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Use of microorganisms with biofertilizer potential in basil (*Ocimum basilicum* L.) crop

Uso de microorganismos con potencial biofertilizante en el cultivo de albahaca (*Ocimum basilicum* L.)

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ABSTRACT

Basil (*Ocimum basilicum* L.) cultivation in Colombia is highly versatile in the market and requires technologies to increase its competitiveness and sustainability. The use of biofertilizers represents an efficient strategy to improve productivity. This study aimed to evaluate the effect of inoculating arbuscular mycorrhizal fungi (AMF): *Claroideoglomus etunicatum* and *Gigaspora rosea*; and the biofertilizers of plant growth-promoting bacteria (PGPB): *Rhizobium leguminosarum*, *Azospirillum brasilense,* and *Herbaspirillum frisingense*, Fosfotal®, and monibac®, individually and in mixtures on crop production. The experiment was carried out on a commercial farm using a randomized complete block design, three replications, and nine treatments. Results show higher aerial fresh biomass with the inoculation of G. rosea (T3), followed by *C. etunicatum* (T4), and its mixture with PGPB2 (T8 and T7). There were positive correlations between T3 and G. rosea and plant length, leaf area index, number of stems, and phosphorus uptake. Treatments 4 and 7 showed positive relationships with *C. etunicatum* and fresh and dry biomass, chlorophyll, nitrogen uptake, and stomatal conductance. Treatments 2 and 8 showed affinity with *Azospirillum brasilense*, *Herbaspirillum* sp., and *Rhizobium* sp., and the physiological variables of intrinsic and extrinsic water use efficiency and the ratio of net photosynthesis to intercellular carbon. It is concluded that AMF, individually or in mixture with PGPB, promotes the growth and development of basil plants, leading to increased fresh biomass production.

Keywords: AMF; bacteria; fertilization; mycorrhizae; PGPB; sustainability

RESUMEN

El cultivo de albahaca (*Ocimum basilicum* L.) en Colombia presenta alta versatilidad en el mercado y requiere de tecnologías que incrementen su competitividad y sostenibilidad. El uso de biofertilizantes es una estrategia eficiente para mejorar la productividad. El objetivo fue evaluar el efecto de la

inoculación de hongos micorrizícos arbusculares (HMA): *Claroideoglomus etunicatum* y *Gigaspora rosea*; y los biofertilizantes de bacterias promotoras de crecimiento vegetal (BPCV): *Rhizobium leguminosarum*, *Azospirillum brasilense y Herbaspirillum frisingense*, Fosfotal® y monibac®, de forma individual y en mezcla sobre la producción del cultivo. La investigación se desarrolló en una finca comercial con un diseño en bloques completos al azar, tres repeticiones y nueve tratamientos. Los resultados reflejan mayor biomasa fresca aérea con la inoculación de *G. rosea* (T3), seguido de *C. etunicatum* (T4) y en mezcla con las BPCV2 (T8 y T7). Se presentaron correlaciones positivas del T3 y *G. rosea* con la longitud de planta, el índice de área foliar, el número de tallos y la absorción de fósforo. Los tratamientos 4 y 7 presentaron una relación positiva con *C. etunicatum* y la biomasa fresca y seca, la clorofila, la absorción de nitrógeno y la conductancia estomática. Los tratamientos 2 y 8 presentaron afinidad con *Azospirillum brasilense*, *Herbaspirillum* sp. y *Rhizobium* sp. y las variables fisiológicas de eficiencia en el uso de agua intrínseco y extrínseco y la relación de fotosíntesis neta y carbono intercelular. Se concluye que los HMA, individualmente o en mezcla con BPCV, promueven el crecimiento y desarrollo de plantas de albahaca con aumento en la producción de biomasa fresca.

Palabras clave: Bacterias; BPCV; fertilización; HMA; micorrizas; sostenibilidad

INTRODUCTION

Basil (*Ocimum basilicum* L.) is an annual herbaceous plant of culinary use belonging to the Lamiaceae family and native to India and other Asian regions (Makri & Kintzios, 2008). It possesses great importance due to its versatility and market demand, making it one of the most important aromatic plants used to flavor foods as a condiment, both fresh and dried (Palermo *et al.*, 2024). In addition, its extracts have medicinal properties that enhance its value in natural and alternative medicine (Filip, 2017; Kisa *et al.*, 2021).

In Colombia, basil cultivation covered 232.5 hectares in 2022, yielding 4.47 t ha-1 (Agronet, 2024). In the same year, according to ANALDEX (2023), the country reached a record figure for exports of aromatic plants, with external sales of USD 49.5 million and a growth of 15.8% compared to 2021. Meanwhile, the weight of sales to foreign markets in 2022 recorded 12,281.9 net tons, with an increase of 13.2% compared to 2021.

The department of Tolima, in Colombia, offers favorable conditions for basil cultivation due to its climate and geographic characteristics. This production system represents opportunities for employment generation and requires technologies that increase productivity, favoring the region's sustainable development. One of these technologies is the use of biofertilizers, which are efficient strategies that have been shown to improve crop yields under stress conditions (Rad *et al.*, 2022).

