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Article

Arbuscular mycorrhizal fungi inoculation for coffee seedling production with commercial and conventional substrates

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ABSTRACT: Coffee seedlings are commonly produced on substrate composed of a mixture of soil and cattle manure, supplemented with chemical fertilizers. Alternatives to reduce production costs and produce seedlings of greater quality and health include the use of commercial organic substrates, which require less handling. The use of beneficial microorganisms such as arbuscular mycorrhizal fungi (AMF) can be considered a good alternative for production of more vigorous coffee seedlings. The main goal of this study was to evaluate the effect of the inoculation of AMF isolates on coffee seedlings development in a commercial organic substrate (based on coconut fiber) and conventional substrate (mixture composed of soil and cattle manure compost). Ten AMF were tested: *Rhizophagus irregularis*, *Glomus macrocarpum*, *Claroideoglomus etunicatum*, *Rhizophagus clarus*, *Glomus* spp., *Gigaspora margarita*, *Acaulospora morrowiae*, *Acaulospora scrobiculata*, *Acaulospora* spp., and *Dentiscutata heterogamma*. Plant growth, shoot P content, mycorrhizal colonization, extraradical mycelium length, phosphatase activity, and photosynthetic pigments were evaluated. The effects of mycorrhization depended on both the inoculated fungal species and the substrate for seedling cultivation. Inoculation of *G. margarita*, *Acaulospora* spp., and *Glomus* spp. in the conventional substrate conferred the best growth plant responses, increasing shoot biomass by 160 to 320%. In the commercial substrate, the most efficient AMF were *R. clarus*, *Glomus* spp, *A. morrowiae* and *A. scrobiculata*, with up to 149% of shoot biomass increase. The commercial organic substrate and the inoculation of some of the AMF isolates were highly beneficial to coffee seedlings development and can replace the use of the conventional substrate. These results open new opportunities for the use of AMF as an inoculant to improve coffee seedling production in commercial organic substrates.

Key words: *Coffea arabica*, arbuscular mycorrhiza, external mycelium, photosynthetic pigments, P nutrition.

INTRODUCTION

Coffee is one of the most traded commodities in the world and an important source of income for many developing countries in Asia, Africa, and Latin America (Andrade et al. 2009), and Brazil is the largest producer and exporter of coffee in the world (Conab 2021). The formation of coffee trees for agricultural purposes implies the use of high-quality seedlings, with good vegetative growth and vigor. The production of coffee seedlings is an important step for the development and longevity of the crop, and the seedlings are produced in commercial nurseries with substrates (Vallone et al. 2010).

The common substrate used for coffee seedling production is composed of soil and bovine manure compost (70/30, v/v) with chemical fertilizers that allows for adequate seedling growth. However, over the past few years, many coffee producers have adopted the use of commercial substrates (without soil and cattle manure), in an attempt to increase seedlings quality.

Nowadays, there is a perspective that the use of commercial organic substrates will be mandatory for the production of coffee seedlings. The main advantage of using commercial substrates is the increase of seedlings sanitation since they are less likely to be infected by soil-borne phytopathogens and nematodes (Andrade et al. 2009). The commercial substrate based on coconut fiber is a substrate of increasing economic importance, as Brazil is a major producer of green coconut; the use of this fiber is a promising alternative for more sustainable crop management due to its high-water retention capacity and nutrient availability (Brigida et al. 2009). The coffee market plays an essential role in Brazilian agribusiness (Carvalho et al. 2017).

Several biological products, the so-called biostimulants or biofertilizers, are composed of substances or microorganisms that improve overall plant performance and productivity (Díaz et al. 2016, Valverde-Lucio et al. 2020). The use of inoculants with plant growth-promoting microorganisms is an available alternative for nursery production of commercial species such as coffee, because currently used substrates commonly lack an efficient community of beneficial microbes (Vallejos-Torres et al. 2021). The use of microbial biofertilizers is an environmentally friendly alternative to the continued input of mineral fertilizers. Microbes with potential to be used as biofertilizers can promote coffee growth by improving plant mineral nutrition and phytohormones production or by inhibiting plant pathogens and pests (Vallejos-Torres et al. 2021). Plant growth-promoting bacteria and arbuscular mycorrhizal fungi (AMF) are the most promising microorganisms for sustainable agriculture (Cipriano et al. 2021, Rossetto et al. 2021). AMF can enhance the uptake of relatively immobile nutrients, particularly phosphorus (P), but also nitrogen (N) and other nutrients, from the soil to the host plant via the extraradical mycelium (ERM) (Bennett and Groten 2022). Besides plant nutrition, other benefits triggered by AMF are better soil aggregation, biogeochemical cycles optimization and increased plant tolerance to biotic and abiotic stresses (Giovannini et al. 2020).

Different systems such as forestry, conventional and agroecological coffee cultivation have shown a wide diversity of AMF in Brazilian soils, such as *Acaulospora* sp., *Acaulospora morrowiae*, *Acaulospora scrobiculata*, *Glomus* spp., *Glomus macrocarpum*, *Acaulospora* spp., *Rhizophagus fasciculatus*, *Gigaspora* sp., among others (Prates Júnior et al. 2019). This AMF diversity opens a range of opportunities to explore the potential of these microorganisms as bioinoculants for seedlings coffee production. In this context, the AMF *Rhizophagus clarus* showed promising potential as inoculant in coffee genotypes, such as Catuaí IAC 144, H 29-1-8-5 and Catigua MG2, due to improvement in biomass production and better plant P nutrition (Fonseca et al. 2019). *R. clarus* and *Claroideoglomus etunicatum* also provided a greater benefit to coffee plants growth when cultivated in a soil with low phosphate fertilization (Moreira et al. 2019).

The arbuscular mycorrhizal (AM) symbiosis is important for the production of coffee seedlings, as this plant is highly dependent on mycorrhizal colonization, especially in low fertility soils (Lopes et al. 1983, Colozzi-Filho et al. 1994). Low P availability in soils can promote greater mycorrhizal beneficial effects for coffee plants (Saggin-Júnior et al. 1994), but the P concentration in the soil solution should be kept at an intermediate level to guarantee an adequate coffee seedling growth (González-Osório et al. 2022). The supply of commercial substrates to produce coffee seedlings is increasing in the market, but substrates are usually poor in nutrients, and additional fertilization is required, which increases production costs. Some substrates do not provide all the nutrients for proper plant development, which can influence seedling quality (Andrade et al. 2021).

