South African Journal of Botany 113 (2017) 164–169

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Acclimatization of micropropagated plants of *Etlingera elatior* (Jack) R. M. Sm. inoculated with arbuscular mycorrhizal fungi

A.R. da Silva [a], N.F. de Melo [b], A.M. Yano-Melo [c,*]

[a] Laboratório de Microbiologia, Programa de Pós-Graduação em Agronomia – Produção Vegetal, Universidade Federal do Vale do São Francisco, Campus Ciências Agrárias, 56300-990 Petrolina, PE, Brazil
[b] Laboratório de Biotecnologia, Centro de Pesquisa Agropecuária do Trópico Semiárido, Embrapa Semiárido, 56302-970 Petrolina, PE, Brazil
[c] Laboratório de Microbiologia, Colegiado Acadêmico de Zootecnia, Universidade Federal do Vale do São Francisco, Campus Ciências Agrárias, 56300-990 Petrolina, PE, Brazil

ARTICLE INFO

Article history:
Received 2 October 2016
Received in revised form 5 July 2017
Accepted 21 August 2017
Available online 30 August 2017

Edited by WC Otoni

Keywords:
AMF
Horticulture
Ex vitro culture
Ornamental species

ABSTRACT

The traditional method of propagating *Etlingera elatior* (Jack) R.M. Sm. promotes the proliferation of pathogens, reducing the quality of its output. However, as an alternative, one can use *in vitro* culture, making it possible to obtain plants free of pathogens on a large scale. Moreover, inoculation of plants with arbuscular mycorrhizal fungi (AMF) can be critical to ensure greater survival and development of these plants under *ex vitro* conditions. Thus, the present study examined the effect of AMF inoculation (*Gigaspora albida* and *Claroideoglomus etunicatum*) on success in the establishment and development of *E. elatior* on the acclimatization phase. Plants were inoculated with *G. albida*, *C. etunicatum* or MIX (both AMF isolates) and after cultivation in a greenhouse for 60 days were evaluated for survival percentage, height, leaf area, biomass production of plants, spores number and mycorrhizal colonization. The effect of AMF on plant survival could be observed from 30 days after inoculation; and by 60 days, there was an increase of up to 50% in survival compared to non-inoculated plants. In addition, mycorrhizal inoculation also provided better performance in plant growth, compared to those not inoculated. Finally, different effects were observed between the AMF species studied (*Gigaspora albida* and *Claroideoglomus etunicatum*). It follows from this work that plants of *E. elatior* respond differently to inoculation with different AMF species and show better development when acclimatized with *G. albida*. This finding highlights the importance of selecting the most effective isolates for specific plants.

1. Introduction

Originating from Southeast Asia, *Etlingera elatior* (Jack) R.M. Sm. is a tropical plant that belongs to the Zingiberaceae family. The family includes several species with applications in horticulture, particularly for their use in cooking, medicine, ornamentation, and landscaping. This plant is conventionally propagated from rhizomes, which hampers the large-scale production of commercial quality plants due to the ease in spreading disease with this technique (Loges et al., 2008; Wong, 2008). Therefore, *in vitro* culture becomes an alternative to this method and allows for the large-scale propagation of plants under high phytosanitary conditions (Faridah et al., 2011; Yunus et al., 2012).

One of the steps of *in vitro* cultivation is the acclimatization phase, which consists of a transition in the plant from a heterotrophic condition (*in vitro*) to an autotrophic (*ex vitro*) one. However, due to the strict environmental control under which plants in *in vitro* culture may be, plants may fail to overcome the adverse conditions related to *ex vitro* cultivation. During this transition, it is important that the crucial physiological processes for the plant's survival are enhanced. The acclimatization phase may affect the plant's photosynthetic efficiency, reduce its defense against pathogens and harm proper development of the root and sap conduction systems (Kapoor et al., 2008).

