

Effect of Arbuscular mycorrhizal fungi (AMF) and *Azospirillum* on growth and nutrition of banana plantlets during acclimatization phase

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ABSTRACT

The effect of the co-inoculation of arbuscular mycorrhizal fungi (AMF) and *Azospirillum* on micro-propagated banana seedlings development during their adaptation phase was determined. At the time of transplanting, banana seedlings were inoculated with an indigenous mycorrhizal inoculum containing 10 spores/g at four doses: 0, 50, 100 and 200 g. Seventy days after fungal inoculation, 20 ml of *Azospirillum* in four concentrations (0, 10⁶, 10⁷ and 10⁸ CFU/ml) were applied. Finally, after 98 days from the start of the experiment a second dose (40 ml) of *Azospirillum* in the concentrations mentioned above was inoculated. Plants were harvested 5 months after transplanting and the growth and nutritional parameters were evaluated. The analysis of the data showed that banana plants co-inoculated with 200 g of AMF and 1.5E⁸ CFU/ml of *Azospirillum* presented greater development, an increase of 7 times in height, 4 times in perimeter, 16 times in leaf area, 12 times in aerial biomass, and 8 times in root biomass relative to control plants. The results achieved were due to synergism between fungus-bacteria when inoculated at higher doses, with lower doses stimulating growth is minimal. The co-inoculation in high doses demonstrates adequate support and cooperative effect between HMA and *Azospirillum* crops. In addition, co-inoculation promotes optimal nutritional status because microorganisms allowed plants achieve greater absorption of phosphorus and nitrogen relative to those treated with single inoculation and the control.

INTRODUCTION

The excellent climatic and ecological condition which owns Ecuador, have become one of the largest producers and exporters of bananas worldwide, with a cultivated area of 230,000 hectares (32 % of world production) located mainly in the provinces of El Oro, Guayas and Los Rios (AEBE, 2010). However, as in most countries where bananas are grown, the application of chemical fertilizers on a large scale has not changed throughout history. This poses an environmental

problem in the long term because the synthetic compounds containing nitrogen are a source of contamination of soil and groundwater; the fact that is aggravated due to the low efficiency of banana roots to absorb synthetic fertilizers. It is therefore, necessary improve the management of banana cultivation, not only to reduce the impact of agriculture on the environment, but also to improve productivity through sustainable management; using products based on own soil microorganisms fertilizers. These microorganisms are able to promote growth, productivity and maintain soil fertility. In this context, both arbuscular mycorrhizal fungi (AMF) as plant growth promoting bacteria *Azospirillum* genus have been studied as a great adaptability to environmental conditions and soil of banana plantations (Loredo *et al.*, 2004).

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Arbuscular mycorrhizae are defined as the mutual association between a fungus and the roots of the plant, where both benefit and participate actively in the uptake and translocation of nutrients (Bolan, 1991). Among the benefits that this partnership is: better uptake of nutrients and water, increased plant growth, tolerance to stress conditions and resistance to pathogens. All these benefits enable the plant to improve its ability to adapt to environmental conditions which give it a competitive advantage over non-mycorrhizal and finally its further development results in increased productivity plants (Maldonado and Ramirez, 1997).

The genus *Azospirillum* includes free-living bacteria (Okon and Labandera, 1994) which are distributed in temperate and tropical regions, presenting greater abundance in the latter (Döbereiner *et al.*, 1976). Its importance lies in its potential to colonize the areas of cell elongation of roots present in the base of the root hairs (Bashan and Holguin, 1997a). These bacteria fix nitrogen and produce growth regulating hormones such as indole acetic acid, which is responsible for stimulating the formation of lateral roots and root hairs.

Bashan and Holguin (1997b) determined that *Azospirillum* inoculation is more successful when other microorganisms are co-inoculated. The co-inoculation with HMA has a synergistic relationship where both microorganisms grow in tandem due to involved in providing nutrients, remove inhibitory products and facilitate this process in plant development.

Although there is research in the field of co-inoculation of *Azospirillum* HMA and in some plant species, is very little information regarding this subject banana plants.

The objective of this experiment was to evaluate the effect of combined inoculation of HMA and *Azospirillum* on growth and nutrition of micro propagated banana plantlets during the acclimatization.

MATERIALS AND METHODS

Biological material

The research was conducted using *in vitro* banana plantlets (Var. Williams) 5cm high. This biological material was provided by Plant Tissue Culture Laboratory, Universidad de las Fuerzas Armadas-ESPE. Plantlets were transplanted into 32 hoxpots containing a sterile substrate composed of 30% sand, 10% black peat PROMIX PGX and 60% black soil.