In this respect, the use of efficient AMF inoculum with organic commercial substrates is an alternative to improve the efficient use of fertilizers and decrease the amount needed to ensure the proper development of coffee seedlings (Tristão et al. 2006). The promotion of coffee plant growth by AMF inoculation has been mainly attributed to the nutritional effects of this symbiosis, especially in relation to P (Siqueira and Saggin-Júnior 2001, Souza et al. 2017). However, studies evaluating the effects of AMF inoculation on plants grown with commercial substrates are scant, especially in Brazil, where this technological innovation is more recent. Moreover, although mycorrhizal fungi are found naturally in coffee plantations (Posada et al. 2018), the use of efficient mycorrhizal inoculum in nurseries could be a promising technology to produce healthy and vigorous coffee seedlings, increasing survival after transplanting to the field.

Overall, there is little evidence on the influence of AMF inoculation on coffee seedling performance under the recent guidelines recommended to producers that promote the use of organic soil-free substrates. This study aimed to evaluate different AMF isolates effects on growth and P nutrition of coffee seedlings in a coconut-fiber based commercial organic substrate, as an eco-friendlier alternative to improve coffee seedlings production.

MATERIAL AND METHODS

Arbuscular mycorrhizal fungi

The ten strains of AMF tested were:

- IAC-FM43 (*Rhizophagus irregular*);
- IAC-FM50 (*Glomus macrocarpum*);
- IAC-FM44 (*Claroideoglomus etunicatum*);
- IAC-FM16 (*Rhizophagus clarus*);
- IAC-FM28 (*Glomus* spp.);
- IAC-FM1 (*Gigaspora margarita*);
- IAC-FM14 (*Acaulospora morrowiae*);
- IAC-FM10 (*Acaulospora scrobiculata*);
- IAC-FM13 (*Acaulospora* spp.);
- IAC-FM2 (*Dentiscutata heterogamma*).

They belong to the collection of Beneficial Microorganisms, Instituto Agronômico (IAC), Campinas, SP, Brazil.

Root fragments of *Brachiaria decumbens* plants colonized by each AMF and sand-soil mixture with AMF propagules were used as inoculum, resulting in approximately 500 spores per pot. A non-mycorrhizal inoculation treatment was maintained as control. To standardize microbiota associated with AMF inoculum, the non-mycorrhizal inoculation treatment received washings of the soil-inoculum mixture filtered through Whatman no. 42 filter paper.

Greenhouse experiment: coffee seedlings

The experiment was conducted under greenhouse conditions. The temperature ranged from 15 to 30°C, and relative humidity from 50 to 80%, measured using a digital thermo-hygrometer placed inside the greenhouse, in the Central Experimental Center, IAC, in Campinas. The experiment was performed as a 2 × 11 factorial, consisting of two substrates and the inoculation or not of 10 AMF species, in a completely randomized design, with five replications, totaling 110 experimental plots, with each plot composed by one plant. Two substrates were tested: a mixture composed of 70% soil (Oxisol) and 30% cattle manure compost, named "conventional" substrate; and an organic commercial substrate based exclusively on coconut fiber, named "commercial".

The fertility analysis of the conventional substrate showed pH in $CaCl_2$ – 5.6; P – 405, and K – 1,142 $mg \cdot dm^{-3}$; Ca – 5.8, and Mg – 3.1 $mmol_c \cdot dm^{-3}$; B – 0.5, Cu – 6.4, and Fe – 58 $mg \cdot dm^{-3}$; organic matter – 48 $g \cdot dm^{-3}$, and the commercial organic substrate showed pH in water – 4.5; P – 84, and K – 459 $mg \cdot dm^{-3}$; Ca – 0.3, and Mg – 0.1 $mmol_c \cdot dm^{-3}$; B – 0.9, Cu – 0.1, and Fe – 0.7 $mg \cdot dm^{-3}$; organic carbon – 388 $g \cdot kg^{-1}$; and C/N – 55, performed according to Sonneveld et al. (1974).

The conventional substrate used an unsterilized soil sample, with propagules of unknown native AMF species, and this approach was performed in order to reproduce the on-farm conditions carried out by Brazilian coffee seedlings producers. Seeds of *Coffea arabica* var. Catuaí amarelo IAC-62 were germinated in vermiculite and grown until the seedlings developed cotyledonary leaves, and then were transplanted to 3-L pots with the respective substrate. Irrigation was based on the water retention capacity, which was maintained at 60%, previously determined for each substrate. During the experiment, 150 mg of N were added per pot as fertigation once a week. At 175 days after transplanting, photosynthetic pigment contents and phosphatase activity in leaves were determined.

For the determination of photosynthetic pigments, 50 mg of foliar tissue from the second and third pairs from the orthotropic branch were collected. Chlorophyll (Chl) *a* and *b* and carotenoids were extracted with 100% dimethyl sulfoxide and measured spectrophotometrically (Hitachi U-2000) directly in the extracts (Hiscox and Israelstam 1979); the results were expressed in $\mu g \cdot mL^{-1}$ of extract. Acid phosphatase (APase) (EC3.1.3.2) activity in leaves was determined "in vivo"

(Besford 1979). A foliar sample of 0.1 g of fresh tissue was taken from the second and third pairs from the orthotropic branch and incubated with p-nitrophenyl phosphate in 0.1 $mol \cdot L^{-1}$ sodium acetate buffer (pH 4) for 20 min at 30°C. The p-nitrophenol (p-NP) formed was measured spectrophotometrically at 410 nm. The activity of APase was expressed in μg p-NP $g^{-1} \cdot h^{-1}$ of fresh weight (FW).

