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ENDOPHYTIC BACTERIA USED AS BIOINOCULANTS IN MICROPROPAGATED BANANA SEEDLINGS[1]

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ABSTRACT- The use of bio-fertilizers and microbial inoculants that promote plant growth and increased yield has been accepted as an alternative to reduce the use of chemical fertilizers. The objective of the present study was to evaluate the effect of plant growth promoting bacteria inoculation on growth and quality of micropropagated banana seedlings 'Prata Anã'. The experimental design was completely randomized, with four replications and the treatments consisted of 24 isolates of endophytic bacteria. The isolates EB-50 (*Bacillus* sp.) and EB-133 (*Bacillus amyloliquefaciens*) characterized as diazotrophic, the EB-51 (*Bacillus pumilus*) indicate for inorganic phosphate solubilization and EB-55 (*Bacillus subtilis*) and EB-40 (*Bacillus* sp.) indole-3-acetic acid producers have provided significant increases for length, pseudostem diameter, fresh masses and dry masses in 'Prata Anã' micropropagated banana seedlings.
Index terms: *Musa* sp., nitrogen fixation, phosphate solubilization, IAA, plant growth promoting bacteria

BACTÉRIAS ENDOFÍTICAS COMO BIOINOCULANTES PARA MUDAS MICROPROPAGADAS DE BANANEIRA

RESUMO - A utilização de biofertilizantes ou inoculantes microbianos capazes de promover o crescimento e incrementar a produtividade das plantas têm sido aceita como alternativa a redução do uso de adubos químicos. Diante do exposto objetivou-se avaliar o o crescimento e qualidade de mudas micropropagadas de bananeira 'Prata Anã' após a bioinoculação de bacterias endofíticas promotoras de crescimento. O delineamento experimental adotado foi inteiramente casualizado, com três repetições, e os tratamentos foram compostos por 24 isolados de bactérias endofíticas. Os isolados EB-50 (*Bacillus* sp.), e EB-133 (*Bacillus amyloliquefaciens*) caracterizados como fixadores biológicos de nitrogênio, o EB-51 (*Bacillus pumilus*) solubilizador de fosfato inorgânico e os isolados EB-55 (*Bacillus subtilis*) e EB-40 (*Bacillus* sp.) sintetizadores do ácido indol-3-acético, propiciaram os maiores incrementos para o comprimento, diâmetro do pseudocaule, massas frescas e massas secas nas mudas micropropagadas de bananeira 'Prata Anã'.
Termos para indexação: *Musa* sp.*,* fixação biológica, solubilização de fosfato, AIA, bactérias promotoras do crescimento de plantas.

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INTRODUCTION

The micropropagated banana seedlings originate from small shoot apices, called explants, that after the aseptic phase are grown under laboratory aseptic conditions in artificial culture media and controlled conditions of temperature, photoperiod and luminosity (NGOMUO et al. 2014). The micropropagation technique provides a clearing of the pathogenic microbiota as well as of the natural associated with the plantlets. This condition is generally extended when plants are transferred to soils or substrates in the initial stages of acclimatization (AHMED et al., 2014).

The absence of a diversified microbiota associated with the rhizosphere of plants can reduce growth and vigor and increase the mortality of seedlings in the field. Studies also indicate an increase in susceptibility to pests and diseases, indirectly increasing production costs during the early stages of plant development (DUBOIS et al., 2006).

The microorganisms are able to confer certain characteristics to the plants, such as greater resistance to conditions of water stress; change their physiological properties; produce plant hormones and other compounds of secondary metabolism (SOUZA et al., 2015). On the other hand, the endophytic organisms find in the plant a habitat with nutrients and less competition with other microorganisms (PEIXOTO NETO et al., 2004).

The growth promotion in plants by endophytic bacteria is due to direct mechanisms such as biological nitrogen fixation (CAVALCANTE et al., 2007), solubilizing phosphates (BALDOTTO et al., 2010) and phytohormones production (KUSS et al., 2007). Such processes improve the development of plants and help in the increase of production. These organisms are also called Plant Growth Promoting Bacteria (PGPB) (LUCY et al., 2004).

