of means tests. **Results**: Significant effects (Tukey, $P \leq 0.05$) of the mycorrhizal consortium Cerro del Metate were found, followed by *R. intraradices* and lastly *A. brasilense,* which proved that these microorganisms promoted plant growth. Mycorrhization significantly increased ($P \leq 0.05$) plant growth rate, as well as dry biomass, observing a mycorrhizal colonization from 16 to 30%. Fertilization only showed a significant interaction (Tukey, $P \leq 0.05$) with *A. brasilense*. **Implications**: The use of native microorganisms and preferably consortia may have better adaptability than commercial ones, which can be explained in part by the effects found in this study. **Conclusion**: Mexican lime plants showed a significantly positive ($P \leq 0.05$) response to inoculation with AMF, showing greater plant growth than the other treatments.

Key words: beneficial microorganisms; growth promoting bacteria; chemical fertilization; rhizosphere.

RESUMEN

Antecedentes: La mayoría de las plantas terrestres han evolucionado en asociación con microorganismos benéficos que los ayudan a adquirir minerales que se encuentran escasos en el suelo como el fósforo y en algunos casos nitrógeno; por ello, el desarrollo y uso de biofertilizantes a base de microorganismos benéficos es importante por la sustitución parcial o total de los fertilizantes químicos. El uso de los hongos micorrízicos arbusculares (HMA) y *Azospirillum brasilense* ayuda a mejorar el crecimiento de plantas de limón mexicano (*Citrus aurantifolia*), haciéndolas más vigorosas y productivas. **Objetivo**: Evaluar el efecto de microorganismos benéficos en el crecimiento de plantas de limón mexicano a nivel de invernadero. **Metodología**: Se estableció un experimento con limón mexicano bajo un diseño experimental completamente al azar bajo condiciones de invernadero. El diseño de tratamientos fue bifactorial:

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factor (A) microorganismo benéfico, con cuatro niveles: consorcio de HMA Cerro del Metate, *Rhizophagus intraradices* (Ri); *Azospirillum brasilense* (Ab) y sin inoculo microbiano; factor (B) fertilización química N-P-K (nitrógeno-fósforo-potasio), con cuatro niveles: alto (180-180-180 kg ha^{-1}), intermedio (90-90-90 kg ha^{-1}), bajo (45-45-45 kg ha^{-1}) y sin fertilización. Teniendo 16 tratamientos totales, con 7 repeticiones. Se evaluaron variables de respuesta del crecimiento vegetal y variables microbiológicas, con las cuales se realizó análisis de varianza y prueba de comparación múltiple de medias de Tukey. **Resultados**: Se encontró un efecto significativo (Tukey, $P \leq 0.05$) del consorcio micorrízico Cerro del Metate, seguido de *R. intraradices* y por último *A. brasilense*, con lo cual se comprueba que estos microorganismos promueven el crecimiento vegetal. La micorrización incrementó significativamente ($P \leq 0.05$) la tasa de crecimiento, así como la biomasa seca de la planta y se observó una colonización micorrízica del 16 al 30%. La fertilización sólo mostró interacción significativamente (Tukey, $P \leq 0.05$) con *A. brasilense*. **Implicaciones**: El empleo de microorganismos nativos y preferentemente consorcios puede tener una mejor adaptabilidad que los microorganismos comerciales lo cual puede explicar en parte los efectos encontrados en este estudio. **Conclusión**: Las plantas de limón mexicano mostraron una respuesta positiva significativa ($P \leq 0.05$) a la inoculación con los HMA, presentando mayor crecimiento vegetal que los otros tratamientos.
Palabras clave: microorganismos benéficos; bacterias promotoras de crecimiento; fertilización química; rizosfera.

INTRODUCTION

The cultivation of Mexican lime (*Citrus aurantifolia* (Christm.) Swingle) has reached an important development in Mexico in the last years, which has benefited different economic sectors, such as producers and workers involved in sanity and certification processes, packaging enterprises, agriculture industry, traders, and researchers. The Mexican lime is placed in characteristic positions, finding it within the first places in global production and exports (González and Silva, 2003). For example, by the closing of the year 2020, the harvested surface had been reported at 81 829.85 ha, with 1 222 344.64 t harvested (SIAP, 2021), which shows its importance at economic level.

From the beginning of human history until the 1940s, plants were cultivated without the help of chemicals. Later, during the green revolution, chemical products were introduced to agriculture at great scale, which caused an increase in crop yield and quality. However, it also brought progressive ecological deterioration as a consequence due to inadequate management of chemical fertilizers, generating preoccupation for ecosystem contamination. At world level, crop sustainability has been searched through more economic and biological alternatives that improve crop feasibility and avoid damaging the environment. This situation has led to research and development of new products to be biologically viable with a minimum impact to the ecosystem and human health, allowing the increase in nutritional quality and crop yield. The biofertilizers based on microorganisms, such as mycorrhizal fungi and beneficial bacteria are an important alternative to boost crop quality since they contribute to capturing nutrients for plants (Aguado-Santacruz *et al*., 2012; Caballero-Mellado *et al*., 2009).