AMF spores of (*Rhizophagus irregularis*) isolated from root-zone soils of banana plants of Central Finance HDINEAGROS SA farm soils, Gold province were multiplied over 5 month's period in the sterilized soils of the same location. For spore's multiplication, oats (*Avena sativa*) plants were used as host and seeded on soil-sand (1:1 w/w) and ground banana substrate, containing 1 spore/g. At the end of one multiplication period 10 spores/g mycorrhizal inoculum was obtained.

Azospirillum (G4b) strain was selected from 8 isolates obtained in mixtures of soil and banana roots based on production

capacity indole acetic acid (IAA) (6µg / ml) by reagent Salkowski (BRIC *et al.*, 1991). *Azospirillum* inoculum developed from axenic amid Malic Acid Red Congo and involved a bacterial suspension of sterile saline (0.85% NaCl) to be adjusted to three levels: 10⁶, 10⁷ and 10⁸ CFU / ml.

Experimental Design and Inoculation

For testing, 16 treatments with a factorial arrangement of two factors were used: mycorrhizal doses (0, 50, 100 and 200 g) and *Azospirillum* concentration (0, 106, 107 and 10⁸ CFU / ml). Nine replicates per treatment were performed (experimental unit: 1 plant. Total 144 plants) and finally plants were organized using a completely randomized design in a growth chamber.

AMF inoculation was performed only once in the transplant and bacterial inoculation was effected twice, first dose (20 ml/plant) *Azospirillum* was applied at 70 days after transplantation and second (40 ml/plant) was performed at 98 days. Acclimation took five months in which the plants were kept in a growth chamber at 25° C, 90% relative humidity and Hewitt nutrient solution irrigation (during first 6 weeks). All plants were watered with distilled water according to the requirements of irrigation.

Monitoring and Evaluation of the variables

Growth variables: height, pseudostem perimeter and leaf area were monitored at 0, 70 and 98 days. At 150 days final evaluation was made and additionally recorded.

Root and foliar biomass: This variable was analyzed at 150 days using general and mixed linear models. Nutrient content (N, P, K) in both soil and leaf tissue, using wet mineralization for Nitrogen by the Kjeldahl method; Phosphorus and Potassium by atomic absorption method. HMA spores final population (Genderman and Nicholson, 1963, Herrera *et al.*, 2004), mycorrhizal colonization percentage (Philips and Hayman, 1970) and *Azospirillum* final population in soil and banana roots.

Statistical analysis

Growth, nutrition and symbiosis variables were analyzed by InfoStat statistical software. Linear and Mixed Models with a comparison test of Fisher LSD statistical averages 5% was used. Finally R language regression analysis was used.

RESULTS AND DISCUSSION

HMA multiplication

For large scale spores multiplication in a short time, a trap crop using host plant, soil and / or plants roots containing mycorrhiza spores are established (Bever *et al.*, 1996). Oat plants (*Avena sativa*) as host planted in suitable soil and inoculated with 1 spore/g soil were used. After 5 months culture, 10 spores/g (89.3% increases) was obtained, which were applied in banana plantlets using different doses (Figure 1).

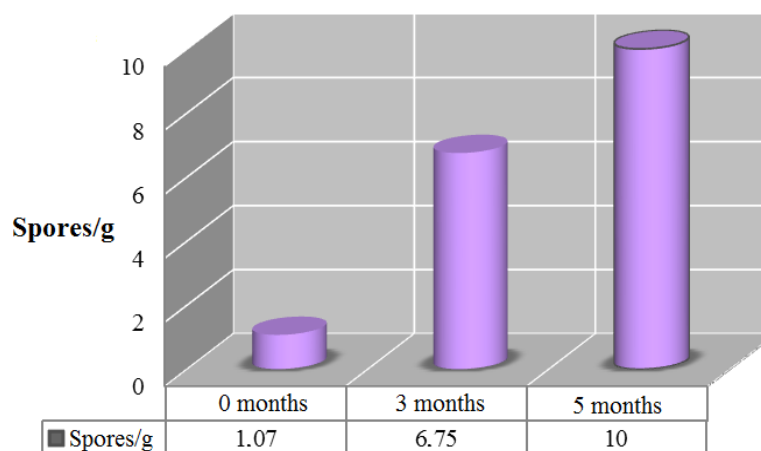


Fig. 1: Increased density of AMF spores in oats trap crop.