Thus, arbuscular mycorrhizal fungi (AMF) may represent a crucial tool to be used at this stage, as they provide several benefits to the plants with which they are associated, such as protection against biotic and abiotic stresses as well as improvement of plant development (Smith and Read, 2008). These fungi are obligatory biotrophs and the symbiosis basically involves an exchange of nutrients, in which the plant provides C through the products of photosynthesis and the fungi transfer nutrients from the soil, especially P, to the plant (S.E. Smith and Smith, 2011). The absorption of nutrients to the plants from the soil may be through direct contact of the roots with soil (direct uptake), or via the mycelium of mycorrhizal fungi (AMF uptake). In general, the latter pathway is more efficient due to the ability of the fungi to extend their hyphae through soil for distances up to 40 times greater than plant roots can (Giovannetti et al., 2001). Furthermore, this pathway is a

* Corresponding author.
E-mail addresses: angelica.ricarte@hotmail.com (A.R. da Silva), natoniel.melo@embrapa.br (N.F. de Melo), amymelo17@hotmail.com (A.M. Yano-Melo).

http://dx.doi.org/10.1016/j.sajb.2017.08.014
0254-6299/

highly regulated and fast transfer system, while direct uptake occurs through immediate contact of the root with the soil fraction (S. E. Smith and Smith, 2011).

Although arbuscular mycorrhizal symbiosis is non-specific, some plant-fungus compatibility has been found with higher efficiency of promoting plant growth. In this sense, there may be differences in plant response to mycorrhization when inoculated with different fungi (Novais et al., 2014). Variation in compatibility was observed with inoculation of two AMF isolates, while *Glomus mosseae* increased the P content and growth in the associated plant, *Gigaspora rosea* had the opposite effect (Burleigh et al., 2002). Similarly, other studies have shown different responses for growth and *ex vitro* establishment of plants inoculated with different species of AMF (Campanelli et al., 2014; de Oliveira et al., 2011). Thus, the selection of the fungus to be inoculated is related to the response of plant growth to mycorrhization (Smith et al., 2009). Furthermore, the combination of compatible fungus-plant-substrate is also an essential criterion in this selection in order to obtain the maximum benefits derived from mycorrhizal inoculation (Azcón-Aguilar and Barea, 1997).

For decades, the benefits provided by AMF in horticulture have already been known, at least in terms of which exert influence on various plant species of ornamental importance. Studies performed under controlled conditions have shown the benefits these fungi provide plants in various aspects of their growth and development (Koltai, 2010). In this sense, AMF inoculation for the *ex vitro* establishment of plants in horticulture has been widely recommended as they enable better adaptation of micropropagated plants to the adverse conditions they face during this phase (Azcón-Aguilar and Barea, 1997; Kapoor et al., 2008).

A previous study demonstrated the widespread occurrence of AMF in plants of the Zingiberaceae family (Uma et al., 2010), except that the effect of these fungi on the *ex vitro* cultivation of these plants was modulated by the conditions of the study. For example, the use of substrates with high fertility, *i.e.* vermicompost, reduced the benefit of the AMF in the growth of *Alpinia purpurata* and *Zingiber spectabile* (Silva et al., 2006). On the other hand, the use of a substrate composed of coconut husk powder can promote the benefits of mycorrhizal inoculation in plants of *Z. spectabile* (de Oliveira et al., 2010). Thus, the use of these fungi can be an alternative to application of P during the acclimatization phase, as observed for the growth of *Z. officinale* grown in a soil- and sand-based substrate (dos Santos et al., 2010; da Silva et al., 2008).

Therefore, inoculants containing AMF are a viable biotechnological tool, especially for cultures that undergo a propagation phase, such as those in which horticultural plants are grown in nurseries, pots or *in vitro* (Azcón-Aguilar and Barea, 1997). Moreover, it is also possible to associate two biotechnological tools for obtaining plants with high added value while ensuring more sustainable production in horticulture: micropropagation and mycorrhizal symbiosis (Campanelli et al., 2014).

The hypothesis to be tested in this study is that the inoculation with compatible AMF species is essential for survival and development of *E. elatior* plants in the acclimatization phase. Thus, this study aimed to verify the effect of inoculation of three treatments of AMF (*Claroideoglomus etunicatum*, *Gigaspora albida* and MIX) on the *ex vitro* establishment of micropropagated plants of *E. elatior* in order to evaluate the effectiveness of the use of these fungi in the acclimatization phase of this plant.

2. Materials and methods

2.1. Micropropagation

Plants of *E. elatior*, previously established *in vitro* in the Biotechnology Laboratory of the Embrapa Semi-Arid (Petrolina, PE, Brazil) were subcultivated in a semi-solid MS medium (Murashige and Skoog, 1962) supplemented with 3% (*w*/*v*) sucrose and 2.0 mg/L BAP (6-benzylaminopurine) with 0.45% agar and pH adjusted to 5.8. The culture medium had been previously autoclaved at 121 °C and 1 atm for 20 min. The subcultures were performed by transferring the explants (average 1 cm), taken from the basal portion between the roots and shoot and containing shoot meristems. Cultures were maintained in a growth chamber at 25 ± 2 °C, with a photoperiod of 16/8 h (light/dark) and irradiance around 35 μmol m^{-2} s^{-1}.

