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Original article

Arbuscular mycorrhizal fungi and *Azotobacter chroococcum* in obtaining coconut seedlings

Hongos micorrizógenos arbusculares y *Azotobacter chroococcum* en la producción de posturas de cocotero

Karen Alvarado-Ruffo[1], Albaro Blanco-Imbert[2], Gloria M. Martín-Alonso[3], Yoania Ríos-Rocafull[4], Ramón Capdesuñer-Rojas[5], Blanca M. de la Noval-Pons[3]*

[1]Universidad de Guantánamo, ave. Ernesto Che Guevara, km 1 ½ carretera a Jamaica. Reparto San Justo, Guantánamo, Cuba. CP 95100

[2]Instituto de Suelos Guantánamo, Departamento de Investigación, carretera 8 este, Ciudad Deportiva, Guantánamo, Cuba. CP 95100

[3]Instituto Nacional de Ciencias Agrícolas (INCA), carretera San José-Tapaste, km 3½, Gaveta Postal 1, San José de las Lajas, Mayabeque, Cuba. CP 32 700

[4]Instituto de Investigaciones Fundamentales en Agricultura Tropical (INIFAT), calle 188 esq. 397, Santiago de la Vegas, Boyeros, La Habana, Cuba. CP10800

[5]Empresa Agropecuaria y Coco Baracoa, La Playa, Baracoa, Guantánamo. CP 95100, Cuba.

ABSTRACT: The coconut is a widely spread crop, with importance in food, cosmetics and industry; however, its production is affected, among other factors, by the aging of the plantations, which affects productivity levels, for which it is essential to obtain quality postures for the repopulation of the production areas. With the aim of studying the combined effect of arbuscular mycorrhizal fungi (AMF) and *Azotobacter* strains in the production of high-quality coconut seedlings, an experiment was carried out in two nurseries in Baracoa, Guantánamo, Cuba, of which two repetitions in time. The domesticated coconut ecotype "Indio Verde-1" was used in two soils, Arenosol háplico (ARh) and Gleysol Flúvico háplico (GFLh), with the inoculation of three strains of AMF (*Glomus cubense*, *Rhizophagus irregularis* and *Funneliformis mosseae*) and two of *A. chroococcum* (CDM-1 isolated from ARh soil and CDM-2 from GFLh soil). A differential response of AMF species was observed in both soils. The inoculation with *R. intraradices* favored the increase of the variables seed germination, height, stem diameter and number of leaves, in the ARh soil when its inoculation was combined with the strain *A. chroococcum* CDM-1, while *G. cubense* was the best soil treatment GFLh, combined with *A. chroococcum* CDM-2. Although high levels of autochthonous AMF strains were quantified in both soils (control), the inoculated strains proved to be more efficient than these for obtaining quality seedlings.

Key words: *Cocos nucifera*, Mycorrhizae, Rhizobacteria.

RESUMEN: El cocotero es un cultivo extendido, con importancia en la alimentación, la cosmética y la industria; sin embargo, su producción se ve afectada, entre otros factores, por el envejecimiento de las plantaciones, lo cual disminuye los niveles de productividad, por lo cual se hace imprescindible la obtención de posturas de calidad para la repoblación de las áreas de producción. Con el objetivo de estudiar el efecto combinado de los hongos micorrizógenos arbusculares (HMA) y cepas de *Azotobacter* en la producción de posturas de cocotero de alta calidad, se desarrolló un experimento en dos viveros de Baracoa, Guantánamo, Cuba, del cual se realizaron dos repeticiones en el tiempo. Se empleó el ecotipo domesticado de cocotero "Indio Verde-1" en dos suelos: Arenosol háplico (ARh) y Gleysol Flúvico háplico (GFLh), con la inoculación de tres cepas de HMA: *Glomus cubense, Rhizophagus irregularis* y *Funneliformis mosseae* y dos cepas de *Azotobacter*: *A. chroococcum* (CDM-1 aislada del suelo ARh y CDM-2 aislada del suelo GFLh). Se observó una respuesta diferencial de las especies de HMA en ambos suelos. La inoculación con *R. intraradices* favoreció el incremento de las variables germinación de las semillas, altura, diámetro del vástago y número de hojas, en el suelo ARh al combinarse su inoculación con la cepa *A. chroococcum* CDM-1, mientras que *G. cubense* fue el mejor tratamiento en el suelo GFLh, combinado con *A. chroococcum* CDM-2. A pesar de que se cuantificaron elevados niveles de las cepas autóctonas de HMA, en ambos suelos, las cepas inoculadas demostraron ser más eficientes que estas para la obtención de posturas de cocotero de calidad.