Arbuscular mycorrhizal fungi (AMF) [Glomeromycota (Schüßler *et al*., 2001)] are the greatest components of rhizosphere microflora in natural ecosystems and are essential for sustainability of plant-soil system (Sharma *et al*., 2009). The arbuscular mycorrhizal association helps the plant in nutrient transport from soil to plant, acting especially in phosphorus (P) absorption besides having a bearing in the uptake of other nutrients, such as Zn, Cu, NO_3^- (nitrate) and NH_4^+ (ammonium) (Smith and Read, 2008). In turn, plants grant carbon and lipids to AMF (Chen *et al*., 2018; Keymer *et al*., 2017). In this manner, mycorrhized plants have an advantage over those that are not since in the first ones, the external mycelium extends to a greater distance in soil, compared with root hairs of the non-mycorrhized plants (Barrer, 2009), which allow them to have a greater surface for nutrient absorption. The use of AMF can also reduce the use of chemical fertilizers up to 50%, although this value depends on plant species (Begum *et al*., 2019). In the particular case of citrus plants, AMF have demonstrated to increase plant growth, which is reflected in height, root length, and dry biomass (Watanarojanaporn *et al*., 2011). Moreover, beneficial effects of mycorrhization have been reported in orange (*Citrus aurantium*) plants and in rootstock of different citrus species (Timmer and Leyden, 1980; Back *et al*., 2016). Furthermore, mycorrhized plants have shown advantages when confronting to different abiotic stress conditions (Chen *et al*., 2018), such as hydric, salinity, and high temperature stress (Abdel-Salam *et al*., 2017; Rodríguez *et al*., 2008), because AMF improve dry matter accumulation and water absorption (Begum *et al*., 2019). On the other hand, it is important to highlight that plants inoculated with consortia with a greater diversity in AMF species richness show greater growth when compared with inocula of less diversity in AMF single species (Sharma *et al*., 2009). Nevertheless, the abundance of each species that conforms a consortium may be determinant in plant growth promotion (Trejo *et al*., 2011).

On the other hand, *Azospirillum* comprises plant growth promoting bacteria. Its beneficial effects are

given mainly not only by biological fixation of molecular nitrogen (N_2) in the rhizosphere but also by the capacity of phytohormone (Garcia *et al*., 2010), phosphate solubilization, and siderophore production besides the existing reports of antipathogenic activity (Mehnaz, 2015). Because a limiting factor in crop growth and development is the scarce availability of nitrogen, therefore these plant-bacteria associations are of great importance in the processes of nitrogen transformation (N_2) to forms available (NH_4^+) to plants (García-Olivares *et al.*, 2012). *Azospirillum* spp. has been isolated in a many parts of the world, mainly in the rhizosphere of Gramineae, including rice, wheat, maize, and sugar cane as the most common hosts. It is associated to coffee, fruit trees, and orchids although less frequently in comparison with Gramineae. The two species with greater distribution worldwide are *A. brasilense* and *A. lipoferum* and thus more studied (Mehnaz, 2015). Diverse reports have demonstrated the beneficial effects of *A. brasilense*. For example, a study performed in soy demonstrated the potential these bacteria to synthesize hormones and this way boost radicle growth (Molla *et al*., 2001). In another study performed in maize, the authors obtained high yields in plants inoculated with *A. brasilense* (Saikia *et al.*, 2007). Most recently, seed inoculation with *A. brasilense* also favored growth in maize plants, improving biochemical characteristics and increasing efficiency in the use of N under fertility deficit (Zeffa *et al*., 2019).

The relationships between plants and microorganisms may reduce the use of chemical products because they promote absorption and transformation of the nutrients found in less amount in soil. Thus, there is important to know the benefits that these associations provide to plants in general, in order to apply this knowledge to citrus production in terms of quality and yield. Therefore, the objective of this research is to determine the effect of arbuscular mycorrhizal fungi and *Azospirillum brasilense* in Mexican lime on plant growth with respect to chemical fertilization in greenhouse conditions.

MATERIALS AND METHODS

Plant material

Seeds from Mexican lime fruit were obtained from Tecomán, Colima, México, seed between paper, and incubated at 28°C until germination (ISTA, 2016); lime seedlings were transplanted to germination trays with peat moss (Sunshine® Mix #3, Sun Gro Horticulture, Agawam, MA. USA) sterilized and set in a plant growth chamber at 26°C with a photoperiod of 16/8 h (light/darkness) for 45 days (modified from Martínez *et al.*, 2010).

Experimental conditions

The experiment was established in greenhouse conditions under a completely randomized bifactorial design: (A) beneficial microorganism with four levels: consortium AMF Cerro del Metate (CM) comprised with the species *Acaulospora excavata, A. mellea, A. rehmii, A. scrobiculata, A. spinosa, Acaulospora* sp., *Septoglomus deserticola, Glomus glomerulatum*, and *Oehlia diaphana* (Sin. *Rhizophagus diaphanum*) according to Trinidad-Cruz *et al.*, 2017; *Rhizophagus intraradices* (Ri); *Azospirillum brasilense* (Ab), and without microbial inoculum (WI); (B) chemical fertilization N-P-K (nitrogen-phosphorus-potassium) with four levels: recommended for greenhouse = high (180-180-180 kg ha^{-1}), intermediate (90-90-90 kg ha^{-1}), low (45-45-45 kg ha^{-1}), and without fertilization. The combination of the four levels of each factor made up the 16 treatments in study, each one with seven replicates. The substrate used consisted of river sand, peat moss and soil (5:3:2) from Parácuaro, Michoacán, production area of Mexican lime, plus 1% of vermicompost sterilized in autoclave (121°C, 1.265 kg cm^{-2}, 8 h). Plants were transplanted from germination trays to pots with the substrate previously mentioned. During transplant, the microbial biofertilizer was inoculated, which was the only one performed. Each experimental unit was inoculated with 90 spores in total for the AMF. The CD strain of *Azospirillum brasilense* was obtained from the bacterial collection of the Phytopathology Laboratory (-80°C) at CIATEJ (Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, A.C.). For its reactivation, the strain was grooved in malic acid and Congo Red culture (Rodríguez, 1982) and incubated at 30°C until growth. Once grown, the strain was transferred to Nutrient Broth (Difco®, Franklin Lake, NJ, USA) culture medium and set to grow in an incubator at 30°C and 200 rpm for 24 h; bacterial concentration was quantified by turbidimetry (optical density at 600 nm -OD_{600}-) in spectrophotometry (Madigan *et al*., 2009). To prepare the inoculum for the experiment, the liquid culture was centrifuged at 4700 rpm at 15°C for 20 min, and the supernatant was discarded; the pellet was resuspended in distilled water and then inoculated with $1x10^7$ colony forming units (CFU)/g^{-1} of soil (García-Olivares *et al.*, 2012). After that, triple 16 (Howard Johnsons, USA) N-P-K fertilizer was applied 10 days after the experiment was established around de plant (in the drip area) accordingly to each treatment.

