Beneficial microorganisms enhance the growth of basil (*Ocimum basilicum* L.) under greenhouse conditions

Yonger TAMAYO-AGUILAR¹, Porfirio JUAREZ-LOPEZ¹*, Jose A. CHAVEZ-GARCIA¹, Iran ALIA-TEJACAL¹, Dagoberto GUILLEN-SANCHEZ¹†, Jesus O. PEREZ-GONZALEZ², Victor LOPEZ-MARTINEZ¹, Maria C. RUEDA-BARRIENTOS¹, Odira BAQUE-FUENTES³

¹Autonomous University of the State of Morelos, Graduate Program in Agricultural Sciences and Rural Development, Faculty of Agricultural Sciences, Av. Universidad 1001, 62210 Cuernavaca, Morelos, Mexico; yongertamayo@gmail.com; porfirio.juarez@uaem.mx (*corresponding author); jose.chavez@uaem.mx; iran.alia@uaem.mx; dagoguillen@yahoo.com; victor.lopez@uaem.mx; claudia.rueda@uaem.mx

²Technological University of the South of Morelos State, Carretera Puente de Ixtla, km 2.35, Colonia 24 de febrero, 62665 Mazatepec, Morelos, Mexico; jopgonzalez1998@gmail.com

³University of Guantanamo, Faculty of Humanistic Sciences, Av. Che Guevara, km 1.5, Carretera a Jamaica, 95100, Guantanamo, Cuba; odiraB@cug.co.cu

†Deceased – August 13, 2021

**Abstract**

The integration of healthy management alternatives continues to be a challenge in the organic production of aromatic and medicinal plants, including of basil (*Ocimum basilicum* L.). The objective of this work was to evaluate the effects of three beneficial microorganisms (1) *Trichoderma harzianum* (TH), (2) *Bacillus subtilis* (BS), (3) *Glomus cubense* (GC) and their combinations on the growth of basil. A completely randomised design was used with a control and seven treatments with six repetitions. The control (1) was with no microorganism inoculation and the seven treatments were inoculations with the single or the combined microorganisms as follows: (2) TH, (3) BS, (4) GC, (5) TH+BS, (6) TH+GC, (7) BS+GC and (8) TH+BS+GC. Three harvests of fresh biomass were made and a number of growth variables were recorded: fresh and dry biomass, leaf area, number of commercial stems, stem length and thickness, Leaf length and width, relative chlorophyll concentration (SPAD readings) and the levels of N, P, K, Ca and Mg. Overall growth increased by 58% with TH+GC compared with the control and by 55% compared with the single inoculations (TH, BS and GC) and with the triple inoculation (TH+BS+GC). A growth increase of 51% was obtained with BS+GC compared with the control and of 38% compared with the other treatments. These results indicate co-inoculation of TH+GC or of BS+GC are useful alternative managements to increase greenhouse production of basil.

**Keywords:** arbuscular mycorrhizal fungi; aromatic plants; *Bacillus subtilis*; biofertilisers; *Glomus cubense*; *Trichoderma harzianum*
Introduction

Because of their perceived nutraceutical (health related) and organoleptic (aroma, flavour) benefits, the use and consumption of medicinal and aromatic plants (MAP) has increased across the world. This increase has contributed to economic development in the agricultural sectors of many nations. The new focus has also delivered important environmental and social benefits (Kala, 2015; Salome-abarca et al., 2015).

Furnell et al. (2019) reported that the three main MAP exporting countries are Mexico, Cameroon and South Africa, while the five main MAP importing countries (77% of total MAP consumption/utilisation) are France (26%), USA (16%), Japan (15%), Germany (11%) and Spain (7%). In Mexico, MAP comprise 8% of total production of organic plants. Of these, basil (Ocimum basilicum L.) is the main species produced for export with an average fresh biomass yield of 8.45 t ha⁻¹ and a planted area of 417 ha, of which 208 ha are produced under organic systems, a further 5 ha is produced in greenhouses and the rest under conventional (i.e. non-organic) systems (CONAGUA, 2018; Chiquito-Contreras et al., 2018). The main producing states in Mexico are Baja California (greenhouse), Morelos, Nayarit (conventional) and Baja California Sur (organic), the latter being the largest producer of basil (Sánchez-Verdugo and Avilés-Quevedo, 2012; Ojeda-Silvera et al., 2015).

Plants of all species have developed a diversity of strategies to cope with the biotic and abiotic challenges faced in their natural habitats. A major one of these, relevant to most plant species, involves symbiotic relationships established with fungal and bacterial microorganisms. These microorganisms interact directly with the plant host, to increase its tolerance of these challenges and also to facilitate their access to belowground resources (water and minerals). In so doing, they result in a stimulation of the plant’s physiological processes, and these are reflected in increases in yield. In a commercial environment, these symbioses often allow reduced rates of application of chemical fertilisers (López-Valenzuela et al., 2019; Tamayo et al., 2019).

The arbuscular mycorrhizal fungi (AMF) with their attributes of association with cultivated species, generate substantial improvements in plant growth, with positive effects on plant nutrition, on the uptake of water and nutrients, on soil stability, and systemic resistance to fungal diseases and other pests. These benefits create important and economically viable alternatives to more conventional methods of cultivation (Baum et al., 2015; Esquivel-Quispe, 2020). It has been reported that Trichoderma harzianum is an opportunistic symbiont fungus of plants, able to produce elicitors that induce plant defences against pathogens and insects, solubilisation of soil-bound P, Mg, Fe, Mn and plant growth promoters (Sharma et al., 2017; Hernández et al., 2019).

Of the bacteria, it has been reported that Bacillus subtilis stimulates plant growth through the synthesis of plant growth regulators such as indole acetic acid, also enabling biological N fixation, solubilisation of P and production of siderophores, (Corrales et al., 2017; Leal-Almanza et al., 2018).

The integration of certain symbiotic microorganisms (Glomus cubense, Trichoderma harzianum and Bacillus subtilis) within organic management systems for basil cultivation, is expected to generate significant benefits over conventional methods. It should reduce disease and increase growth, resulting in increased production per unit area and reductions in the use of synthetic fertilisers. The objective of this work was to evaluate the effects of Trichoderma harzianum, Bacillus subtilis, Glomus cubense and their combinations on growth of basil under greenhouse conditions.