2.2. Acclimatization and experimental design

Micropropagated plantlets with developed roots and leaves were previously selected based on height and acclimatized for 60 days in a greenhouse, with an average temperature of 30.5 ± 3 °C and relative humidity of 57 ± 9%. The roots were washed to remove excess culture medium and transplanted into polyethylene conical shape pots (11.5 cm × 13.5 cm larger diameter × 9 cm smaller diameter) containing sand: vermiculite-based substrate, which had been previously sterilized at 121 °C and 1 atm for 1 h.

At transplanting, inoculation was carried out with AMF species (*Claroideoglomus etunicatum* – 10 mL, *Gigaspora albida* – 9 mL or MIX Ce and Ga – 5.5 and 4.5 mL, respectively), via soil-inoculum (containing spores, colonized root fragments and AMF hyphae), deposited directly on the roots and standardized to 1500 infective propagules/cm^3 in each pot. To standardize the microbiota of the substrate, all plants were supplemented with 2 ml of a filtrate (AMF-free) prepared from screening the dilution of the two inocula (1500 infective propagules/cm^3 of each inoculum in 100 mL of distilled water) through a 45-μm sieve. Irrigation was done with distilled water, and once a week was replaced by 40 mL of nutrient solution prepared with half-strength of the MS salts concentration.

The experiment was randomized with four inoculation treatments (NI – non-inoculated, Ga – inoculated with *G. albida*, Ce – inoculated with *C. etunicatum* and MIX – inoculated with mixture of Ga and Ce) with 10 replicates (one plant per replicate).

2.3. Mycorrhizal inoculum

The AMF inocula, *C. etunicatum* [URM AMF 03] and *G. albida* [URM AMF 11], were provided by the Laboratory of Mycorrhiza of the Federal University of Pernambuco (Recife/PE). The AMF inocula were cultivated in a greenhouse from September to December 2014, using a sterilized soil:sand-based substrate (1:1, *v*/v) and maize (*Zea mays* L.) as a host plant. The inoculum of *C. etunicatum* and *G. albida* had, respectively, 50.3 spores g^{-1} soil and 140 propagules cm^{-3} substrate and 8.8 spores g^{-1} soil and 170 propagules cm^{-3} substrate. Spores were extracted from the substrate by the wet-sieving technique (Gerdemann and Nicolson, 1963) and centrifuged in water and sucrose (Jenkins, 1964; modified with 50% sucrose), and then quantified in a channeled plate with the aid of a stereomicroscope. Quantitation of the Most Probable Number (MPN) of infective propagules of AMF (propagules cm^{-3} substrate) was made by the technique of Feldmann and Idczak (1994), from inoculum dilutions in tubes with a capacity of approximately 250 mL of substrate, which were performed in order of 0, 1/10, 1/100 and 1/1000 in five replicates, using washed and sterilized sand and maize as a test plant whose roots were clarified and stained by the technique of Phillips and Hayman (1970) after 30 days, for analysis of the presence of mycorrhizal colonization. Data were analyzed with the Cochran Table (Cochran, 1950).

2.4. Plant growth measurements

During the 60 days of acclimatization, for each treatment, the plants were evaluated every two weeks in terms of percentage of survival. At the end of the experiment, the following parameters were evaluated: height increment, number of leaves, leaf area, fresh and dry biomass of the shoot, fresh biomass of the root, estimated total root length,

number of spores and percentage of mycorrhizal colonization. For the survival percentage 10 replicates were considered, while for the other variables five replicates were considered.

The increase in height was determined from the difference between final height (measured at 60 days) and initial height (at transplantation). Leaf area was measured with the aid of a Cl-202 LASER AREA METER (CID Bio-Science). To determine dry biomass, shoots were kept in a forced air circulation oven at 60 °C until constant weight and plant dry biomass was measured.