Palabras clave: *Cocos nucifera*, Mycorrhizae, Rizobacterias.

*Author for correspondence. bdelanov65@gmail.com

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INTRODUCTION

The coconut palm is a crop with a wide range of uses, which makes it a unique plant. It has applications in the food industry (1, 2), as an oil substitute and sweetener (3), as well as for its high nutritional value (4, 2). It also has wide applications in the cosmetic industry (5-7), in the timber and handicraft industries (8). By-products such as fiber are used in agriculture for the preparation of substrates (9, 10), and in the livestock industry for the preparation of feed additives (11). It also has important applications in medicine for skin diseases (12-14), cardiovascular diseases (15-17) and multiple sclerosis (18). Its use has spread to more than 94 countries around the world, providing livelihoods for millions of people in the Asia-Pacific regions (19). However, in most of these countries there is an aging of plantations that constitutes the main limitation for obtaining high yields (20), an effect that is also observed in Cuban plantations. This produces economic effects for the producers and for the companies that use its derivatives, such as coconut oil, since they have to import it from an external market. Because of this, it is necessary to apply technologies for the production of quality seedlings, as a crucial step to obtain vigorous and productive populations. The quality of the planting material determines to a great extent the final yields of the coconut, being the basis for a successful production (21).

The use of microorganisms, active components of biofertilizers, may represent a feasible alternative technology to improve plant yields (22, 23). Effective formulations of agriculturally important microorganisms such as nitrogen fixers, plant growth-promoting rhizobacteria (PGPR), and arbuscular mycorrhizal fungi (AMF) have been developed as valuable inputs for crop production (21).

AMF are the most widely geographically distributed microbial group of all biofertilizing microorganisms with a high adaptation to diverse soil conditions (24). They play an important role, contributing most efficiently to seedling survival and growth by playing a crucial role in plant nutrition (25, 26). Studies conducted in the rhizosphere of adult coconut plantations have demonstrated the rich diversity of this species, whose roots are colonized by AMF (27).

Among the PGPR is the genus *Azotobacter*. These are free-living associative bacteria, with potentialities among which N fixation and phytohormone production stand out (28). They are also antagonists to soil pathogens (29), as well as inducers of systemic resistance in plants against foliar and root pathogens (30).

In crops other than coconut, the joint application of AMF and *Azotobacter* has been shown to promote plant growth (31, 32), by different mechanisms such as the regulation of plant growth hormones (28) and the improvement of nutrition (26), and constitutes an extremely useful approach for sustainable agriculture, including in ecosystems affected by some stress (33-35). The joint use of both microbial groups may constitute a technology for obtaining quality seedlings, leading to rejuvenated plantations with high productivity.

For these reasons, the objective of the present work was to study the combined effect of AMF and *Azotobacter* strains in the production of high quality coconut seedlings in the nursery.

MATERIALS AND METHODS

The experiment was developed in the nurseries of Playa Duaba with an Arenosol haplic soil (ARh), and Cabacú with a Gleysol Fluvic haplic soil (GFh), both in Baracoa municipality, Guantánamo, Cuba. The substrate used was the combination Soil: Earthworm Humus: Coconut Fiber (S:H:FC), in a 4:1:1 ratio (v/v/v/v), taking into account the results of previous works (36). In each nursery, local soil was used to prepare the substrates. As a control, substrates made up of the combination of soil and organic sources were used in the ratio 1:1:1 v/v/v/v as recommended in the Technical Instructions for coconut cultivation (37). Partial characterization of the substrates was carried out (38) and the autochthonous populations of AMF in each type of soil were quantified (39). Table 1 shows the partial characterization of the substrates and the results of the quantification of the autochthonous AMF populations of the soils.