Materials and Methods

The study was carried out in a tunnel greenhouse with a whitish plastic covers above (30% shading) and anti-aphid mesh on the side walls, located at the Autonomous University of Morelos, Mexico (18° 58’ 51” N, 99° 13’ 55” E) at an altitude of 1.866 m. During the experiment, environmental data were recorded in the
greenhouse with a data logger (Hobo® datalogger, model MX2301A, USA); the long-term average temperature was 22.4 °C and long-term average relative humidity was 53.0%.

Transplanting and application of beneficial microorganisms

Transplanting was carried out on March 1, 2020 into 20.3 cm plastic pots containing 2.8 kg of agricultural soil. Basil seedlings cv. ‘Nufar’ F1 produced in styrofoam trays with 200 cavities, from the company Fusión Mexicana Agropecuaria S.A. de C.V. located in Jojutla de Juárez, Morelos. The topsoil used in the experiment was obtained from a depth of 0-20 cm from the aforementioned company, it had a silty clay texture with the following chemical characteristics: pH 6.32; high CEC of 27.1 mEq 100 g⁻¹; normal contents of: organic matter 2.3%, EC 2.7 dS m⁻¹, total N 0.6 cmol kg⁻¹, available P (Bray-Kurtz) 5.1 ppm; high contents of K (7.4 cmol kg⁻¹), Ca (107.0 cmol kg⁻¹) and Na (14.7 cmol kg⁻¹); low contents of of Mg (9.0 cmol kg⁻¹) and normal Cu (0.03 cmol kg⁻¹), Mn (0.07 cmol kg⁻¹), Zn (0.04 cmol kg⁻¹) and B (0.39 cmol kg⁻¹). The soil was sterilised by solarisation (Katan and Gamliel, 2012), for this, it was covered with transparent plastic and exposed to sunlight for 30 days.

The inoculation of the basil seedlings with the species *T. harzianum* and *B. subtilis* obtained from the Technological University of the South of Morelos State (UTSEM), was carried out in a soil drench. Prior to inoculation these were grown on PDA culture media and nutrient agar at 25 ± 2 °C; the first inoculation was carried out at the time of transplanting and the second 15 days later, at a concentration of 1 x 10⁵ spores per mL⁻¹ and 1 x 10⁵ bacteria per mL⁻¹ prepared with sterile distilled water in a 1000 mL beaker. They were applied with a 1000 mL batlle 730061UNID model spray that guaranteed the uniformity of the application to all plants. The species *G. cubense* with 70 spores per gram of inoculant and 50% radical colonisation, non-toxic and free of pathogens obtained through the State Council of Organic Fertilisers (CEFO) located in Oaxaca, Mexico, was prepared in a plastic container, in which the roots were immersed in a fluid paste of the mycorrhizal inoculant with a dose of 0.5 kg ha⁻¹ of the product per 800 mL of distilled water at the time of transplanting (Fernández et al., 2000).

Irrigation and mineral nutrition

Every two days 1 L of 50% Steiner’s nutrient solution was supplied per pot. The solution was prepared from soluble commercial fertilisers: Ca (NO₃)₂, KNO₃, MgSO₄, K₂SO₄ and KH₂PO₄ (Steiner, 1984). The pH of the nutrient solution was adjusted between 5.5 and 5.7 with 95% sulfuric acid. As a source of micronutrients, ultrasol Micro Mix, SQM® was used at 20 g per 500 L of nutrient solution.

Experimental design and treatments

The three beneficial microorganisms were: *Trichoderma harzianum* (TH), *Bacillus subtilis* (BS) and *Glomus cubense* (GC). A completely randomised design was used with a control and seven treatments with six repetitions. The control (1) was with no microorganism inoculation and the seven treatments were inoculations with the single or the combined microorganisms as follows: (2) TH, (3) BS, (4) GC, (5) TH+BS, (6) TH+GC, (7) BS+GC and (8) TH+BS+GC (Table 1). The experimental unit was a pot containing one basil plant.

Harvests and variables measured

Basil is a perennial herbaceous plant and three harvests (cuts) of fresh stems were made. The first was on March 31, 2020 (30 days after transplanting), the second on April 14 and the third on April 29, all of the same year. For each harvest, the following variables were evaluated so the cumulative effects of the three harvests could be determined: the number of commercial stems; stem length from the base to apex (precision 0.01 cm ruler); stem thickness 1 cm above the base of the commercial stem cut (digital vernier, Traceable® Model 97152-16, USA); leaf length and width, measured below the first internode of the stem cut (digital vernier); relative chlorophyll concentration in mature, fully-expanded leaves (SPAD reading, Minolta® Model 502 Plus, Japan);
leaf area (all leaves were removed from the branches and stems and placed on a leaf area meter, (LI-COR® Model LI-3100C, USA); fresh and dry biomasses of each cut of commercial stems, separated into leaves and stems (digital scale OHAUS® Model Scout Pro SP401, USA, sensitivity 0.01 g). After the fresh weight had been obtained, organs were placed in brown paper bags and dried in a force ventilated oven (Luzeren® Model DHG9070A, China) at 60 °C for three days to constant weight. The macronutrient concentrations (N, P, K, Ca and Mg) were then determined in each organ. For N, the micro-Kjeldahl method was used (Alcántar and Sandoval, 1999), while for P, K, Ca and Mg, wet digestion was carried out with a mixture of perchloric and nitric acids 2:1 ratio (Alcántar and Sandoval, 1999). Macronutrient extraction was carried out from the dried biomass of each organ (leaves and stems) and the % concentrations (N, P, K, Ca and Mg) determined as:

\[ \text{Extraction of macronutrients (kg ha}^{-1}\text{)} = [\text{DM aerial part (g per plant)} \times \text{concentration (\%) of the element in the DM of the aerial part}] \times 10. \]

| Table 1. Description of the treatments applied to basil plants (Ocimum basilicum L.) |
|-----------------------------------------------|
| Treatment | Concentration of inocula |
| Control | Without inoculation |
| TH | 1 x 10^5 CFU mL\(^{-1}\) |
| BS | 1 x 10^5 CFU mL\(^{-1}\) |
| GC | 70 spores g\(^{-1}\) of soil |
| TH+BS | 1 x 10^5 CFU mL\(^{-1}\) + 1 x 10^5 CFU mL\(^{-1}\) |
| TH+GC | 1 x 10^5 CFU mL\(^{-1}\) + 70 spores g\(^{-1}\) of soil |
| BS+GC | 1 x 10^5 CFU mL\(^{-1}\) + 70 spores g\(^{-1}\) of soil |
| TH+BS+GC | 1 x 10^5 CFU mL\(^{-1}\) + 1 x 10^5 CFU mL\(^{-1}\) + 70 spores g\(^{-1}\) of soil |

CFU = Colony formation unit. TH = Trichoderma harzianum, BS = Bacillus subtilis, GC = Glomus cubense.