Plant growth response variables

The evaluations were taken monthly; the quantified growth variables were stem diameter, plant height, and leaf number. When the experiment concluded (180 days after microorganism inoculation -DAI-) the fresh weight of root and foliage, total fresh weight, dry

weight of root and foliage and total dry weight were determined (the dry weight was taken after drying the plant material in a drying oven at 60ºC).

Microbiological variables

At 180 DAI soil and roots of the eight treatments inoculated with AMF were sampled to determine presence/absence and final abundance of AMF spores by extraction and counting spores by wet sieving and decanting. Root thinning and staining were performed by Phillips and Hayman's (1970) modified technique, with subsequent assessment in optical microscope to determine mycorrhizal colonization. Colonization percentage was determined following Mcgonigle *et al*. (1990) modified technique. Once the roots were stained, they were cut in 1 cm fragments in length; 20 roots were placed per slide (three replicates were performed), obtaining a total of 60 roots per sample. Each root was observed in three different fields, obtaining 180 observations of the sample in total.

Spore density was performed by extraction by wet sieving and decanting according to Gerdemann and Nicolson (1963) with spore counting in stereoscopic microscope (VE-S5C Velab MX), starting from soil samples of 50 g of dry weight. The spores were centrifuged at 2500 rpm for 5 min; the supernatant was discarded. Sucrose was added at 50%, and it was centrifuged again at 2000 rpm for 1 min. The supernatant was passed through a 45 µm sieve; spores were recovered with distilled water and counted (Walker, 1997).

In the case of *A. brasilense*, soil sampling was performed to determine presence/absence and abundance of the four inoculated treatments with *A. brasilense*. Four samplings were performed (1) one day before fertilization; (2) one week after fertilization; (3) two months after fertilization; (4) at the end of the experiment. Serial decimal dilutions (up to 10^{-5}) were performed starting from the samples; once the dilutions were performed, 100 µL of the dilutions 10^{-4} and 10^{-5} were sown in malic acid Congo Red culture medium and left at room temperature until bacterial growth was observed. Subsequently, the CFU g^{-1} of soil were determined (modified from Guigón and González, 2004). The rhizosphere was sampled in three plants at random of each treatment in each sampling date.

Statistical analysis of data

A multifactorial and one way analysis of variance (ANOVA) were performed with the data of plant growth and microbiological response variables at a level of significance of 5% ($P \leq 0.05$); Tukey's multiple comparison of means was performed using the statistical program Statgraphics® Centurion XV version 15.2.06 (StatPoint Technologies Inc. Warrenton, VA, USA).

RESULTS

Mexican lime shows growth in response to beneficial microorganism inoculation

The evaluations of the different plant growth variables performed along the experiment (Tables 1 and 2), as well as plant biomass accumulation (Tables 3 and 4) showed significant ($P \leq 0.05$) differences in the treatments. In general, AMF showed a significant ($P \leq 0.05$) increase in Mexican lime plant growth. The effects of AMF on plant height, stem diameter, and leaf number were observed more significant in the last evaluations performed. However, a greater significant effect was observed for AMF of the CM consortium compared to *R. intraradices*, whose inoculated plants were more vigorous (Figure 1).

Arbuscular mycorrhizal fungi increased stem diameter in more than 30%, and dry weight in more than 110% with respect to the control without AMF inoculation, which means a greater biomass production, thus greater yield when extrapolated to crop fields.

With respect to the application of *A. brasilense*, although it was not the best microorganism used, a beneficial effect was observed when compared to the control (Figure 2), showing and increase of 16% in stem diameter and 54% in dry weight.

Chemical fertilization effect on Mexican lime growth

In the treatments without inoculation, fertilization on its own favored the plants more as the quantity applied was greater, and the benefits decreased as the fertilization levels were lower. However, this effect was observed very slightly. The statistical analysis showed that no significant differences existed in the fertilization effect with respect to the control without fertilizer (Figure 3). Furthermore, fertilization did not show interaction with any microorganism.

Microorganisms interact with Mexican lime plants

Positive results were observed in spore density in all treatments inoculated with AMF. *R. intraradices* produced 320% more spores than the CM consortium, while CM consortium showed 52% greater radicle colonization than *R. intraradices* (Tables 3 and 4; Figure 4). However, the CM consortium favored plant height and dry biomass with values of 15 and 19%, respectively, greater than *R. intraradices*. When spore density was compared among the 4 treatments inoculated with CM consortium, as the lower fertilization dose was used, the greater number of

spores was observed. This result suggests that fertilization has a bearing on decreasing AMF sporulation and inhibiting it with high fertilization doses.

Table 1. Evolution of Mexican lime (*C. aurantifolia*) plant growth in response to a bifactorial design over time under greenhouse conditions.

