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Plant Growth-Promoting Effects of *Azotobacter* as a Biofertilizer During the Acclimatization Process of Plantains Cultivars

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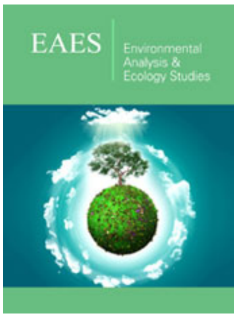

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Abstract

The objective of this work was to determine the effect of a new *Azotobacter*-based biofertilizer on the development of plantain plantlets obtained by *in vitro* culture, during their acclimatization. Application were made weekly according to a completely randomized design (in 20x12cm polyethylene bags) with five treatments: 0 (absolute control), 1,2,3 and four applications of the biofertilizer. After one week of acclimatization, survival percentage was determined on the two commercial cultivars studied: 'INIVIT PB-2012' (ABB) and 'INIVIT PV 06-30' (AAB). Measurements were made in 20 randomly selected plantlets per treatment at 50 and 60 days after planting (dap): plant height, number of leaves, petiole length, width and length of the developed leaf and its leaf area was calculated. The survival plantlets at seven dap always behaved above 98 %. Evaluations at 60 dap, demonstrated that *Azotobacter* enhanced transplant vigor and stimulated the morphological development of both cultivars. A substantial increment of the size and health of surviving plantlets (height, longest and widest leaves) was determined, which were significantly different to the control (without biofertilizer). Plantlets inoculated with the new biofertilizer for three weeks were ready for transplanting, at least, nine days before to the control, and that has a favorable economic value.

Keywords: Biofertilizer; Biological nitrogen fixation; *in vitro* culture; Plant growth-promoting rhizobacteria; Sustainable agriculture

Introduction

The negative effects of the climatic change, especially on the agriculture, have gotten the attention about the necessity to promote environmentally friendly agricultural practices. Humanity needs an alternative agricultural development paradigm, able to produce enough healthy foods for millions of people. According to Ranjan S et al. [1], due to the exorbitant cost of nitrogen fertilizers and the environmental damages on use of chemical fertilizers, biofertilizers could be the best alternative to chemical inputs and will help in reducing the rate of ecological disturbance to a great extent. In general, the high cost of chemical fertilizers and difficulties to access them are very common problem for farmers around the world. At the same time, Nicholls CI et al. [2] considered that the ecological resilience of agroecosystems is closely associated to social resilience; because of that, appropriate government and academic strategies should be integrated to fulfill the objectives of sustainable development outlined by the Food and Agriculture Organization of the United Nations.

In current agricultural ecosystems, the poor soil fertility is one of the major threats to crops productivity. The application of beneficial soil microorganisms offers an important alternative for restoring, maintaining and improving soil health and fertility, and at the same time, crop health and productivity for a sustainable agriculture [3,4]. Among of them, biofertilizers such as micorhizas, *Azospirillum*, *Pseudomonas*, *Bacillus* or *Azotobacter* can add and compensate the nutrient loss from soil [5,6]. Two recent papers [1,7], explained some reasons why biofertilizers have become in a novel tool for enhancing soil fertility and crop productivity. In general, a wide group of plant growth-promoting rhizobacteria has shown an important role in the sustainable agriculture [8,9]. Among the most important beneficial microorganisms, *Azotobacter chroococcum* has been recognized for its positive activity as a free-living nitrogen fixing bacterium; this bacteria also produce plant growth regulators, increase the solubility of mineral phosphates. *Azotobacter* is the most commonly isolated and researched free living nitrogen fixer contributing to the natural vegetation and soil fertility [1]; this highly aerobic and heterotrophic bacterium has an interesting anti-fungi activity [10] and produce antibiotics [11]. Among the most important crops around the world, plantains have a high demand on the global food market, but their yield depends on several factors. For example, fertilization, watering and the quality of the planting material are critical inputs for a successful production of this crop. Inputs may represent 70% of the production cost and the plantlets alone account for 36.49% of those costs [12].