For the total root length of each replicate, the same samples were used which had been processed and evaluated for determination of the percentage of mycorrhizal colonization (Giovannetti and Mosse, 1980; Phillips and Hayman, 1970). After evaluation, total root length was estimated according to eq. 1 (Newman, 1966):

$$R = \frac{\pi NA}{2H} \quad (1)$$

wherein R is the total root length (cm), using a sample of about 0.5 g root; N is the number of intersections between the root and the grid lines; A is the area of the quadrant (cm^2); and H is the circumference of the quadrant (cm).

2.5. Mycorrhizal measurements

The extraction of spores was performed from 100 g of fresh substrate as previously described (Section 2.3).

To evaluate root colonization by AMF, roots were rinsed in running tap water, dried at room temperature (about 28 °C) for approximately 1 h and a sample of 0.5 g was collected. The process was followed by diaphanization with KOH (10%) for 24 h and clarification with H_2O_2 (10%) + KOH (10%) (1:1 *v*/v) for 15 min, followed by acidifying the roots with HCl (1%) for 5 min and staining with trypan blue (0.05%) in lactoglycerol overnight (Phillips and Hayman, 1970 modified). After processing, the roots were placed under a stereomicroscope to quantify the percentage of mycorrhizal colonization by the grid-line intersect method, in which all intersections were observed (Giovannetti and Mosse, 1980).

From the shoot dry biomass, the percent plant growth response to mycorrhization (GRM) was calculated, according to Eq. 2 (Hetrick et al., 1992):

$$GRM = \frac{100x(I - NI)}{NI} \quad (2)$$

wherein, I and NI represent the plant biomass with and without AMF, respectively.

2.6. Statistical analysis

The data, except for the GRM and percentage of survival variables, were submitted to the Shapiro–Wilk normality and Bartlett homogeneity of variance tests at 5% significance. Subsequently, the data, when presenting normal and homogeneous variances, were submitted to ANOVA ($p < 0.05$) and to Tukey's multiple comparison of means test ($p < 0.05$), with the aid of the ExpDes.pt. package (Ferreira et al., 2013). In the absence of normality or homogeneity of variance, the non-parametric Kruskal–Wallis test was performed at 5% probability, using the dunn.test package (Dinno, 2016). The Pearson linear correlation coefficients were also calculated between the variables analyzed at the end of the experiment, using the agricolae package (Mendiburu, 2015). Analyses were performed using R software version 3.2.1 (R CORE TEAM, 2015).

3. Results

The plants inoculated with AMF, compared to non-inoculated ones, had a higher percentage of survival (Fig. 1). Mycorrhized plants had

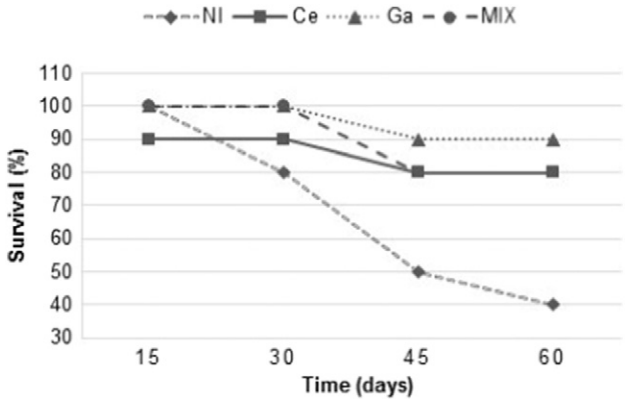

Fig. 1. Survival percentage of *Etlingera elatior* plantlets after 15, 30, 45, and 60 days of acclimatization without mycorrhizae (NI) or inoculated with *Claroideoglomus etunicatum* (Ce), *Gigaspora albida* (Ga) or both (MIX).

reduced survival between the 30th and 45th days of acclimatization, with values stabilizing after this period; whereas non-inoculated plants (NI) had reduced survival over the 60-day duration of the experiment. In this period, there was survival of only 40% among non-inoculated plants, while those with *G. albida* (Ga) had the highest percentage (90%), followed by those inoculated with *C. etunicatum* (Ce; 80%) and both fungi (MIX; 80%) (Fig. 1).

After 60 days of acclimatization, there was a significant positive linear correlation between the variables related to the vegetative growth of *E. elatior* (increase in height, leaf number, leaf area, fresh biomass of the root and shoot, dry biomass of the shoot and total root length), however there was no significant correlation between the increase of height and leaf number. Likewise, total root length was not correlated with the leaf number nor the fresh biomass of the root. In addition, the variables concerning mycorrhizal association (percentage of colonization and number of spores) were positively correlated, but only among themselves (Table 1).