Factors	Plant height (cm)			Stem diameter (mm)			Leaf number		
	30 DAI	90 DAI	180 DAI	30 DAI	90 DAI	180 DAI	30 DAI	90 DAI	180 DAI
(A) Inoculant									
CM	5.05 a	19.7 a	37.9 a	1.02 a	2.14 a	4.19 a	6.93 ab	20.3 a	45.0 a
Ri	4.44 a	19.1 a	33.0 ab	1.01 a	2.19 a	4.03 a	6.75 ab	19.1 ab	38.9 a
Ab	4.83 a	15.4 ab	27.2 bc	1.05 a	2.05 a	3.59 ab	7.71 a	16.1 bc	35.5 ab
Wi	4.93 a	13.6 b	22.6 c	1.05 a	1.93 a	3.09 b	6.18 b	14.4 c	25.3 b
(B) Fertilization									
180-180-180	4.70 a	16.7 a	31.6 a	1.01 a	2.1 a	3.69 a	6.39 a	17.1 a	44.6 a
90-90-90	4.80 a	18.0 a	32.5 a	1.02 a	2.1 a	3.94 a	7.04 a	18.5 a	37.3 ab
45-45-45	4.38 a	15.5 a	28.8 a	0.99 a	1.95 a	3.50 a	6.71 a	16.5 a	34.0 ab
Wf	5.35 a	17.5 a	27.8 a	1.11 a	2.14 a	3.77 a	7.43 a	17.9 a	28.9 b
Interaction AxB									
F	1.17	1.06	0.49	1.50	1.11	0.47	1.16	1.09	0.56
P-value	0.3221	0.4005	0.8805	0.1589	0.3666	0.8882	0.3325	0.3754	0.8301

DAI = days after microorganism inoculation. Different letters in the same column for each factor indicate significant differences according to Tukey's test ($P \leq 0.05$). Consortium Cerro del Metate (CM); *Rizophagus intraradices* (Ri); *Azospirillum brasilense* (Ab); without microorganism inoculation (Wi) and without fertilization (Wf).

Table 2. Evolution of Mexican lime (*C. aurantifolia*) plant growth inoculated with different microorganism and fertilizer levels over time under greenhouse conditions.

Treatment		Plant height (cm)			Stem diameter (mm)			Leaf number		
Inoculant	Fertilization	30 DAI	90 DAI	180 DAI	30 DAI	90 DAI	180 DAI	30 DAI	90 DAI	180 DAI
CM	180-180-180	5.23 a	17.9 ab	37.1 ab	1.10 a	2.10 a	3.99 ab	6.57 ab	18.7 ab	48.1 ab
Ri		4.76 a	16.0 ab	37.6 ab	1.00 a	2.20 a	4.27 ab	6.43 ab	18.9 ab	50.6 a
Ab		4.74 a	20.1 ab	27.9 ab	0.94 a	2.10 a	3.56 ab	7.14 ab	16.1 ab	51.6 a
Wi		4.09 a	12.9 ab	23.8 ab	1.00 a	2.02 a	2.95 ab	5.43 b	14.5 ab	28.2 ab
CM	90-90-90	5.37 a	21.5 ab	40.3 a	1.04 a	2.40 a	4.47 a	7.86 ab	22.1 a	47.7 ab
Ri		4.47 a	17.9 ab	32.8 ab	0.95 a	2.00 a	4.19 ab	6.57 ab	20.0 ab	42.4 ab
Ab		4.00 a	16.8 ab	31.7 ab	1.03 a	1.99 a	3.74 ab	7.43 ab	16.3 ab	30.9 ab
Wi		5.40 a	15.7 ab	25.3 ab	1.05 a	2.02 a	3.35 ab	6.29 ab	15.6 ab	28.1 ab
CM	45-45-45	4.57 a	18.2 ab	37.3 ab	0.94 a	1.86 a	4.01 ab	6.14 ab	18.0 ab	45.6 ab
Ri		3.30 a	16.4 ab	28.6 ab	0.85 a	2.18 a	3.55 ab	6.14 ab	17.6 ab	34.0 ab
Ab		5.36 a	17.3 ab	29.0 ab	1.13 a	2.14 a	3.72 ab	8.86 a	19.0 ab	36.0 ab
Wi		4.30 a	10.1 b	20.2 b	1.04 a	1.63 a	2.73 b	5.71 ab	11.4 b	20.5 b
CM	Wf	5.04 a	21.1 ab	36.8 ab	0.99 a	2.20 a	4.30 ab	7.14 ab	22.3 a	38.7 ab
Ri		5.71 a	21.9 a	33.0 ab	1.23 a	2.36 a	4.09 ab	7.86 ab	20.1 ab	28.6 ab
Ab		4.74 a	11.6 ab	20.4 ab	1.11 a	1.95 a	3.33 ab	7.43 ab	12.9 ab	23.6 ab
Wi		5.91 a	15.5 ab	21.1 ab	1.12 a	2.05 a	3.35 ab	7.29 ab	16.3 ab	24.6 ab

DAI = days after microorganism inoculation. Different letters in the same column for each treatment indicate significant ($P \leq 0.05$) differences according to Tukey's test. Consortium Cerro del Metate (CM); *Rizophagus intraradices* (Ri); *Azospirillum brasilense* (Ab) and without fertilization (Wf).

Table 3. Mexican lime (*C. aurantifolia*) dry biomass and presence of beneficial microorganisms in plants under greenhouse at 180 days after inoculation in each study factor.