Before harvest, 200 days after transplantation, the following growth parameters were evaluated: plant height, stem diameter, and leaf number. Shoot and root were harvested and separated, washed, and dried at 65°C. Shoot was weighed and ground for determination of phosphorus concentration by inductively plasma optical emission spectrophotometry (ICP-OES) (JobinYvon, JY50P Longjumeau, France), after nitric-perchloric acid digestion. The symbiotic efficiency of different AMF species was determined through the difference in biomass production, in percentage, between the AMF inoculated and the non-inoculated plants. Root replicates were thoroughly washed in running water, dried in absorbing paper, and weighed to determine root fresh weigh.

About 1 gram of thin roots was collected for mycorrhizal colonization determination. Mycorrhizal colonization was evaluated by the root slide method (Giovannetti and Mosse 1980) by first clearing the roots with KOH and staining with trypan blue (Phillips and Hayman 1970). The extraction of external AMF mycelium, from 5 g of the substrate suspended in 1,500 mL of water, and the determination of total external mycelium length were performed according to Melloni and Cardoso (1999).

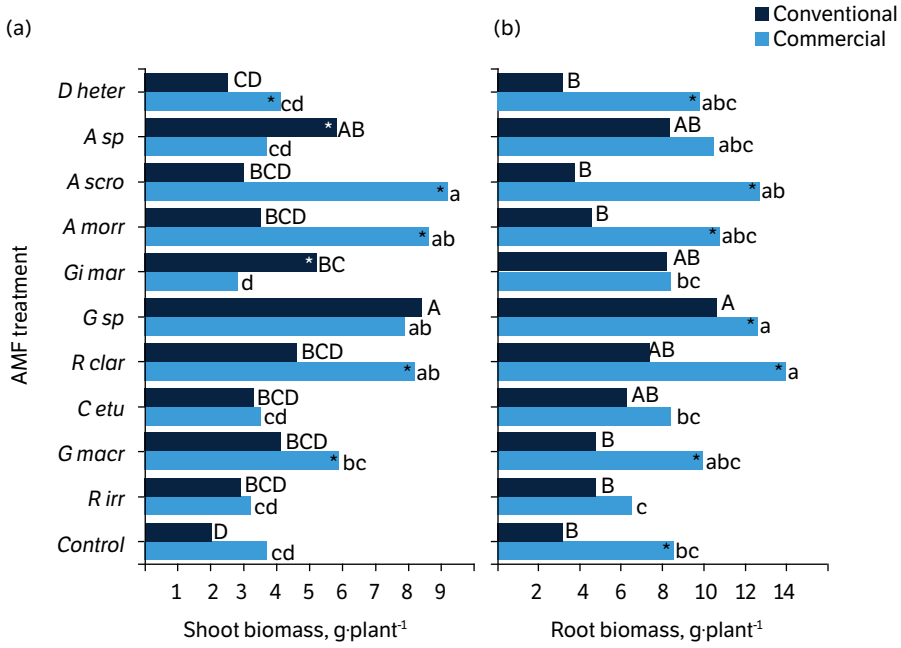
Data were submitted to analysis of variance (two-way ANOVA) to test the main effects of AMF inoculation, substrate, and their interaction. Tukey test at 5% significance was used for comparison of means, using the software SISVAR (Ferreira 2011). Data expressed as percentage were arcsine-square-root transformed, and the data relative to counts were log x + 1 transformed before the statistical analyses. Multivariate analysis was performed by principal component analysis (PCA) and Pearson's correlation using Minitab Statistical version 17.1.0.

RESULTS

Growth parameters were significantly influenced by the growth-substrate for coffee seedling cultivation and also by the AMF species used as inocula (Fig. 1 and Table 1). Plants grown in the commercial substrate showed the highest shoot biomass production, stem diameter, plant height and number of leaves when A scro, A morr, R clar and G sp were inoculated, with up to a 149% of increase in shoot biomass compared to the control (Fig. 1A and Table 1). With the conventional substrate, the inoculation of Gi mar, A sp and G sp increased seedling shoot mass, stem diameter, plant height and number of leaves, with increases of 160, 190 and 320% in shoot biomass, respectively (Fig. 1A and Table 1). Greater root biomass was observed in plants colonized by R clar, G sp and A scro in the commercial substrate (Fig. 1B) with increases up to 68%. Plants cultivated in the conventional substrate and with G sp inoculation showed higher root growth (Fig. 1B), increasing root mass by 242%. In general, with the commercial substrate, root biomass was 77% greater than with the conventional substrate (Fig. 1).

The mycorrhizal colonization of coffee roots ranged from 17 to 60% in plants grown in the conventional substrate, and the highest rates were obtained with A morr and G mac inoculation (Fig. 2A). However, in the commercial substrate, the mycorrhizal colonization was relatively low, from 4 to 7%, and significantly lower than in the conventional substrate (Fig. 2A).

In the commercial substrate, the different AMF isolates did not show a significant difference in the length of total ERM (Fig. 2B), but they differed from the control treatment, which did not show intraradical colonization or extraradical mycelium (Figs. 2A and 2B). In the conventional substrate, the C etu isolate showed the longest ERM length compared to the control treatment. The length of total ERM did not differ between substrates, except for the D heter and the control (without AMF inoculation), which showed higher ERM in the conventional substrate (Figs. 2A and 2B).