Plant growth-promoting bacteria (PGPB) and arbuscular mycorrhizal fungi (AMF) colonize plant roots, enhancing growth and development through direct and indirect mechanisms (Khalediyan *et al.*, 2021). Inoculation of productive systems with free-living nitrogen-fixing bacteria such as *Azotobacter chroococcum* and *Azospirillum lipoferum* not only fixes atmospheric nitrogen but also releases phytohormones such as indole-3-acetic acid (IAA) essential for root development in plants nutrient uptake, and improves the photosynthesis process. In addition, PGPB and AMF can release phosphorus (P) from inorganic and organic reserves of total P through solubilization and mineralization (Cappellari *et al.*, 2013). Direct inoculation of medicinal and aromatic plants (*Origanum majorana*, *Origanum* × *majoricum*, *Tagetes minuta, Mentha piperita*, and *O. basilicum*) with different PGPB and AMF species has shown a significant increase in plant development, secondary metabolite production, and phenolic compound synthesis (Yilmaz & Karik, 2022).

Research evaluating the effect of inoculation of PGPB as *Bacillus amyloliquefaciens* GB03 on basil plants demonstrated significant improvements in plant growth parameters, including shoot fresh/dry weight and leaf area index, as well as higher essential oil yield (Tahami *et al.*, 2017). Finally, studies by Emmanuel & Babalola (2020) have shown that the joint application of PGPB and AMF enhance crop productivity. Olanrewaju *et al.* (2017) and Nadeem *et al.* (2017) demonstrated that PGPB promotes plant development, and that AMF establishes symbiosis with plant roots to enhance nutrient uptake and alter their physiology to resist biotic and abiotic factors. Therefore, evaluating the effect of AMF and PGPB inoculation in a consortium and individually on the basil (*Ocimum basilicum* L.) crop may be beneficial to improve physiological conditions and biomass yields under field conditions.

MATERIAL AND METHODS

Location

In the municipality of Espinal, Tolima, Colombia, an experiment was established in a commercial production lot at 313 meters above sea level (m.a.s.l.). A crop cycle was established using basil plant material (*Ocimum basilicum* L., cv. Nufar) from the second half of 2023 to the first half of 2024 with a total of 7 productive cuts. A randomized complete block experimental design was used, with three replications and nine treatments (Table 1). Each experimental unit consisted of thirty-three plants per treatment.

Table 1. *Inoculation treatments applied to the basil crop*

Treatment	Acronym	Description
1	PGPB 1	*Rhizobium pusense* "B02" (Fosfotal®), *Azotobacter chroococcum* "AC1 & AC10" (Monibac®).
2	PGPB 2	*Rhizobium leguminosarum* "T88", *Azospirillum brasilense* "D7" & *Herbaspirillum frisingense* "AP21".
3	AMF 1	*Gigaspora rosea*
4	AMF 2	*Claroideoglomus etunicatum*
5	PGPB 1 + AMF 1	*Rhizobium pusense* "B02" (Fosfotal®), *Azotobacter chroococcum* "AC1 & AC10" (Monibac®), *Gigaspora rosea*.
6	PGPB 1 + AMF 2	*Rhizobium pusense* "B02" (Fosfotal®), *Azotobacter chroococcum* "AC1 & AC10" (Monibac®), *Claroideoglomus etunicatum*.
7	PGPB 2 + AMF 1	*Rhizobium leguminosarum* "T88", *Azospirillum brasilense* "D7" & *Herbaspirillum frisingense* "AP21", *Gigaspora rosea*.
8	PGPB 2 + AMF 2	*Rhizobium leguminosarum* "T88", *Azospirillum brasilense* "D7" & *Herbaspirillum frisingense* "AP21", *Claroideoglomus etunicatum*.
9	Control	Not inoculated

PGPB: Plant Growth-Promoting Bacteria.
AMF: Arbuscular Mycorrhizal Fungi.

Microorganisms

For the inoculation treatments, the AMF, *Claroideoglomus etunicatum* (Becker & Gerdemann, 1977; Schüßler & Walker, 2011) and *Gigaspora rosea* (Nicolson & Schenck, 1979), were used due to their potential reported in other

studies (Copetta et al., 2006; De Almeida Silva et al., 2021; Hazzoumi et al., 2015). In addition, strains *Rhizobium pusense* (B02), *Azospirillum brasilense* (D7), *Herbaspirillum frisingense* (strain AP21), *Rhizobium leguminosarum* (T88), and *Azotobacter chroococcum* strains (AC1 and AC10) were used for PGPB. These strains were provided by Agrosavia microorganism germplasm bank and are registered under accession number 129 in the biological collections of the Instituto de Investigación de Recursos Biológicos Alexander Von Humboldt in Bogotá, Colombia.