Factors	Dry biomass (g)			Bacteria (10^6 CFU g^{-1} soil)	Mycorrhiza	
	Root	Stem	Total		Spores density (100 g dry soil)	Root colonization (%)
(A) Inoculant						
CM	1.07 a	2.81 a	3.88 a	0.0 b	407 b	26.9 a
Ri	0.97 a	2.28 ab	3.25 ab	0.0 b	1710 a	17.6 b
Ab	0.79 ab	1.56 bc	2.34 bc	10.9 a	0.0 c	0.0 c
Wi	0.54 b	0.99 c	1.52 c	0.0 b	0.0 c	0.0 c
(B) Fertilization						
180-180-180	0.73 a	2.15 a	2.88 a	2.58 ab	284 a	10.5 a
90-90-90	0.96 a	2.17 a	3.13 a	1.00 b	469 a	10.4 a
45-45-45	0.80 a	1.77 a	2.57 a	3.42 ab	731 a	11.8 a
Wf	0.88 a	1.54 a	2.42 a	3.92 a	633 a	11.8 a
Interaction AxB						
F	0.61	0.40	0.43	3.64	0.11	0.52
P-value	0.7835	0.9335	0.9167	0.0032	0.9511	0.8562

Different letters in the same column for each factor indicate significant differences according to Tukey's test ($P \leq 0.05$). Colony forming units (CFU); Consortium Cerro del Metate (CM); *Rizophagus intraradices* (Ri); *Azospirillum brasilense* (Ab); without microorganism inoculation (Wi) and without fertilization (Wf).

Table 4. Mexican lime (*C. aurantifolia*) dry biomass and presence of beneficial microorganisms in plants under greenhouse at 180 days after inoculation in different treatments.

Treatment		Dry biomass (g)			Bacteria (10^6 CFU g^{-1} dry soil)	Mycorrhiza	
Inoculant	Fertilization	Root	Stem	Total		Spores density (100 g dry soil)	Root colonization (%)
CM	180-180-180	0.84 ab	2.95 ab	3.79 ab	0.0 c	191 b	23.3 ab
Ri		1.00 ab	2.73 abc	3.73 ab	0.0 c	946 ab	18.8 ab
Ab		0.65 ab	1.84 abc	2.49 ab	10.3 ab	0.0 c	0.0 c
Wi		0.42 b	1.09 abc	1.51 ab	0.0 c	0.0 c	0.0 c
CM	90-90-90	1.30 a	3.14 a	4.44 a	0.0 c	299 b	25.4 ab
Ri		1.03 ab	2.56 abc	3.59 ab	0.0 c	1580 ab	16.3 b
Ab		0.80 ab	1.74 abc	2.55 ab	4.0 b	0.0 c	0.0 c
Wi		0.71 ab	1.23 abc	1.93 ab	0.0 c	0.0 c	0.0 c
CM	45-45-45	0.97 ab	2.68 abc	3.66 ab	0.0 c	407 ab	30.4 a
Ri		0.83 ab	1.80 abc	2.62 ab	0.0 c	2520 a	16.7 b
Ab		1.01 ab	1.87 abc	2.89 ab	13.7 a	0.0 c	0.0 c
Wi		0.37 b	0.74 c	3.65 ab	0.0 c	0.0 c	0.0 c
CM	Wf	1.18 ab	2.47 abc	1.11 b	0.0 c	733 ab	28.3 ab
Ri		1.01 ab	2.03 abc	3.04 ab	0.0 c	1800 ab	18.8 ab
Ab		0.67 ab	0.78 c	1.45 ab	15.7 a	0.0 c	0.0 c
Wi		0.64 ab	0.90 c	1.54 ab	0.0 c	0.0 c	0.0 c

Different letters in the same column for each treatment indicate significant differences according to Tukey's test ($P \leq 0.05$). Colony forming units (CFU); Consortium Cerro del Metate (CM); *Rizophagus intraradices* (Ri); *Azospirillum brasilense* (Ab) and without fertilization (Wf).

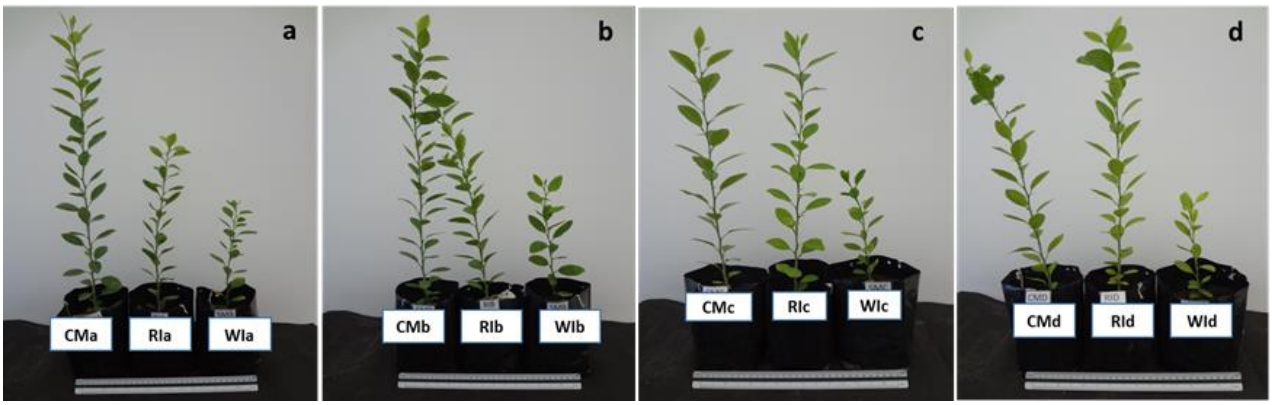

Figure 1. Visual aspect of Mexican lime (*C. aurantifolia*) plant growth by inoculation effect of arbuscular mycorrhizal fungi (consortium CM = CM; *R. intraradices*= RI; without microorganism inoculation = WI) and the application of nitrogen-phosphorus-potassium (NPK) fertilization (a = High, 180 kg ha^{-1}; b = Intermediate, 90 kg ha^{-1}; c = Low 45 kg ha^{-1}; d = without fertilization) at 180 days after microorganism inoculation under greenhouse conditions. The scale below each photograph is equal to 30 cm.