In this sense, plant biotechnology has many advantages, as compared to the traditional method to produce good planting material (higher multiplication rate and better phytosanitary quality [13], but the last stage of the *in vitro* process is technically so complex because it determines when the plantlets are ready to their transplantation to the field. In this important phase, usually defined as acclimatization, the plantlets are grown in a controlled environment (greenhouse) with high demand for fertilizer, water and labor [14] before they reach the appropriate characteristics to their successful planting at farm. Recently, researchers from the Cuban Business Group LABIOFAM developed a biofertilizer based on an *A. chroococcum* strain nitrogen solubilizer [15]; the new product is in the process of technical validation for its future commercial use in the agriculture. However, the impact of this bioproduct during the acclimatization phase of most important crops, including plantains and bananas, is not known. For that reason, the objective of this work was to determine the effect of the *Azotobacter*-based biofertilizer on the development of plantain plantlets obtained by *in vitro* culture, during their acclimatization.

Materials and Methods

All the experiments were carried out in the greenhouse area from the Biotechnology Unit at the Research Institute of Tropical Roots and Tubers Crops (INIVIT), in Santo Domingo, Cuba.

Plant material and the experimental design

Two commercial plantains cultivars from the germplasm bank of INIVIT were chosen as the initial plant material: ‘INIVIT PB-2012’ (ABB) and ‘INIVIT PV 06-30’ (AAB); both were obtained in the Cuban breeding program of Musa spp. Plantlets produced by *in vitro* culture were obtained from elite and healthy plants selected on farm and grown under controlled conditions at the greenhouse (phase 0 for *in vitro* culture). A relative humidity of 85-90 % was guaranteed into the greenhouse; it was covered by an agricultural anti-aphids mesh and a plastic mesh that allowed to reduce sunlight by up to 70%. *In vitro* plantlets were transplanted, at the stage of 2-3 leaves and 3-5cm plant height, into polyethylene bags (20 x 12.5cm) with a conventional mixed substrate (70 % organic matter (filter cake) and 30 % soil). The novel biofertilizer, produced from an *Azotobacter* strain isolated and studied by Pérez MC et al. [15], was used at a recommended unique doses of 20L ha^{-1}. Its effect was evaluated by the aspersion on the substrate and plantlets. The experimental design include five treatments according with the number of bioproduct applications every seven days.

Fifty plantlets for each treatment were grown in the greenhouse conditions and experiments were conducted in a completely randomized design with five treatments (replicated two times):

T1. Absolute control (without biofertilizer)

T2. One application

T3. Two applications

T4. Three applications

T5. Four applications

Measurements and statistical analysis

After one week of acclimatization, survival percentage was determined. At 50 and 60 days after planting, (dap) the measurements were recorded on twenty randomly selected plantlets from each treatments. At data collection, the following variables were evaluated: plant height (cm), number of leaves, petiole length (cm), width and length of the developed leaf (cm) and its leaf area (cm^2) was calculated. The collected data were subjected to the analysis of variance (ANOVA) appropriate to the completely randomized design. All statistical procedure was according to the experimental design and using the tools from the IBM.SPSS/PC$^+$ statistical package version 23.0 for Windows® [16]. Whenever differences existed among means values, the comparison of them was carried out with the test of Tukey for P≤ 0.05.

Results

The results showed that *Azotobacter* mostly enhanced transplant vigor and stimulated vegetative growth. Observations of the survival plantlets percentage seven days after planting always behaved above 98 %. All plants, regardless of the treatment received, maintained the morphological characteristics of each cultivar during the experimental period. Evaluations at 60 dap allowed to quantify the stimulating effect of the new biofertilizer on the morphological development of both cultivars. Table 1 show the observed effect on the evaluated variables in the cultivar ‘INIVIT PB-2012’.