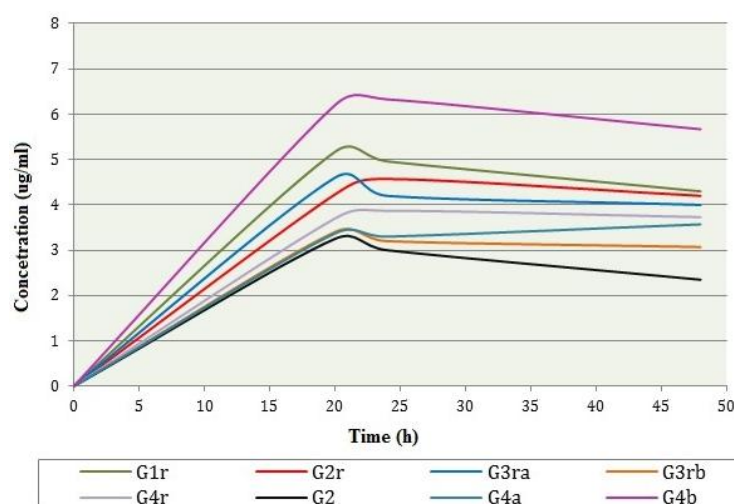


Fig. 2: IAA concentration produced by *Azospirillum* strains.

Selecting *Azospirillum* strain inoculum

Diazotrophic bacterias isolated from soil and roots were plated on solid Malic Acid Congo-red selective medium obtained strains were tested for primary and secondary biochemical identification. For selection of seed strain, we worked with identified and codified 8 strains, and with them the ability to produce IAA in tryptophan medium supplemented with different incubation times were evaluated. Results show that the maximum concentration of IAA between *Azospirillum* strains ranges from 2.5 to 6.33ug/ml, values within the range reported for production gender and corresponding to concentrations of 1 to 25 ug/ml (Tien *et al.*, 1979; Crozzier *et al.*, 1988) (Figure 2). The selected strain showed increased production of IAA (6.33ug/ml) and corresponded to the isolation G4b.

Growth

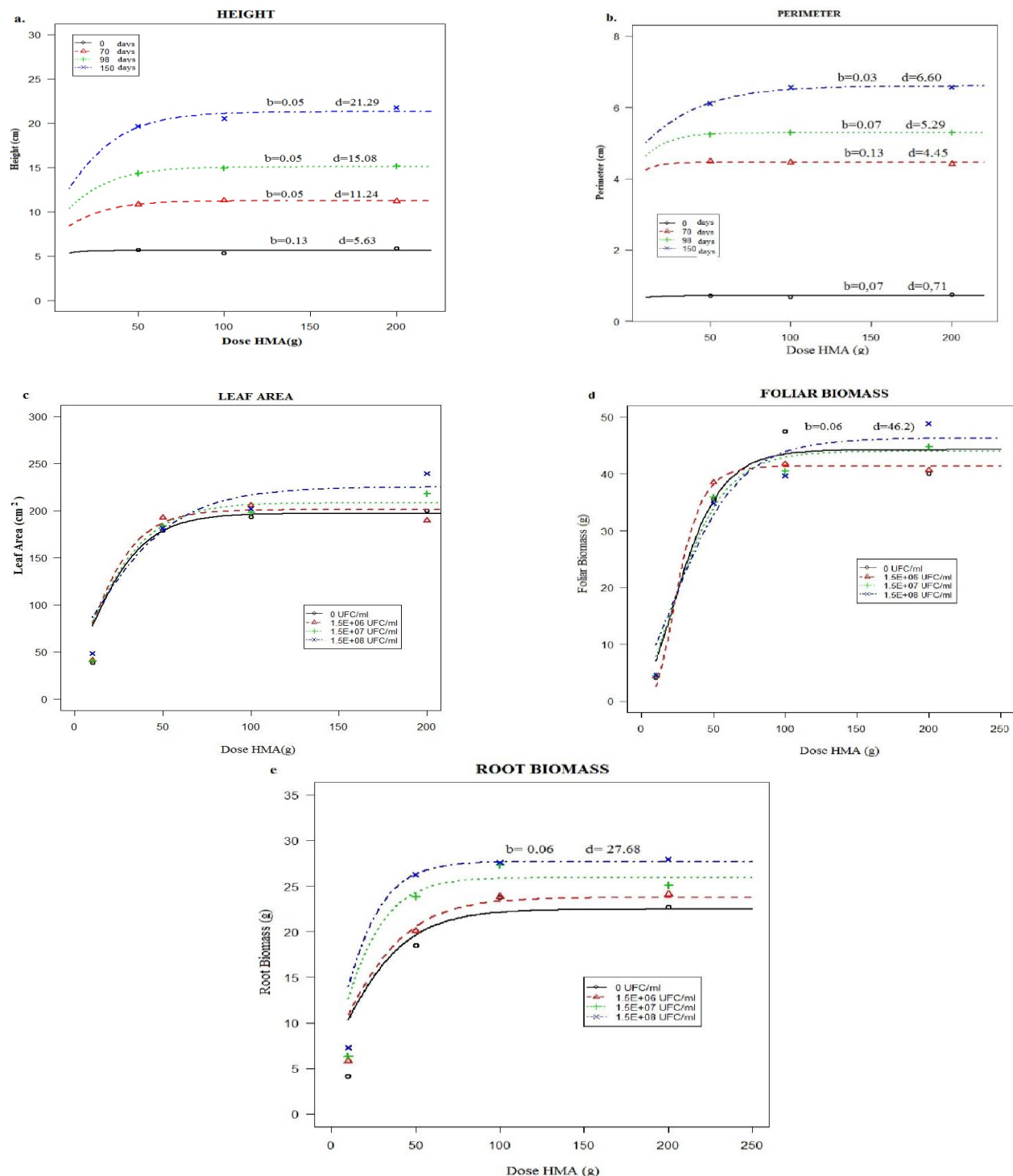
HMA simple inoculation, HMA co-inoculation and *Azospirillum* allowed benefits plant growth. However seedlings treated with 200 g of HMA and 10^8 CFU / ml *Azospirillum* showed the largest increases in all evaluated growth variables. Table 1 shows the highest statistical measures. Data recorded for all