*Significant difference between substrates within each arbuscular mycorrhizal fungi (AMF) inoculation treatment, capital letters compare means of AMF inoculation in the "conventional" substrate and small letters compare means of AMF inoculation in the "commercial" substrate, by the Tukey test (P < 0.05); Control: without AMF inoculation; *R irr: Rhizophagus irregularis; Gi mar: Gigaspora margarita; C etu: Claroideoglomus etunicatum; A morr: Acaulospora morrowiae; R clar: Rhizophagus clarus; A scro: Acaulospora scrobiculata; G sp: Glomus sp.; D heter: Dentiscutata heterogama; G macr: Glomus macrocarpum; A sp: Acaulospora sp.*

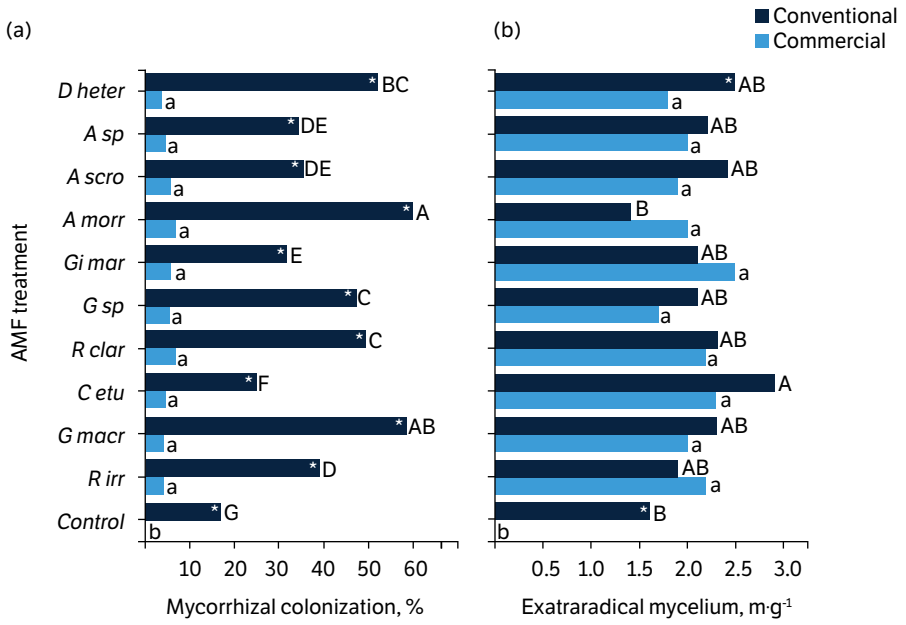
Figure 1. (a) Shoot and (b) root biomass production of *Coffea arabica* seedlings under the influence of different arbuscular mycorrhizal fungi isolates and grown in conventional and commercial substrates.

Table 1. Stem diameter, height and number of leaves in *Coffea arabica* seedlings, 200 days after transplanting, under the influence of different arbuscular mycorrhizal fungi inocula and grown in conventional and commercial substrates.

Substrate	Arbuscular Mycorrhizal Fungi										
	Control	***R irr***	***G macr***	***C etu***	***R clar***	***G sp***	***Gi mar***	***A morr***	***A scro***	***A sp***	***D heter***
Stem diameter (mm)											
Commercial	2.9 d	3.1 d	3.5 bcd *	3.1 d	4.3 a *	4.0 ab *	3.2 cd	3.9 abc *	3.9 abc *	3.6 bcd	3.3 bcd *
Conventional	2.5 C	2.7 BC	3.0 ABC	2.7 BC	3.1 ABC	3.4 AB	3.2 ABC	2.9 ABC	2.7 BC	3.5 A	2.6 C
Height (cm)											
Commercial	23.8 e *	24.2 e	27.9 bcd	27.3 bcd *	34.3 a *	34.1 ab *	25.6 de	32.7 abc *	33.0 abc *	27.0 bcd	26.0 cde *
Conventional	15.7 D	20.2 BCD	24.2 ABC	20.5 BCD	26.4 AB	24.0 ABC	25.9 ABC	21.3 BCD	19.4 BCD	27.5 A	18.7 CD
Leaf number											
Commercial	18.8 bc	18.8 bc	26.0 ab *	18.8 bc	31.6 a *	31.6 a *	17.2 c	31.6 a *	31.2 a *	18.4 bc	22.8 bc *
Conventional	14.4 B	16.8 AB	18.4 AB	16.8 AB	21.6 AB	23.6 A	22.8 A *	18.8 AB	18.0 AB	22.0 AB	16.4 AB

*Significant difference between substrates within each arbuscular mycorrhizal fungi (AMF) inoculation treatment, capital letters compare means of AMF inoculation in the "conventional" substrate and small letters compare means of AMF inoculation in the "commercial" substrate, by the Tukey test (P < 0.05); Control: without AMF inoculation; *R irr: Rhizophagus irregularis; Gi mar: Gigaspora margarita; C etu: Claroideoglomus etunicatum; A morr: Acaulospora morrowiae; R clar: Rhizophagus clarus; A scro: Acaulospora scrobiculata; G sp: Glomus sp.; D heter: Dentiscutata heterogama; G macr: Glomus macrocarpum; A sp: Acaulospora sp.*

Regarding photosynthetic pigments of coffee leaves, the inoculation of AMF isolates did not influence the contents of chlorophyll *a*, *b*, *a+b* and carotenoids in seedlings grown on the commercial substrate (Table 2). However, in the conventional substrate, the inoculation of R clar, Gi mar and A sp promoted higher levels of chlorophyll *a*, chlorophyll *a+b* and carotenoids than the non-inoculation treatment (Table 2).



*Significant difference between substrates within each AMF inoculation treatment, capital letters compare means of AMF inoculation in the "conventional" substrate and small letters compare means of AMF inoculation in the "commercial" substrate, by the Tukey test (P < 0.05); Control: without AMF inoculation; *R irr: Rhizophagus irregularis; Gi mar: Gigaspora margarita; C etu: Claroideoglomus etunicatum; A morr: Acaulospora morrowiae; R clar: Rhizophagus clarus; A scro: Acaulospora scrobiculata; G sp: Glomus sp.; D heter: Dentiscutata heterogama; G macr: Glomus macrocarpum; A sp: Acaulospora sp.*

Figure 2. (a) Mycorrhizal root colonization and (b) total length of arbuscular mycorrhizal fungi (AMF) extraradical mycelium of *Coffea arabica* seedlings under the influence of different arbuscular mycorrhizal fungi isolates and grown in conventional and commercial substrates.

