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BIOTECHNOLOGY

Inoculation of arbuscular mycorrhizal fungi as a strategy to improve annatto (*Bixa orellana* L.) growth

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ABSTRACT. This study aimed to assess the occurrence of arbuscular mycorrhizal fungi (AMF) in annatto (*Bixa orellana* L.) cultivars and their response to AMF inoculation using biometric parameters. The occurrence surveys were conducted in annatto fields in three municipalities from Pernambuco Forest Zone: Lagoa de Itaenga, Gloria de Goitá, and Vitoria de Santo Antão, and in four cultivars (Red Piave, Green Piave, Red Peruvian Paulista, and Green Peruvian Paulista). In a greenhouse, biometric parameters of annatto seedlings of Red Piave, Red Peruvian Paulista, Embrapa-36, and Embrapa-37 cultivars inoculated with AMF isolated from annatto fields. The Red Piave cultivar exhibited greater root colonization than the Green Peruvian Paulista in the Lagoa de Itaenga and Vitoria de Santo Antão municipalities. The cultivar Red Piave showed a more beneficial association with AMF in plants and soil than cultivar Green Peruvian Paulista did, in both Lagoa de Itaenga and Vitoria de Santo Antão. AMF inoculation was effective in promoting the growth of annatto plants, particularly those inoculants with *S. heterogama* and *C. etunicatum*.

Keywords: spores; inoculation; colonization; AMF.

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Introduction

Annatto (*Bixa orellana* L.) belongs to the Bixaceae family and is a native plant species found in Brazil, more specifically in the Amazon region. This plant species is widely distributed and is currently found in several regions, such as Peru, Mexico, Ecuador, Indonesia, India, Kenya, and East Africa (Dequigiovanni et al., 2018). It is a perennial and pioneer species, with an average height of 5 m, being used to restore degraded areas (Lorenzi, 2008).

Annatto is also economically important to agriculture, particularly for small and medium farmers (Mazzani, Marín, & Segovia, 2000). The seeds produce pigments that are used as a natural dye in the food, medicine, textile, pharmaceutical, and cosmetic industries. This is important because there are some legal restrictions imposed on synthetic dyes, and therefore plant-based dyes could be in greater demand (Pratibha et al., 2013). The production of annatto is important, but needs to include sustainable practices. Among the current sustainable practices in agriculture, the inoculation of arbuscular mycorrhizal fungi (AMF) is ecologically and economically important. AMF is particularly beneficial to annatto as it can increase the plant's resistance to drought and promote higher nutrient uptake by roots.

Previous studies have reported the contribution of AMF to plant growth (Barbieri, Braga, Sousa, & Roque, 2011; Feijen, Vos, Nuytinck, & Merckx, 2018). For example, Feijen et al. (2018) reported that about 3,617 plant species, including Bixaceae, are favored by mycorrhizal fungi. However, there are few studies that specifically emphasize the contribution of AMF to the growth of annatto plants. In this study, we investigated the occurrence of AMF in annatto plants and their responses to AMF inoculation using biometric parameters. Our findings serve as guidelines for strategies aiming to promote the sustainable growth of annatto plants.

Material and methods

Evaluation of native AMF

Native AMF were quantified in soil cultivated with annatto in Pernambuco state. Based on their importance in annatto cultivation, three municipalities in the Pernambuco Forest Zone were selected, namely

Lagoa de Itaenga, Gloria do Goitá, and Vitoria de Santo Antão. Fields with the cultivars Red Piave, Green Piave, Red Peruvian Paulista, and Green Peruvian Paulista, with five years of establishment, were evaluated for the occurrence of AMF. In each location, ten plants were selected, and the surrounding soil (0-20 cm deep) and roots were collected to evaluate soil characteristics. Physical and chemical analyses were performed using standard laboratory protocols.

In the laboratory, soil samples were separated from the roots. Roots were then washed and stored in alcohol (50% concentration). Root colonization by AMF was estimated using the clarification and staining method (Phillips & Hayman, 1970), followed by the method of Giovannetti and Mosse (1980). Briefly, 1cm of stained root segments was randomly selected and assembled in groups of 5 blades per plant. Each 10-segment blade was evaluated under a microscope for the presence or absence of hyphae, shrubs, vesicles, and spores.

The number of native AMF spores in the soil was counted by the decantation and wet sieving method (Gerdemann & Nicolson, 1963) complemented by the sucrose centrifugation and flotation method (Jenkins, 1964). The identification of AMF present in the soil was performed according to Schenck and Pérez (1990).

