Ornamental Horticulture
ISSN 2447-536X | HTTPS://ORNAMENTALHORTICULTURE.EMNUVENS.COM.BR/RBHO
SBFPO Sociedade Brasileira de Floricultura e Plantas Ornamentais

Scientific Article

Establishment and production of Torch Ginger plants associated with arbuscular mycorrhizal fungi inoculation

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Abstract

Traditionally, Torch Ginger is commercially propagated via rhizomes. Micropropagation (M) is a viable alternative that ensures the genetic and phytosanitary quality of plantlets. However, *in vitro* cultivation conditions can lead to morphophysiological disorders resulting in death or difficulties in the acclimatization process and establishment of seedlings/plantlets in field conditions. Thus, Arbuscular Mycorrhizal Fungi (AMF) has been used in some crops in order to mitigate the drastic effects during acclimatization and establishment of micropropagated plantlets in the field. In this sense, the objective of this study was to evaluate the implantation forms and efficacy of micropropagation and AMF inoculation on the establishment and production of Torch Ginger plants. The planting was carried in shading screens (50%) and different implantation forms were used; through rhizome (RIZ) and plantlets micropropagated with (M+AMF) and without (M-AMF) inoculation with arbuscular mycorrhizal fungi. Evaluations of growth, phenology and mycorrhizal colonization were carried out for one year. Micropropagation, independently of AMF inoculation, favoured a better development in height and number of tillers when compared to RIZ plants It is concluded that micropropagated plants of *E. elatior* showed earlier tiller emission, better development and initial establishment in the field. Additionally, the forms of implantation of *E. elatior* via rhizome and via micropropagation with or without AMF inoculation produce inflorescences with the minimum characteristics required for commercialization.

Keywords: *Etlingera elatior*, Glomeromycota, *in vitro* cultivation, tropical flowers, rhizomes.

Resumo

Estabelecimento e produção de plantas de bastão-do-imperador associadas a inoculação com Fungos Micorrízicos Arbusculares

Tradicionalmente, a forma de propagação comercial do bastão-do-Imperador é por meio de rizomas. A micropropagação (M) é uma alternativa viável que garante a qualidade genética e fitossanitária das plântulas. No entanto, as condições do cultivo *in vitro* podem causar distúrbios morfofisiológicos resultando na morte ou dificuldades no processo de aclimatização e estabelecimento das mudas sob condições de campo. Dessa forma, Fungos Micorrízicos Arbusculares (FMA) têm sido utilizados em algumas culturas com o intuito de amenizar os efeitos drásticos durante a aclimatização e estabelecimento de plântulas micropropagadas no campo. Nesse sentido, o objetivo desse estudo foi avaliar as formas de implantação e eficácia da micropropagação e da inoculação com FMA no estabelecimento e produção de plantas de bastão-do-Imperador. O plantio foi realizado em telado com sombreamento (50%) e utilizou-se diferentes formas de implantação; via rizoma (RIZ) e através de plântulas micropropagadas com (M+FMA) e sem (M-FMA) inoculação com fungos micorrízicos arbusculares. Foram realizadas avaliações de crescimento, fenologia e colonização micorrízica ao longo de um ano de cultivo. A micropropagação, independente da inoculação com FMA, favoreceu melhor desenvolvimento em altura e número de perfilhos quando comparada às plantas obtidas de mudas via RIZ. Conclui-se que plantas micropropagadas de *E. elatior* apresentaram emissão de perfilhos mais precoce, melhor desenvolvimento e estabelecimento inicial no campo. Adicionalmente, as formas de implantação de *E. elatior* via rizoma e via micropropagação com ou sem inoculação de FMA produzem inflorescências com as características mínimas exigidas para comercialização.

Palavras-chave: *Etlingera elatior*, Glomeromycota, cultivo *in vitro*, flores tropicais, rizomas.