Table 1: Effect of an Azotobacter-based biofertilizer on the general plant development during the acclimatization phase of plantain plantlets cv. 'INIVIT PB-2012'.

Treatments	PH (cm)	NL	PeL (cm)	LDL (cm)	WDL (cm)	LA (cm^2)
T1 (control)	9,89 c	3,27 b	1,99 b	14,57 c	5,43 c	64,65 c
T2	12,62 b	3,75 ab	2,21 ab	17,65 b	6,99 b	99,67 b
T3	13,99 ab	3,95 a	2,36 ab	18,92 ab	7,20 ab	110,74 ab
T4	14,52 a	4,05 a	2,47 a	20,47 a	8,06 a	134,49 a
T5	14,16 ab	3,90 a	2,42 a	18,63 ab	7,84 ab	117,62 ab
S$\bar{x}$	0,497*	0,150*	0,113*	0,683*	0,251*	7,321*

*Means followed by the same letter in a same column are not significantly different according to Tukey's HSD test (P≤ 0.05).

Legend: PH: Plant height, NL: Number of leaves, PeL: Petiole length, length (LDL) and width (WDL) of the developed leaf, LA: Leaf area.

T1-absolute control (without biofertilizer); T2-with one application of Azotobacter; T3-with two applications; T4- with three applications; T5- with four applications.

With more than two applications of the new biofertilizer most of the evaluated variables were significantly different from the absolute control, but without statistical differences among the treatments with some application of the bioproduct. After three applications, plant height increased in 4.63cm (46.81%), but petiole and leaves improved their size too. Due to an increment in the leaves size (length and width of the developed leaf): 5.9cm (40.49%) and 2.63cm (48.43%), respectively, the leaf area grew significantly regarding to the control, in more than 69cm^2 (108%) when the *Azotobacter* solution was applied three times each seven days. During the experiments with the cultivar 'INIVIT PV 06-30' (a cooking plantain called as "plátano macho" in Cuba, AAB group) weekly applications induced the same stimulant effect on the plantlets with lightly superior values to those of cultivar 'INIVIT PB-2012', which was positive in some essential variables related with their quality for farm planting: plant height, length and width of the developed leaf, and in consequence, for its leaf area (Table 2).

Table 2: Effect of an Azotobacter-based biofertilizer on the general development during the acclimatization phase of plantain plantlets cv. 'INIVIT PV 06-30'.

Treatments	PH (cm)	NL	PeL (cm)	LDL (cm)	WDL (cm)	LA (cm2)
T1 (control)	9,72 c	3,47 b	1,94 b	12,05 c	5,56 c	53,80 d
T2	10,85 c	3,80 ab	2,30 ab	15,47 b	6,14 c	76,41 c
T3	13,28 b	3,85 ab	2,29 ab	17,31 b	7,11 b	99,19 b
T4	15,60 a	4,10 a	2,58 a	20,28 a	8,17 a	133,90 a
T5	13,94 b	4,00 a	2,31 ab	17,23 b	7,36 b	102,14 b
S$\bar{x}$	0,415*	0,137*	0,107*	0,559*	0,183*	5,300*

*Means followed by the same letter in a same column are not significantly different according to Tukey's HSD test (P≤ 0.05).

Legend: PH: Plant height, NL: Number of leaves, PeL: Petiole length, length (LDL) and width (WDL) of the developed leaf, LA: Leaf area

According to measurements, the optimum treatment was T4 (three applications) for both cultivars, with significant differences observed with the control but without statistical differences with two or four applications. Despite this, had no significant differences in number of leaves and petiole length for all treatments with the bioproduct. When three applications were made, increments were significantly bigger than the absolute control for plant height, length and width of the developed leaf and leaf area, was 60.49, 68.29, 46.94 and 148.88 %, respectively. For the leaf area, there were not statistical differences among the treatments with two (110,74cm^2), three (134,49cm^2) or four applications (117,62cm^2) of *Azotobacter*-based biofertilizer in 'INIVIT PB-2012' cultivar, but in 'INIVIT PV 06-30' the fourth combination of three inoculations each seven days was clearly the best treatments. General observations showed that plantlets inoculated with the new biofertilizer for three weeks (every seven days) were ready for transplanting, at least, nine days before to the control, and that has a favorable economic value (data not shown). It means that size and health of surviving plantlets (height, longest and widest leaves) was significantly different to the absolute control (without biofertilizer) and it demonstrated that three applications were enough to achieve the ideal plants height (12-15cm) in 50 dap, and other desirable characteristics for planting in farm: three true developed leaves or more, pseudostem diameter superior to 1.0-1.5cm.