The domesticated ecotype of coconut palm “Indio Verde-1” (40) was used, whose seeds were obtained from healthy mother plants, which were prepared and sown as reported (36).

Three species of AMF were selected, which came from the stock of the National Institute of Agricultural Sciences (INCA): *Glomus cubense*, Y. Rodr. and Dalpé (41) strain INCAM-4 (DAOM241198), with 92 spores g-1 of inoculum; *Rhizophagus irregularis* (Blaszk, Wubet, Renker and Buscot) Walker and Shüβler (42), strain INCAM-11 (DAOM711363), with 73 spores g^{-1} of inoculum and *Funneliformis mosseae*, (Nicol. and Gerd.) Walker and Schüßler (42), strain INCAM-2, with 57 spores g^{-1} inoculum. Inocula were adjusted to ensure 215 spores per seed, (43). For the application, the inoculant was mixed with 600 mL of water, in which the seed was immersed, by the part of the notch, using the seed coating method (44) with the referred modification.

All treatments inoculated with AMF were co-inoculated with strains of *A. chroococcum* belonging to the strain collection of the National Institute for Fundamental Research in Tropical Agriculture (INIFAT), isolated from the nurseries that were the object of the study. In the Playa Duaba nursery treatments, strain CDM-1 was inoculated and in the Cabacú nursery, strain CDM-2, in both cases, after the inoculation of AMF species, taking into account the results of previous studies (36). The strains were prepared as described in the aforementioned work.

No inoculation of microorganisms was performed in the control. An additional treatment was used, conformed by mineral fertilization (NPK 100 %) for which the complete formula 9:13:17 was used at a rate of 45 g per seed, fractioned at 33 % at 30 days after sowing (das) and the rest at 90 das, to evaluate the effect of AMF with respect to the control of complete fertilization (37).

Table 1. Partial chemical characterization of substrates and native AMF populations of the soils used

Substrate	pH (H_2O)	OM ($g\ kg^{-1}$)	C/N	Resident AMF populations (spores 50 g^{-1} soil)
		Nursery Playa Duaba (ARh)		
S: HL: FC (1:1:1)	7.46	320.4	17.21	923.33
S: HL: FC (4:1:1)	7.6	214	14.43	
		Nursery Cabacú (GFh)		
S: HL: FC (1:1:1)	7.63	344.4	18	2676.67
S: HL: FC (4:1:1)	7.77	256	16.32	

Methods: pH (H_2O), OM: organic matter; C/O Carbon/Nitrogen ratio (38); Resident AMF populations (39)

A randomized block design with three replications was used. Each experimental plot within the block contained 20 seeds sown at 0.05 x 0.20 m spacing.

The experiment had a duration of 180 days. At 120 das, the germination velocity index (GVI) was calculated (36), according to the formula:

$$GVI = \sum(number\ \ of\ \ plants\ \ germinated\ \ at\ \ time\ \ n/time\ \ n)$$

For this variable, the percent increase of the inoculated treatments relative to the control was calculated.

At 180 das, fifteen plants per replicate were sampled and height, diameter at the stem base, number of leaves, leaf area, and total dry mass were evaluated (36). In the aerial part, N, P and K contents were quantified (45).

The quantification of mycorrhizal variables, percentage of colonization (%) (46), and intensity of mycorrhizal colonization (%) and native AMF populations in the rhizosphere, evaluated as number of spores (39), was also carried out.

The experiment was repeated for two years, using a randomized block design. The data were processed by Simple Classification Analysis of Variance and the comparison of means by Duncan's Multiple Range Test with a significance level of 95 %. Data for the variable percentage of mycorrhizal colonization were transformed by the formula arcseno√x. The statistical package STATGRAPHIC version 15.2 was used.

RESULTS

When analyzing the results of the different indicators evaluated, no interaction was observed between the repetition factor and time in any of the nurseries, so the means of both repetitions are analyzed.

Regarding the GVI, it was observed that co-inoculation with AMF with *A. chroococcum* strains accelerated the germination process, regardless of the AMF strain inoculated (Figure 1). The control and the fertilized treatment (NPK) showed similarities between them, but differences in relation to the inoculated ones, in the two soils studied.