growth evaluated parameters show the greatest increases in the inoculated treatment with higher doses of the two microorganisms, reaching 7 times height, 4 times perimeter and 16 times leaf area relative to control plants. Equally significant effect on plant biomass of co-inoculated banana seedlings was observed, showing 12 times foliar biomass increase and 8 times root biomass compared to the control group. In addition, regression analysis shows that the model that fits all growth variables evaluated correspond to Gompertz logistic regression. Only to analyze the data of height and perimeter a previous calculation of the growth rate was performed and it was determined that the height and width of plant is directly related to HMA amount, since mycorrhiza higher number increased leaf development. For leaf area and plant biomass HMA co-inoculation and *Azospirillum* are directly influenced. Highest values were recorded in 10^8 CFU/ml of *Azospirillum* +200 g of HMA treatment. These results are consistent with that described by Jaizme-Vega *et al.* (2004) who determined that applying *Glomus manihotis* and *Bacillus* sp cocktail to banana plantlets, higher values were obtained for all growth variables evaluated for the simple inoculation with each microorganism separately and the control group (Figure 3).

Table 1: Effect of HMA co-inoculation and *Azospirillum* the growth of micro propagated banana plantlets (150 days).

Treatment	Height (cm)	Perimeter (cm)	Leaf area (cm ²)	Foliage Biomass (g)	Root Biomass (g)
Control	1.59 a	0.76 a	12.11 c	4.27 c	4.37 d
<i>Azospirillum</i> (10 ⁸ CFU/ml)	3.00 a	1.04 a	55.67 b	4.57 c	7.88 c
HMA (200 g)	9.82 a	2.19 a	158.56 ab	36.59 b	20.37 b
<i>Azospirillum</i> + HMA (10 ⁸ UFC/ml+200 g)	10.83 a	2.68 a	193.44 a	51.07 a	34.82 a

In the same column, data with the same letter have no statistical differences according to Fisher LSD test at 5% ($p \leq 0.05$).