Inoculation of AMF in annatto cultivars

The effect of AMF on annatto cultivars was evaluated in a greenhouse. The experimental design was a randomized block design with three replicates and the following treatments: (a) plants of the cultivars Red Piave, Red Peruvian Paulista, Embrapa-36, and Embrapa-37 annatto cultivars inoculated and without inoculation; (b) the cultivars were inoculated with each AMF species (*Scutellospora heterogama, Claroideoglomus etunicatum,* and native species), individually, from commercial annatto plantations, located in Pernambuco, and (c) in association, inoculated with native spores, *S. heterogama,* and *C. etunicatum* species. The biometric parameters evaluated 150 days after planting included plant height, stem diameter, number of spores, root colonization, total nitrogen (N) content of the shoot and root, and fresh and dry shoot biomass.

First, each pot was filled with autoclaved soil (for 1 hour at 120°C and 101 kPa) collected from the municipality of Goiana, state Pernambuco, Brazil. The chemical characteristics of this soil are summarized in Table 1.

Table 1. Chemical characteristics of the soil used to grow annatto plants varieties (*Bixa orellana L.*) in a greenhouse.

Depth	P (mg.dm^{-3})	pH (H_2O)	cmolc dm^{-3}								%	
			Ca	Mg	Na	K	Al	H	S	CTC	V	m
0-20 cm	7	6.20	1.35	0.55	0.02	0.02	0.00	1.89	1.9	3.8	51	0

The soil was sieved (5 mm mesh) and autoclaved. The seeds were inoculated at sowing and the plants were kept for a period of 150 days. Distilled water was used for irrigation, and a nutritive solution without P was applied weekly. The plants were measured monthly for height and diameter.

At 150 days after sowing, plants were collected and weighed. Shoots and roots were separated and dried for 72 hours at 65°C. The estimation of N in plants was performed using the Kjeldahl method (Teixeira, Donagemma, Fontana, & Teixeira, 2017)

Root colonization by AMF was estimated using the clarification and staining method (Phillips & Hayman, 1970), followed by the method of Giovannetti and Mosse (1980). Briefly, 1cm of stained root segments was randomly selected and assembled in groups of 5 blades per plant. Each 10-segment blade was evaluated under a microscope for the presence or absence of hyphae, shrubs, vesicles, and spores.

Analysis of variance was performed, and means were compared by Tukey's test at 5% probability.

Results and discussion

Native AMF community

The physical and chemical properties of the soil obtained from the areas wherein annatto plants were cultivated in Lagoa de Itaenga, Gloria do Goitá, and Vitoria de Santo Antão are shown in Table 2 and 3, respectively.

Based on spore depletion, the cultivar Red Piave exhibited the highest root colonization rates in all municipalities, except for Glória de Goita wherein no significant differences among the cultivars were observed (Table 4). These results suggest that some AMF characteristics, such as diversity and community composition, can be affected by biogeography and cultivation system (Turrini et al., 2017).

Table 2. Soil physical properties of fields cultivated with annatto (*Bixa orellana* L.) from Lagoa de Itaenga, Gloria do Goitá, and Vitória de Santo Antão, state Pernambuco.

depth (cm)	Density (g cm^{-3})		Granulometry (%)				Nat. Clay[a] (%)	Floc deg[b] (%)	Text. Clas.[c]	Mosture (%)			Available water	
	Dap[e]	Dr[f]	Coarse sand	Fine sand	Silt	Clay				Residual	0.33 Atm	15 Atm	%	mm cm^{-1}
Lagoa de Itaenga														
0-20	1.48	2.57	48	23	20	9	2	78	S[d]	2.20	11.17	4.97	6.20	0.92
Glória do Goitá														
0-20	1.46	2.59	59	18	8	15	6	60	S	1.45	11.83	5.42	6.41	0.94
Vitória de Santo Antão														
0-20	1.31	2.57	62	14	15	9	2	78	S	2.30	19.69	9.06	10.63	1.39

[a]Nat Clay = Natural clay; [b]Floc deg. = Flocculation degree; [c]Text. Clas. = Textural class; [d]S = Sandy franc; [e]Dap = Bulk density; [f]Dr = particle density.

Table 3. Soil chemical properties of fields cultivated with annatto (*Bixa orellana* L.) from Lagoa de Itaenga, Gloria do Goitá, and Vitória de Santo Antão, state Pernambuco.