The use of biofertilizers or microbial inoculants that are capable of promoting the plants' growth and increasing their productivity is internationally accepted as an alternative to chemical fertilization. It is an environmentally correct practice that can confer sustainability to banana farming by reducing the use of agricultural inputs (ZUM FELDE et al., 2009). In Brazil, few studies have been conducted with the use of endophytic bacteria and rhizobacteria as bioinoculants (bacterial inoculants) in the banana crop.

Thus, in the last decades, much has been invested in researches to provide alternative forms of nutrients to plants, in promoting growth and alternative methods of pathogen control, with a focus on the use of endophytic microorganisms (BARROSO, NAHAS, 2008; KUSS et al. ., 2007). In view of the above, the objective of this study was to evaluate and select the best isolates of endophytic bacteria in the growth promotion of 'Prata Anã' banana micropropagated seedlings.

MATERIAL AND METHODS

Selection of the isolates and preparation of bacterial suspensions

The endophytic bacteria used in the present study were isolated and identified by Souza et al. (2013) and the evaluation of their *in vitro* biotechnological potential was carried out by Andrade et al. (2014). For the accomplishment of the present study 24 strains with different abilities were selected (Table 1).

The bacteria were cultured in 150 mL of TSB (*Triptic Soy Broth*) liquid medium for 48 hours at 28 °C to obtain the bacterial suspensions. The suspensions were prepared in saline solution at 0.85% of sodium chloride (NaCl) from TSB medium cultures. The bacterial cell concentration was adjusted at optical density of 1.0 absorbance at wavelength at 540 nm.

Microbiolization of micropropagated banana seedlings

The micropropagated banana seedlings were produced by the Biotechnology Laboratory of the State University of Montes Claros - UNIMONTES, Campus Janaúba. Seedlings approximately 7 cm long and with at least three fully expanded leaves were transplanted into plastic tubes (50 cm3) containing Bioplant® (Ponte Nova, Brazil) sterilized commercial substrate (Table 2). Then the seedlings were taken to the acclimatization chambers with temperature between 25ºC to 35ºC and 90% relative humidity. After two weeks the seedlings were transplanted to 3L pots containing the same sterilized substrate and taken to greenhouse where they remained for 120 days.

A total volume of 100 ml of bacterial suspensions at the concentration of 10^8 CFU mL^{-1} was applied every 15 days. The first application of the bacterial suspension was performed 15 days after transplantation to the pots. The controls received a solution of 100 mL of sterile water.

Three types of Hoagland solution were prepared. The complete solution (HOAGLAND; ARNON, 1950) and two modified. The complete with pH 6.8, containing the following components:N=

210.1 mg L^{-1}, P= 31.0 mg L^{-1}, K=234.6 mg L^{-1}, Ca=200.4 mg.L^{-1}, Mg=48.6 mg L^{-1}, S= 64.2 mgL^{-1}, B= 500 µg L^{-1}, Cu= 20 µg L^{-1}, Cl=648 µg L^{-1}, Fe=5022 µg L^{-1}, Mn=502 µg L^{-1}, Mo= 11 µg L^{-1} and Zn= 50 µg L^{-1}. The modified Hoagland solution 1 had all components except the N and the modified Hoagland solution 2, with all components except P.

Three experimental assays were performed. In assay 1 and 2, two controls were used: SSN (application of Hoagland 1 solution and without bacterial inoculation) and SCN (application of Hoagland complete solution and without bacterial inoculation). In test 3 only the SCN control was used.

In trial 1, the treatments that received the endophytic bacteria with biological nitrogen fixation ability received the Hoagland 1 solution. In trial 2, with P-solubilizing bacteria the seedlings received the Hoagland 2 solution and 275 g of calcium phosphate (Algeria reactive natural phosphate with 29% P_2O_5) was added to the substrate in each pot.

For trial 3, treatments that received the IAA-synthesizing bacteria were fertilized with the complete Hoagland solution. In all three assays a 25 mL volume of one of Hoagland's three nutrient solutions was applied at 15 day intervals. The plants were irrigated daily according to water needs.