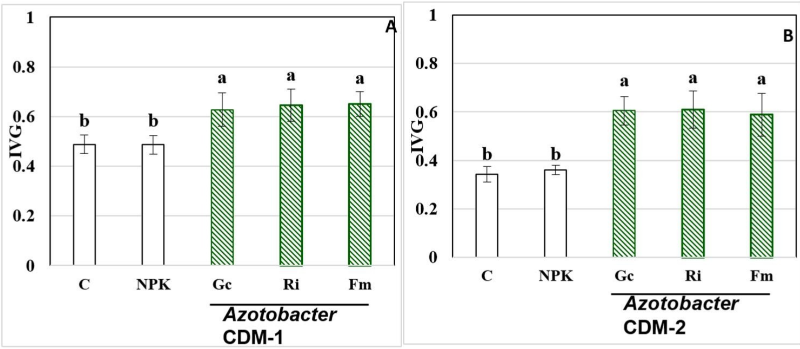
In the Cabacú nursery (GFh soil), higher percentages of increase were observed for the inoculated treatment than those obtained in the Playa Duaba nursery (ARh soil) (71-77 and 28-33, respectively), due to the fact that both the control and the fertilized treatment showed lower values in the latter, which shows that a greater mycorrhizal effect was obtained in the GFh soil.

When analyzing the results of vegetative development (Table 2), a differential response of the AMF strains studied in the two nurseries, corresponding to two types of soil, was observed. In the Playa Duaba nursery, the co-inoculated *R. intraradices* - *A. chroococcum* CDM-1 treatment showed significantly higher levels than the rest of the variants in all the indicators evaluated (height, stem diameter, number of leaves, leaf area and total dry mass). With the use of *G. cubense* and *F. mosseae*, values of height, stem diameter and leaf area were statistically similar to those of the control; however, in the rest of the indicators it was lower than the control. The fertilized treatment showed the lowest levels in all indicators.

Unlike the Playa Duaba nursery, in Cabacú the best results were obtained with the combination *G. cubense* - *A. chroococcum* strain CDM-2, which significantly outperformed the rest of the treatments in all the indicators evaluated. The treatments inoculated with *R. irregularis* and *F. mosseae* achieved values similar to or lower than the control in all the indicators evaluated, with the exception of leaf area, where they exceeded the control. In all cases, the fertilized treatment showed the lowest levels.

When analyzing the contents of N, P and K in the plant, in the Playa Duaba nursery (ARh soil), the *R. irregularis* - *A. chroococcum* CDM-1 co-inoculation treatments stood out, with an increase in the contents of the three elements. In the treatments where *G. cubense* and were used, intermediate levels were reached, being statistically superior to the control. The control and the fertilized treatment showed statistical similarity, obtaining the lowest values.

In the Cabacú nursery (GFLh soil), the combination of *G. cubense* - *A. chroococcum* CDM-2 had a positive effect on N, P and K contents, while the treatments using *R. irregularis* and *F. mosseae* obtained intermediate values, with significant differences between them. The control and the fertilized treatment showed the lowest levels; however, the organic fertilizer (control) outperformed the NPK fertilization.



Means with different letters for the same soil differ from each other, according to Duncan's Multiple Range Test (p≤0.05). Vertical lines above the bars indicate standard deviation. AMF: *G. cubense* (Gc), *R. irregularis* (Ri) and *F. mosseae* (Fm). *Azotobacter*: *A. chroococcum*, strain CDM-1 in Nursery Playa Duaba (A) and strain CDM-2 in Nursery Cabacú (B)

Figure 1. Germination rate index (GRI) of coconut palm seeds, Indio Verde-1 ecotype, inoculated with three AMF and two *Azotobacter* strains

Table 2. Vegetative development and foliar nutrient content of coconut seedlings, ecotype Indio Verde-1, inoculated with three strains of AMF and two strains of *A. chroococcum* (CDM-1 at nursery Playa Duaba and CDM-2 at Cabacú)