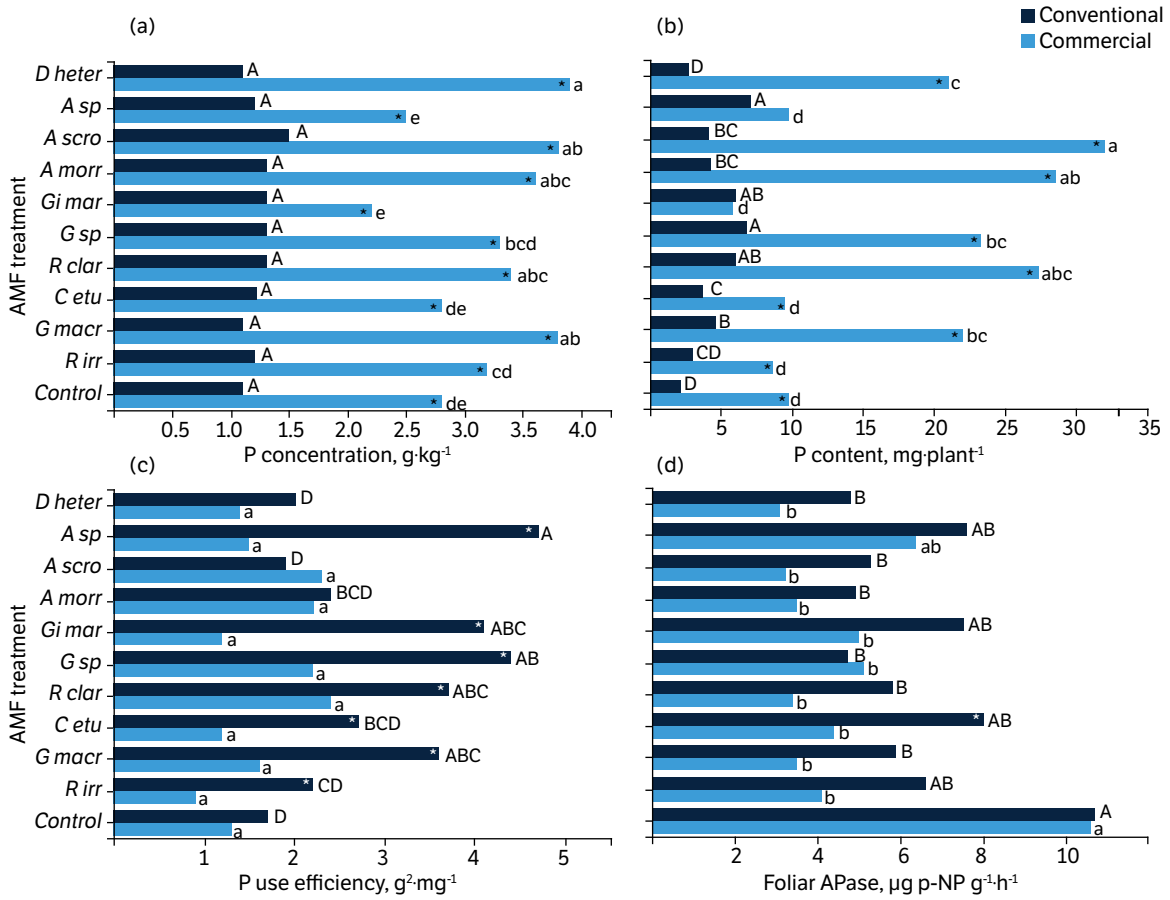
Table 2. Contents of photosynthetic pigments in leaves of *Coffea arabica* seedlings inoculated with different arbuscular mycorrhizal fungi isolates and uninoculated controls, growing in different substrates.

Substrate[1]	Arbuscular Mycorrhizal Fungi[2]										
	Control	*R irr*	*G macr*	*C etu*	*R clar*	*G sp*	*Gi mar*	*A morr*	*A scro*	*A sp*	*D heter*
Chlorophyll *a* (mg·mL^{-1}plant extract)											
Commercial	13.3 ab	13.3 ab	15.1 ab	13.1 ab	17.2 a	16.5 ab	10.6 b	16.8 a	14.9 ab	11.7 ab	14.4 ab
Conventional	13.4 B	18.8 AB *	18.9 AB *	17.1 AB *	20.1 A	18.1 AB	20.0 A *	17.3 AB	18.2 AB	20.9 A *	18.1 AB *
Chlorophyll b (mg·mL^{-1}plantextract)											
Commercial	3.8 a	3.8 a	3.6 a	3.9 a	4.4 a	4.3 a	3.5 a	3.9 a	3.4 a	3.2 a	3.3 a
Conventional	3.3 A	4.5 A	4.1 A	4.2 A	4.4 A	3.7 A	4.4 A	4.1 A	3.8 A	4.5 A	3.8 A
Chlorophyll *a*+*b* (mg·mL^{-1} plant extract)											
Commercial	17.1 a	17.1 a	18.6 a	17.0 a	21.6 a	20.8 a	14.1 a	20.8 a	18.3 a	15.0 a	17.7 a
Conventional	16.7 B	23.3 AB *	23.0 AB	21.2 AB	24.5 A	21.8 AB	24.4 AB *	21.4 AB	22.0 AB	25.5 A *	21.9 AB
Carotenoids (mg·mL^{-1} plant extract)											
Commercial	3.7 a	3.7 a	4.2 a	3.7 a	4.4 a	4.4 a	2.9 a	4.5 a	4.1 a	3.4 a	4.0 a
Conventional	3.8 B	4.9 AB *	4.9 AB	4.9 AB *	5.1 AB	4.7 AB	5.4 A *	4.5 AB	4.7 AB	5.5 A *	4.7 AB

*Significant difference between substrates within each arbuscular mycorrhizal fungi (AMF) inoculation treatment, capital letters compare means of AMF inoculation in the "conventional" substrate and small letters compare means of AMF inoculation in the "commercial" substrate, by the Tukey test (P < 0.05); Control: without AMF inoculation; *R irr: Rhizophagus irregularis; Gi mar: Gigaspora margarita; C etu: Claroideoglomus etunicatum; A morr: Acaulospora morrowiae; R clar: Rhizophagus clarus; A scro: Acaulospora scrobiculata; G sp: Glomus sp.; D heter: Dentiscutata heterogama; G macr: Glomus macrocarpum; A sp: Acaulospora sp.*

The highest shoot P concentrations and contents were observed in plants grown on the commercial substrate, mainly with G macr, A scrob, A morr and D heter inoculation (Figs. 3A and 3B). Concerning the P use efficiency (PUE) index, it was observed that plants grown on the conventional substrate and with G macr, R clar, G sp, Gi mar and A sp inoculation showed higher index values than control plants and than plants grown on commercial substrate (Fig. 3C). Mycorrhization had no significant effect on PUE index in the commercial substrate (Fig. 3C). In general, the foliar APase activity was

significantly higher in the control plants (without AMF inoculation) grown in both substrates used for coffee seedlings cultivation (Fig. 3D).