In general, plants inoculated with *G. albida* (Ga) showed the greatest development, compared to the other treatments (Table 2). While plants mycorrhized with Ga showed greater average leaf area (102.13 ± 9.20 cm^2) and shoot fresh biomass (4.57 ± 0.79 g), there were no differences in height, root fresh biomass and shoot dry biomass between Ga and the treatment without inoculation (NI). However, leaf number and total root length did not show any significant effect of inoculation with AMF.

Moreover, in general, inoculation with Ce and the MIX of both AMF species did not promote development of *E. elatior* during acclimatization (Table 2). In this sense, there was a positive plant growth response to mycorrhizae (GRM) only in the treatment with *G. albida* (9.0%), the opposite of what was observed for *C. etunicatum* (−47.9%) and MIX with both inocula (−25.2%).

Table 1
Pearson's correlation between height increment (HI), leaf number (LN), leaf area (LA), shoot fresh biomass (SFB) and shoot dry biomass (SDB), root fresh biomass (RFB), total root length (TRL), mycorrhizal colonization (MC), and number of spores (NS) after 60 days of acclimatization of *Etlingera elatior* plantlets inoculated or not with *Claroideoglomus etunicatum*, *Gigaspora albida* or both.

	LN	LA	SFB	SDB	RFB	TRL	MC	NS
HI	0.25^{ns}	0.71^{*}	0.80^{*}	0.67^{*}	0.79^{*}	0.73^{*}	0.39^{ns}	0.11^{ns}
LN		0.56^{*}	0.49^{*}	0.50^{*}	0.65^{*}	0.09^{ns}	-0.07^{ns}	-0.23^{ns}
LA			0.91^{*}	0.68^{*}	0.81^{*}	0.52^{*}	0.07^{ns}	0.00^{ns}
SFB				0.68^{*}	0.84^{*}	0.56^{*}	0.30^{ns}	0.22^{ns}
SDB					0.63^{*}	0.57^{*}	0.20^{ns}	-0.15^{ns}
RFB						0.40^{ns}	0.15^{ns}	-0.06^{ns}
TRL							0.25^{ns}	0.12^{ns}
MC								0.48^{*}

*: significant at 5% probability by T test. ns: not significant at 5% probability.

Table 2
Effect of inoculation or not (NI) with *Claroideoglomus etunicatum* (Ce), *Gigaspora albida* (Ga) or both (MIX) on height increment, leaf number, leaf area, shoot and root fresh biomass, shoot dry biomass, total root length, mycorrhizal colonization, and number of spores after 60 days of acclimatization of *Etlingera elatior* plantlets.

Treatment	Height increment	Leaf number	Leaf area	Fresh biomass		Dry biomass	Total root length	Mycorrhizal colonization	Number of spores
	(cm)	–	(cm^2)	Shoot (g)	Root (g)	Shoot (g)	(mm)	(%)	($100\ g^{-1}$ substrate)
NI	13.73 ± 4.53 ab	5.27 ± 0.27 a	80.44 ± 10.90 b	2.99 ± 0.51 b	1.28 ± 0.20 a	0.33 ± 0.09 a	353.16 ± 39.30 a	0.00 ± 0.00	0.00 ± 0.00
Ce	6.40 ± 1.23 c	4.00 ± 0.29 a	38.45 ± 12.16 c	1.09 ± 0.27 c	0.59 ± 0.24 b	0.17 ± 0.06 b	291.71 ± 50.75 a	6.51 ± 0.18 b	20.31 ± 1.14 ab
Ga	19.02 ± 0.95 a	5.20 ± 0.19 a	102.13 ± 9.20 a	4.57 ± 0.79 a	1.58 ± 0.24 a	0.36 ± 0.09 a	394.79 ± 67.24 a	38.39 ± 8.38 a	25.95 ± 4.03 a
MIX	12.84 ± 4.02 b	3.80 ± 0.21 a	41.36 ± 9.91 c	1.75 ± 0.56 c	0.79 ± 0.21 b	0.25 ± 0.09 ab	351.99 ± 60.62 a	43.50 ± 11.74 a	15.72 ± 6.72b
p-Value	<0.001	0.080	<0.001	<0.001	<0.001	0.008	0.066	<0.001	0.014
CV (%)	24.06	23.03	16.16	21.72	21.13	29.12	15.95	30.4	22.13

Means (±SD) followed by the same letter do not differ between the treatments of inoculation by Tukey's test at 0.05 significance level.