Evaluation of seedlings growth characteristics

The pseudostem length and diameter characteristics were evaluated from 30 days to 120 days after transplanting. The determination of the length was carried out by measuring the limiting area of the planting until the point of emergence of the leaves with the aid of a tape measure. In the evaluation of the diameter of the pseudostem performed with the aid of a pachymeter, the measurement was obtained at the base of the seedling at the planting limit.

At 120 days, the fresh mass and the dry mass of the aerial part, the root system and the total seedlings were obtained by means of a semi-analytical balance. The material was placed in a temperature controlled fan ventilated oven at 65^{O}C and the dry mass was measured every day until its mass did not present a constant variation.

Experimental design and statistical analysis

Three bioinoculation assays were performed on endophytic bacteria. Trial 1: twenty N-fixing isolates and two controls. Assay 2: three phosphate solubilizers and two controls. Assay 3: four isolated IAA synthesizers and one control (Table 1). In all assays the design was completely randomized with four replicates. The results were submitted to analysis of variance by the statistical program Sisvar and, when significant, were submitted to the Scott-Knott tests at 5% probability for means comparison.

RESULTS AND DISCUSSION

Through the variance analysis of assay 1 (diazotrophic isolates) significant differences were observed ($p < 0.05$) for plant length characteristics, pseudostem diameter, fresh shoot matter, fresh root matter, total fresh matter and shoot dry matter (Figure 1a, b and c).

All components of fresh mass evaluated were affected by the inoculation of the endophytic bacteria described as biological fixatives of N. Significant effect was observed in the total fresh mass and fresh shoot mass, in which all 20 isolates promoted significant gains when compared to both controls (SSN and SCN). For the component, fresh root mass, eleven isolates presented superior performance to both controls (Figure 1a). Among the isolates, the following stand out: EB-23, EB-50, EB-50, EB-64, EB-87, EB-88, EB-126, EB-127, EB-133, EB-136 and EB-169, among them nine belong to the genus *Bacillus* sp., one to the genus *Klebsiella* sp. and the other to the genus *Sporolactobacillus* sp.

For the dry mass characteristic evaluated, significant differences were observed for total dry mass and shoot dry mass (Figure 1 b). Three isolates had dry mass lower than both controls (EB-56, EB-136 and EB-144) and for dry shoot mass only EB-04 and EB-25 isolates were significantly lower than controls. All other treatments presented similar behavior to the controls (SCN and SSN) (Figure 1b).

The pseudostem diameter and plant length characteristics were significantly affected by the bioinoculation of the endophytic bacteria (Figure 1c). For the pseudostem diameter, 17 isolates determined higher diameters in the micropropagated seedlings at 120 days after transplanting. While for the length, 14 isolates presented superiority when compared to the controls (Figure 1c). Eleven isolates promoted positive effects for both characters, namely: EB-04, EB-45, EB-47, EB-49, EB-50, EB-51, EB-56, EB-64, EB-88, EB-126 and EB-133. Of these 11 isolates, ten belong to the genus *Bacillus* sp. and only one to the genus *Lysinibacillus* sp.

Previous studies of diazotrophic bacteria of the genus *Herbaspirillum* sp. and *Burkholderia* sp. inoculated in isolation or in combined manner showed the potential of their use in banana crop. The authors observed significant increases in the

growth of micropropagated seedlings of the cultivars Caipira and Prata Anã at 75 days after transplanting (TSAVKELOVA et al., 2000).

In the present study a large part of the most promising diazotrophic isolates belong to the genus *Bacillus* sp.. This genus has also been reported in other studies demonstrating several biotechnological abilities such as biological N fixation, nutrient solubilization, growth hormone synthesis and indirectly acting on plant growth via pathogen control (TSAVKELOVA et al., 2006; ARAUJO et al., 2012; LUO et al., 2012).