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https://doi.org/10.1590/2447-536X.v29i3.2639
Received Mar 21, 2023 | Accepted Aug 1st, 2023 | Available online Sept 19, 2023

Editor: Maurecilne Lemes da Silva Carvalho

Introduction

The Torch Ginger [*Etlingera elatior* (Jack) R.M. Smith] is well known among the tropical flowers. It is a robust, rhizomatous plant, and produces inflorescences of vibrant colour, resembling a torch (Yunus et al., 2021). Like most species of the tropical flower group, *E. elatior* is commonly propagated via rhizome, as it is a method that ensures greater plant homogeneity and faster onset of inflorescence production (between 11 and 18 months after planting) compared to seed propagation (Araújo et al., 2018). However, phytosanitary quality is compromised due to the risk of disease propagation (Pinheiro et al., 2021).

Micropropagation emerges as a viable alternative that ensure both genetic and phytosanitary quality, since the *in vitro* cultivation of plant tissues is carried out using aseptic containers and under controlled conditions (Silva et al., 2020). However, the supply of sugars, nutrients and growth regulators, as well as the high relative humidity of the air and the low levels of irradiation and CO_2 concentrations, typical of *in vitro* cultivation, can result in the appearance of plantlets with structural and physiological alterations, making them more vulnerable to transplant shock when transferred to field conditions (Figueiredo et al., 2021; Souza et al., 2021).

Thus, Arbuscular Mycorrhizal Fungi (AMF) have been used as a tool to mitigate the drastic effects of transferring plants grown *in vitro* to field conditions (Begum et al., 2019). The AMF have capacity for mutualistic symbiotic interaction with plant roots, favouring the absorption of water and nutrients, especially those with low mobility in the soil, such as phosphorus. They can also increase plant tolerance to biotic and abiotic stress, favouring their adaptation to different ecosystems (Brito et al., 2017; Lopes et al., 2019). Several reports have shown that AMF inoculation increases growth, nutrients contents, and rate of photosynthesis during the acclimatization of the seedlings/plantlets (Kapoor et al., 2008). Sometimes the choice of AMF isolate can be important for the success of the acclimatization of the micropropagated plantlets. For example, Torch Ginger plantlets colonized by *Gigaspora albida* displayed better development than plants associated with *Claroideoglomus etunicatum* (= *Entrophospora etunicata*) after 60 days of the acclimatization (Silva et al., 2017). Therefore, micropropagation together with AMF inoculation may represent a sustainable and efficient strategy to improve the establishment and field performance of plants.

In this sense, the objective of this study was to evaluate the implantation forms and efficacy of micropropagation and AMF inoculation on the establishment and production of Torch Ginger plants.

Material and Methods

Plant material and growth conditions

The micropropagated plantlets of *Etlingera elatior* (cv. Red Torch) were obtained following protocols of Silva et al. (2017) and previously established *in vitro* in the Biotechnology Laboratory of the Embrapa Semiárido. Explants of about 1 cm, taken from the basal region between the root and stem containing shoot meristems of plants established *in vitro* were cultivated for 62 days in MS medium (Murashige and Skoog, 1962) added with 2 mg L^{-1} of BAP, 3% of sucrose and 0.6% of agar. After this period, transplantation was carried out in pots containing autoclaved sand and vermiculite (1:1). The plantlets presented an average of 3.7 cm (± 1.35) in height and 60% of rooting. One day after transplanting, part of the plantlets was inoculated with a suspension containing 200 glomerospores of *Gisgaspora albida*. In both treatments – with and without inoculation – 2 mL of a microbial filtrate were added, derived from the sieving of the inoculum soil previously used in the extraction of spores, in order to re-establish the soil microbiota.

Acclimatization was carried out in a greenhouse for 150 days. During this period the plants were irrigated daily with 50 mL of distilled water and fertilized with nutrient solution composed of one-half concentration MS salts once a week.

The rhizomes, also obtained from Embrapa Semiárido, were standardized with 20 cm in length and with at least one bud. The roots were washed, removed, and disinfected in a hypochlorite solution (0.1% active chlorine) for 5 minutes.