Treatments with AMF inoculation were colonized, while none of the plants of the NI treatment formed associations with AMF, showing that inoculation was effective and that there was no contamination. Mycorrhizal colonization was higher in plants subjected to the Ga and MIX treatments, while the number of spores differed only between these treatments. It was found that Ce colonized only 6.5% of the root, possibly indicating poor compatibility considering the negative GRM in plants of *E. elatior* (Table 2).

4. Discussion

The observed survival percentage of plants inoculated with *G. albida* (90%), *C. etunicatum* (80%), and both fungi (80%) showed increased survival by at least 50% as compared to non-inoculated ones (40%). This response, however, may vary according to the plant species, as *Alpinia purpurata* showed only 50% survival when inoculated with *G. albida*, 25% with *C. etunicatum* and 87.5% in the absence of AMF. For *Zingiber spectabile* this rate was 100% in the treatments with *G. albida* and without AMF and 85% with *C. etunicatum*, both acclimatized in soil with high fertility of up to 209 mg dm^{-3} P and 92.5 g kg^{-1} of organic matter (Silva et al., 2006). Similarly, there was no effect of mycorrhizal inoculation on the survival rate of *Tapeinochilos ananassae*, where *G. albida* had a lower percentage (80%) compared to the control treatment without AMF and with *C. etunicatum* (both 100%), as observed by de Oliveira et al., 2011.

The increased survival of *E. elatior* plants inoculated with AMF, especially with *G. albida* (Ga), may have been a result of the benefits provided by the symbiosis. In *Heliconia* species, despite the high rate of colonization by *Gigaspora margarita* (55.95%), there was no significant effect of mycorrhizal inoculation, including other AMF, such as *Glomus clarum* (= *Rhizoglomus clarus*) and *Glomus etunicatum* (= *Claroideglomus etunicatum*) on the growth of that plant during acclimatization (Sato et al., 1999). On the other hand, in *Z. officinale* higher values were observed of growth measurements when inoculated with AMF compared to the treatment without mycorrhiza (dos Santos et al., 2010).

The results clearly showed the influence of inoculation with AMF on the success of acclimatization of *E. elatior*, highlighting the *G. albida* inoculum, whose effect can already be seen 45 days after inoculation. In the same way, benefits of mycorrhizal inoculation on the acclimatization of *T. ananassae* were more evident from 45 days after inoculation (de Oliveira et al., 2011). In general, for AMF species of the Glomeraceae and Gigasporaceae families, this is sufficient time for effective colonization in roots of the host plant to occur (Hart and Reader, 2002). According to F.A. Smith and Smith (2011), the percentage of mycorrhizal colonization is the most convenient and common measure in field-based study that indicates the activity of AMF and its benefit for plants. However, variation promoted by mycorrhization in medicinal plants was demonstrated in a review by Zeng et al. (2013). Their studies indicated differential mycorrhizal colonization among distinct AMF species that affect the accumulation of secondary metabolites. Plants inoculated with *G. albida* had higher mycorrhizal colonization than *C. etunicatum*, but did not differ from MIX treatment, which suggests greater contribution of *G. albida*. Synergistic interactions can be found between different species of AMF when they were co-inoculated, *e.g.* higher frequency of the *S. castanea* and *G. rosea* in the roots when *Glomus* species were inoculated (van Tuinen et al., 1998). Recently, differences among mycorrhizal colonization promoted by distinct AMF inoculum were observed by Tarraf et al. (2017) in *Salvia officinalis* L. plants.

In this study, plants inoculated with Ga showed improvements in terms of height, leaf area and fresh biomass of the shoot, observing positive GRM with this AMF isolate. On the other hand, there was no positive effect of inoculation with Ce and no synergism of the two species of AMF (MIX treatment) on these variables, highlighting Ga compared to Ce. Neither synergism nor competition was found between Ga and Ce in promoting increase on the analyzed variables. It is possible that differential compatibility between each fungi and plant had been established (Novais et al., 2014). Harmonious co-existence between members belonging to these genera (*Glomus intraradices* and *G. margarita*) was observed by Tiwari and Adholeya (2002), which can be detected by the normal development of the structures and sporulation of these fungi. In addition, the influence of the AMF on plant growth depends strongly on its genotype. While *Acaulospora morrowiae* has stimulated the growth of *Rudbeckia hirta* and reduced the growth of *Plantago lanceolata*, the opposite effect was observed with *G. rosea* (Klironomos, 2003). Baum et al. (2015) reported that the probability of an AMF isolate to promote growth and quality of the host plant was also determined by genotype of the fungi, genotype of the plant and their interaction. Thus, although the GRM has been negative in plants inoculated with MIX, this treatment did not reduce dry shoot biomass as observed with Ce inoculation.