Discussion

Plantlets with three applications of the *Azotobacter*-based biofertilizer, 60 days after planting (dap) exceeded the control with significant differences in most of the evaluated variables. According to Zhou J et al. [17], the dynamic of plants growth is related with genetic and environmental factors, in that sense, the phenotypic analysis of plant growth variables is an important approach to understand how plants interact with environmental changes as well as respond to different treatments. During a recent research, to evaluate three organic inputs during the acclimatization of banana (cv. Williams) micro propagated seedlings, authors informed that best results were found with a bioproducts combination increasing the height by 29.6 % and the pseudostem diameter by 19.9 %, while in the leaf area the best treatment overcame in 84.7 % the remain treatments [18]. In all cases, values were smaller than the ones obtained in the present research, but both bioproducts stimulated the plantlets development.

Among the causes of these results, in absence of others possible reasons associated to the observed differences, the applications of the new biofertilizer were responsible of them. A few years ago, the exact mode of action behind the growth promoting activity of *Azotobacter* was not very clear, but there is a coincidence about several probable mechanisms related with growth hormone production (gibberellins, Indole Acetic Acid (IAA) and cytokinins) or the presence of siderophores [19] due to a bigger access to the sparingly soluble Fe in the environment. At the same time, this microorganism is one of the most important a non-symbiotic N2-fixing bacteria with a high diversity and dispersion in soils, and therefore, significant improvements in crop productivity and soil fertility [7].

One of the most important finding was the significantly increment on the leaf area in those treatments with two or three applications of the biofertilizer, in both cultivars. This variable determines the light interception (photosynthesis process efficiency) and is an important parameter in determining plant productivity [20], especially in its biomass production [21]. Higher photosynthesis provides better growth of plants because around 90% of plant biomass is a consequence of the CO_2 assimilation through photosynthesis [22]. The reduction of acclimatization phase in around nine days was economically important, because in less time the plantlets were ready for farm transplant. Theoretically, plantains and bananas plantlets require between 45 and 60 days to be ready to transplant to the farm, it depend of many factors related with the acclimatization conditions, the cultivar, etc. Nevertheless, when some microorganisms are using as a biofertilizer, due to several causes, it should be expected that its effect could not be observed before 50-60 days after planting.

Evaluations at 60 dap are justified because after the microorganisms exogenous application and they enter in contact with the root, they should colonize and adhere to the radicular tissue before they can materialize their action. Some researchers found that this kind of colonization requires time and it only happens when there is compatibility between the microorganisms and intrinsic factors of the plant, as those perspired of root [23]. All those factors could influence in a lack of inoculant's effect during the first 45-50ddp. These results coincided with previous findings published by Beovides Y et al. [24], which didn't find any effect of the natural bioestimulant VIUSID Agro®, before 45-50 days of its application, during the acclimatization of taro (Colocasia esculenta Schott) plantlets.