The cultivation of micropropagated plantlets (inoculated and non-inoculated with *Gigaspora albida*) and rhizomes was carried out under shading screens (50%) conducted at the geographical coordinates 09°19’ South latitude and 40°32’ West longitude, in a semiarid climate (BSh according to the KÖPPEN classification), with an annual rainfall of approximately 400 mm, majorly distributed between November and April (Dubreuil et al., 2018). The soil was classified as an Orthic Quartz-Sand Neosol (Embrapa, 2013). The data on temperature, relative air humidity and solar radiation were obtained from the Davis Automatic Weather Station, model Vantage PRO 2, with temperature (± 0.5%) and air humidity (± 35%) sensors, located 500 m from the growing area (Figure 1).

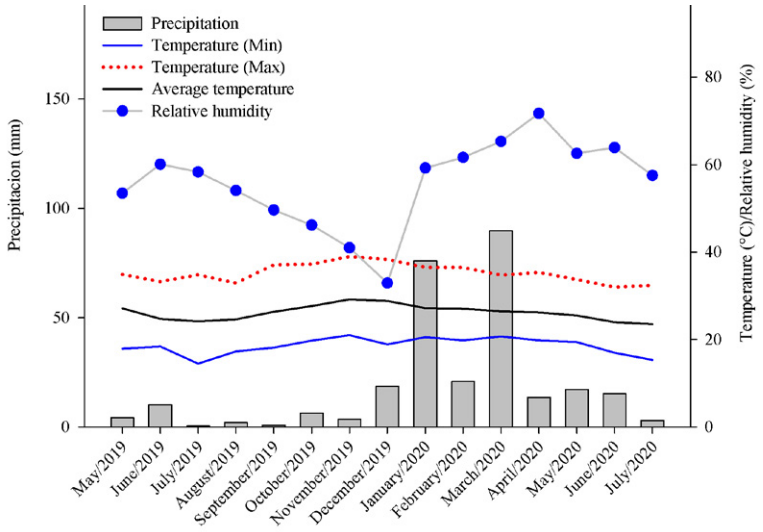

Figure 1. Temperature (ºC), relative air humidity (%), and precipitation (mm) data at the experiment site.

The soil of the experimental area was characterized as sandy loam texture, composed of 82% sand, 9% silt, and 9% clay; 3.26 dag kg^{-1} of organic matter (dry extraction method); pH (H_2O) of 5.47; phosphorus of 5.52 mg dm^{-3} (extraction with anion exchange resin); calcium of 1.70 $cmol_c$ dm^{-3} and magnesium of 1.37 $cmol_c$ dm^{-3} (KCl extraction); potassium of 0.48 $cmol_c$ dm^{-3}, sodium of 0.31 $cmol_c$ dm^{-3}, iron of 365.46 mg dm^{-3}, manganese of 12.64 mg dm^{-3}, copper of 0.11 mg dm^{-3}, zinc of 0.63 mg dm^{-3} and boron of 1.86 mg dm^{-3} (Mehlich 1 method).

The planting of the plantlets was done one per hole (20 cm wide and 20 cm deep) with 1.5 m plant spacing and 1.5 m between rows. The fertilization was carried out according to the soil analysis, using 50% of the recommendation for irrigated banana plants. A drip irrigation system was adopted with a nominal flow rate of 4 L h^{-1} per plant and irrigation was conducted daily.

The experimental design used was randomized block design with three treatments and eight repetitions with one plantlet per plot. The treatments used consisted of implantation forms of Torch Giger through rhizome (RIZ), and plantlets via micropropagation with (M+AMF) and without (M-AMF) inoculation with arbuscular mycorrhizal fungi.

Growth and phenology

Growth assessments were performed every two months for one year (cultivation on June 6th, 2019). The evaluated variables were plant height (m); number of tillers and area occupied by clump (AOC, m^2), calculated using the width L1 (side between rows) and L2 (side between plants). In addition, the chlorophyll index was measured using a portable chlorophyll meter (Chlorophyll Meter, SPAD-501, Minolta Co. Japan) on two pairs of fully expanded leaves in the middle third of the plants in the morning.