In this sense, it is possible that only the Ga effect had occurred, evidencing the compatibility of this isolate. Some plants simultaneously inoculated with different AMF species present further development during acclimatization, compared to those inoculated with isolated species, as observed by Moreira et al. (2015) in substrate with low levels of P (≤40 mg kg^{-1}).

The growth response of mycorrhizal plants can be highly positive, neutral or negative, which can be influenced by factors controlled as much by the plant as by the fungus, together or separately (F.A. Smith and Smith, 2011; S. E. Smith and Smith, 2011). In addition, growth responses of mycorrhized plants may be related to the inoculated fungus, and consider the cost in C for the plant to maintain the symbiosis, as well as to the differentiated balance in nutrient absorption via direct (roots) and indirect (external mycelium) means, as reviewed by Smith et al. (2009) and S. E. Smith and Smith (2011). Colonization of roots by less effective fungal species has a high cost in C for the plant (Kiers et al., 2011). This high cost of C is mainly verified when mycorrhized plants have lower growth than non-mycorrhized counterparts, and this C supply to the fungi has been allocated to sporulation and production of biomass (inter and extra-radical) (Smith and Read, 2008). Species of Glomeraceae have been reported to display high internal mycorrhizal colonization (Hart and Reader, 2002), however, radicular colonization by Ce was low (6.51%). This fact suggests that if

drain of C has occurred, this allocation of C could have been used for sporulation (20.3 spores) and not for mycorrhizal colonization. Evaluation of extra-radical mycelium should help to clear our understanding.

The absence of significant correlation between variables of growth of the *E. elatior* and mycorrhizal colonization observed in this study disagrees with studies made with other plants. Gladiolus varieties (*Gladiolus grandiflorus* L.) showed highest positive correlation between mycorrhizal colonization and plant vegetative and reproductive growth in early growth stage (Javaid and Riaz, 2008a); similarity, positive correlation between dry biomass and mycorrhizal colonization were found for maize (Javaid and Riaz, 2008b) and lentil (*Lens culinaris* cv. Laird) (Xavier and Germida, 2002). However, as emphasized by de Novais et al. (2014), some AMF isolates can colonize roots without promoting benefits to growth or uptake of nutrients, suggesting that functionality of mycorrhizal association is not strictly related to the percentage of radicular colonization.

Considering that micropropagation and acclimatization should ensure the survival and vigor of the plants, resulting in better quality of leaves and flower (Silva et al., 2015), the hypothesis of this study was confirmed because plants of *E. elatior* were benefited by mycorrhizal inoculation of *G. albida* (URM AMF 11), which increased the survival of plants by 50%, displaying more compatibility with *E. elatior* plants. Other studies have also shown the effectiveness of using AMF in acclimatization to obtain higher rates of survival and growth, ensuring the *ex vitro* establishment of other horticultural plants, such as *Gerbera* sp. (Sato et al., 1999), *Curcuma zedoaria* (Miachir et al., 2004), *Gloriosa superba* (Yadav et al., 2013), *Cynara cardunculus* (Campanelli et al., 2014), *Musa* spp. (Kavoo-Mwangi et al., 2013; Koffi and Declerck, 2015; Yano-Melo et al., 1999) and three cultivars of *Paeonia* (Wen et al., 2016), among others. This confirms the importance of using AMF as a biotechnological tool for the sustainable production of plants of high quality and with the potential of large-scale production in order to obtain plant material that can be recommended for implementation in the field.

5. Conclusions

This study shows that micropropagated plants of *E. elatior* may respond differently to inoculation with *C. etunicatum* and *G. albida*, which have varying effects on the performance of this plant. Therefore, inoculation of this plant with *G. albida* for the plant's *ex vitro* establishment, given the potential of this fungus to foster establishment and plant growth during the acclimatization phase, is recommended.