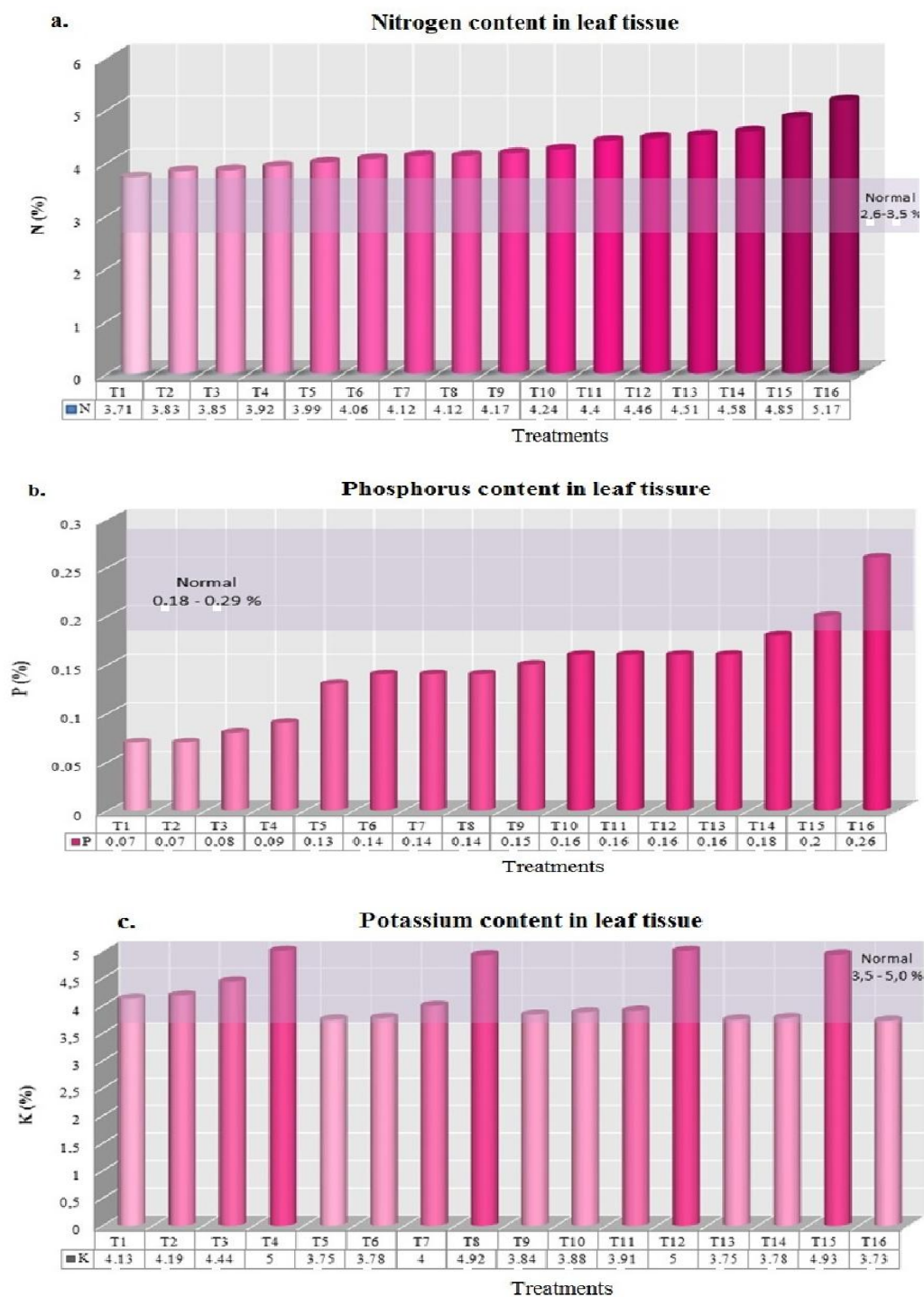
**Fig. 3:** Gompertz model for banana growth variables. a) Height b) Perimeter c) Leaf area d) Foliar biomass e) Root Biomass; Notation- b: slope d: upper limit.