The development and phenology of the plants were evaluated by means of daily observations (days after planting - DAP) and recording as number of days to emission the first tiller (NDET), number of days to emission the first inflorescence (NDEI) and number of days to harvest the first inflorescence (NDH). In addition, the number of days of inflorescence development, from emission to harvest, and the production of stems per clump were calculated. From the beginning of the emission of floral stems, each stem was marked, and the date of emission was noted. And the harvest was performed when the inflorescences reached the E stage (lower bracts open, central part semi-open) (Loges et al., 2008). The inflorescences were always harvested in the morning and then sent to the laboratory to measure the length of the floral stem (LFS), considering the distance between the base of the pseudo stem and the apex of the inflorescence (cm); length of the inflorescence (LI), distance between the colored part of the peduncle and the apex of the inflorescence (cm); and stem diameter (SD), 20 cm below the inflorescence (mm), using a digital caliper.

Assessment of mycorrhizal colonization

Prior to planting, soil analysis was carried out to assess the number of AMF glomerospores present in the area. For this purpose, the soil was collected and then proceeded with wet sieving by the method of Gerdemann and Nicolson (1963) and centrifugation in water and 50% sucrose (Jenkins, 1964 modified). In a stereomicroscope, the number of glomerospores was counted and expressed in glomerospores 100 g^{-1} of soil.

At the end of the experiment, soil collection was also carried out in the root region at four points around the plants to quantify the spores. In addition to the soil, fine roots were collected in the rhizosphere of the plants, which were separated, washed in running water, dried on a paper towel and weighed (0.5 g) to determine the mycorrhizal colonization (%). Roots bleaching was performed with a 10% potassium hydroxide (KOH) solution for 24 hours, washing with water, plus a bleaching step with a 10% KOH and 10% hydrogen peroxide (H_2O_2) solution (1:1 v v^{-1}) for 5 minutes. After this period, the roots were

washed and acidified with a hydrochloric acid solution (HCl 1%) for 5 minutes and stained with 0.05% Trypan blue in lactoglycerol for 24-hours at room temperature (Phillips and Haymam, 1970), and then stored in water for the assessment. The percentage of root colonization was evaluated by the methodology proposed by Giovannetti and Mosse (1980).

Data were tested for normality of residuals (Kolmogorov-Smirnov) and then were subjected to analysis of variance and, when significant, to an "F" test ($p < 0.05$). The means of the treatments were compared by SNK (Student-Newman-Keuls) test ($p > 0.05$), and the averages obtained in the analyses of growth and chlorophyll index over time were submitted to regression analysis ($p > 0.05$).

Results and Discussion

There was a significant difference between treatments for the number of tillers and plant height, from 6 months after planting. Were it is verified that plants the M+AMF treatment presented superior results when compared to M-AMF and RIZ plants (Figure 2). In addition, an increasing quadratic behaviour was observed throughout the experimental period for the regression curves in all treatments studied.