Inoculum preparation

The Arbuscular Mycorrhizal Fungi (AMF) *C. etunicatum* and *G. rosea* were multiplied for 6 months by "trap culture" (Morton *et al.*, 1995) using onion hosts (*Allium fistulosum* L.) in 500 g pots, with sterile soil: sand substrate (3:1 v/v). To ensure quality control of the AMF inoculum, spore quantification was performed by the sieving and wet decantation technique proposed by Gerdemann & Nicolson (1963), with a concentration of 105 spores/g substrate. Meanwhile, the production of biomass of plant growth-promoting bacteria was carried out as follows: for strains *A. brasilense* (D7) and *H. frisingense* (strain AP21), the DYGS culture medium was used (Baldani *et al.*, 2014). For strains *A. chroococcum* (AC1 and AC10), EMR culture medium was used (Moreno-Galvan *et al.*, 2011), and for strains, *R. pusense* (B02) and *R. leguminosarum* (T88), YM culture medium was used (Vincent, 1970). Standard fermentation conditions were 48 h at 30°C and 120 rpm.

Inoculation in basil plants (*Ocimum basilicum* L.)

AMF inoculation was performed at a concentration of 70 spores/g substrate. For PGPB, bacterial concentration was standardized to an optical density (OD) of 600nm = 0.5 (~108 colony-forming units (CFU) mL-1). Bacterial strains for co-inoculations were mixed at a 1:1 ratio (v/v) to obtain 5 mL of mixed inoculants just before plant inoculation. For uninoculated treatments, a sterile liquid medium was used. The inoculation of each of the microorganisms and their respective co-inoculations (Table 1) were applied at the time of sowing, which was carried out in nursery conditions from basil seeds using peat as substrate in trays of 200 cells. The AMF was inoculated once at sowing in the nursery, while PGPB inoculation occurred at both sowing and planting in the field.

Fertilization was managed at 75% in all treatments according to crop requirements and soil analysis. The agronomic management of the crop was uniform in all blocks.

Data variables and analysis

The quantification of the growth variables as plant fresh weight (PFW) and plant dry weight (PDW) were carried out in seven cutting stages with commercial value using a Sartorius Entris precision balance. During the first cutting (called "Pinch"), the variables related to gas exchange were measured, including net photosynthesis (A - lmol CO2 m2 s-1), transpiration rate (E - mmol H2O m2 s-1), stomatal conductance (gs - mol H2O m2 s-1), intercellular carbon (Ci - lmol CO2 m2 s-1). These measurements were taken using an Infra-Red Gas Analyzer (IRGA, LI-6400XT, Li-COR Inc., Lincoln, NE).

Gas exchange variables were evaluated at 45 days after inoculation (DAI) when flowering started, and the plant had greater photosynthetic activity. The analysis was realized with a portable infrared gas analyzer (IRGA, model LI-6400XT, LI-COR, Nebraska, USA). The airflow was 300 mL min-1, and photosynthetically active radiation (PAR) was 1200 mmol m2 s1. Data collection was performed

between 9:00 and 10:00 a.m. The following variables were measured: stomatal conductance (gs – mol H2O m2 s-1), net CO2 assimilation rate (A – lmol CO2 m2 s-1), transpiration (E – mmol H2O m2 s-1), internal CO2 concentration (Ci – lmol CO2 m2 s-1), water use efficiency (WUE ¼ A/E), intrinsic water use efficiency (iWUE ¼ A/gs), and intrinsic carboxylation efficiency (iCE ¼ A/Ci).

Agronomic variables of fresh aerial biomass were taken during the seven cuttings throughout the production cycle. Physiological variables of leaf area index, intrinsic (WUEi) and extrinsic (WUEe) water use efficiency, net photosynthesis to intercellular carbon ratio (Pn/Ci), and stomatal limitation (Ls) were recorded at the first cut. Nitrogen and phosphorus uptake were recorded at the fourth cut. For the fifth cutting, destructive sampling of the whole plant was carried out with records of height, number of stems, fresh leaves, and root biomass. Finally, in cuts 1, 4, 6, and 7, measurements of chlorophyll and dry weight were made in basil plants.

For the statistical analysis, the assumptions of normality and homoscedasticity were verified. Analysis of variance (ANOVA) and Fisher's least significant difference (LSD) mean comparison test ($p<0.05$) were conducted. Additionally, a canonical correspondence analysis was performed (Chen *et al.*, 2019). Data were analyzed using R® statistical software, version 4.3.1 (R Core Team, 2020).

RESULTS

Biomass production

The results indicate that, in the total of the seven cuts carried out on average, the production of fresh biomass of the basil crop was significantly higher ($p<0.05$) in the inoculated treatments compared to the control treatment without inoculation. The treatment T3 inoculated with Arbuscular Mycorrhizal Fungi (AMF), *G. rosea,* presented an increase of 17%, followed by T4 (16%), T8 (12%), and T7 (10%) in comparison with the control without inoculation (Figure 1a). Likewise, in the dry biomass variable, the treatments that presented significant differences ($p<0.05$) were T7, T3, and T4 compared to the control without inoculation (Figure 1b), with T7 being the mixture of the bacteria *A. brasilense*, *H. frisingense, and R. leguminosarum,* with the AMF *G. rosea* being the one with the best result.