Treatments	Height		Stem diameter		Number of leaves		Leaf area		Total dry mass		N		P		K	
	(cm)		(cm)				(m^2 $plant^{-1}$)		(g $plant^{-1}$)				(g $plant^{-1}$)			
Nursery Playa Duaba																
Control	108.77	b	3.02	b	3.11	d	1119.81	b	139.80	c	7.60	D	0.66	c	4.88	C
NPK	104.31	c	2.81	c	2.93	e	1111.81	c	140.69	c	7.50	D	0.63	c	4.92	C
G. cubense	107.74	b	3.02	b	3.98	b	1120.71	b	167.29	b	8.51	B	0.77	b	5.74	B
R. intraradices	115.19	a	3.21	a	4.24	a	1380.00	a	283.01	a	15.61	A	1.70	a	10.14	A
F. mosseae	108.72	b	2.97	b	3.71	c	1119.41	b	166.36	b	8.14	C	0.75	b	5.57	b
SEx:	1.18		0.04		0.05		0.49		0.89		0.1		0.02		0.07	
Nursery Cabacú																
Control	108.54	b	2.98	b	2.93	c	1110.06	b	145.42	c	8.66	D	0.70	c	4.99	d
NPK	97.70	c	2.41	d	2.82	c	1085.70	d	143.80	c	7.43	E	0.36	d	4.7	e
G. cubense	117.55	a	4.19	a	4.31	a	1338.69	a	269.01	a	15.36	A	1.16	a	9.07	a
R. intraradices	109.02	b	3.03	b	4.20	a	1110.06	b	159.41	b	10.00	B	0.96	b	5.87	b
F. mosseae	95.81	c	2.68	c	3.13	b	1108.42	c	157.31	b	9.02	C	0.65	c	5.25	c
SE x:	1.19		0.04		0.06		0.49		0.89		0.11		0.02		0.07	

Means with different letters for the same soil differ from each other, according to Duncan's Multiple Range Test (P<0.05). SE x: standard error of mean

Regarding mycorrhization indicators, in the Playa Duaba nursery (ARh soil) the control showed similar colonization levels to those obtained by the mycorrhized treatments (Figure 2); however, when analyzing the CI, the *R. irregularis* strain surpassed all treatments, similar to what was observed in the vegetative development study. This behavior was similar to that observed in the analysis of spore abundance, an indicator in which all treatments reached high values.

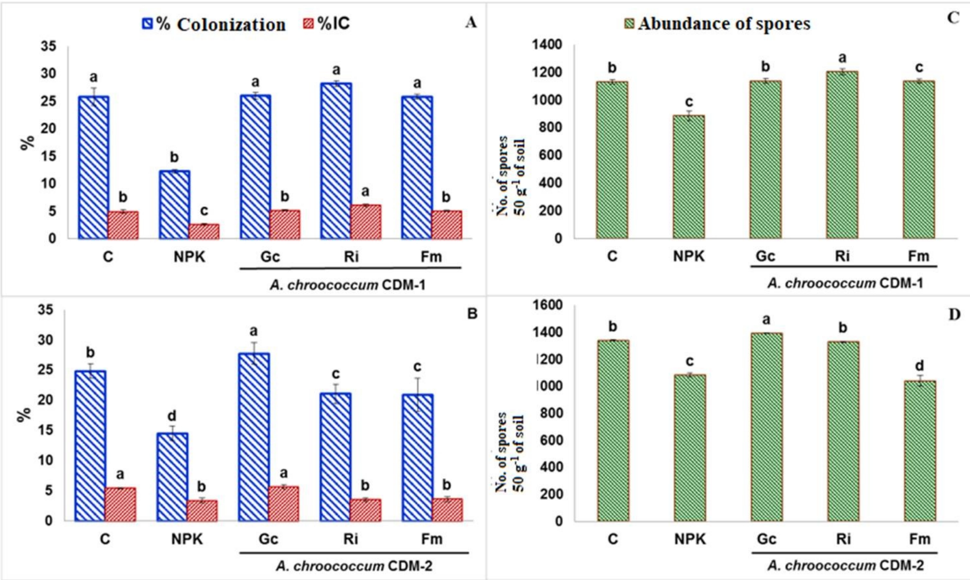
In Cabacú nursery (GFLh soil), a behavior similar to that obtained in the analysis of vegetative development was observed for the three indicators evaluated; however, the co-inoculation *G. cubense* - *A. chroococcum* CDM-2 significantly outperformed all treatments.