Recent studies indicate a close association between species of the genus *Bacillus* sp. and the banana tree (SOUZA et al., 2013). The application of twenty different triple combinations of PGPB in banana plants indicates that the isolates EB-40, EB-51 and EB-194, all belonging to the genus *Bacillus* sp., promoted significant increases in the nitrogen content in the shoot dry matter of banana seedlings (SOUZA et al., 2016).

It should be noted that in the present study, the treatments that received the endophytic bacteria did not receive any nitrogen fertilization during the evaluation period (120 days). Several authors report that the inoculation of microorganisms, when associated to the absence or reduced dose of nitrogen fertilizer, can provide plant production similar to that observed under conditions of high N application via fertilizer (GUIMARÃES et al., 2010; HUNGRIA, 2011) .

Through the analysis of variance of assay 2 (solubilizers isolates of P), significant differences were observed ($p < 0.05$) for all characteristics evaluated (Figure 2 a, b and c). The EB-51 isolate (*Bacillus pumilus*) significantly influenced the total fresh and dry masses, the fresh and dry root and shoot masses in relation to other treatments and controls (Figure 2a and b).

The seedlings length and diameter of the pseudostem characteristics presented similar behavior. Only the control that received the solution of Hoagland 1 (without addition of N) and without the bacterial inoculation showed significantly lower averages than the other treatments (Figure 2c). It is noteworthy that all treatments that received the bacterial inoculation also received the solution of Hoagland 2 (absence of high solubility phosphorus), replaced by the application of calcium phosphate of low solubility, applied directly to the commercial substrate.

There are few reports in the literature on the application of phosphorus solubilizing bacteria in association with micropropagated banana seedlings. The isolates evaluated belong to the genus *Lysinibacillus* sp. (EB-53) and the species of *Bacillus pumilus* (EB-51) and *Bacillus amyloliquefaciens* (EB-44). Among the isolates evaluated, the EB-51 isolate deserves special attention. The species *B. pumilus* (EB-51) promoted increments and superior performance for all evaluated characters. This superior performance can be attributed to the biochemical abilities already described above. According to Andrade et al. (2014) study, this isolate besides showing the ability to fix the N, also demonstrated the ability to solubilize phosphorus of low solubility under *in vitro* conditions.

Souza et al. (2016) report that the combination of diazotrophic bacteria, phosphate solubilizing bacteria and indole acetic acid synthesizers promoted significant increases in the length, pseudostem diameter, number of leaves, shoot and root fresh mass and dry mass of 'Prata Ana' banana seedlings.

A significant difference ($p < 0.05$) was observed for pseudostem diameter, total fresh mass, and fresh root mass (Figure 3a, b and c) through variance analysis of assay 3 (IAA synthesizers).

The shoot fresh mass and the total fresh mass were significantly influenced by the isolates EB-38 (*Stenotrophomonas* sp.), EB-40 (*Bacillus* sp.) and EB-55 (*Bacillus subtilis*) (Figure 3a). Of the four isolates evaluated, only EB-38 did not promote an increase in the pseudostem diameter of the micropropagated banana seedlings (Figure 3c).

The bioinoculation of the 24 isolates with biological N fixation, P solubilization and IAA synthesis showed positive results for most of the evaluated traits, demonstrating the great potential of this practice use in order to increase the quality of the micropropagated banana seedlings. In this culture, studies are more recent and also indicate that bioinoculation should be considered as one of the most efficient biological strategies for improving the quality of micropropagated seedlings, field plant development, disease control and productivity increase (TSAVKELOVA et al., 2000, MIA et al., 2005, KAVINO et al., 2007).

The results of the present study reaffirm the possibility of developing a commercial bioinoculant to be applied in micropropagated banana seedlings. The isolates EB-50, EB-51 and EB-55 of the genus *Bacillus* sp. and the species *Bacillus pumilus* and *Bacillus subtilis*, respectively, could be combined in a bioproduct to improve the quality of the seedlings and reintroduce a beneficial microbiota, which in addition to the already demonstrated effects could also act indirectly in reducing diseases and pests.