*Significant difference between substrates within each arbuscular mycorrhizal fungi (AMF) inoculation treatment, capital letters compare means of AMF inoculation in the "conventional" substrate and small letters compare means of AMF inoculation in the "commercial" substrate, by the Tukey test ($P < 0.05$); Control: without AMF inoculation; *R irr: Rhizophagus irregularis; Gi mar: Gigaspora margarita; C etu: Claroideoglomus etunicatum; A morr: Acaulospora morrowiae; R clar: Rhizophagus clarus; A scro: Acaulospora scrobiculata; G sp: Glomus sp.; D heter: Dentiscutata heterogama; G macr: Glomus macrocarpum; A sp: Acaulospora sp.*

Figure 3. (a) Shoot phosphorus concentration and (b) content, (c) phosphorus use efficiency index and (d) foliar acid phosphatase (APase) activity in *Coffea arabica* seedlings inoculated or not with different arbuscular mycorrhizal fungi isolates and grown in conventional and commercial substrates.

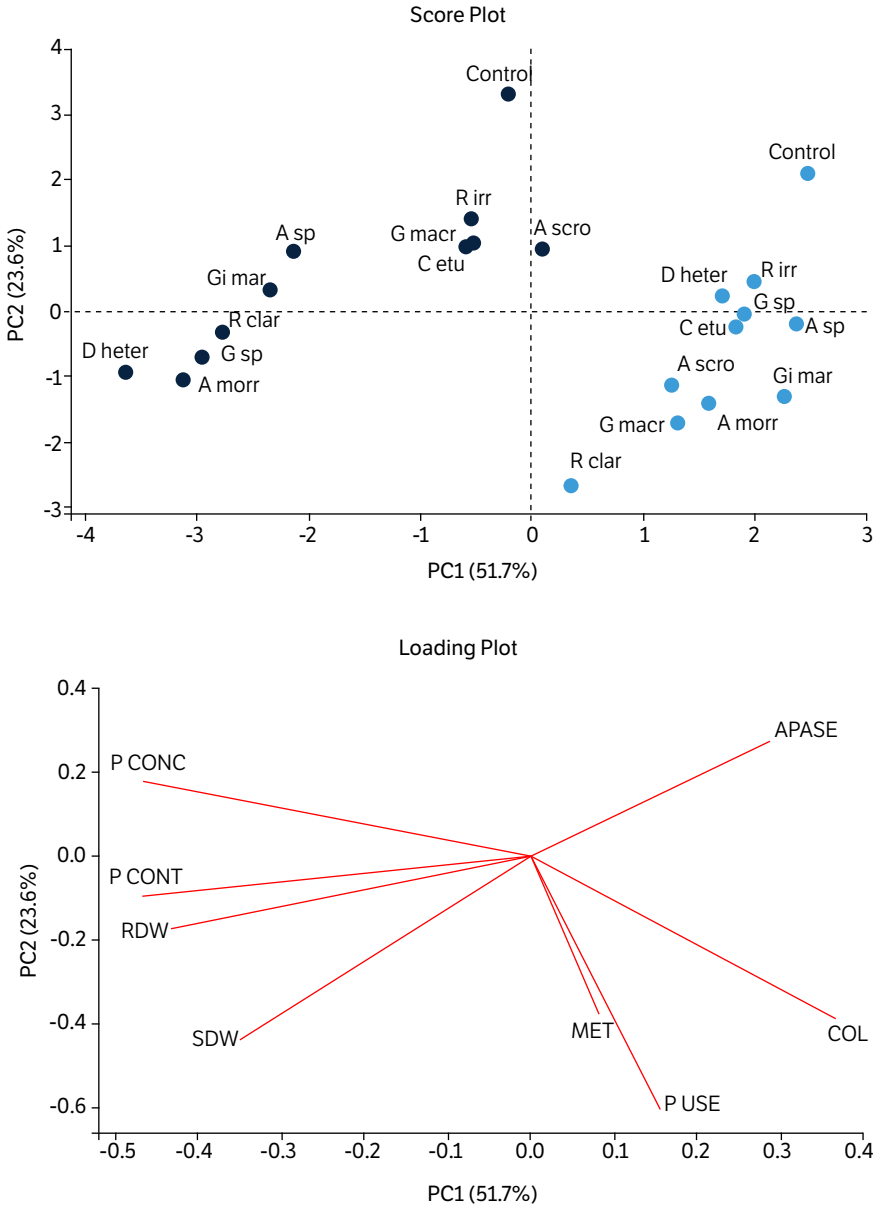
The highest values of symbiotic efficiency in the commercial substrate were observed in plants with A scro, A morr, R clar and G sp inoculation. In the conventional substrate, inoculation of G sp, Gi mar, A sp, R clar and G macr showed higher symbiotic efficiency (Table 3).

Table 3. Symbiotic efficiency of the different arbuscular mycorrhizal fungi isolates inoculated in commercial and conventional substrates for *Coffea arabica* seedling cultivation.

Substrate[1]	*R irr*	*G macr*	*C etu*	*R clar*	*G sp*	*Gi mar*	*A morr*	*A scro*	*A sp*	*D heter*
	Symbiotic Efficiency (%)									
Commercial	-13.5	59.5	-5.4	121.6	113.5	-24.3	132.4	148.6	0.0	10.8
Conventional	45.0	105.0	65.0	130.0	320.0	160.0	75.0	50.0	190.0	25.0

R irr: Rhizophagus irregularis; Gi mar: Gigaspora margarita; C etu: Claroideoglomus etunicatum; A morr: Acaulospora morrowiae; R clar: Rhizophagus clarus; A scro: Acaulospora scrobiculata; G sp: Glomus sp.; D heter: Dentiscutata heterogama; G macr: Glomus macrocarpum; A sp: Acaulospora sp.