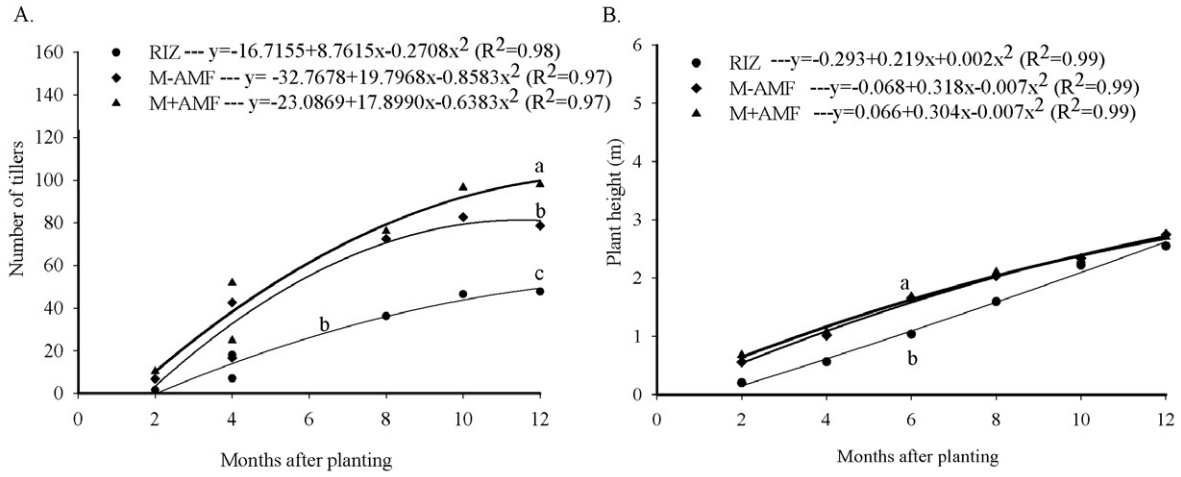

Figure 2. Number of tillers per clump (A) and height (B) of plants of *E. elatior* cv. Red Torch. in function of the experimental period (12 months) and the forms of culture implantation.

Micropropagation independently of AMF inoculation favoured a better development in height when compared to RIZ plants, regardless of the evaluation date (Figure 2B). This better growth performance of micropropagated may be related to the presence of a root system in the plants at the time of planting, which allows the absorption of water and nutrients, providing greater advantage during acclimatization and establishment of the plants. in the field (Souza et al., 2021). Similar results were verified in an experiment with heliconia, in which the height of the plant and the number of tillers were higher when derived from *in vitro* cultivation, in relation to propagation by rhizome (Ulisses et al., 2018).

An increase in the number of tillers was also observed by Kalamulla et al. (2022) in rice with the application of biofertilizer composed of indigenous AMF (*Claroideoglomus* sp., *Glomus* sp. and *Acaulospora* sp.) and *Azospirillum* sp., mainly in sterilized soil. However, under field conditions, soils present a native community that can be effective in suppressing the advantages of pre-inoculation with AMF, as they can be more adapted and efficient in providing benefits to plant development, as reported in *Salix miyabeana* (Pray et al. 2018). The increase in the number of tillers (Figure 2A) observed in plants micropropagated and inoculated with AMF may indicate that pre-inoculation with *G. albida* was effective in competing or acting synergistically with the native AMF community.

The AOC (Figure 3) did not differ in relation to the implantation forms of the plants (RIZ, M-AMF and M+AMF). A quadratic behaviour was verified in all treatments, whose maximum values estimated in time to reach the maximum area were 31 months after planting (MAP) and 8.05 m^2 for RIZ, 15 MAP and 9 m^2 for M-AMF and 12 MAP and 9 m^2 for M+AMF (Figure3).

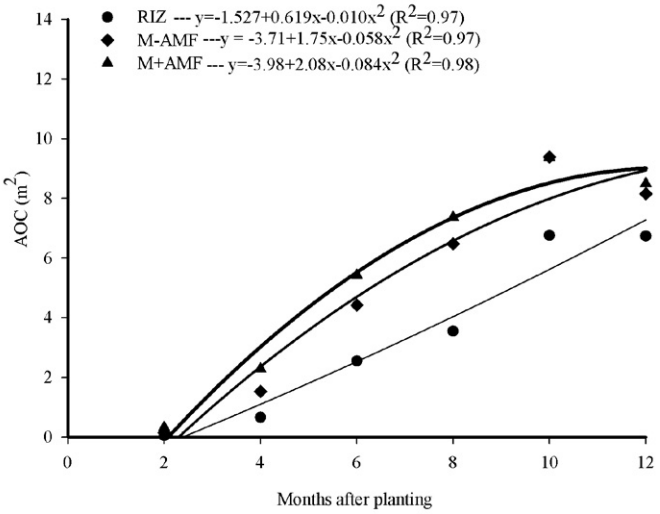

Figure 3. Area occupied by clump (AOC) of *E. elatior* plants cv. Red Torch in function of the experimental period (12 months) and the forms of culture implantation.