TABLE 1- Isolates of endophytic bacteria identified as nitrogen fixers, phosphorus solubilizers and producers of 3-indole acetic acid and two controls (SSN and SCN) used in the three bioinoculation assays in 'Prata Anã' banana micropropagated seedlings, Janaúba, Minas Gerais, Brazil.

Isolates	Genus / Species	***In vitro*** **Skills Tested**	**Germplasm Accession Number**
EB-133	*Bacillus amyloliquefaciens*	N Fixer	AB301022.1
EB-25	*Bacillus cereus*	N Fixer	GU451184.1
EB-88	*Bacillus flexus*	N Fixer	DO870687.1
EB-49	*Bacillus licheniformis*	N Fixer	EU366371.1
EB-51	*Bacillus pumilus*	N Fixer P Solubilizer	HQ218993.1
EB-64	*Bacillus pumilus*	N Fixer	JF271873.1
EB-169	*Bacillus pumilus*	N Fixer	FJ189791.1
EB-40	*Bacillus* sp.	N Fixer IAA Producer	GQ340516.1
EB-47	*Bacillus* sp.	N Fixer	FJ611939.1
EB-50	*Bacillus* sp.	N Fixer	HM769816.1
EB-56	*Bacillus* sp.	N Fixer	GU269573.1
EB-194	*Rhizobium* sp.	N Fixer	FJ405377.1
EB-04	*Bacillus subtilis*	N Fixer IAA Producer	AY741264.1
EB-126	*Bacillus subtilis*	N Fixer	HM769817.1
EB-136	*Bacillus subtilis*	N Fixer	AB301012.1
EB-87	*Bacillus tequilensis*	N Fixer	HM770882.1
EB-23	*Klebsiella pneumoniae*	N Fixer	JN201948.1
EB-45	*Lysinibacillus sphericus*	N Fixer	EF178460.1
EB-144	*Paenibacillus* sp.	N Fixer	D16282.1
EB-127	*Sporolactobacillus* sp.	N Fixer	AB301022.1
EB-53	*Lysinibacillus* sp.	P Solubilizer	JN215512.1
EB- 44	*Bacillus amyloliquefaciens*	P Solubilizer	GU122948.1
EB-38	*Stenotrophomonas* sp.	IAA Producer	JN215502.1
EB-55	*Bacillus subtilis*	IAA Producer	HQ334981.1
SSN	-	Absence of N and bacterial inoculum	-
SCN	-	Presence of N and bacterial inoculum	-