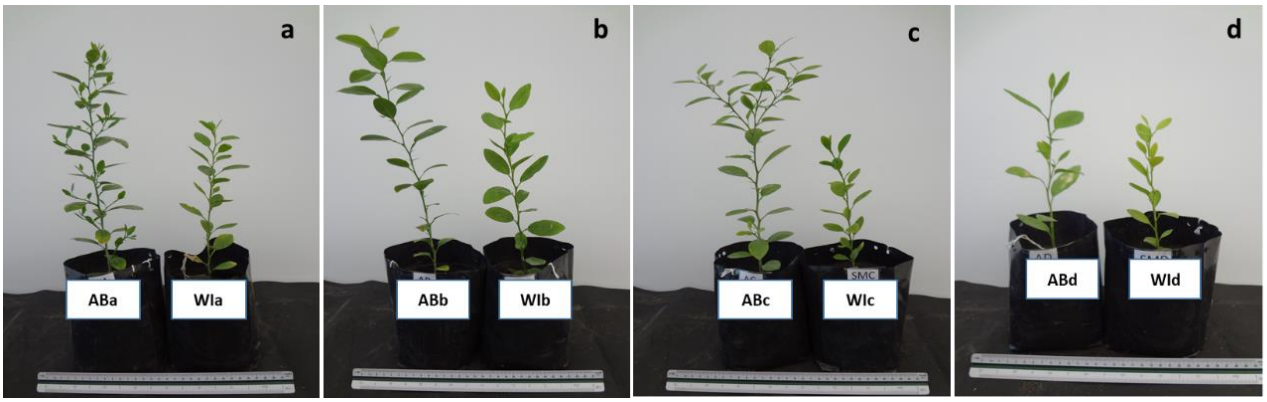

Figure 2. Effect on Mexican lime (*C. aurantifolia*) plant growth by inoculation with bacteria (*Azospirillum brasilense* = AB; without microorganism inoculation = WI) and application of nitrogen, phosphorus, and potassium (NPK) fertilization (a = High, 180 kg ha^{-1}; b = Intermediate, 90 kg ha^{-1}; c = Low, 45 kg ha^{-1}; d = without fertilization) at 180 days after microorganism inoculation under greenhouse conditions. The scale below each photograph is equal to 30 cm.

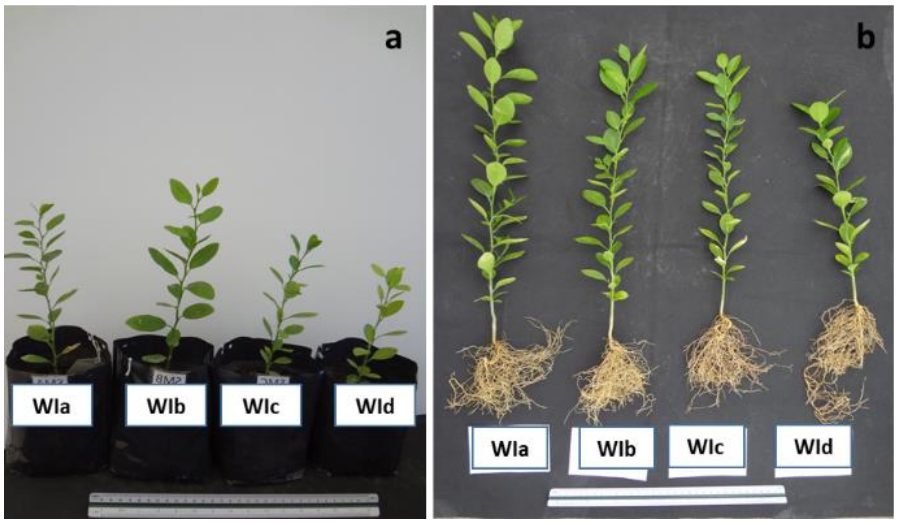

Figure 3. Stem (a) and root (b) growth of Mexican lime (*C. aurantifolia*) as a result of the application of different doses of nitrogen-phosphorus-potassium (NPK) fertilizer (a = High, 180 kg ha^{-1}; b = Intermediate, 90 kg ha^{-1}; c = Low, 45 kg ha^{-1}; d = without fertilization; without microorganism inoculation = WI) at 180 days after microorganism inoculation in greenhouse. The scale below each photograph is equal to 30 cm.

On the other hand, mycorrhizal colonization was greater in the case of the CM consortium (Figure 4), which supports that consortium are more beneficial than monospecific strains. When the statistical analysis was performed, not interaction was observed between fertilization and microorganisms for any of the eight treatments, with respect to the plant response. However, the consortium treatments with less fertilization (CM + 45-45-45 and CM + 0-0-0) showed a greater colonization than those with a high dose (Table 4).

When soil samplings were performed, *Azospirillum* was present, but no response was observed by the plant, which indicated that it did not respond adequately to inoculation. Figure 5 shows the population dynamics of the bacteria throughout the experiment. A decrease in population levels of *A. brasilense* existed through time, which could be explained because a second inoculation was not performed. The presence of 34% more CFU of *A. brasilense* was observed in the treatment without fertilization compared with the high fertilization (180-180-180) treatment. The same trend was observed for intermediate (90-90-90) and low (45-45-45) fertilization treatments, which suggests that in this manner the bacteria is affected negatively with greater doses of the fertilizer (Tables 3 and 4).