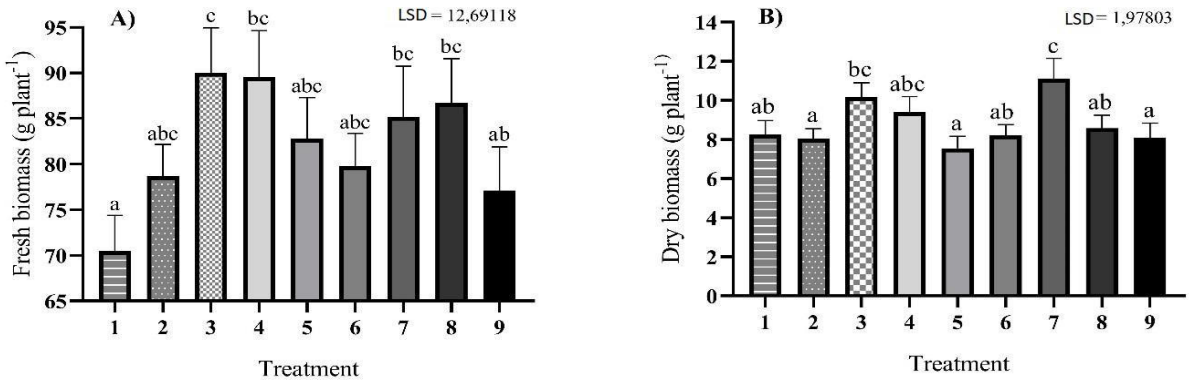

Figure 1. *A. Biomass fresh weight of seven cuttings and B. Biomass dry weight of four cuttings in basil crops. Means with different letters indicate significant differences between treatments (LSD Fisher, p≤ 0.05). LSD: Least Significant Difference. Treatment is explained in Materials and Methods.*

Nitrogen and phosphorus uptake in basil plants did not present significant differences (p<0.05), but a higher nitrogen uptake in treatment 8, which is the mixture of PGPB with AMF (Figure 2a), and a higher phosphorus uptake in treatment 4 (Figure 2b) compared to the other treatments stand out.

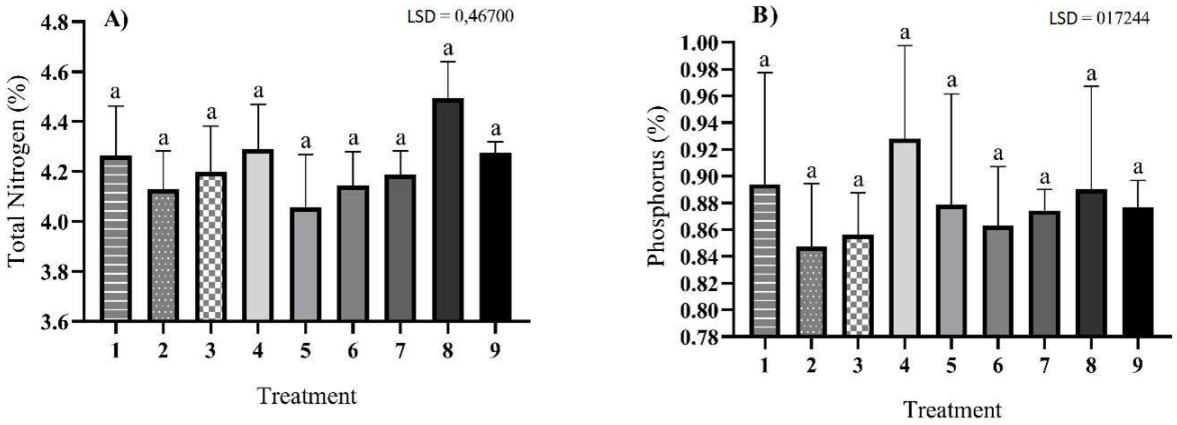

Figure 2. *Nutrient uptake in basil crops. A. Nitrogen. B. Phosphorus. Means with different letters indicate significant differences between treatments (LSD Fisher, p≤ 0.05). LSD: Least Significant Difference. Treatment is explained in Materials and Methods.*

At the fifth cutting, basil plants in the different treatments showed visual differences, as shown in Figure 3. Fresh leaf biomass showed significant differences (p<0.05), with AMF1 (T3) outperforming all except T1 (Figure 4a). In plant height, AMF1 (T3) outperformed T2 by 23%, T5 by 21%, and T8 by 18% (Figure 4b). In fresh root biomass, AMF1 significantly outperformed several treatments and the control (Figure 4c). There were also significant differences in the number of stems between T5 and T6, T7, and T8 (Figure 4d).