Statistical analyses
These were carried out with the software IBM SPSS® Statistics for Windows vs 25 (IBM Corp, Armonk, New York, USA). The normality and homogeneity of variance were verified using the Levene and Kolmogorov-Smirnov test. Subsequently, the analysis of variance and the Tukey mean comparison test (p ≤ 0.05) were carried out.

Results and Discussion

Fresh and dry biomasses
Significant differences (p≤0.05) were observed between the treatments in fresh and dry biomasses (Figure 1). The co-inoculation TH+GC increased fresh biomass by 67% compared with the control, the single inoculations (TH, BS and GC) and the three combined microorganisms (TH+BS+GC). Likewise, TH+GC increased dry biomass by 63% compared with the same treatments before mentioned. Similarly, the combined inoculation BS+GC increased fresh biomass by 45% and dry biomass by 55% compared with the control, the single inoculations (TH, BS and GC) and the three combined microorganisms (TH+BS+GC).

The results show that a direct interaction with these beneficial microorganisms in the rhizoplane of basil plants, has increased the physiological activity and other biotic and abiotic factors so as to increase plant biomass. The ascending biomass increments for TH+GC and BS+GC are likely due to some different specificity of the symbiotic activities of these microorganisms with basil, as new stems appear between harvests throughout the experimental period. This idea is also suggested in the report by Chiquito-Contreras et al. (2018) where mixed inoculations between mycorrhizae and bacteria of the genus Stenotrophomonas rhizophila, stimulated the processes involved in plant growth.
Álvarez et al. (2018) note that co-inoculated microbes encourage plant growth through synergism between the distinct benefits they each provide in terms of the metabolism of the plant. In contrast, Moncada et al. (2021) found a decrease in plant biomass of *Ocimum basilicum* L. plants grown in pots inoculated with *Bacillus* spp. compared with a 100% Steiner solution control. These authors hypothesised that the temperature of 15 °C that was recorded during the experiment could have delayed root growth and the activity of plant growth-promoting rhizobacteria (PGPR), which in turn limited the interaction of the *Bacillus* spp. with the plant host and any increase in the uptake of nutrients.

Meanwhile, Khalediyah et al. (2021) found a significant increase in fresh and dry weight of the aerial and root biomasses of *Ocimum basilicum* L. in plants co-inoculated with *G. mosseae* and *B. subtilis* compared to the control treatment and plants treated with mineral fertiliser. These authors attributed their results to the increase in the uptake of water and nutrients by the fungal and bacterial structures that also penetrate the plant cells and so stimulate the plant’s physiological activity.

Riahi et al. (2020) reported that *Pseudomonas rhizophila* S211, *Halomonas desertis* G11 and *Oceanobacillus iheyensis* E9 significantly increased various biomass growth parameters of leaves and roots compared to the control in *Pelargonium graveolens* L’Hér plants. They also reported a 43.34% increase in biomass weight with the dual microorganism inoculation over the control. These effects were attributed to the production of siderophores, the solubilisation of minerals and the diffusion of substances that promote direct plant growth.

Hernández-Montiel et al. (2020) reported an increase of 19.29% in plant biomass in *Capsicum annuum* L. plants co-inoculated with rhizobacteria compared to the control. This positive effect was attributed to the ability of the bacterial inoculants to produce plant growth hormones which stimulate cell division and differentiation and thus an increase in biomass. The beneficial effects of *T. harzianum* combined with rhizobacteria have also been shown to increase plant survival and biomass production in *Arachis hypogaea* L. plants. However, their effects are less when applied separately (Neelipally et al., 2020).

**Leaf area**

There were significant (p≤0.05) differences in leaf area between the evaluated treatments (Figure 2). TH, BS and TH+BS+GC did not show differences between them, but they did with the control. A similar effect was observed between GC and TH+BS. However, compared to the control, leaf area did show an upward
trend of 55 and 45% with TH+GC and BS+GC, respectively. This could be due to synergisms between the microorganisms that stimulate the physiological systems of plants.

These results suggest that the effects of the plant-microbe interaction may have been to stimulate the uptake of water, essential nutrients and the production of metabolites such as enzymes, plant growth-promoting compounds, organic acids etc that promote the leaf area of the plants. Bhat et al. (2020) suggests the increase in leaf area in plants inoculated with microbes is likely the production of a wide range of beneficial strategies and mechanisms in the rhizosphere that make nutrients more available to plants. Rivera et al. (2020) also found that the leaf area of Pennisetum purpureum and Nicotiana tabacum L. increased gradually due to benefits from associated mycorrhizal inoculants under a range of edaphic and environmental conditions.

Pan et al. (2020) found positive effects on aerial biomass accumulation in Elaeagnus angustifolia L. with applications of AMF and PGPR. Simple inoculation of Glomus mosseaese increased the aboveground biomass by 64% compared with the control. They too reported a similar response with the addition of Bacillus amyloliquefaciens and attribute the results to the specificity of the plant to the AMF strain, rather than to the richness or microbial diversity in the plant’s rhizosphere.

The contrasting responses between our inoculation treatments, could be because beneficial microorganisms do not always have the same responses in different host plant species due to their different functional interactions and edaphic environments. However, when correctly applied their symbiotic interactions with the host stimulate its growth (Tian et al., 2020).