Nutrient content

Nitrogen

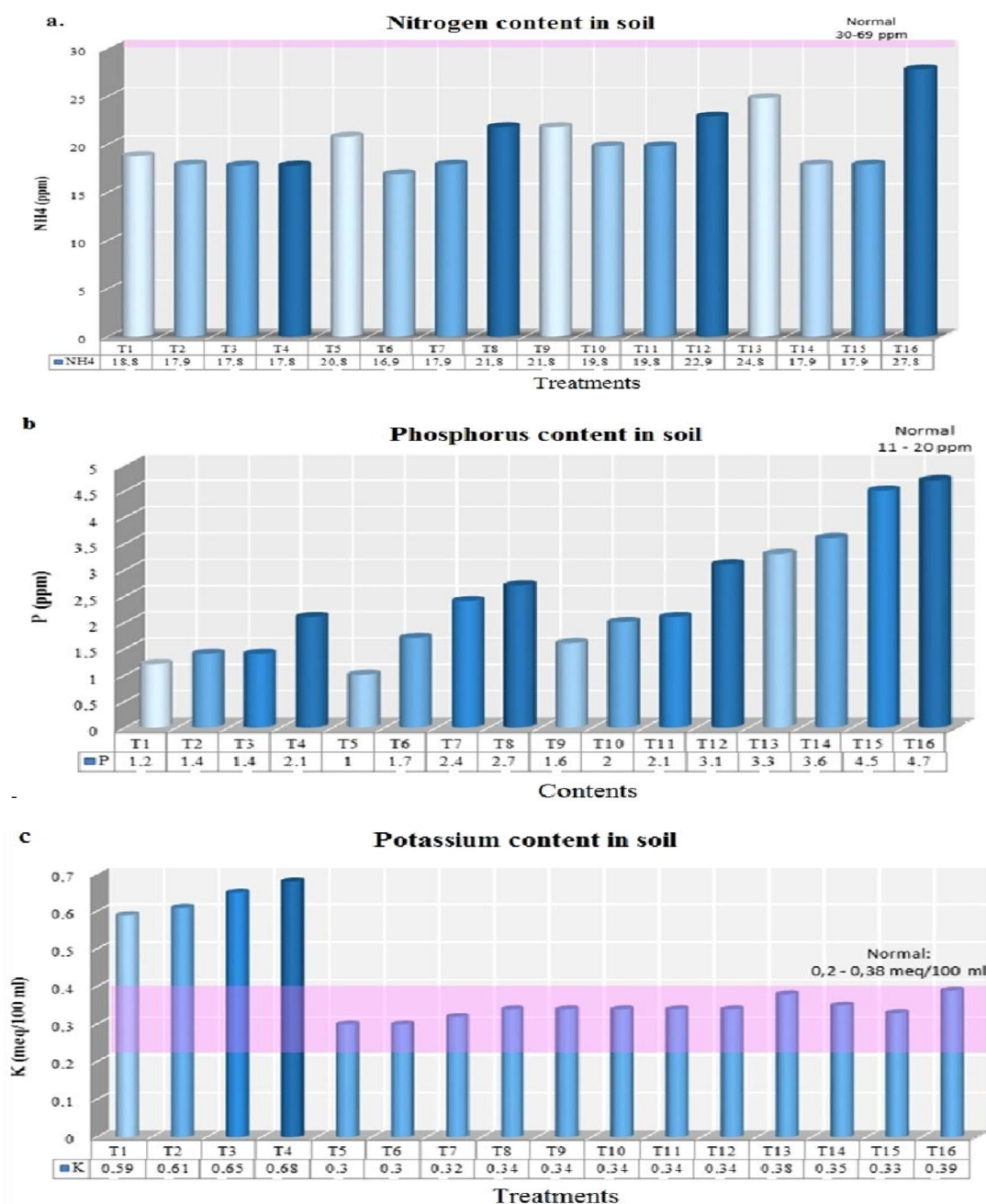
In leaf tissue nitrogen content is above normal in all plants, since when HMA and bacteria nitrogen content increases, in the plant also increases, reaching highest value (5.17%) when HMA 200g and *Azospirillum* 10^8 CFU/ml was applied (Figure 4a).

Nitrogen content in soil inoculated with *Azospirillum* only increases when microorganism concentration is increased (Figure 5a). Furthermore the figure shows soil with HMA different doses and *Azospirillum* highest concentration, these soils presents higher nitrogen concentration, reaching its highest level (27.8 ppm) when 200 g and 10^8 CFU / ml HMA is applied.



HMA dose treatments (grams): *Azospirillum* concentration (CFU/ml); T1 (0:0), T2 (0: 10^6), T3 (0: 10^7), T4 (0: 10^8), T5 (50:0), T6 (50: 10^6), T7 (50: 10^7), T8 (50: 10^8), T9 (100:0), T10 (100: 10^6), T11(100: 10^7), T12 (100: 10^8), T13 (200:0), T14 (200: 10^6), T15 (200: 10^7), T16 (200: 10^8).

Fig. 4: Effect of HMA and *Azospirillum* inoculation on nutrient content in leaf tissue. a) Nitrogen b) Phosphorus c) Potassium.



Treatment dose HMA(g): concentration *Azospirillum* (UFC/ml); T1(0:0), T2(0:10⁶), T3(0:10⁷), T4(0:10⁸), T5(50:0), T6(50:10⁶), T7(50:10⁷), T8(50:10⁸), T9(100:0), T10(100:10⁶), T11(100:10⁷), T12(100:10⁸), T13(200:0), T14(200:10⁶), T15(200:10⁷), T16(200:10⁸).

Fig. 5: Effect of HMA and *Azospirillum* inoculation on nutrient content in soil. a) Nitrogen b) Phosphorus c) Potassium.