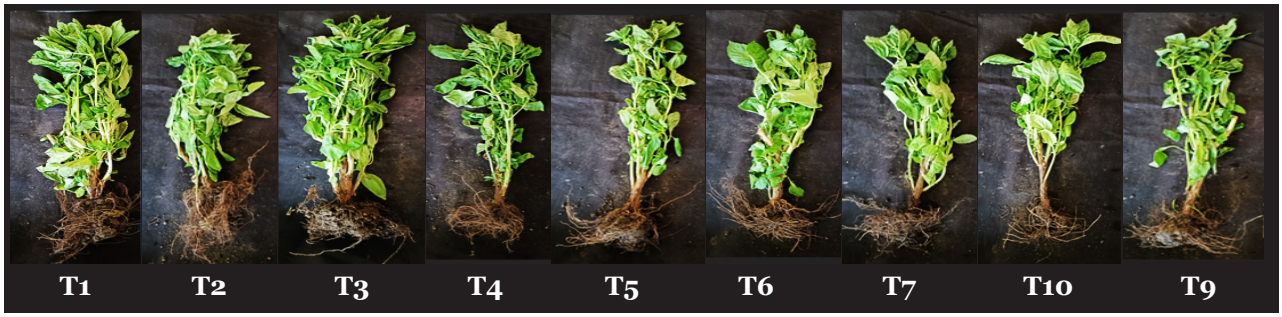

Figure 3. *Basil plants at the fifth cutting of the crop. Treatment is explained in Materials and Methods. T1: PGPB1. T2: PGPB2. T3: AMF1. T4: AMF 2. T5: PGPB1+ AMF1. T6: PGPB1+ AMF2. T7: PGPB 2+ AMF1. T8: PGPB2+ AMF2. T9: Control.*

In cut 5, the fresh leaf biomass showed significant statistical differences (p<0.05), mainly with the inoculation of AMF1 (*G. rosea*), concerning the other treatments (Figure 4a), except for treatment 1, which surpassed by 26%. In plant height, the inoculation treatments stood out, with AMF1 (*G. rosea*) significantly exceeded treatment 2 by 23%, PGPB1 + AMF1 (T5) by 21%, and PGPB2 + AMF2 (T8) by 18%.. Regarding fresh root biomass, significant differences (p<0.05)

were presented with higher values in the inoculation with AMF1 (*G. rosea*) with treatments 2, 4, 5, 8, and 9, exceeding the control by 38%, 58%, 52%, 58%, 58%, and 46%, respectively. No significant differences were observed in treatments with PGPB1 (T1), T6 (PGPB1 + AMF2), or T7 (PGPB2 + AMF1) (Figure 4c). There were significant differences (p<0.05) in the number of stems in T5 (PGPB1+AMF1) to T6 (PGPB1+AMF2) and T7 (PGPB2 + AMF1) (Figure 4c). There were significant differences (p<0.05) in the number of stems in T5 (PGPB1+AMF1) compared to T8 (Figure 4d).