**Growth variables**

The statistical analysis showed significant differences (p≤0.05) among the treatments in the various parameters we measured (Table 2). There was a gradual trend in all variables with the co-inoculation of TH+GC followed by with BS+GC. In contrast, GC and TH+BS showed similar responses in all growth variables. Likewise, the application of TH+BS+GC and the single inoculations TH and BS were not significantly different. The sequential harvests during the experimental period and the relationships with TH+GC and BS+GC increased the number and commercial quality of the stems harvested. It is assumed that the plants took up water and soil minerals via the associated microorganism structures, with the microorganism better able to obtain carbon compounds from the host while also enhancing the development of the host’s aerial biomass (Arango et al., 2013). Abdollahi Arpanahi et al. (2020) found the morphological parameters were significant in the plants treated with PGPR compared to the controls. In contrast, an inappropriate
selection of rhizobacteria in a culture can inhibit host plant growth, due to the deficient or excessive concentration of hormones, as well as to incompatibility between microorganism and host plant (Anguiano-Cabello et al., 2019; Rivera et al., 2020).

### Table 2. Growth variables evaluated in basil inoculated with beneficial microorganisms

| Treatment   | Number of commercial stems | Stem length (cm) | Stem thickness (mm) | Leaf length (mm) | Leaf width (mm) |
|-------------|----------------------------|------------------|--------------------|------------------|-----------------|
| Control     | 10.25 e                    | 21.55 e          | 5.05 f             | 90.34 e          | 51.26 e         |
| TH          | 12.50 d                    | 23.45 d          | 5.77 d             | 94.09 d          | 53.99 d         |
| BS          | 12.25 d                    | 23.01 d          | 5.53 e             | 93.31 d          | 54.14 d         |
| GC          | 14.00 e                    | 24.44 c          | 6.18 c             | 97.55 c          | 57.89 c         |
| TH+BS       | 15.00 c                    | 24.63 c          | 6.31 c             | 98.72 c          | 58.54 c         |
| TH+GC       | 19.00 a                    | 27.15 a          | 7.24 a             | 106.17 a         | 64.93 a         |
| BS+GC       | 16.75 b                    | 26.02 b          | 6.84 b             | 102.65 b         | 61.46 b         |
| TH+BS+GC    | 12.25 d                    | 23.25 d          | 5.68 de            | 94.21 d          | 54.23 d         |
| Es χ        | 0.50*                      | 0.27*            | 0.09*              | 0.79*            | 0.68*           |
| CV (%)      | 19.71                      | 7.13             | 11.36              | 5.24             | 7.70            |

TH = Trichoderma harzianum; BS = Bacillus subtilis; GC = Glomus cubense; Es χ = standard error of the mean; CV = Coefficient of variation. Different letters in the same column indicate significant differences according to Tukey’s multiple range test (p≤0.05).

Abdel-Rahman et al. (2011) found a growth stimulation of three varieties of Ocimum basilicum L. treated with B. subtilis and AMF. Likewise, they indicate that mycorrhizal colonisation was superior with respect to PGPR and they also reported that a duality of microorganisms provided a greater response compared to individual ones. In this context, Mohamed et al. (2019) in a trial carried out under greenhouse conditions, demonstrated the effect of single or combined inoculations of mycorrhizae, Bacillus subtilis and Pseudomonas fluorescens with Phaesolus vulgaris L. They report that the combined treatments with microorganisms were more effective than the individual treatments for increasing growth. Other studies indicate that the use of Trichoderma contributes to the balance of hormones such as indole acetic acid, gibberellic acid, ethylene, which is reflected in the quality and safety aspects for the commercialisation of these species (Stewart and Hill, 2014; Peccatti et al., 2019).

Makarov et al. (2020) indicate that the growth of plants inoculated with microorganisms gradually increases compared with non-inoculated controls, regardless of the soil medium in which they are established due to increases in the activity of microorganism exoenzymes that stimulate plant growth. Similarly, Singh et al. (2021) report that plants have evolved in close association with the beneficial microorganisms that are involved in the biosynthetic pathway of plant metabolism. Moreover, they have numerous secondary functions in plant survival and growth. The physiology and biochemistry of inoculated crops in different ecosystems are reflected in the photosynthetic pigmentation of the leaves (carotenoids and chlorophyll), antioxidant potential, root volume, greater efficiency in the uptake of nutrients due to appropriate use of endophytic fungi and rhizobacteria (Malik et al., 2021).

**Relative chlorophyll concentration**

There were significant (p≤0.05) differences in the SPAD readings in our basil plants inoculated with TH, GC or BS (Figure 3). Co-inoculation with TH+GC and BS+GC increased chlorophyll concentration by 23 and 19%, respectively, compared to the control. These responses can be attributed to the synergy between the inoculants and the plants, by promoting the photosynthetic activity with a coefficient of variation of 6.17%. Sánchez et al. (2018) and Ajeng et al. (2020) indicate that the greater intensity of green pigmentation is due, among other factors, to the greater uptake of N, P and K that are all involved in the photosynthetic machinery.
The combined use of these organisms increases the uptake of nutrients from the soil and thus the leaf chlorophyll content, regardless of the chemical and physical characteristics of the soil.

On the other hand, the intensity of the greenness of the leaves may be related to the mechanisms involved in the species of fungi and bacteria in the elicitation process that interacts in the plant biochemical pathways to produce secondary metabolites in large quantities. In this way, the also raise the quality of the crop in terms of growth, aroma, flavour and colour (Aguirre-Becerra et al., 2021).

Figure 3. SPAD readings for leaves of basil plants inoculated with beneficial microorganisms

\[ \text{TH} = \text{Trichoderma harzianum; BS} = \text{Bacillus subtilis; GC} = \text{Glomus cubense}; \text{Es} \chi = \text{standard error of the mean; CV} = \text{Coefficient of variation. Bars indicate standard error. Different letters in the same column indicate significant differences according to Tukey's multiple range test (p≤0.05)}. \]

Favourable trends have been reported in chlorophyll content of basil plants inoculated with AMF consortia and combined with PGPR, due to the direct or indirect mechanisms in which microbes produce antibiotics, metabolites, phytohormones and organic compounds that stimulate nutrient uptake and plant growth (Chiquito-Contreras et al., 2018; Anguiano-Cabello et al., 2019). Likewise, Emmanuel and Babalola (2020) and Suchitra et al. (2020) mention that mycorrhizal fungi increase mineral uptake and provide protection from abiotic stresses in plants to changes in photosynthetic products. Similarly, the microbe effects are greater when combined with PGPR suggesting that, by intervening in the physiology of plants, control the levels of plant hormones or indirectly reduce the inhibitory effects of pathogens.