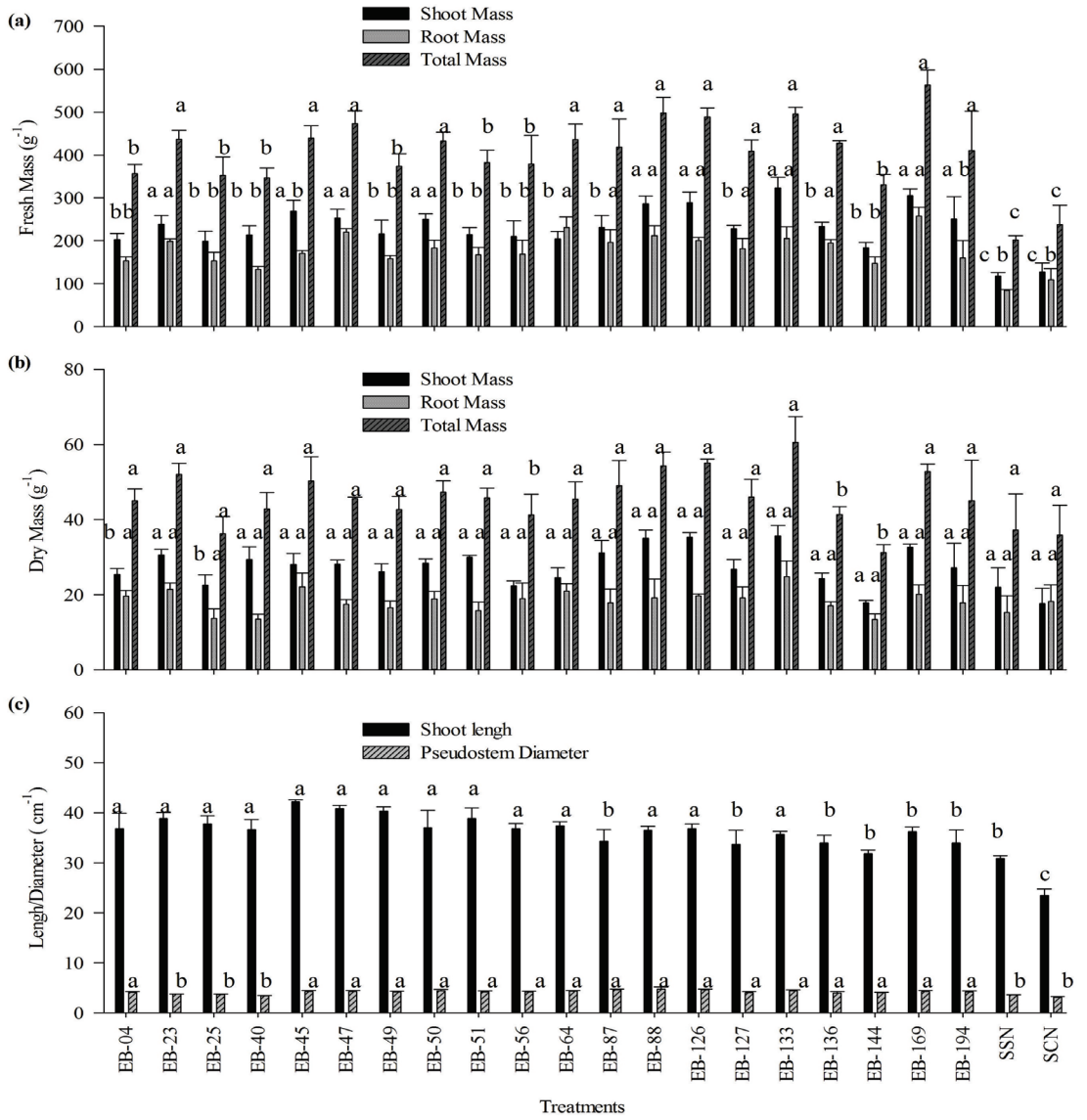

FIGURE 1- Effect of inoculation of 20 isolates of diazotrophic endophytic bacteria on micropropagated 'Prata Anã' banana seedlings on fresh mass (a), dry mass (b), length and diameter of pseudostem (c). Vertical bars indicate the mean standard error and equal letters belong to the same group by the Scott-Knott test, at 5% probability. [Hoagland Solution 1 (absence of N) and without bacterial inoculum (SSN), Complete Hoagland Solution without bacterial inoculum (SCN)].

TABLE 2 - Nutrient contents resulting from the chemical analysis of the substrate used in the bioinoculation assays of endophytic bacteria in micropropagated seedlings of 'Prata Anã' banana tree, Janaúba, Minas Gerais, Brazil*.

Substrate	pH[1]	P[2]	K[2]	Ca[3]	Mg[3]	Al[3]	Al+H[4]	TB	Cu[2]	Zn[2]	OM[5]
	H_2O	--mg/dm^3--		---------- cmolc/dm^3 ----------					-mg/dm^3-		dag/kg
	4.9	549.7	1306	11.2	4.3	0.1	7.7	19.5	1.9	18.3	19.7

* Analyzes carried out by the Agricultural Research Company of Minas Gerais - EPAMIG, Regional Unit from North of Minas,Nova Porteirinha – MG. [1]pH in water; [2]Extractor: Mehlich-1; [3]Extractor: KCl 1 mol/L; [4]pH SMP; [5]Colorimetry. TB: Total Base; OM: Organic Matter.