Phosphorus

Fig. 4b shows low phosphorus levels in all plants, when HMA was increased, phosphorus reached the normal level when 200g HMA and *Azospirillum* 10⁸ CFU/ml was applied.

Regarding the content of phosphorus in soil (Figure 5b), although this element is below the normal range in all treatment, when HMA and *Azospirillum* dose increases, phosphorus soil content reaches higher levels when HMA (200 g) and *Azospirillum* (10⁸ CFU/ml) maximum quantities were applied.

Potassium

The potassium content in leaf tissue (Figure 4c) showed a normal level in all plants, but by increasing *Azospirillum* concentration, a gradual potassium increase was registered, reaching values close to 5% when 10⁸ CFU/ml was inoculated. The potassium content in soil (Figure 5c) showed normal values in all treatments, reaching higher values (0.68 meq/100 ml) when 10⁸ CFU/ml of *Azospirillum* were applied.

Table 2: Effect of AMF and *Azospirillum* inoculation on increasing HMA spores final population.

HMA (g)	AZO (UFC/ml)	Spore population (spore/g)
200	1.50E ⁸	62.67 a
200	1.50E ⁶	60.33 b
200	1.50E ⁷	60.33 b
200	0	58.67 b
100	1.50E ⁸	41.33 c
100	1.50E ⁶	40.33 c
100	0	40.33 c
100	1.50E ⁷	40.00 c
50	1.50E ⁷	36.00 d
50	1.50E ⁸	36.00 d
50	1.50E ⁶	33.67 e
50	0	32.33 e
0	1.50E ⁸	0.00 f
0	1.50E ⁷	0.00 f
0	1.50E ⁶	0.00 f
0	0	0.00 f

HMA: dose HMA, AZO: dose *Azospirillum*. In the same column, data with the same letter have no statistical differences according to Fisher LSD test at 5% ($p \leq 0.05$)

Mycorrhizal colonization percentage

Mycorrhizal colonization was analyzed within cell cytoplasm roots of banana plantlets (Figure 6).

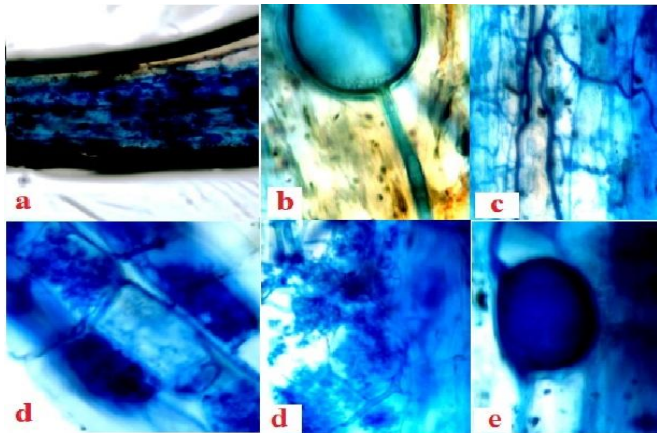


Fig. 6: HMA Structures found inside banana roots. a) HMA Root colonized, b) HMA Spore with supporting hyphae, c) Infective mycelial hyphae, d) Arbuscules HMA, e) Gall HMA.

Table 3. HMA inoculation effect and *Azospirillum* on mycorrhizal colonization percentage

MA (g)	AZO (UFC/ml)	% colonization
200	1.50E ⁸	96.67 a
100	1.50E ⁸	95.33 ab
200	1.50E ⁷	95.33 ab
200	0	95.00 bc
200	1.50E ⁶	95.00 bc
100	1.50E ⁷	94.67 bc
100	0	94.00 bc
100	1.50E ⁶	93.67 cd
50	1.50E ⁸	92.33 de
50	1.50E ⁷	91.33 ef
50	1.50E ⁶	91.33 ef
50	0	90.00 f
0	1.50E ⁸	0.00 g
0	1.50E ⁷	0.00 g
0	1.50E ⁶	0.00 g
0	0	0.00 g

HMA: dose HMA, AZO: dose *Azospirillum*

When HMA-*Azospirillum* interactions on colonization percentage (Table 3) was analyzed, it is observed that HMA (100 to 200 g) higher doses treatment and *Azospirillum* (10^7 and 10^8 CFU/ml) highest concentrations, promote further increase in banana roots colonization. Additionally, the combined inoculation of two microorganisms, allows higher percentage of arbuscular mycorrhizal colonization, showing that these bacteria promote higher percentage of colonization up to 96.67% when 200g HMA and 10^8 CFU/ml *Azospirillum* was applied. In the same column, data with the same letter have no statistical differences according to Fisher LSD test at 5% ($p \leq 0.05$)

Azospirillum final population

Table 4 shows T2 to T4 treatments without HMA inoculated, the bacterial population in soil and roots is not increased. However, treatment with HMA 50, 100 and 200 g promote *Azospirillum* growth in roots and soil; all HMA doses promote bacterial growth.