Bordoloi and Shukla (2020) reported that the chlorophyll concentration in \textit{Piper mullesua} plants grown under greenhouse conditions varied significantly between isolates of arbuscular mycorrhizal fungi. Likewise, they report that there was a higher trend (p > 0.001) in mycorrhizal treatments compared to non-inoculated controls in sterile soil. In turn, Gómez-Bellot et al. (2020) in \textit{Viburnum tinus} L. plants found leaf chlorophyll concentration as well as the stomatal conductance were increased with inoculation of a microbial complex of AMF, compared to simple applications of mycorrhizae. These authors associated these effects with the specificity of the microbial complex in the rhizosphere under different irrigation conditions.

In consideration of the benefits of \textit{T. harzianum} in combination with PGPR, Singh et al. (2020) showed in greenhouse-grown \textit{Ocimum sanctum} L. that the combined effect of these improved the uptake of soil nutrients and the photosynthetic efficiency of the leaves to generate increased fresh biomass weight (83.78%), compared to uninoculated control plants. Neelipally et al. (2020) demonstrated that \textit{Arachis hypogaea} L. plants inoculated with \textit{T. harzianum + Bradyrhizobium spp} in the greenhouse generated higher dry biomass and chlorophyll concentration (p <0.0001) compared to simple applications and the control treatment. However, they found that the chlorophyll index and the accumulation of N in plant tissues was higher with
the simple inoculation of *Bradyrhizobium* with respect to *T. harzianum*, due to the interaction effects between endophytic fungi and rhizobacteria, as well as the particular functions of each in the growth of plants.

**Macronutrient extraction**

Compared to the control, the uptake of macronutrients increased 80% in basil plants treated with beneficial microorganisms (Table 3), with significant differences (p≤0.05) due to inoculation with TH+GC and BS+GC which showed increases of 82 and 71%, respectively. A similar effect was also observed between GC and TH+BS for all macronutrients. The inoculations with TH, BS, and with TH+BS+GC behaved similarly. The response found in this variable coincided with those described previously, in relation to the fact that the basil plants established a direct relationship between the mixed applications of the inoculants TH+BS and TH+GC unlike the single inoculations with TH, BS and GC and with the triple inoculation TH+BS+GC.

Likewise, a tendency for increased N and K uptake was observed (expressed as kg ha⁻¹) compared with the other macronutrients. Similarly, P was the element most strongly taken up by the plants. This could be associated with the participation of N, P and K in photosynthesis, respiration, photosynthate translocation, protein synthesis and activation of key enzymes for various biochemical functions in plants (Delgado-Ospina et al., 2012); In addition to the fact that microorganisms also exert effects on the uptake of macronutrients, which coincides with that reported by Bordoloi and Shukla (2020) who argue that microorganisms also participate in the supply and uptake of nutrients by plants.

Table 3. Uptake of macronutrients by basil plants inoculated with beneficial microorganisms

| Treatment     | N  | P  | K  | Ca | Mg |
|---------------|----|----|----|----|----|
| Control       | 1.66 e | 1.66 e | 5.15 c | 0.56 e | 0.54 e |
| TH            | 3.44 d | 3.44 d | 10.62 d | 1.17 d | 1.07 c |
| BS            | 3.03 d | 3.03 d | 9.35 d | 1.03 d | 0.80 d |
| GC            | 6.80 c | 6.80 c | 20.48 c | 2.26 c | 1.09 c |
| TH+BS         | 6.48 c | 6.48 c | 20.98 c | 2.31 c | 1.15 c |
| TH+GC         | 10.43 a | 10.43 a | 32.17 a | 3.54 a | 1.96 a |
| BS+GC         | 8.10 b | 8.10 b | 24.97 b | 2.73 b | 1.54 b |
| TH+BS+GC      | 3.07 d | 3.07 d | 9.48 d | 1.04 d | 0.79 d |
| Es χ          | 0.15* | 0.15* | 0.48* | 0.04* | 0.03* |

*TH = Trichoderma harzianum; BS = Bacillus subtilis; GC = Glomus cubense; Es χ = standard error of the mean. Different letters in the same column indicate significant differences according to Tukey’s multiple range test (p≤0.05).*

Arango et al. (2012) reported a significant increase in the level of macronutrients in mycorrhizal plants of *Menta piperita* L. grown in a greenhouse, with a direct relationship between fresh and dry matter and leaf area at 60 days after transplantation. Studies conducted by Delgado-Ospina et al. (2012), indicate that the uptake of nutrients can vary according to the species and the phenological phases of the crop. They reported K as the element with the highest foliar extraction requirement and P and Mg with lower demands in *Lippia organoides* H.B.K plants grown in a greenhouse.

As has also been reported, the use of *T. harzianum* as a disease control fungus and growth promoter in aromatic and medicinal plants mixed with other beneficial microorganisms or inorganic fertilisers increase the uptake of essential soil nutrients, and plant growth and yield (Quiroga et al., 2015). On the other hand, Sun et al. (2020) reported a significant increase in the content of N, P, K, Ca and Mg in tea plants (*Camellia sinensis* (L.) O. Kuntze inoculated with *Glomus etunicatum* under shaded conditions. At the same time, they indicate that the results could be due to the involvement of mycorrhizae in the induction of plant resistance to stress, increased root biomass and uptake of nutrients and the hormone content of the root, actively regulating the transport pathway and synthesis.
Ortas et al. (2021) suggest mycorrhizal colonisation regulates the expressions of chloroplast genes in leaves and improves plant water status. They also confirm that essential soil nutrients are taken up by extraradical hyphae and the differences in their acquisition, as well as in transpiration and stomatal conductance are related to the mycorrhizal efficiency of different species or the combination with other microorganisms, which is related with the results obtained in the present study.