The PCA showed that the first (PC1) and second (PC2) components explained 51.7 and 23.6% of the total variation, respectively (Fig. 4). According to the PCA, the substrates used for coffee seedling growth were clearly separated in the PC axes. The separation of commercial and conventional substrates had a great influence on the measured variables. The separation of plants grown in the conventional substrate in the PC axes was mainly determined by mycorrhizal colonization, P use efficiency and the length of ERM, while in the commercial substrate it was mainly influenced by shoot and root biomass production, and shoot P concentration and content. Control treatments in the commercial and conventional substrates were more dispersed than treatments with AMF inoculation (Fig. 4).



Control: without AMF inoculation; *R irr: Rhizophagus irregularis; Gi mar: Gigaspora margarita; C etu: Claroideoglomus etunicatum; A morr: Acaulospora morrowiae; R clar: Rhizophagus clarus; A scro: Acaulospora scrobiculata; G sp: Glomus sp.; D heter: Dentiscutata heterogama; G macr: Glomus macrocarpum; A sp: Acaulospora sp.*; P CONC: P concentration; P CONT: P content; P USE: P use efficiency index; RDW: root biomass; SDW: shoot biomass; COL: mycorrhizal colonization; ERM: extraradical mycelium length; APase: foliar acid phosphatase activity.

Figure 4. (a) Score and loading (b) plots of the first two components (PC1 and PC2) of the principal component analysis for some of the determined variables in *Coffea arabica* seedlings inoculated or not with different arbuscular mycorrhizal fungi isolates and grown in conventional (dark blue dots) and commercial substrates (light blue dots)

DISCUSSION

In general, AMF inoculation increased coffee seedling growth, corroborating the importance of the mycorrhizal association for coffee, which is a species highly dependent on mycorrhizae at the nursery and under field conditions (Lopes et al. 1983, Colozzi-Filho et al. 1994, Andrade et al. 2009).

Both substrates were suitable for coffee seedling production, but the effects were clearly different, as shown by PCA analysis (Fig. 4). The substrates showed great influence on plant mycorrhization and effect on growth and P nutrition. The commercial organic substrate influenced biomass production and P uptake, while the conventional substrate mainly influenced mycorrhization (intra and extraradical mycelium). The AMF inoculation in both substrates caused an evident effect on the plants, clearly standing out from the effect of non-inoculation (Fig. 4).

The growth parameters showed that AMF may be more efficient in promoting plant development depending on the substrate (Fig. 1, Table 1). The type of substrate caused different effects on the development of coffee seedlings, what may be mainly related to differences in the fertility levels of each substrate, concerning the P, K, Ca, Mg, Cu and Fe concentrations. The conventional substrate showed a higher P concentration than the commercial substrate, but in both substrates the P concentration was very high and, even so, did not inhibit the establishment of mycorrhizal association in the seedlings roots and the plant growth promotion, indicating the importance of mycorrhization to coffee seedlings.

This result contrasts with the observation that mutualistic symbiosis in coffee occurs in the range of 10 to 100 $mg{\cdot}kg^{-1}$ of available P in the soil (Saggin-Júnior and Siqueira 1996). Coffee seedlings produced with less phosphate fertilization and inoculated with *R. clarus* and *C. etunicatum* produced more biomass (Moreira et al. 2019). Low soil P concentration (5.3 to 18.4 $mg{\cdot}kg^{-1}$) and pH (5.7 to 6.6) are important characteristics that may favor the presence of specific AMF in the coffee crop (Bertolini et al. 2020). The response of different mycorrhizal fungi regarding P availability in the substrate may be related to differences in their symbiotic efficiency (Bennett and Groten 2022). As already mentioned, there is a relationship between the range of available P concentration in which AMF achieve a mutualistic relationship and its optimal symbiotic efficiency (Smith et al. 2011). The mycorrhization of coffee with *G. margarita* and *Acaulospora* spp. was efficient in promoting seedlings growth in the conventional substrate, which showed a high P concentration, corroborating previous reports in mycorrhizal coffee (Colozzi-Filho and Siqueira 1986, Saggin-Júnior et al. 1994).

In the present study, the seedlings of control plants (without AMF inoculation) produced on commercial substrate showed growth comparable to the control seedlings in the conventional substrate (Fig. 1A; Table 1), pointing out that this substrate can be used to replace conventional coffee seedling production. Commercial substrates based on coconut fiber favor rooting possibly due to their high porosity (Rosa et al. 2001). Coffee seedlings produced with the commercial substrate showed in general greater root biomass production, mainly with *R. clarus* and *Glomus* spp. inoculation (Fig. 1B), which stimulated P uptake, indicated by higher shoot P concentration and content and lower phosphatase activity (Figs. 3A, 3B and 3D), and, consequently, greater shoot growth (Fig. 1A). Seedlings grown in the commercial substrate had better growth especially when *R. clarus*, *Glomus* spp. and *A. scrobiculata* were inoculated, highlighting these isolates as candidates for new inoculants for coffee seedlings.

Tristão et al. (2006) compared different substrates for coffee seedlings production, including the conventional substrate and this coconut fiber-based substrate with *R. irregularis*, *C. etunicatum*, and *G. margarita* inoculation, and concluded that both, substrate and AMF, influenced seedling growth. Regarding mycorrhization, *G. margarita* inoculation, considered an efficient species for coffee growth promotion (Colozzi-Filho and Siqueira 1986), caused higher P use efficiency, greater shoot biomass and symbiotic efficiency, favoring the concentration of photosynthetic pigments in the conventional substrate than in commercial organic substrate, even though the P concentration was higher in the conventional substrate. Moreover, varieties of coffee also can influence the mycorrhization efficiency, and there are more than 100 species of AMF that are known to associate with coffee (Hernández-Acosta et al. 2021), indicating that it may be an effective variety – AMF species interaction to improve P use efficiency, under a range of growing conditions.