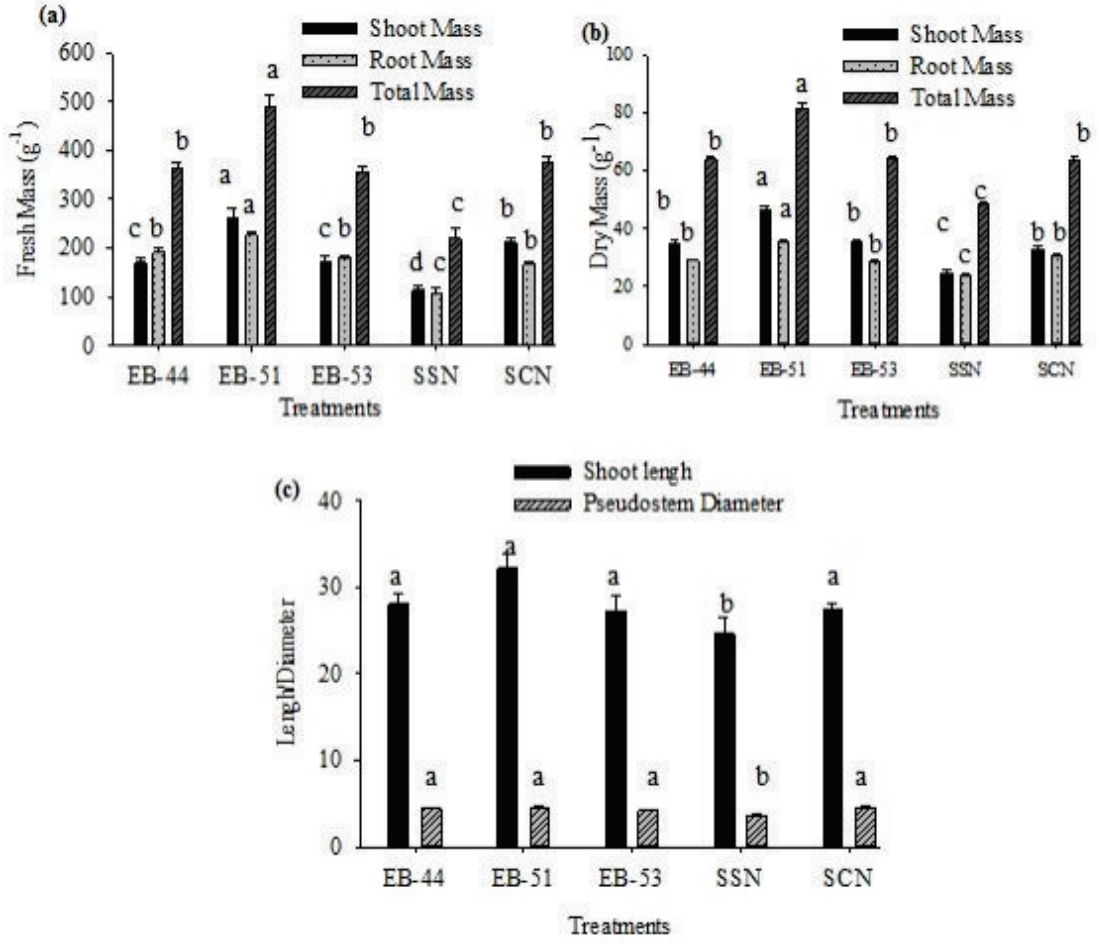

FIGURA 2- Effect of the inoculation of three isolates of P solubilizing endophytic bacteria on micropropagated 'Prata Anã' banana seedlings in fresh mass (a), dry mass (b), length and diameter of pseudostem (c). Vertical bars indicate the mean standard error and equal letters belong to the same group by the Scott-Knott test, at 5% probability. [Hoagland solution 2 (absence of P) and without bacterial inoculum (SSN), Hoagland solution complete and without bacterial inoculum (SCN)].