Conclusions

Compared with the control, a 58% increase in growth of basil was obtained with inoculation with the combination Trichoderma harzianum + Glomus cubense, and a 55% increase in growth with respect with single inoculations of Bacillus subtilis, Glomus cubense, Trichoderma harzianum and with the triple inoculation of these microorganisms. Secondly, an increase of 51% in the growth of this fine herb was obtained with the co-inoculation of Bacillus subtilis + Glomus cubense with respect to the control and 38% with the other treatments, respectively. These plant growth responses are reflected in increases in both fresh and dry biomasses, leaf area, various morphological variables, the concentration of chlorophyll and the amounts of the main macronutrients. In contrast, the effects found with the joint application of the three inoculants and simple applications were lower in all the variables evaluated. These results suggest that the combined inoculation of beneficial microorganisms Trichoderma harzianum + Glomus cubense or Bacillus subtilis + Glomus cubense, is a useful and more sustainable alternative management for cultivation of basil under greenhouse conditions.

Authors’ Contributions

Conceptualisation: PJL; JACG, YTA; execution of the investigation: YTA; drafting-original draft: YTA, PJL, JCHG; Methodology, review and supervision: PJL, IAT; advice and review: DGS, JOPG, VLM, MCRB, OB-F. All authors read and approved the final manuscript.

Acknowledgements

The first author thanks the National Council of Science and Technology (CONACYT) of Mexico for support received through a national scholarship 2019-2 CVU 1010338 to carry out doctoral studies.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

Abdel-Rahman SSA, Abdel-Kader AAS, Khalil SE (2011). Response of three sweet basil cultivars to inoculation with Bacillus subtilis and arbuscular mycorrhizal fungi under salt stress conditions. Nature and Science 9(6):93-111.
Abdollahi-Arpanahi A, Feizian M, Mehdipourian G (2020). Influence of drought stress and plant growth promoting rhizobacteria (PGPR) inoculation on morphological characteristics, essential oil yield and composition of
Thymus daenensis Clack. Iranian Journal of Medicinal and Aromatic Plants Research 36(3):417-428.
https://doi.org/10.22092/ijmapr.2020.342064.2731

Aguirre-Becerra H, Vázquez-Hernández MC, Sáenz de la OD, Alvarado-Mariana A, Guevara-González RG, García-Trejo JF, Feregrino-Pérez AA (2021). Role of stress and defense in plant secondary metabolites production. In: Pal D, Nayak AK (Eds). Bioactive Natural Products for Pharmaceutical Applications. Springer International Publishing pp 151-195. https://doi.org/10.1007/978-3-030-54027-2_5

Ajeng A A, Abdullah R, Malek MA, Chew K W, Ho YC, Ling TC, Lau BF, Show PL (2020). The effects of biofertilizers on growth, soil fertility, and nutrients uptake of oil Palm (Elaeis Guineensis) under greenhouse conditions. Processes 8(12):1681. https://doi.org/10.3390/pr8121681

Alcántar GG, Sandoval VM (1999). Manual de Análisis Químico de Tejido Vegetal. Guía de muestreo, preparación, análisis e interpretación [Manual of chemical analysis of plant tissue. Guide to sampling, preparation, analysis and interpretation]. Publicación especial Núm. 10. Sociedad mexicana de la Ciencia del Suelo, A. C. Chapingo, México pp 155.

Álvarez M, Tucta F, Quispe E, Meza V (2018). Incidencia de la inoculación de microorganismos benéficos en el cultivo de fresa (Fragaria sp.) [Incidence of the inoculation of beneficial microorganisms in the strawberry (Fragaria sp.) Crop]. Scientia Agropecuaria 9(1):33-42. https://doi.org/10.17268/sci.agropecu.2018.01.04

Anguiano-Caballo JC, Flores-Olivas A, Olalde-Portugal V, Arredondo-Valdés R, Laredo-Alcalá EJ (2019). Evaluation of Bacillus subtilis as promoters of plant growth. Revista Bio Ciencias 6(e418):1-13. https://doi.org/10.15741/revbio.06.e418

Arango MC, Ruscitti M, Betran J (2013). Alternativa para aumentar la producción en plantas de Mentha x piperita L [Alternative to increase production in plants of Mentha x piperita L]. Contacto Rural 3:6-7.

Arango MC, Ruscitti MF, Ronco MG, Beltrano J (2012). Mycorrhizal fungi inoculation and phosphorus fertilizer on growth, essential oil production and nutrient uptake in peppermint (Mentha piperita L.). Revista Brasileira de Plantas Medicinais 14(4):692-699. https://doi.org/10.1590/S1516-05722012000400018

Baum C, El-Tohamy W, Gruda N (2015). Increasing the productivity and product quality of vegetable crops using arbuscular mycorrhizal fungi: A review. Scientia Horticulture 187:131-141. https://doi.org/10.1016/j.scienta.2015.03.002

Bhat MA, Kumar V, Bhat MA, Wani IA, Dar FL, Farooq I, ... Jan AT (2020). Mechanistic insights of the interaction of plant growth-promoting rhizobacteria (PGPR) with plant roots toward enhancing plant productivity by alleviating salinity stress. Frontiers in Microbiology 11. https://doi.org/10.3389/fmicb.2020.01952

Bordoloi A, Shukla AK (2020). Effect of mycorrhizal application on plant growth and nutrient uptake of Piper mullesua plantlets under sterilized, unsterilized and field soil condition. International Journal of Current Microbiology and Applied Sciences 9(5):2948-2960. https://doi.org/10.20546/jcmas.2020.905.338

Chiquito-Contreras RG, Solís-Palacios R, Reyes-Pérez JJ, Reyes J, Murillo-Amador B, Alejandro-Rosas J, Hernández-Montiel LG (2018). Promoción del crecimiento de plantas de albahaca utilizando hongos micorrícos arbusculares y una bacteria marina [Promotion of basil plant growth using arbuscular mycorrhizal fungi and marine bacteria]. Acta Universitaria 28(6):68-76. https://doi.org/10.15174/au.2018.2086