The differences observed in the intensity of the answer in some variables for each cultivar could be associated to significant specificity between microbe and plant genotype already observed by Anbi AA et al. [25] for endophytes like *Azotobacter sp*. In general, many aspects are related with the complex process of colonization by those kind of beneficial microorganisms. In an interesting review, Trivedi P et al. [23] referred recently about interactions among the host, environment and microbes that take place both, above and below ground; they mentioned too, some findings associated to the requirements of common adaptation mechanisms for an effective colonization. Probable, there are genes involved in motility, adhesion and biofilm production help to the plant colonization. Studies based on comparative genomics [26] informed about the identification of some homologs of known bacterial genes involved in colonization, pathogenesis or provision of nutrients to plants. On the other hand, Huang AC et al. [27] affirmed that root metabolome changes could determine a specialized microbial communities that alter plant performance. In this sense, studies made by Leach JE et al. [28] and Trivedi P et al. [23] showed that metabolomics could contribute with information for the detection and quantification of small molecules, such as benzoxazinoids and strigolactones that impact on plant-microbiome interactions.

Results of this research showed that the evaluated new biofertilizer promoted the growth of plantlets and the used number of applications had a direct effect on the development of acclimatized plantain plants. *Azotobacter* is said to contribute in a substantially way to the nitrogen content of soil, a number of beneficial characters are present in this organism such as higher nitrogen fixation, ammonia excretion, production of vitamins and growth promoters, production of siderophores, production of antifungal antibiotics. It was reported that *Azotobacter* secretes substances that inhibit the growth of certain root pathogens and improve root growth and uptake of plant nutrients [29].

Additionally, these positive traits offer promising possibilities to ecologically engineer *Azotobacter* species likely providing significant N inputs, while reducing reliance to N-containing fertilizers such as urea [30,31]. All that is in correspondence with the special interest of advancing applied research on *Azotobacter* species as both agriculturally important plant growth promoting N2-fixing rhizobacterium that can be used for improving plant N nutrition and a biofertilizer based products at large scale, having significant improvements in crop productivity and soil fertility. They are able to synthetized plant growth hormones and these hormones can not only improve plant growth and nutrient uptake, but can also indirectly protect host plants from phytopathogens and stimulate other beneficial rhizosphere microorganisms [32,33].

Azotobacter species are able to influence directly plant growth by synthesizing plant growth hormones. These hormones can not only enhance plant growth and nutrient uptake but can also indirectly protect host plants from phytopathogens and stimulate other beneficial rhizosphere microorganisms [33]. Several works on drought stress tolerance using *Azotobacter* species as a solution demonstrated the efficacy of their use [34]. Maybe, it is caused because they acts as antagonists and suppress the incidence of soil borne plant pathogens and usually, bio-fertilizer liberates growth promoting substances and vitamins and helps in maintaining the soil fertility [35].

On the other hand, in case of biofertilizers, is important to consider possible interactions among the contained physiologically active compounds, and because of that, their effects on plants may depend on dose, time of treatment, general or specific growth conditions, and even, plant species. Some researchers [1,7] pointed out *Azotobacter* based-biofertilizers, possess unique characteristics such as cyst formation containing novel liquid (more than one type of nitrogenase, extreme tolerance to oxygen) conferring resistance to environmental stresses, an idea mentioned before by [36]. Moreover, the abundance of *Azotobacter* species in the soil could improve the availability not only of N through the biological nitrogen fixation processes [37], but also P as well [38].

In general, results confirmed the possibility to reduce the dependency on synthetic fertilizers with the use of effective biological-based alternatives like *Azotobacter*-based biofertilizers, as an effective component of integrated plant nutrition strategy, which contributes positively to sustainable agricultural production. In this sense, *Azotobacter*-based biofertilizer is a new input for the plantain acclimatization that can reduce the application rates of chemical fertilizers toward a more sustainable agriculture. Current findings during this research, may help to implement an applicable and cost-effective micropropagation protocol for plantains and bananas plantlets, using the new biofertilizer during the acclimatization phase.

Conclusion

Applications of the new *Azotobacter*-based-biofertilizer (three times, every seven days), increase significantly the plant growth (plant height, length and width of the developed leaf, and leaf area) of plantain plantlets during their acclimatization. It reduce in nine days the required period for transplanting using a conventional and non-sterile mixed substrate (70 % organic matter and 30 % soil).

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