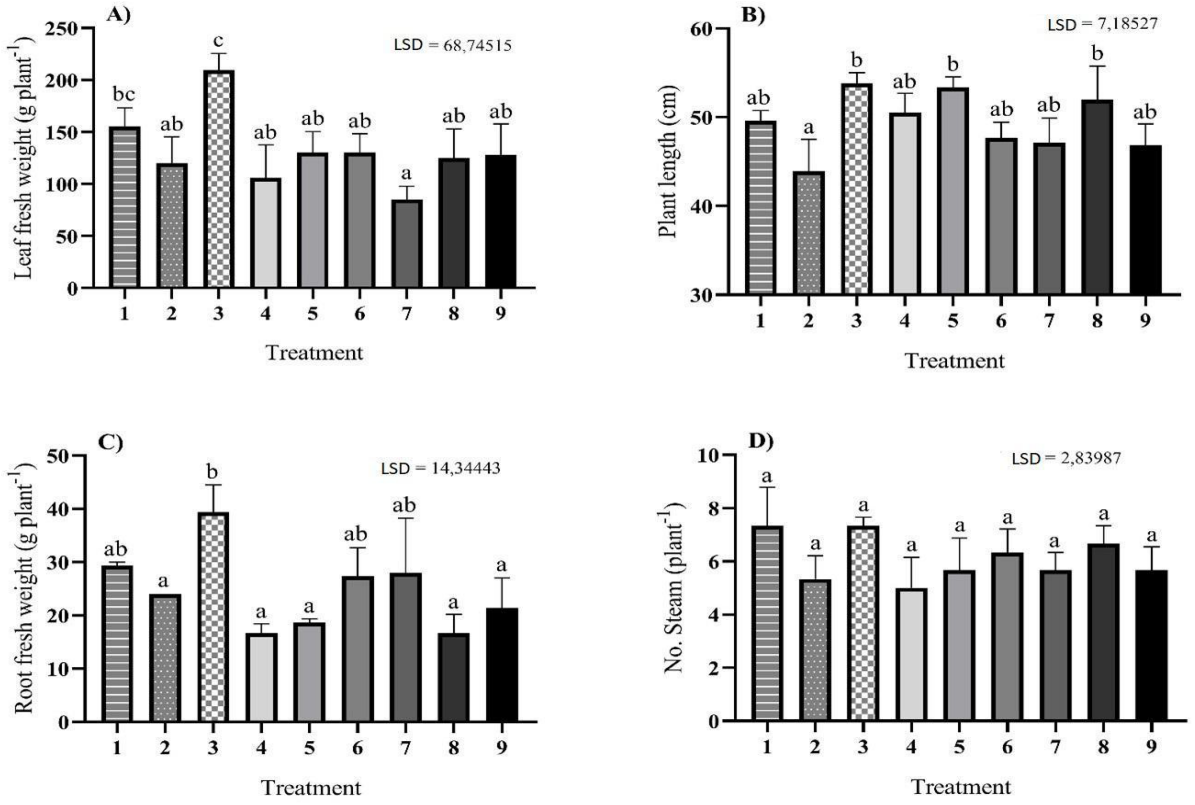

Figure 4. *Agronomic response of basil plants at the fifth cutting of the crop. A. Fresh aerial biomass. B. Plant height. C. Root fresh biomass. D. Number of stems. Means with different letters indicate significant differences between treatments, according to Fisher's LSD test (p≤ 0.05). LSD: Least Significant Difference. Treatment is explained in Materials and Methods.*

Physiological response

At the time of the first cutting, physiological data were recorded. The leaf area index showed significant differences (p<0.05), with a higher value in the AMF1 inoculation (T3) with all other treatments except T3 and T7 (Figure 5a). Although chlorophyll content did not vary significantly, but an increase of 4% is highlighted in treatment 4 (AMF2) concerning the control treatment (T9) (Figure 5b). There were no significant differences in the other physiological variables. However, it is noteworthy that in intrinsic water use efficiency (WUEi), inoculation treatments 5, 6, 7, and 8 stood out over the control treatment and the individual treatments of AMF and PGPB (Figure 5c). For extrinsic water use efficiency (WUEe), T8 was the highest value (Figure 5d). In the ratio of net photosynthesis to intercellular carbon (Figure 5e), treatments 5, 7, and 8. Finally, all inoculation treatments showed higher stomatal limitation than the control (Figure 5f).