CONAGUA (2018). Estadísticas Agrícolas de las Unidades de Riego. Año agrícola 2016–2017. (Edición 2018; pp 925). Comisión nacional del agua [Agricultural Statistics of the Irrigation Units. Agricultural year 2016–2017. (2018 Edition; p. 925). Comisión nacional del agua. México. Retrieved 2020 December 11 from https://files.conagua.gob.mx/conagua/publicaciones/Publicaciones/SGIH-3-18.pdf

Corrales LC, Caycedo-Lozano L, Gómez-Méndez MA, Ramos-Rojas SJ, Rodríguez-Torres JN (2017). Bacillus spp: Una alternativa para la promoción vegetal por dos caminos enzimáticos [Bacillus spp: An alternative for plant promotion by two enzymatic pathways]. Nova 15(27):45. https://doi.org/10.22490/24629448.1958

Emmanuel OC, Babalola OO (2020). Productivity and quality of horticultural crops through co-inoculation of arbuscular mycorrhizal fungi and plant growth promoting bacteria. Microbiological Research 239:126569. https://doi.org/10.1016/j.micres.2020.126569

Esquivel-Quispe R (2020). Propagación de hongos micorrizógenos arbusculares nativos y su influencia en la producción de maíz amiláceo en Paquée – Ayacucho. Segunda parte: Hacia una agricultura sostenible [Propagation of native arbuscular mycorrhizal fungi and their influence on the production of starchy corn in Paquée-Ayacucho. Second two: Towards sustainable agriculture]. Journal of the Selva Andina Biosphere 8(1):53-63.
Fernández F, Gómez R, Vanegas LF, Martínez MA, de la Noval BM, Rivera R (2000). Producto inoculante micorrízógeno [Mycorrhizal inoculant product]. (Patent No. 22641). Habana Cuba.

Furnell S, Timoshyna A, Harter D (2019). Voluntary certification standards and the implementation of CITES for trade in medicinal and aromatic plant species. Traffic Bulletin 31(2):79-88.

Gómez-Bellot MJ, Ortuño MF, Álvarez S, Sánchez-Blanco MJ (2020). Influence of mycorrhizal or microbial complex inoculation on laurustinus plants irrigated with reclaimed water. The Journal of Horticultural Science and Biotechnology 95(5):661672. https://doi.org/10.1080/14620316.2020.1727781

Hernández-Melchor DJ, Ferrera-Cerrato R, Alarcón A (2019). Trichoderma: Importancia agrícola, biotecnológica, y sistemas de fermentación para producir biomasa y enzimas de interés industrial [Trichoderma: Agricultural, biotechnological importance, and fermentation systems to produce biomass and enzymes of industrial interest]. Chilean Journal of Agricultural and Animal Sciences 35(1):98-112. https://doi.org/10.4067/S0719-38902019005000205

Hernández-Montiel LG, Murillo-Amador B, Chiquito-Contreras CJ, Zúñiga-Castañeda CE, Ruiz-Ramírez J, Chiquito-Contreras RG (2020). Respuesta morfo-productiva de plantas de pimiento morrón biofertilizadas con Pseudomonas putida y dosis reducida de fertilizantes sintéticos en invernadero [Morpho-productive response of bell pepper plants biofertilized with Pseudomonas putida and reduced dose of synthetic fertilizers in the greenhouse]. Revista Técnica Latinoamericana 38(3):583-596. https://doi.org/10.28940/terra.v38i3.651

Kala CP (2015). Medicinal and aromatic plants: Boon for enterprise development. Journal of Applied Research on Medicinal and Aromatic Plants 2 (4): 134-139. https://doi.org/10.1016/j.jarmap.2015.05.002

Katan J, Gamliel A (2012). Soil solarization for the management of soilborne pests: the challenges, historical perspective, and principles. In: Gamliel A, Katan J (Eds). Soil Solarization: Theory and Practice. The American Phytopathological Society. St Paul Minnesota, Chapter 5, USA, pp 45-52.

Leal-Almanza J, Gutiérrez-Coronado MA, Castro-Espinoza L, Lares-Villa F, Cortes-Jiménez JM, Santos-Villalobos S de los (2018). Microorganismos promotores de crecimiento vegetal con yeso agrícola en papa (Solanum tuberosum L.) bajo casa sombra [Plant growth promoting microorganisms with agricultural gypsum in potato (Solanum tuberosum L.) under shade house]. Agrociencia 52(8):1149-1159.

López-Valenzuela EB, Armenta-Bojorquez AD, Hernández-Verdugo S, Apodaca-Sánchez MA, Samaniego-Gaxiola JA, Valdez-Ortiz A (2019). Trichoderma spp. and Bacillus spp. as growth promoters in maize (Zea mays L.). Phyton 88 (1):37-46. https://doi.org/10.32604/phyton.2019.04621

Malik A, Moz VS, Tokas J, Punia H, Malik S, Malik K, ... Karwasra A (2021). Biostimulant-treated seedlings under sustainable agriculture: A global perspective facing climate change. Agronomy 11(1):14. https://doi.org/10.3390/agronomy11010014

Mohamed I, Eid KE, Abbass MHH, Salem AA, Ahmed N, Ali M, … Fang C (2019). Use of plant growth promoting Rhizobacteria (PGPR) and mycorrhizae to improve the growth and nutrient utilization of common bean in a soil infected with white rot fungi. Ecotoxicology and Environmental Safety 171:539-548. https://doi.org/10.1016/j.ecoenv.2018.12.100

Neelipally RTKR, Anoruo AO, Nelson S (2020). Effect of co-Inoculation of Bradyrhizobium and Trichoderma on growth, development, and yield of Arachis hypogaea L. (Peanut). Agronomy 10(9):1415. https://doi.org/10.3390/agronomy10091415