Depth	P (mg dm^{-3})	pH (H_2O)	cmolc dm^{-3}								%	
			Ca	Mg	Na	K	Al	H	S	CEC	V	m
Lagoa de Itaenga – state Pernambuco												
0-20 cm	6	5.50	3.05	1.35	0.03	0.26	0.00	3.46	4.7	8.2	58	0
Glória do Goitá – state Pernambuco												
0-20 cm	25	7.00	2.75	1.75	0.03	0.26	0.00	1.15	4.8	5.9	81	0
Vitória de Santo Antão – state Pernambuco												
0-20 cm	9	4.90	2.00	1.75	0.10	0.27	0.35	7.07	4.1	11.5	36	8

CEC = cation exchange excel.

Table 4. Spore density (g) and root colonization rate (%) in soil samples collected in annatto fields (*Bixa orellana* L.), in municipalities of Lagoa de Itaenga, Glória do Goitá and Vitória de Santo Antão – state Pernambuco.

Cultivars	Spore density (50 g soil)	Root colonization rate (%)
Lagoa de Itaenga – state Pernambuco		
Red Piave	171.50a	75.00a
Green Piave	157.50a	57.50ab
Red Peruvian Paulista	143.75a	52.50ab
Green Peruvian Paulista	125.50a	47.50b
CV (%)	17.99	19.39
Glória do Goitá – state Pernambuco		
Red Piave	244.50a	65.00a
Green Piave	227.75a	60.00a
Red Peruvian Paulista	222.00a	77.50a
Green Peruvian Paulista	240.50a	80.00a
CV (%)	9.54	15.16
Vitória de Santo Antão – state Pernambuco		
Red Piave	160.25a	72.50a
Green Piave	182.75a	72.50a
Red Peruvian Paulista	200.00a	52.50ab
Green Peruvian Paulista	178.00a	47.50b
CV (%)	19.07	15.63

Means followed by the same letter do not differ statistically from each other by Tukey's test ($p < 0.05$).

Effect of AMF inoculation on annatto cultivars

The Red Peruvian Paulista cultivar inoculated with *S. heterogama, C. etunicatum,* and native fungi presented higher values ($p > 0.05$) in all biometric parameters measured than the control (Red Peruvian without inoculation) after 150 days of cultivation (Table 5).

Means followed by the same letter do not differ statistically from each other by Tukey's test ($p < 0.05$).

The Peruvian cultivar inoculated with *S. heterogama* alone also had higher values than the control, except for total N content of shoots. Upon inoculation with *C. etunicatum,* this cultivar had larger stem diameter than the control. The cultivar Red Piave presented superior characteristics in all variables studied when inoculated with *S. heterogama* species alone or in association with *C. etunicatum* + native fungi compared with the control (Red Piave without inoculation). Furthermore, when inoculated with *C. etunicatum,* Red Piave grew higher and showed larger stem diameters than the control.

AMF inoculation promoted higher N accumulation in both the shoots and roots. In contrast, uninoculated plants showed lower N accumulation. Interestingly, higher N accumulation were obtained following inoculation of *S. heterogama* associated to *S. heterogama* + *C. etunicatum* + native fungi (Table 5; Cobb, Wilson, Goad, & Grusak, 2018).

Table 5. Plant height, stem diameter, shoot and roots dry biomass, and total N content of the shoot and roots of annatto plant (*Bixa orellana* L.) cultivars inoculated with AMF and grown for 150 days in a greenhouse.