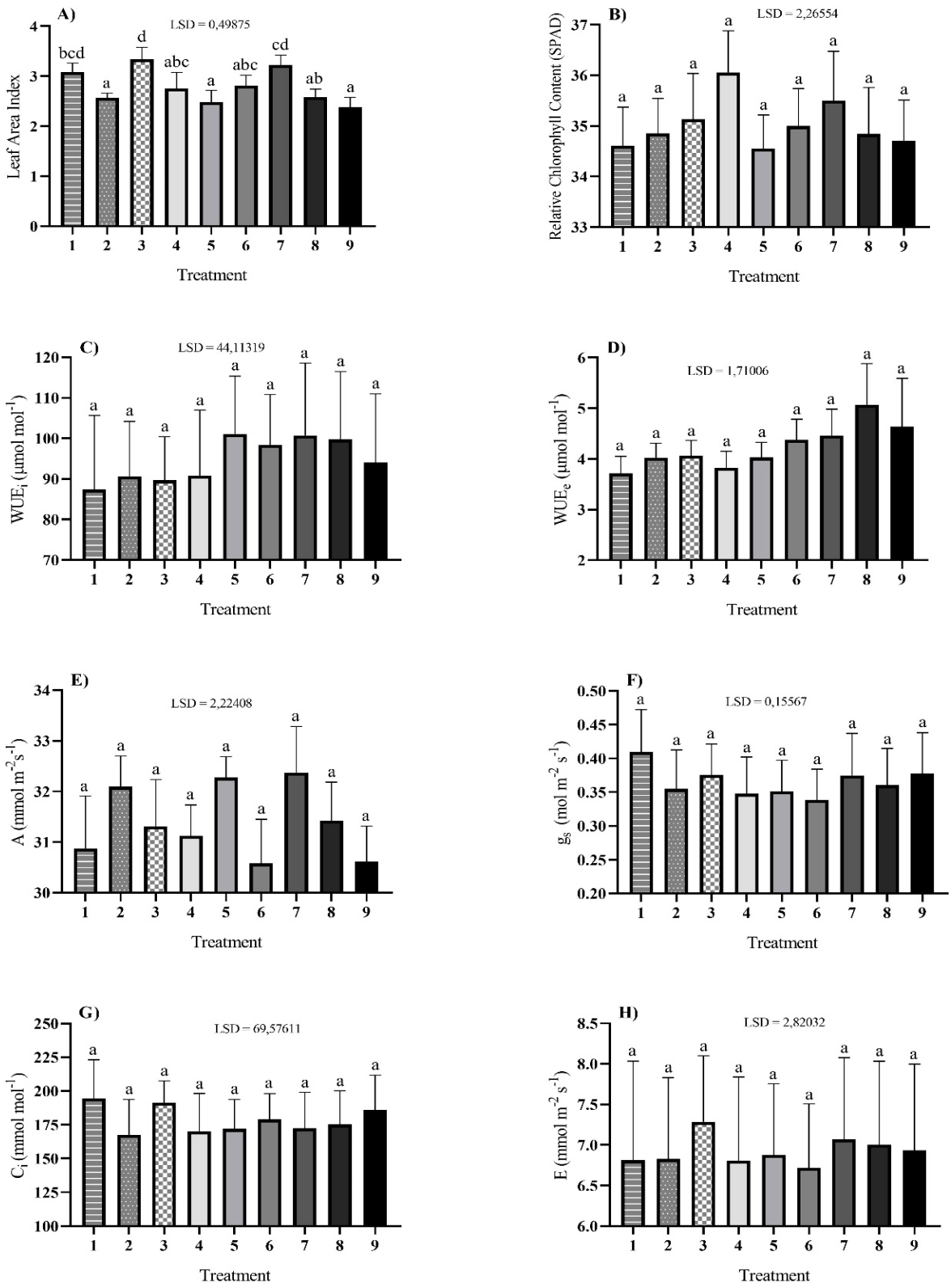

Figure 5. *Physiological variables in the basil crop. A. Leaf area index. B. Relative chlorophyll content (SPAD). C. WUEi: intrinsic water use efficiency. D. WUEe: extrinsic water use efficiency. E. photosynthetic rate. F. gs: stomatal conductance. G. Ci: intercellular CO2 concentration. H. E: Transpiration rate. Means with different letters indicate significant differences between treatments (LSD Fisher, p≤ 0.05). LSD: Least Significant Difference. Treatment is explained in Materials and Methods.*

Response to the inoculation of basil crop

Figure 6 shows the behavior and interaction of the basil crop with the different treatments. Through the canonical correspondence analysis (CCA) with a total variance of 83.4%, the positive correlations between variables, microorganisms, and treatments were confirmed, which are enclosed in circles within the graph, highlighting the high affinity of treatment 3 and its inoculation of *G. rosea* with the variables of height, leaf area index, number of stems, and phosphorus absorption. Treatments 4 and 7 showed a positive relationship with the inoculation of *C. etunicatum* and the variables of fresh and dry biomass, chlorophyll, nitrogen uptake, and stomatal limitation. Treatments 2 and 8 showed an affinity with the inoculations of *R. leguminosarum*, *A. brasilense,* and *H. frisingense,* with a positive relationship with the physiological variables of intrinsic and extrinsic water use efficiency and with the ratio of net photosynthesis and intercellular carbon.

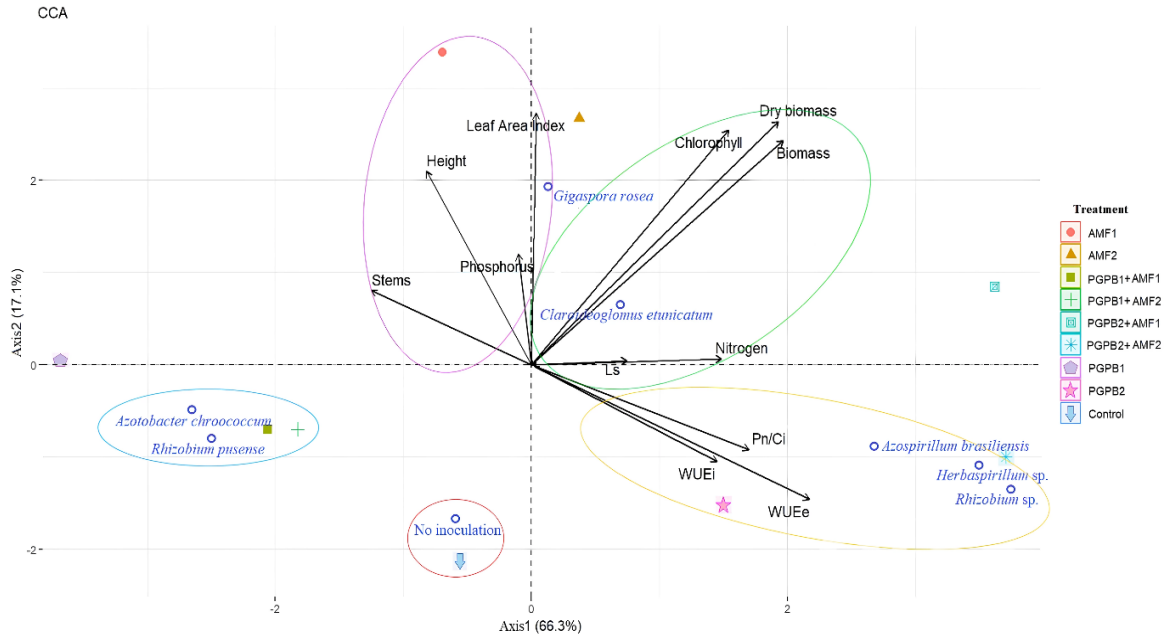

Figure 6. *Canonical correspondence analysis of inoculation with biofertilizing microorganisms and physiological and production variables in basil crops. Treatment is explained in Materials and Methods.*