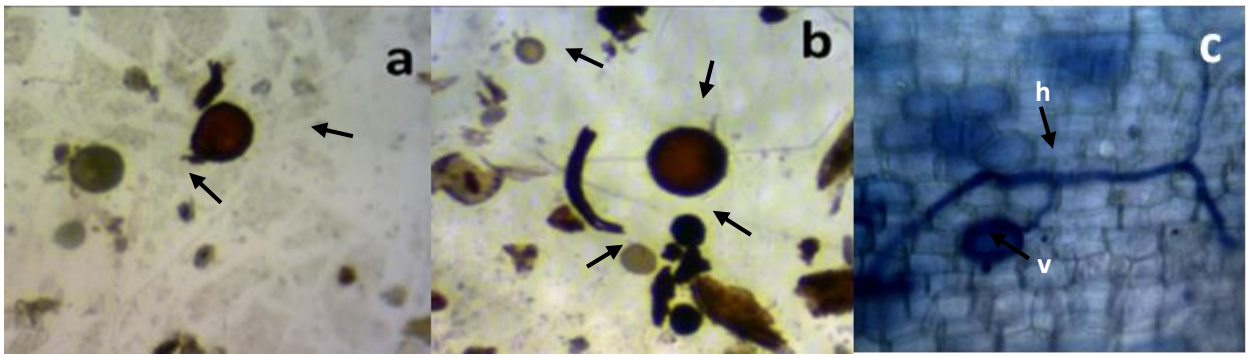

Figure 4. Spores of arbuscular mycorrhizal fungi of the CM consortium from the rhizosphere of Mexican lime plants under greenhouse conditions 180 days after microorganism inoculation; views under the stereoscopic microscope (a and b), note the diversity in color and size (arrows). (c) Mycorrhizal colonization of the CM consortium in Mexican lime roots under an optical microscope with a 40x objective, (hyphae=h, vesicles=v).

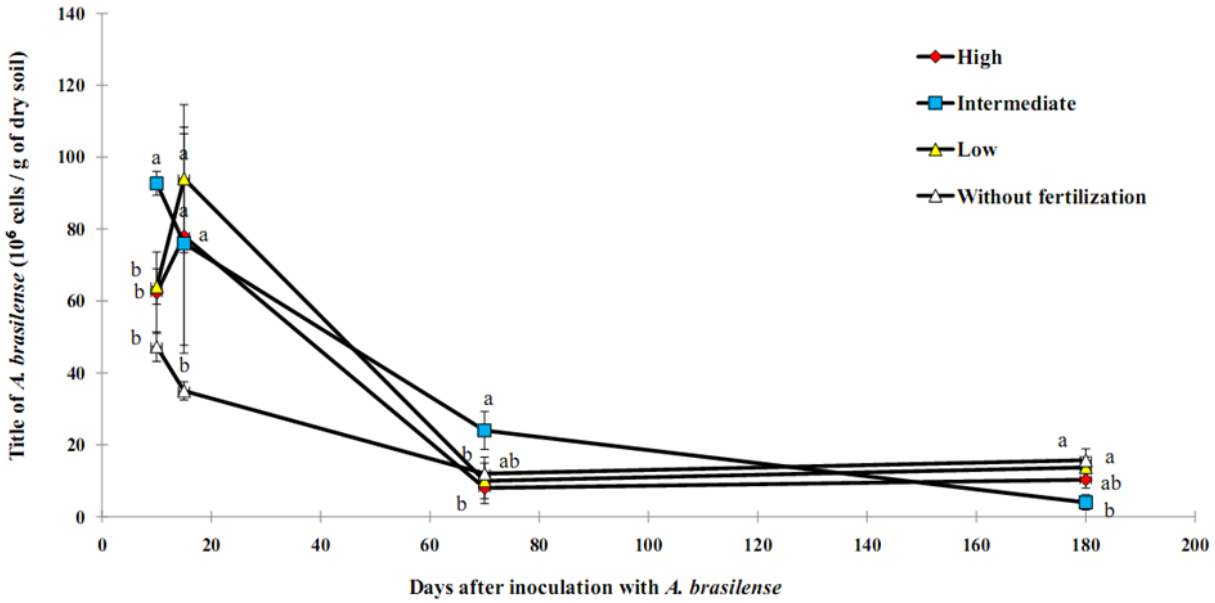

Figure 5. *Azospirillum brasilense* in the rhizosphere of Mexican lime (*C. aurantifolia*) established under greenhouse conditions. Doses of nitrogen-phosphorus-potassium (NPK) fertilizer (High =180 kg ha^{-1}; Intermediate = 90 kg ha^{-1}; Low = 45 kg ha^{-1}). Different letters in each sampling point indicate significant differences according to Tukey's test ($P \leq 0.05$). The bars in each point represent the mean±standard error.

DISCUSSION

When commercial biofertilizers composed by microorganisms previously selected are used, chances are they do not function properly because they are not evolutionarily associated to the plant. Thus, the use of native microorganisms is better. Using consortia is also recommended since they have better adaptability. Additionally, the greater the species diversity is, the more favored the conservation of interspecific relationships performed in the rhizosphere is. In this manner, the different microorganism species occupy distinct ecological niches. For example, some of them provide nutrients and mineral fixation, others growth hormones, and some others protection against pathogens, just to mention some. García *et al*. (2010) support the information previously mentioned, maintaining that despite commercial biofertilizers of *Azospirillum* spp exist, its application is not always effective. Thus, they prefer the use of native microorganisms adapted to climate conditions, which may compete successfully with native microbiota. These authors selected native strains for their study to reach greater indoleacetic acid values and nitrogen fixation, which increased plant development of the variety INIA508-Tinajones of rice in greenhouse condition with indices of effectiveness up to 21.8% in height, as well as 102.1 and 126.1% in dry aerial part and roots.