Cultivars	Plant height (cm)	Diameter of stem (mm)	Shoot dry biomass (g)	Roots dry biomass (g)	Total N in shoot (g)	Total N in roots (g)
Peruvian (*S. heterogama*)[a]	47.70ab	9.30a	17.80abcd	9.11ab	0.80abcdef	0.80abcd
Peruvian (*C etunicatum*)[b]	31.70abcde	7.20abc	7.78cdefg	2.73de	0.90abcd	0.35cdef
Peruvian (natives AMFs)	16.70cde	2.80de	1.55g	0.80e	0.07fg	0.12ef
Peruvian (*S. heterogama* + *C. etunicatum* + natives)	45.50abc	8.60ab	16.69abcde	9.72ab	1.17abc	0.77abcd
Peruvian (control)	9.00e	1.70e	0.15g	0.18e	0.08fg	0.03f
Piave (*S. heterogama*)	55.80a	8.80a	17.97abc	7.30abcd	1.39a	0.72abcd
Piave (*C. etunicatum*)	47.70ab	6.00abcd	8.26bcdefg	3.38cde	0.53bcdefg	0.44bcdef
Piave (natives AMFs)	30.20abcde	3.90cde	4.49fg	1.84de	0.31defg	0.25def
Piave (*S. heterogama* + *C. etunicatum* +natives AMFs)	59.20a	9.40a	20.48a	12.39a	1.17abc	1.07a
Piave (control)	11.20de	1.50e	0.220g	0.243e	0.01g	0.03f
Embrapa 36 (*S. heterogama*)	53.00a	8.70ab	17.90abcd	8.40abc	0.93abcd	0.68abcde
Embrapa 36 (*C. etunicatum*)	57.70a	7.50abc	13.16abcdef	4.50bcde	0.76abcdef	0.34cdef
Embrapa 36 + natives AMFs	35.70abcde	5.10bcde	6.86efg	2.92cde	0.34defg	0.29cdef
Embrapa 36 + (*S. heterogama* + *C. etunicatum*. + natives AMFs)	50.20a	9.50a	18.15 abc	10.21a	0.91abcd	0.86abc
Embrapa 36 (control)	10.80de	1.07e	0.26g	0.14e	0.01g	0.03f
Embrapa 37 (*S. heterogama*)	42.50abc	8.70a	16.38abcde	8.39abc	0.84abcde	0.77abcd
Embrapa 37 (*C. etunicatum*)	40.00abcd	6.00abcd	7.23defg	2.75cde	0.43cdefg	0.25def
Embrapa 37 (natives AMFs)	19.00bcde	2.60de	1.81g	0.59e	0.11efg	0.07f
Embrapa 37 + (*S. heterogama*+*C. etunicatum* + natives AMFs)	49.50a	8.00ab	18.64ab	9.35ab	1.19ab	0.97ab
Embrapa 37 (control)	9.30e	1.60e	0.10g	0.08e	0.010g	0.02f
CV (%)	26.90	19.70		15.62	30.21	23.00

[a]*S. heterogama=AMF Scutellospora heterogama;* [b]*C. etunicatum=AMF Claroideoglomus etunicatum*

The cultivar Embrapa-36 had superior growth compared to the control (uninoculated Embrapa-36) when inoculated with *S. heterogama* species alone or combined with *C. etunicatum* + native AMF for all evaluated variables (Table 5). The cultivar Embrapa-37 had superior growth than the control (uninoculated Embrapa-36) when inoculated with species *S. heterogama* alone or combined with *C. etunicatum* + native AMF for all evaluated variables. When inoculated with *C. etunicatum* alone, they had higher values than the control regarding plant height and diameter of the plant stem.

We found a greater root system volume when root production increased in inoculated plants. The shoot and root dry biomass of plants inoculated either with *S. heterogama* or *C. etunicatum* was also significantly different from that of uninoculated plants and from that of plants inoculated with native AMF (Table 5). Although AMF did not cause any visual morphological alterations in the root system, they can alter the physiology, root architecture, growth, and longevity. Moreover, a previous study has reported that AMF increases the number and length of secondary roots (Motta et al., 2017).

Inoculation with *S. heterogama* alone or with *C. etunicatum* and native AMF resulted in higher spore density in the soil than in that surrounding control plants. Meanwhile, that of *S. heterogama* + *C. etunicatum* + native AMF, as well as that of *S. heterogama* alone, showed significantly more extensive root colonization (Table 6). In the control group, no spores were detected in the soil, and roots showed no sign of infection. These results demonstrated the efficiency of soil sterilization for these treatments.

Among the AMF that provided better growth to annatto cultivars in this research, genus *Scutellospora* can be highlighted, because there was an increase in biometric parameters in its presence. Studies have demonstrated that AMF may improve the supply of nutrients and water, induce tolerance to environmental stress, resistance to root diseases, and can increase tolerance to drought and salt stress (Baum, El-Tohamy, & Gruda, 2015).

Inoculation with *S. heterogama* and *C. etunicatum*, either alone or in combination, was beneficial for annatto plant yield. Regarding height, the inoculated Piave plants had 436% higher yield than those without inoculation after 150 days.

Table 6. Spore density (50 g soil^{-1}) and Root colonization rate (%) of annatto plant (*Bixa orellana L.*) cultivars inoculated with AMF, after 150 days of cultivation in a greenhouse.