Ojeda-Silveira CM, Murillo-Amador B, Nieto-Garibay A, Troyo-Díezeg E, Reynaldo-Escobar IM, Ruiz-Espinoza FH, García-Hernández JL (2015). Emergencia y crecimiento de plántulas de variedades de albahaca (Ocimum basilicum L.) sometidas a estrés hídrico [Emergence and growth of seedlings of varieties of basil (Ocimum basilicum L.) subjected to water stress]. Ecosistemas y Recursos Agropecuarios 2(5):151-161.
Ortas I, Rafique M, Çekiç FO (2021). Do mycorrhizal fungi enable plants to cope with abiotic stresses by overcoming the detrimental effects of salinity and improving drought tolerance? In Shrivastava N, Mahajan S, Varma A (Eds). Symbiotic Soil Microorganisms. Springer International Publishing Vol. 60, pp 391-428. https://doi.org/10.1007/978-3-030-51916-2_23

Pan J, Huang C, Peng F, Zhang W, Luo J, Ma S, Xue X (2020). Effect of arbuscular mycorrhizal fungi (AMF) and plant growth-promoting bacteria (PGPR) inoculations on Elaeagnus angustifolia L. in saline soil. Applied Sciences 10(3):945. https://doi.org/10.3390/app10030945

Peccatti A, Rovedder APM, Steffen GP, Maldaner J, Missio EL, Witt CS, ... Dalcút LP (2019). Effect of Trichoderma spp. On the propagation of Maytenus ilicifolia Mart. former Reissek. Journal of Agricultural Science 11(3):435. https://doi.org/10.5539/jas.v11n3p435

Quiroga M, Agüero D, Zapata R, Busilacchi H (2015). Activadores de crecimiento y biorrefertilizantes como alternativa al uso de fertilizantes químicos en cultivo de chía (Salvia hispanica L.) [Growth activators and biomulitizers as an alternative to the use of chemical fertilizers in Chia (Salvia hispanica L.) cultivation]. Energías Renovables y Medio Ambiente 35:33-40.

Riahi L, Cherif H, Miladi S, Neifar M, Bejaoui B, Chouchane H, ... Cherif A (2020). Use of plant growth promoting bacteria as an efficient biotechnological tool to enhance the biomass and secondary metabolites production of the industrial crop Pelargonium graveolens L’Hér. Under semi-controlled conditions. Industrial Crops and Products 154:112721. https://doi.org/10.1016/j.indcrop.2020.112721

Rivera RA, Martín GM, Simó JE, Pentón G, Garcia-Rubido M, Ramirez JF, ... Bustamante C (2020). Benefits of joint management of green manure and mycorrhizal inoculants in crop production. Tropical and Subtropical Agroecosystems 23(3):3. https://www.revista.ccca.uchy.mx/ojs/index.php/TSA/article/view/3294

Salome-abarca LF, Cruz EC, Vásquez AL, Nava AD, Palemon FA, Castro EH, ... Cabañas JNS (2015). Biochemical characterization, antioxidant and antibacterial activity of aromatic plants from Guerrero, Mexico. Weber Medicinal Plant Research 1(2):239-246.

Sánchez E, Ruiz JM, Romero L, Preciado-Rangel P, Flores-Córdova MA, Márquez-Quiroz C, ... Márquez-Quiroz C (2018). Son los pigmentos fotosintéticos buenos indicadores de la relación del nitrógeno, fósforo y potasio en frijol ejotero? [Are photosynthetic pigments good indicators of the nitrogen, phosphorus and potassium ratio in green beans?] Ecosistemas y Recursos Agropecuarios 5(15):387-398. https://doi.org/10.19136/era.a5n15.1757

Sharma V, Salwan R, Sharma PN (2017). The comparative mechanistic aspects of Trichoderma and probiotics: Scope for future research. Physiological and Molecular Plant Pathology 100:84-96. https://doi.org/10.1016/j.pmpp.2017.07.005

Singh A, Chaubey R, Srivastava S, Kushwaha S, Pandey R (2021). Beneficial root microbota: Transmogrifiers of secondary metabolism in plants. In Singh KP, Jahagirdar S, Sarma BK (Eds). Emerging Trends in Plant Pathology. Springer, pp 343-365. https://doi.org/10.1007/978-981-15-6275-4_16

Singh S, Tripathi A, Chanotiya CS, Barnawal D, Singh P, Pateli VK, Vajpayee P, Kalra A (2020). Cold stress alleviation using individual and combined inoculation of ACC deaminase producing microbes in Ocimum sanctum. Environmental Sustainability 3(3):289-301. https://doi.org/10.1007/s42398-020-00118-w

Steiner A A (1984). The universal nutrient solution. In 6. International Congress on Soilless Culture, Lunteren (Netherlands), 29 Apr-5 May 1984. ISOSC, pp 633-650.

Stewart A, Hill R (2014). Chapter 31-Applications of Trichoderma in plant growth promotion. In Gupta VK, Schmoll M, Herrera-Estrella A, Upadhyay RS, Druzhinina I, Tuohy MG (Eds). Biotechnology and Biology of Trichoderma Elsevier, pp 415-428. https://doi.org/10.1016/B978-0-444-59576-8.00031-X

Suchitra R, Rajaram K, Arunkumar N, Kumar DSS (2020). Contribution of beneficial fungi for maintaining sustainable plant growth and soil fertility. In Varma A, Tripathi S, Prasad R (Eds). Plant Microbe Symbiosis. Springer International Publishing, pp 105-113. https://doi.org/10.1007/978-3-030-36248-5_6
Sun M, Yuan D, Hu X, Zhang D, Li Y (2020). Effects of mycorrhizal fungi on plant growth, nutrient absorption and phytohormones levels in tea under shading condition. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 48(4):2006-2020. https://doi.org/10.15835/nbha48412082

Tamayo-Aguilar Y, Riera-Nelson MC, Terry-Alfonso E, Juárez-López P, Rodríguez-Matos Y (2019). Respuesta de *Vigna unguiculata* (L) Walp ante la aplicación de bioproductos en condiciones de huertos intensivos [Response of *Vigna unguiculata* (L) Walp to the application of bioproducts in conditions of intensive orchards]. Acta Agronómica 68(1):41-46. https://doi.org/10.15446/acag.v68n1.72797

Tian L, Lin X, Tian J, Ji L, Chen Y, Tran L, Tian C (2020). Research advances of beneficial microbiota associated with crop plants. International Journal of Molecular Sciences 21(1792):2-18. https://doi.org/10.3390/ijms21051792