DISCUSSION

The results of this study provide robust evidence supporting the beneficial effects of microbial inoculation on basil (*Ocimum basilicum*) biomass production, nutrient uptake, and physiological performance. In particular, the arbuscular mycorrhizal fungus *Gigaspora rosea* (AMF1, T3) stood out for significantly enhancing both fresh and dry biomass, plant height, and root development. These findings are in line with Copetta et al. (2006), who reported increased basil yield using a mixture of AMF species with *G. rosea*, *Funneliformis mosseae,* and *Gigaspora margarita*, suggesting synergistic effects of multi-species inoculations.

The superior dry biomass performance in treatment T7 (a microbial mixture including *Azospirillum brasilense, Herbaspirillum frisingense, Rhizobium*

leguminosarum, and *G. rosea*) aligns with observations by Singh et al. (2013), who demonstrated a 55% biomass increase in basil through bioinoculant consortia. The present results also complement Rasouli-Sadaghiani et al. (2010), who found that basil plants colonized by mycorrhizal fungi exhibit significantly greater biomass and improved nutrient content, including nitrogen (N), phosphorus (P), and potassium (K). Finally, Bharti et al. (2016) reported improved growth of O. basilicum CIM-Saumya plants when inoculated with Rhizoglomus intraradices, Dietzia natronolimnaea STR1, and vermicompost under salt-stressed soil conditions in both greenhouse and field trials.

While nitrogen and phosphorus uptake did not show statistically significant differences ($p<0.05$), treatment T8 (combining PGPB2: *R. leguminosarum, A. brasilense, and H. frisingense*, with AMF2: *Claroideoglomus etunicatum*) had the highest nitrogen levels, and treatment T4 (AMF2 alone) showed the highest phosphorus uptake. This highlights the nutrient absorption potential of AMF and PGPB associations, as previously reported by Wilches-Ortiz et al. (2022), who found improved N and P uptake in crops colonized by AMF, and Wu et al. (2024), who emphasized AMF's contribution to enhanced root development and nutrient transport efficiency.Morphologically, the pronounced differences observed at the fifth cutting validate the effect of microbial inoculation on shoot and root biomass, plant stature, and stem number. T3 and T5 (PGPB1 + *G. rosea*) especially improved multiple parameters. This aligns with research from Toussaint et al. (2008) and Baum et al. (2015), which established that most vegetable crops are AMF hosts and respond positively in growth and productivity. The increased stem count in T5 is particularly relevant, as Battini et al. (2016) demonstrated that PGPB and AMF co-inoculation enhances both growth and secondary metabolite biosynthesis in *O. basilicum*.

In terms of physiological performance, *G. rosea* (T3) was again the most effective, significantly improving leaf area index, an essential trait linked to photosynthetic potential. This result corroborates Zulueta-Rodríguez et al. (2016), who observed larger leaf area in *O. basilicum* inoculated with *G. rosea* or *Gigaspora* spp. Although chlorophyll content did not differ significantly, the increase in T4 (AMF2) points to subtle physiological enhancements. Improvements in intrinsic and extrinsic water use efficiency (notably in T5–T8) indicate that microbial inoculants positively influence plant water relations, likely through hormonal modulation and improved root architecture.

The CCA analysis reinforced these associations, showing that different microbial inoculants promote distinct yet complementary plant traits. *G. rosea* was highly associated with structural traits and phosphorus uptake, while *C. etunicatum* and PGPB consortia correlated with biomass, nitrogen uptake, chlorophyll content, and photosynthetic performance. These differentiated but synergistic effects highlight the importance of tailoring microbial inoculants to specific agronomic goals.

Finally, the results agree with Emmanuel & Babalola (2020), who emphasized that AMF and PGPB can reduce dependency on chemical inputs by enhancing nutrient availability and yield. Despite their potential, such microbial tools remain underutilized in commercial horticulture. Our findings support broader application of AMF and PGPB to maximize basil productivity, even under moderate environmental constraints.

CONCLUSIONS

The AMF *G. rosea* responded positively to inoculation in basil, showing improved plant growth, biomass, and vigor.

The AMF *C. etunicatum* favors phosphorus uptake in the basil crop, and in mixture with the PGPB-based biofertilizer *R. leguminosarum*, *A. brasilense* and *H. frisingense*, it favors nitrogen uptake.

The use of biofertilizers contributes to the sustainability of basil crops; the integration of AMF and PBGB positively influences the yield of the production system. The present study serves as a valuable reference to the biofertilizing potential of these microorganisms, both individually and in a mixture, in the basil crop.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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