Otherwise, Sharma *et al*. (2009) found that *Curculigo orchioides* inoculated with an AMF consortium of great species richness showed greater effect on growth compared with the consortium that had little AMF diversity and with *R. intraradices*, as a single species. In fact, the plants inoculated with *R. intraradices* did not survive after 20 DAI, which emphasizes the need of protecting AMF diversity and recommend the use of consortia in restoration practices.

Arbuscular mycorrhizal fungi colonize the interior of plant roots, interacting more intimately with them than in the case of *Azospirillum*, which is found in the rhizosphere. Thus, the beneficial effects granted by AMF are greater because such fungi form a more stable relationship with the plants, and assure, so to speak, their place within the radicle cell. On the other hand, because *Azospirillum* has been isolated originally from cereals, it might have caused lower benefits on the Mexican lime. Although the association *Azospirillum*-plant is not considered specific, certain affinity may exist for some plant species – affinity that in this case seems to not exist with *C. aurantifolia.* The previous is sustained by Trejo *et al*. (2011) who found in a study that the effect of seven AMF consortia isolated from coffee plantations was proven in coffee (*Coffea arabica* L.) var. Garnica plant growth in greenhouse and field conditions. Plant growth seemed to be defined by species richness and the origin of their isolation since AMF from coffee plantations favor significantly coffee plant growth more than exogenous fungi. Even though the bacteria *Azospirillum* and AMF are completely different organisms, the origin of the strain is a factor that should be taken into account at the moment of using any microorganism as growth promoter. Since microorganisms and the plant species are associated, they have co-evolved, which is why a certain degree of specificity exists and greater plant response toward the microorganisms isolated from rhizosphere of the same plant species. The fact that the plant response has been greater toward the mycorrhizal consortium than *R. intraradices* – a single species – shows that a greater number of spores is not necessary for an optimum plant-fungus interaction. Nevertheless, the mycorrhizal diversity that consortia grant is highly important for such interaction to benefit the plant in a greater measure, asserting also that using native microorganisms is better than commercial strains. Barrer (2009) mentioned that studies performed on pepper and banana where native and commercial AMF were used for inoculation, the plants subjected to the treatment with native fungi showed better results than those inoculated with commercial AMF. Furthermore, not all combination plant-AMF are compatible, of which some are more beneficial to a certain host and better adapted to specific soil-climate conditions (Linderman and Davis, 2004). In a comparative study between a native strain (*Claroideoglomus claroideum*) and a commercial one (*Rhizophagus intraradices*), Castillo *et al*. (2009) reported significant differences where the native strain increased 15.2% in height more than the commercial strain and 30.8% more than the control. Whereas biomass increased 60 and 116% with respect to the commercial strain and the control without AMF, respectively; these positive effects were also reflected on fruit production, of which the plants treated with native AMF had more earliness.

In the case of fertilization, the effect of chemical fertilization is so little that the cost goes beyond the benefits. This fact supports that the use of chemical fertilizers should be reduced significantly in Mexican lime crop, including eliminating them completely once cultivation soil has been improved and soil microorganisms recovered, especially those that have proven to grant greater benefits at a lower cost. In a comparative study performed with rice by Alam and Seth (2014), the treatment with biofertilizer produced greater height and rice yield. However, in pepper Castillo *et al.* (2009) reported that fertilization should not be completely eliminated, but with the use of AMF, fertilization is more efficient, saving a great amount of fertilizer jointly with better absorption of the nutrients available in soil. Based on the previous, the plant species of interest should be taken into account since the strategy that functions for a species not necessarily works for another one. Thus, it is important to carry out specific studies for the species of interest in this case

of Mexican lime. Nevertheless, despite all the benefits reported on the use of microorganisms, the use of traditional chemical fertilizers still is the most common practice among producers. Therefore, future research should be performed to show the benefits of AMF in agriculture (Chen *et al*., 2018).

CONCLUSIONS

Mexican lime showed a significant response in plant growth associated with AMF and a light one with *A. brasilense* inoculation. In general, despite AMF granted Mexican lime plants relevant benefits in terms of growth and development, the consortium CM was the best treatment since the plant response was significantly greater. By contrast, chemical fertilization did not show significant effects, highlighting the importance of substituting it for beneficial microorganisms as the AMF isolated in the consortium CM. At the end of the experiment all the microorganisms evaluated were isolated from Mexican lime rhizosphere although not all showed beneficial effects, which indicates that the simple presence of the microorganism is not enough. Thus, the plant and microorganism species should be taken into account to achieve the beneficial effects to boost plant species growth.

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Declarations

Conflict of interest. No conflict of interest to declare.

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Compliance of ethical standards. Because of the nature of study, no national or international ethical or bioethical standards apply.

Availability of data. Data are available in equinones@ciatej.mx con la Dra. Evangelina Quiñones, previous reasonable request.

Author contribution statement: M. Ríos-Sandoval- Data curation, Investigation, Methodology, Formal Analysis, **G. Rincón-Enríquez**- Conceptualization, Formal Analysis, Data curation, Investigation, **M.A. Bautista-Cruz**- Investigation, Formal Analysis, **E.E. Quiñones-Aguilar**- Conceptualization, Formal Analysis, Funding acquisition, Project administration, Investigation.

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