Cultivars	Spore density (50 g soil^{-1})	Root colonization rate (%)
Peruvian (*S. heterogama*)[a]	15.66cd	100.00a
Peruvian (*C. etunicatum*)[b]	15.00cd	60.00bc
Peruvian (natives)	18.33bcd	19.000d
Peruvian (*S heterogama* + *C etunicatum* + natives AMF)	32.66abcd	100.00a
Control	00.00	00.00
Piave (*S. heterogama*)	8.66d	53.33cd
Piave (*C. etunicatum*)	63.33a	36.66cd
Piave (natives AMF)	25.33abcd	23.33cd
Piave (*S. heterogama* + *C. etunicatum* + natives AMF)	31.66abcd	100.00a
Control	00.00	00.00
Embrapa 36 (*S. heterogama*)	7.66d	96.66ab
Embrapa 36 (*C. etunicatum*)	32.33abcd	57.33cd
Embrapa 36 (natives)	27.66abcd	49.00cd
Embrapa 36 (*S. heterogama* + *C. etunicatum* + natives AMF)	56.00abc	96.00ab
Control	00.00	00.00
Embrapa 37 (*S. heterogama*)	15.66cd	100.00a
Embrapa 37 (*C. etunicatum*)	58.66ab	42.66cd
Embrapa 37 (natives AMF)	11.00d	44.66cd
Embrapa 37 (*S. heterogama* + *C. etunicatum* + natives AMF)	33.33abcd	100.00a
Control	00.00	00.00
CV (%)	14.23	18.83

[a]*S. heterogama = AMF Scutellospora heterogama;* [b]*C. etunicatum = AMF Claroideoglomus etunicatum*. Means followed by the same letter do not differ statistically from each other by Tukey's test ($p < 0.05$).

According to Walder and Heijden (2015), several factors, such as environmental conditions and functional diversity, can affect nutrient exchange between fungi and plants. The experimental conditions used in this work may have been favorable for *S. heterogama* or combined with *C. etunicatum* and native AMF species, which significantly improved phosphorus uptake compared to the control (no inoculation). Moreover, native AMF may differ in terms of regulation of genes involved in phosphorus uptake.

Stem diameter increased following inoculation with *S. heterogama*. Similarly, Barbieri et al. (2011) found that annatto seedlings inoculated with *C. etunicatum* developed thicker stems than those of plants grown in the presence of phosphorous 4,200 $g \cdot m^{-3}$.

The cultivar with the best spore density was Piave inoculated with *C. etunicatum* (Table 6). However, similar results were obtained with Peruvian, Emgrapa-36 and Embrapa-37 inoculated with *S. heterogama* + *C. etunicatum* + native as well as Piave and Embrapa-36 associated with AMF native and Piave, Embrapa-36 and Embrapa-37 inoculated with *C. etunicatum.* The ability to sporulate is considered an important criterion for selection of AMF, as sporulation is connected to the persistence of the AMF after inoculation.

Analysis of the percentage of mycorrhizal colonization showed that the inoculated AMF colonized the interior of the annatto roots, where the cultivars of Peruana, Piave, Embrapa-36 and Embrapa-37 generally obtained a high percentage of colonization when inoculated with *S. heterogama* and with *S. heterogama* + *C. etunicatum* + natives. Although AMF *C. etunicatum* and native AMF colonized the roots of the plants, they presented a lower percentage of colonization (Table 6).

Some studies indicate positive effects on plant development with AMF inoculation of several species including *Oryza sativa* (Bernaola et al., 2018), *Carica papaya* L. (Lima, Martins, Freitas, & Olivares, 2011), *Mimosa tenuiflora* (Pedone-Bomfim et al., 2018), *Poncirus trifoliata* L. Raf. (Wang, Srivastava, Wu, & Fokom, 2014), *Jacaranda mimosifolia* D. Don (Zaouchi, Bahri, Rezgui, & Bettaieb, 2013), *Prunus armeniaca* (Dutt, Sharma, & Kumar, 2013) and *Olea europaea* (Meddad-Hamza et al., 2017).

Several agricultural crops and various Brazilian forest and fruit crops respond positively to inoculation with AMF. However, despite its biotechnological and ecological potential, mycorrhizal symbiosis still has several gaps to be explored in response to different growth-promoting fungi (Vergara et al., 2019).

Conclusion

Annatto Red Piave had a better associate with AMF, in relation to the Peruvian, both in the municipality of Lagoa de Itaenga and Vitoria de Santo Antão.

The highest percentage of mycorrhizal colonization was obtained by inoculating with *S. heterogama* in combination natives + *S. heterogama* + *C. etunicatum.*

Larger quantities of nitrogen were obtained from plants inoculated with AMF *S. heterogama* in isolation and in combination natives + *S. heterogama* + *C. etunicatum*.

AMF inoculation was beneficial and effective in promoting the growth of annatto plants, and inoculation was more suitable for the species *S. heterogama* and *C. etunicatum*.

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