In the joint analysis of the variables evaluated, it was observed that when using the ARh soil (Playa Duaba nursery), the best treatment was the co-inoculation of *R. irregularis* - *A. chroococcum* CDM-1, while with the GFLh soil (Cabacú nursery) the combination of *G. cubense* - *A. chroococcum* CDM-2 was the one with which the best results were obtained. In these treatments, the co-inoculation of the microorganisms induced in the plants a better utilization of nutrients, an increase in photosynthetic surface (leaf area) and a greater accumulation of biomass (total dry mass).

DISCUSSION

In the soils used for substrate preparation in both nurseries, high levels of resident AMF propagules were found (923.33 and 2676.67 AMF spores g-1 in ARh and GFLh soils, respectively, Table 1), similar to those found by other authors in similar, undisturbed agroecosystems (47, 48). An effect of mycorrhizal symbiosis on the different indicators evaluated was observed.

The analysis of the results of the AMF-*A. chroococcum* interaction suggests that it is possible that the co-inoculation could have broken the barriers imposed by the competition of the inoculated AMF strains with the autochthonous strains because *Azotobacter* releases active metabolites that allow the growth of the fungus. In addition, it modulates the expression of specific active proteins that play an important role in the physiology and establishment of the symbiosis, since they stimulate sporulation during the vegetative state. On the other hand, these proteins



Means with different letters for the same soil differ from each other, according to Duncan's Multiple Range Test (p≤0.05). Vertical lines above the bars indicate standard deviation. Control (C), *G. cubense* (Gc), *R. irregularis* (Ri), *F. mosseae* (Fm)

Figure 2. Mycorrhizal indicators, colonization and intensity of colonization (%IC) (A and B) and spore abundance (C and D) in the roots of Indio Verde-1 ecotype coconut palm plants inoculated with three AMF strains in the nurseries of Playa Duaba (A and C) and Cabacú (B and D)

regulate the synthesis of ATP and ammonium, which can be used in the processes of active absorption of organic and inorganic nitrogen sources by the mycelium, energy that is also used by the fungus for its growth (49). Similar to the results of the present work, other authors have observed that joint inoculation of three PGPR (*A. chroocococum*, *B. megaterium*, and *B. mucilaginous*) with two AMF resulted in increased mycorrhizal colonization (50).

Coconut is a crop that naturally establishes symbiosis with AMF (51) and is associated with bacteria of the genus *Azotobacter* (36, 52), which was evidenced in plants grown within the evaluated controls (S-H-FC 1:1:1 and NPK 100 %) which were not inoculated, so that the quantified populations of both microbial groups corresponded to the autochthonous ones.

The positive response in relation to the increase of N, P and K contents in the aerial part of the plants with results of the treatments *R. irregularis* - *A. chroocococum* CDM-1 and *G. cubense* - *A. chroocococum* CDM-2, superior to the NPK 100 % and 1:1:1 controls of S-H-FC could be due to the combined effect of the organic fertilizer with the co-inoculation of *Azotobacter chroocococum* and AMF strains. It is known that organic fertilizers improve the physical properties of soils by different mechanisms (53), allowing greater availability of nutrients, which favors better plant development (54) and, consequently, could increase the quantity and quality of root exudates and thus the establishment of the most efficient *A. chroocococum* and AMF strains for each soil.

A. chroocococum produces the secretion of phytohormones that favor plant root growth, allowing greater water absorption and nutrient absorption, in addition, it has the capacity to solubilize phosphorus, fix atmospheric nitrogen and make Fe available through the production of siderophores (29). In this way, plants can have a higher content of nutrients obtained through the nitrogen fixation process, since Fe constitutes an important cofactor for this biological mechanism (55).

Experimental results show that stimulation by plant growth-promoting rhizobacteria is the result of several mechanisms that act together and are activated simultaneously. Plant symbioses with soil microorganisms confer energetic benefits due to improved plant nutrition; the supply of N and P from the microbial symbiont to the plant depends on the availability of ATP in the microbial symbiont, which is regulated by its demand for carbon compounds (56).