Table 4. The final population of *Azospirillum* in the soil and banana roots.

TREATMENT	MICO (g)	AZOS (UFC/ml)	SOIL (UFC/G)	ROOTS (UFC/g)
T1	0	0	0	0
T2	0	1.50E ⁺⁶	9.00E ⁺⁰⁷	9.00E ⁺⁰⁶
T3	0	1.50E ⁺⁷	6.00E ⁺⁰⁷	3.3E ⁺⁰⁷
T4	0	1.50E ⁺⁸	9.00E ⁺⁰⁸	4.20E ⁺⁰⁸
T5	50	0	0	0
T6	50	1.50E ⁺⁶	4.20E ⁺⁰⁸	9.00E ⁺⁰⁷
T7	50	1.50E ⁺⁷	3.45E ⁺⁰⁸	4.20E ⁺⁰⁹
T8	50	1.50E ⁺⁸	3.3E ⁺⁰⁹	4.20E ⁺⁰⁹
T9	100	0	0	0
T10	100	1.50E ⁺⁶	4.20E ⁺⁰⁸	6.00E ⁺⁰⁷
T11	100	1.50E ⁺⁷	3.30E ⁺⁰⁸	4.20E ⁺⁰⁸
T12	100	1.50E ⁺⁸	3.45E ⁺⁰⁸	4.20E ⁺⁰⁹
T13	200	0	0	0
T14	200	1.50E ⁺⁶	3.30E ⁺⁰⁸	4.20E ⁺⁰⁷
T15	200	1.50E ⁺⁷	7.50E ⁺⁰⁷	3.30E ⁺⁰⁸
T16	200	1.50E ⁺⁸	3.30E ⁺⁰⁹	4.95E ⁺⁰⁹

The results described are due to a synergistic action which allows a significant increase in growth and nutrition of the host (Bashan *et al.*, 1996). HMA action associated with further growth and development of banana plants inoculated contribute to the physiological function of the host plant, and allow assimilate easily the nutrients from the soil (Alarcon *et al.*, 2000). Regarding *Azospirillum*, Bashan *et al.* (1996) states that auxin produced by this bacterial genus increase the number and length of lateral roots, increased root biomass accumulates, allow early development of root hairs, increase root surface area and stimulate root exudates. Root system transformation improves mineral absorption and increases development and plant nutrition. Bacteria and fungi interact having a positive effect on plant growth. The interaction between fungi and bacteria cause a positive effect on growth plants. This is related experimental evidence described by Russo *et al.* (2005) who observed that bacteria of *Azospirillum* genus were adhered to the surface of structures such as fungal mycelia, spores and colonized roots. This suggests that fungal structures can be used by bacteria as bridges to reach far into the epidermis radical

(Bianciotto *et al.*, 1996) layers. Furthermore, it is proposed that *Azospirillum* increases HMA-plant symbiosis because of phytohormones production that stimulate root branching. Tisserant *et al.* (1996) suggest that the physiological and enzymatically active mycelium is newly formed lateral roots; since HMA-*Azospirillum* develop a functional interaction mycorrhizosphere (Barea *et al.*, 2005). Finally, the increase in both microorganisms populations evidence a synergistic relationship. According to Packovsky *et al.*, (1985) cortical cells penetration by HMA provide an entry route for *Azospirillum* resulting in bacterium population increase and facilitating their establishment and multiplication. According to Thimann (1979) *Azospirillum* hormones cause morphological changes in the root (increase in the number and length of roots) resulting in an increase of mycorrhizal colonization. In this experiment no adverse effects of microorganisms applied to banana plantlets development were observed, however *Azospirillum* and HMA inoculation increase growth and nutrition plants during acclimatization.

CONCLUSIONS

HMA and *Azospirillum* application (200 g 10⁸cfu/ml respectively), allow greater growth and nutrition of micropropagated banana plantlets, registering increases of 7 times in height, 4 times in perimeter, 16 times in leaf area, 12 times in biomass and 8 times in root biomass, relative to control plants. The final microbial populations of both organisms are favored by co-inoculation, reaching higher values with higher doses of each, which shows that HMA and *Azospirillum* combined application promotes mutual survival and multiplication and plant colonization.

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Conflict Of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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