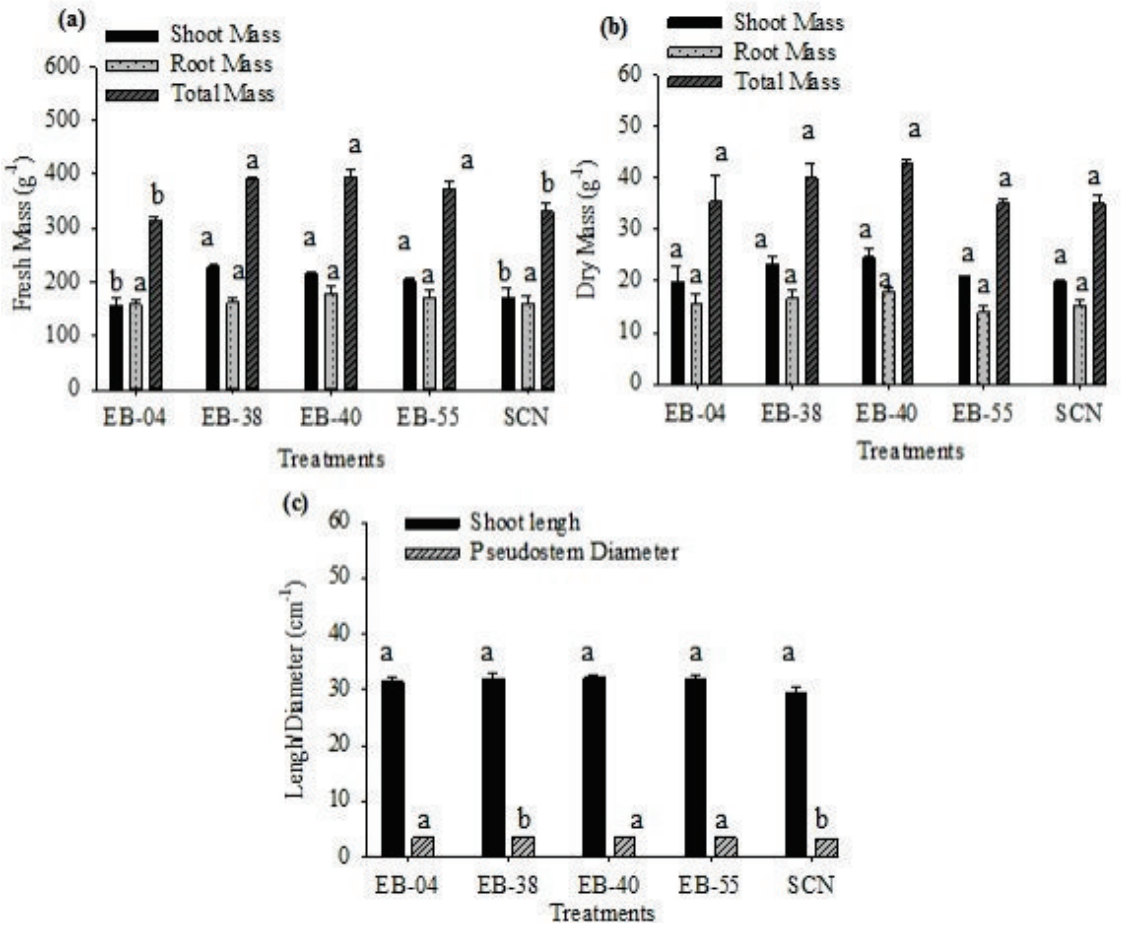

FIGURA 3- Effect of the inoculation of four isolates of endophytic IAA synthesizing bacteria on micropropagated 'Prata Anã' banana seedlings on fresh matter mass (a), dry mass (b), length and diameter (c). Vertical bars indicate the mean standard error and equal letters belong to the same group by the Scott-Knott test, at 5% probability. [Complete Hoagland solution without bacterial inoculum (SCN)].

CONCLUSION

The diazotrophic isolates EB-50 (*Bacillus* sp.) and EB-133 (*Bacillus amiloliquefaciens*), the EB-51 isolate (*Bacillus pumilus*), inorganic phosphate solubilizer and the isolates EB-55 (*Bacillus subtilis*) and EB-40 (*Bacillus* Sp.), indol-3-acetic acid synthesizers, promote growth and higher quality of 'Prata Anã' banana seedlings.

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