Although no difference was observed between the implantation forms for AOC it appears that the M+AMF treatment had a shorter time (12 MAP) estimated to obtain the maximum area. Furthermore, the area occupied by the clump is directly correlated with the number of tillers, which was higher in M+AMF plants (Figure 2A). This response may represent greater gains in productivity and in less time, since the greater the number of tillers, the greater the number of flower stems (Yunus et al., 2021).

The chlorophyll *a* content (Chl a) showed a quadratic behaviour throughout the experimental period and the M+AMF plants showed higher values only 2 months after planting (Figure 4A). Chlorophyll *b* contents (Chl b) also showed quadratic regressions, with maximum estimated period of 6 months after cultivation (Figure 4B). During this period, the Chl b content was similar among the treatments. While at 2 and 4 MAP the total chlorophyll index was higher in M+MAF plants (Figure 4C).

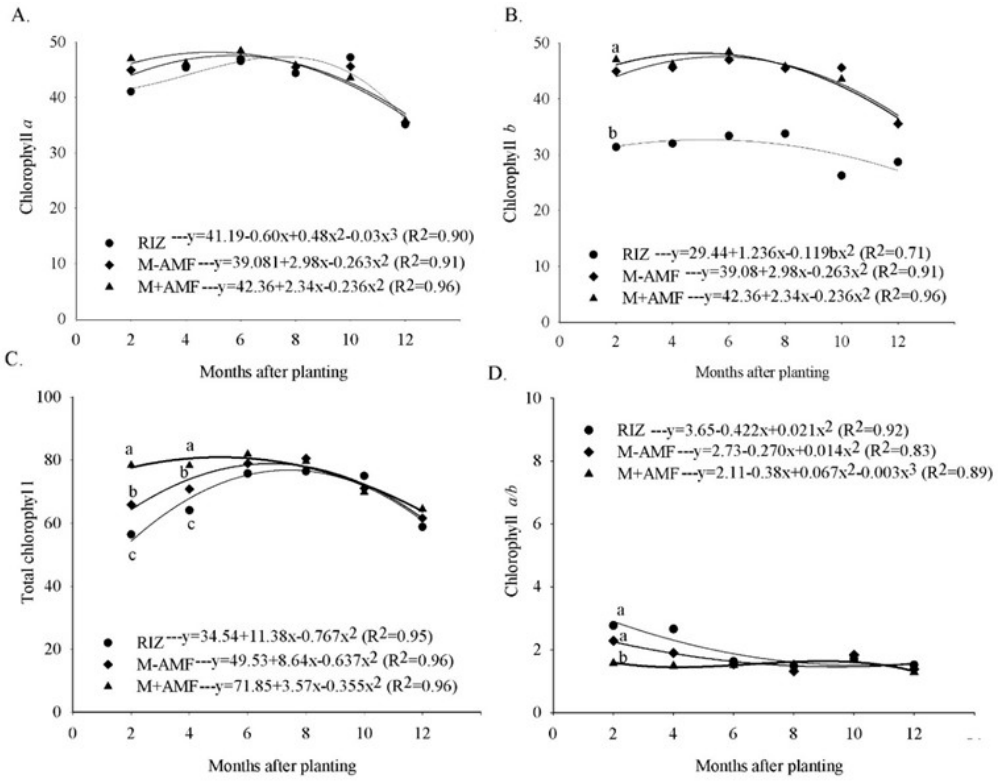

Figure 4. Chlorophyll a (A), chlorophyll b (B), total chlorophyll (C) and chlorophyll a/b (D) ratio in plants of *E. elatior* cv. Red Torch in function of the experimental period (12 months) and the forms of culture implantation.

It is known that the root system already formed in micropropagated plantlets favours the absorption of water and nutrients from the soil, including nitrogen, which is an important component of chlorophyll molecules (Lopes et al., 2019; Souza et al., 2021). In addition, the results demonstrate the symbiotic efficacy of *G. albida* under the studied conditions, since AMF produced beneficial effects on plant growth. This effect may be related to the greater ability of the fungi to extend their hyphae through soil for lower layers, in greater proportions than plant roots without inoculation can reach facilitating the absorption of nutrients by the mycorrhizal pathway (Begum et al., 2019).