It is known that AMF promote plant growth by different mechanisms, among which are the improvement of water status (57) and the increased uptake of mineral elements present in the soil, mainly those of limited diffusion such as P (34), Zn, and Cu (58). It is suggested that there may be a differentiated need based on the nutritional demand of the inoculated AMF strain itself, which could be a possible explanation for the observed results (59).

In a general sense, the results could be conditioned by the relationship established between *A. chroococcum* and AMF. The inoculated *Azotobacter* strains, when established in the substrate, were able to produce phytohormones that stimulated root growth in plants, inducing the secretion of radical exudates that allowed the colonization of the AMF strain, depending on the type of soil used in the substrate (60). On the other hand, the pH of the substrates was in the optimal range for the growth of *Azotobacter*, which favored the establishment of this bacterium (61). At these pH values, the bacteria could have solubilized insoluble phosphates, which apparently was favorable for the establishment of AMF strains, considering that this is one of the factors that regulates their establishment and functioning (62).

The different AMF species studied responded to the substrate depending on the soil that formed it. An effect was observed depending on the high levels of existing autochthonous propagules, in spite of having similarity in pH, as well as in the organic matter content and in the C/N ratio, which indicated that other physicochemical and biological properties of the substrates could be influencing the differential response of the AMF observed, so it is necessary to deepen the studies of mycorrhizal inoculation in these soils, since there were no previously published results.

The extraradical mycelium of AMF can effectively translocate the products of rhizobacterial activity when they coexist in the rhizosphere of the plants, so that their response to mycorrhizal symbiosis also involves the action of rhizospheric microorganisms as a whole (63).

The AMF-Azotobacter interaction favored an increase in the levels of absorption of nutrients such as nitrogen, phosphorus and potassium and their translocation to the interior of the plant, with an increase in photosynthetic capacity and, consequently, an increase in the morphological indicators evaluated, which allowed a reduction in the period of permanence in the nursery.

In all cases, the treatments where the best results were obtained also indicate that a greater capacity to capture solar energy to carry out photosynthesis must have occurred, as they showed a greater number of leaves and leaf area. It has been observed that AMF significantly improve photosynthetic rate and increase chlorophyll content (64, 34), which would have a high impact on growth rate.

The beneficial effect on plant growth has been reported when using inoculants composed of nitrogen-fixing bacteria and AMF, combined with organic fertilizers, with the reduction of the levels of mineral fertilizers used (58).

In a research carried out in coconut postures with the use of AMF, it was found a positive effect on the number, volume and dry mass of primary, secondary, tertiary and quaternary roots, from the fourth month after inoculation, which improved the efficiency in the absorption of water and nutrients and induced increases in the perimeter of the base of the stem and the dry mass of the aerial part from the sixth month (65).

The positive action of interactions between AMF and PGPR on growth promotion has been demonstrated in other crops (22, 23, 66), as well as that of these microbial groups and organic fertilizers (67); however, similar studies have not been carried out in coconut before, where they had only been used independently. Having results of the co-inoculation of AMF and *Azotobacter* in this crop makes it possible to enhance the use of these microbes and, thus, make it possible to obtain quality seedlings and, consequently, to increase yields.

The combined study of organic sources, AMF and nitrogen-fixing bacteria has shown that the combined sources provide a suitable environment for plant growth at an early stage (57).

Organic farming practices with a focus on building biological soil fertility bases through the application of organic inputs and bioproducts, such as AMF and PGPR (*Azotobacter*), allow for sustainable coconut production in an environmentally friendly manner.

CONCLUSIONS

Considering the results, it can be concluded that the co-inoculation of *R. irregularis* with the *A. chroococcum* CDM-1 strain allows obtaining high quality coconut seedlings to be used in the Playa Duaba Nursery with ARh soil, while the combination of *G. cubense* and *A. chroococcum* CDM-2 show the best indicators for the Cabacú Nursery with GFh soil, both using as substrate the combination of S-H-FC (4:1:1), which allows optimizing the use of local organic fertilizers. The growth promoting effect of AMF and *Azotobacter* combined with organic fertilizers to obtain coconut seedlings is demonstrated.

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