A possible explanation for low mycorrhizal colonization rates may be related to high organic matter content and P concentration in the soil (Trindade et al. 2000), what possibly influenced root colonization by AMF in both substrates

(Fig. 2a). Although in both substrates there was high P concentration, especially in the conventional substrate, there was higher colonization rate in plants grown in this substrate (Fig. 2a) and lower mycorrhizal colonization in plants grown in the commercial substrate. As the soil used in the conventional substrate was not disinfected, according to standard in conventional coffee seedling production practices, roots of non-inoculated plants showed the presence of mycorrhizal structures and a 17% colonization rate (Fig. 2a).

However, in this substrate all the AMF inoculated showed positive symbiotic efficiencies, ranging from 25 to 320% (Table 3), indicating that exotic AMF were more effective than native AMF in promoting coffee seedling growth. The length of the total ERM was similar in both substrates (Fig. 2b) and did not correlate to the intraradical colonization rate by AMF ($r = 0.244$, $p = 0.275$), as seedlings on the commercial substrate, with low intraradical colonization, showed similar ERM length to seedlings on the conventional substrate, with relatively higher intraradical colonization (Fig. 2a). This result could be explained by the fact that coffee seedlings produced significantly greater root biomass in the commercial than in the conventional substrate (Fig. 1b). Thus, even with the lower intraradical colonization observed with the commercial substrate, the greater root production could have resulted in similar extraradical mycelium length in both substrates. Organic substrates based on coconut fiber are also known to offer high porosity (Carrijo et al. 2002) and pore size which can positively influence the establishment of extraradical mycelia (Drew et al. 2003).

A significant negative correlation was found between mycorrhizal colonization and shoot P concentration ($r = -0.607$, $p = 0.003$) probably due to the lower mycorrhizal colonization in plants grown on the commercial substrate that showed higher shoot P concentration (Figs. 2a and 3a). However, there was no influence of mycorrhization on shoot P concentration in plants grown on conventional substrate, despite the higher colonization rates. In this study, it is not possible to infer the main route for P uptake, if via mycorrhizal or direct pathway (Smith et al. 2011). Probably the contribution of mycorrhiza to P uptake was higher in the conventional substrate, although shoot P concentrations were higher in seedlings grown in the commercial substrate that also showed high available P concentration. These results confirm the well-known effect that the mycorrhizal colonization may not be related to the fungal ability to absorb P or to the symbiotic efficiency. The activity of the extraradical mycelium can be more related to fungal ability to absorb and translocate P from the substrate to the host (Bennett and Groten 2022, Qin et al. 2022).

Acid phosphatase activity (APase) was negatively correlated to shoot P content ($r = -0.579$, $p = 0.005$), indicating that in leaves of plants with lower P content the APase was increased, in order to mobilize internal P sources (Duff et al. 1994). Although there was a large difference in P availability between the substrates, APase activity was generally not different between plants grown in both substrates, probably because the plants had reached at least the optimal P concentration in the tissues, considered to be between 1.2 and 2 $g \cdot kg^{-1}$ in adult plants (Cantarella et al. 2022). In control plants, without AMF inoculation, APase activity was higher in both substrates than in plants with AMF inoculation, which suggest that the increase in the enzyme activity was more evident in plants with the lowest P contents (Ascencio 1994), mainly in the commercial substrate, evidencing the importance of AMF colonization (Fig. 3).

The contents of photosynthetic pigments may be related to the N concentration in the substrate since this nutrient is the main compound of chlorophyll. As the C/N ratio of the commercial substrate was high and the immobilization of N was likely intense, in spite of the N added by fertigation, there may have been a lack of N to the plants and, consequently, a lower content of photosynthetic pigments in the leaves (Table 3).

It is important to evaluate the effects of different AMF species with different commercial organic substrates in order to expand the range of substrates and AMF inocula for coffee seedling producers. The fertility level and type of the substrates must be carefully considered to ensure the best performance of the mycorrhizal association (Tristão et al. 2006). The inoculation of AMF reduces the use of fertilizers, mainly concerning P. Therefore, it is a promising biotechnological tool for sustainable coffee production, especially because coffee is highly dependent on mycorrhizal association (Andrade et al. 2009). Furthermore, recent evidence has provided new insights into the exchange of nutritional benefits between the symbiotic partners. The great potential for AMF inoculation has given rise to a thriving industry for AMF-related products for agriculture, horticulture, and landscaping (Chen et al. 2018).

CONCLUSION

The efficiency of different AMF in promoting coffee seedlings growth depended on the cultivation substrate. Inoculation of *G. margarita* (IAC-FM1), *Acaulospora* spp. (IAC-FM13) and *Glomus* spp. (IAC-FM28) in the conventional substrate (mixture composed of soil and cattle manure compost) conferred the best growth-plant responses, with increases between 160 and 320%, while in the commercial substrate (organic substrate based on coconut fiber) the most efficient AMF were *R. clarus* (IAC-FM16), *Glomus* spp. (IAC-FM28), *A. morrowiae* (IAC-FM14) and *A. scrobiculata* (IAC-FM10), with up to 149% growth improvement.

Here we showed that commercial substrate based on coconut fiber with the inoculation of some AMF isolates were highly beneficial for coffee seedlings development and can replace the current use of the conventional substrate. These results open new opportunities for the use of arbuscular mycorrhizal fungus inoculants as a key element to improve coffee seedling production with commercial organic substrates.

AUTHORS' CONTRIBUTION

Conceptualization: Silveira, A. P. D. and Tristão, F. S. M.; **Methodology:** Silveira, A. P. D., Tristão, F. S. M. and Andrade, S. A. L.; **Investigation:** Tristão, F. S. M. and Andrade, S. A. L.; **Writing – Original Draft:** Tristão, F. S. M. and Fernandes, A. O.; **Writing – Review and Editing:** Silveira, A. P. D., Cipriano, M. A. P. and Andrade, S. A. L.; **Supervision:** Silveira, A. P. D. and Cipriano, M. A. P..

DATA AVAILABILITY STATEMENT

All dataset were generated or analyzed in the current study.

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