The Chla/Chlb ratio also showed a quadratic behaviour with a decreasing tendency throughout experimental period (Figure 4D). The RIZ treatment showed a higher chlorophyll a/b ratio at the beginning of the experiment, but from the sixth month onwards all treatments showed similar to each other. Higher indices of the chlorophyll a/b ratio indicate a higher content of Chl a in relation to Chl b in the light harvesting complex and express the plasticity of the plant, that is, its ability to adapt to adverse conditions (Souza et al., 2021).

Plants from the M+AMF treatment showed higher levels of chlorophyll b and total, while the chlorophyll a/b ratio was lower. In symbiosis AMF emits a network of hyphae from the root to the rhizosphere, extending the reach of the plant's root system and improving nutrient absorption, providing greater physiological quality and consequently greater plant resistance to stress conditions (Lopes et al., 2019). In addition, it was possible to observe that the M+AMF plants showed greater stability of chlorophyll content in relation to the other treatments, demonstrating that the symbiosis can attenuate stress factors and ensure the acclimatization of the plants to different environments (Begum et al., 2019).

The implantation forms studied here did not interfere with the NDEI or the NDH having an effect only on the NDET (Table 1). The appearance of the first tiller occurred more quickly in micropropagated plants both inoculated (M+AMF) and non-inoculated (M-AMF). While plants from RIZ produced the first tiller more slowly approximately 41 days after implantation (Table 1).

Table 1. Number of days for the emission of the first tiller (NDET), number of days for the emission of the first inflorescence (NDEI) and number of days for the harvest of the first stem (NDH) of *Etlingera elatior* plants in function of the forms of culture implantation.

Factor	NDET	NDEI	NDH
	---------- DAP ----------		
Forms of implantation[1]	6.24*	0.41^{ns}	0.92^{ns}
RIZ	41.50 a	370.75 a	460.25 a
M-AMF	28.67 b	345.17 a	430.67 a
M+AMF	20.63 b	332.63 a	403.00 a
C.V. (%)	34.60	19.91	16.52

The emission of the first inflorescence in plants M+AMF occurred at approximately 330 DAP (11 months) and the harvest of the first inflorescence started at approximately 403 DAP (13 months and 13 days) (Table 1). The average time for the start of production is consistent with that indicated by Yunus et al. (2021), which indicates the average time for the start of production of *E. elatior* stems equal to or greater than 12 months.

As for the characteristics of the stems of *E. elatior*, it appears that the implantation form did not influence the LFS or the production of stems per clump. However, there was an effect on LI and SD, where it is observed that the plants from the RIZ implantation system showed higher values (Table 2).

Table 2. Length of the floral stem (LFS), length of the inflorescence (LI), stem diameter (SD) and stem production of *Etlingera elatior* plants in function of the forms of culture implantation.

Factor	LFS	LI	SD	Production
	-- cm --	-- cm --	-- mm --	stem.clump^{-1}
Forms of implantation[1]	2.58^{ns}	4.78*	8.26**	0.73^{ns}
RIZ	139.41	20.27 a	12.42 a	4.00
M-AMF	120.11	15.21 b	9.74 b	4.67
M+AMF	132.82	16.61 b	10.18 b	5.88
C.V. (%)	10.74	15.16	10.19	53.81

[1]RIZ: rhizome; M-AMF: micropropagated without inoculation of AMF; M+AMF: micropropagated with inoculation of AMF. * Significant at 0.05; ** Significant at 0.01; ^{ns} not significant. C.V. = coefficient of variation. Averages followed by the same letter do not differ by SNK test at 0.05 probability.

The characteristics required for commercialization of a type A floral stem (of better quality) of the Ginger Torch is at least 80 cm in length and 10 mm in diameter (Loges et al., 2008). Therefore, it is possible to affirm that all treatments produced inflorescences with the dimensions (length and diameter of the stem) that meet the requirements for the commercialization of type A stems.

Mycorrhizal activity

At the end of one year of cultivation the number of glomerospores in the rhizosphere of the plants from the different forms of implantation did not differ among them (Table 3). There was difference only for the percentage of mycorrhizal colonization, where a higher percentage of colonization was observed in M+AMF plants compared to RIZ treatment (Table 3).

Table 3. Spore density and mycorrhizal colonization in *Etlingera elatior* plants in function of the forms of culture implantation.

Factor	Spore density	Mycorrhizal colonization
	--- spore 100 g-1 soil ---	-------- % --------
Forms of implantation	0.93 ^{ns}	5.25*
RIZ	425.83	14.50 b
M-AMF	578.33	19.50 ab
M+AMF	480.00	22.25 a
C.V. (%)	36.55	19.93

[1]RIZ: rhizome; M-AMF: micropropagated without inoculation of AMF; M+AMF: micropropagates with inoculation of AMF. * Significant at 0.05; ** Significant at 0.01; ^{ns} not significant. C.V. = coefficient of variation. Averages followed by the same letter do not differ by SNK test at 0.05 probability.

In a field experiment, Lino et al. (2018) also found higher percentages of mycorrhizal colonization in corn roots that had been inoculated at sowing with *Acaulospora longula* and *Claroideoglomus etunicatum* (= *Entrophospora etunicata*) (mean of 72%) compared to non-inoculated ones (mean of 54%). Although mycorrhizal colonization in inoculated plants, both in our study and in Lino et al. (2018), was higher than in non-inoculated plants, it is not possible to attribute these percentages to the inoculum used, since the technique used to evaluate mycorrhizal colonization does not allow the identification of the AMF inoculated. In addition, AMF species from the native community can colonize the roots of these plant species, as observed by the percentages found in non-inoculated plants. We should also consider that the time elapsed, that is, 12 months after the transplant of the plantlets to the field, has been long enough for the establishment of mycorrhizal colonization, even by the native community. The low percentages of mycorrhizal colonization (mean of 20%) in *E. elatior* in the field may indicate that the inoculated AMF has: i. persisted in the roots or has been replaced by native AMF or ii. presented synergism with the native AMF, allowing another AMF to colonize the root.

As the initial glomerospores number found in the soil of the experimental area was 290 spores 100 g^{-1} of soil (data not shown) probably the AMF species of the native community were able to colonize the roots of *E. elatior*. Some studies have shown that native AMF are usually more competitive than commercial inoculum (Basiru et al., 2022), mainly because some mycorrhizal inoculants do not present desirable quantity and quality of the AMF propagules (Salomon et al., 2022). Similar results were obtained by Yamawaki et al. (2013) who also did not observe statistical difference between inoculated and non-inoculated AMF treatments in relation to mycorrhizal colonization in two banana crops, in the field and in the greenhouse. However, the inoculated plants showed better growth performance.

Silva et al. (2017) evaluating the effect of inoculating micropropagated plants of *E. eleatior* with AMF during the

acclimatization process, highlighted the inoculum *G. albida* as being quite effective in colonization and on promoting the growth of treated plants. Still stating that the greatest benefits provided by inoculation were more evident from 45 days after inoculation. In the present study, it was also demonstrated that the benefits on the growth of treated plants were more evident from the 6th month after planting.

Conclusions

Micropropagated plants of *E. elatior* with and without AMF inoculation showed earlier tiller emission, better development and initial establishment in the study conditions. The forms of implantation of *E. elatior* via rhizome and via micropropagation with or without AMF inoculation produce inflorescences with the minimum characteristics required for commercialization.

Author contribution

MDGF: Conceptualization, methodology, investigation, data curation, writing – original draft. **LGL, AMRS:** Investigation, methodology and data curation. **RRS:** Conceptualization, writing – review & editing, visualization. **AMYM:** Methodology, writing – review & editing, visualization. **MZBC:** Conceptualization**,** supervision**,** project administration, writing – review & editing.

Acknowledgements

The authors gratefully thank to Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco, Brazil (PBPG-1476-5.01/16); CNPq to providing PQ granting (AMYM - Process 314078/2021-5).

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