Acknowledgments

The authors thank the Empresa Brasileira de Pesquisa Agropecuária (Embrapa Semi-Arid) for logistical support and infrastructure for execution of the work; and the support teams of the partnering biotechnology laboratories (Embrapa Semi-Arid), especially Elenício Gomes Coelho and Angela Katiussia Nascimento dos S. Coelho, and Microbiologia Geral (Universidade Federal do Vale do São Francisco – Univasf, CCA).

Funding sources

This work was supported by the Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) [grant number IBPG-0246-5.01/14 and APQ-0393-5.01/15]; and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) [grant numbers PQ 309847/2014-1, DT 310335/2014-0].

References

Azcón-Aguilar, C., Barea, J.M., 1997. Applying mycorrhiza biotechnology to horticulture: significance and potentials. Scientia Horticulturae (Amsterdam) 68:1–24. http://dx.doi.org/10.1016/S0304-4238(96)00954-5.

Baum, C., El-Tohamy, W., Gruda, N., 2015. Increasing the productivity and product quality of vegetable crops using arbuscular mycorrhizal fungi: a review. 187, 131–141. http://dx.doi.org/10.1016/j.scienta.2015.03.002.

de Novais, C.B., Borges, W.L., da Jesus, E.C., Saggin Júnior, O.J., Siqueira, J.O., 2014. Inter- and intraspecific functional variability of tropical arbuscular mycorrhizal fungi isolates colonizing corn plants. Applied Soil Ecology 76:78–86. http://dx.doi.org/10.1016/j.apsoil.2013.12.010.

Burleigh, S.H., Cavagnaro, T., Jakobsen, I., 2002. Functional diversity of arbuscular mycorrhizas extends to the expression of plant genes involved in P nutrition. Journal of Experimental Botany 53:1593–1601. http://dx.doi.org/10.1093/jxb/erf013.

Campanelli, A., Ruta, C., Tagarelli, A., Morone-Fortunato, I., de Mastro, G., 2014. Effectiveness of mycorrhizal fungi on globe artichoke (*Cynara cardunculus* L. var. scolymus) micropropagation. J. Plant Interactions 9:100–106. http://dx.doi.org/10.1080/17429145.2013.770928.

Cochran, W.G., 1950. Estimation of bacterial densities by means of the most probable number. Biometrics 6, 105–116.

Dinno, A., 2016. dunn.test: Dunn's Test of Multiple Comparisons Using Rank Sums. R Package Version 1.3.2. Available at: https://cran.r-project.org/web/packages/dunn.test/index.html.

Faridah, Q.Z., Abdelmageed, A.H.A., Julia, A.A., Nor Hafizah, R., 2011. Efficient in vitro regeneration of *Zingiber zerumbet* Smith (a valuable medicinal plant) plantlets from rhizome bud explants. African Journal of Biotechnology 10:9303–9308. http://dx.doi.org/10.5897/AJB11.1182.

Feldmann, F., Idczak, E., 1994. Inoculum production of vesicular-arbuscular mycorrhizal fungi for use in tropical nurseries. In: Norris, J.R., Read, D.J., Varma, A.K. (Eds.), Techniques for Mycorrhizal Research Methods in Microbiology. Academic Press, London, pp. 799–833.

Ferreira, E.B., Cavalcanti, P.P., Nogueira, D.A., 2013. ExpDes.pt: Experimental Designs package (Portuguese). R package Version 1.1.2. Available at: http://cran.r-project.org/package=ExpDes.pt.

Gerdemann, J.W., Nicolson, T.H., 1963. Spores of mycorrhizal endogone species extracted from soil by wet sieving and decanting. Transactions of the British Mycological Society 46:235–244. http://dx.doi.org/10.1016/S0007-1536(63)80079−0.

Giovannetti, M., Mosse, B., 1980. An evaluation of techniques to measure vesicular-arbuscular mycorrhizal infection in roots. The New Phytologist 84:489–500. http://dx.doi.org/10.1111/j.1469-8137.1980.tb04556.x.

Giovannetti, M., Fortuna, P., Citernesi, A.S., Morini, S., Nuti, M.P., 2001. The occurrence of anastomosis formation and nuclear exchange in intact arbuscular mycorrhizal networks. The New Phytologist 151:717–724. http://dx.doi.org/10.1046/j.0028-646x.2001.00216.x.

dos Santos, R., Girardi, C.G., Pescador, R., Stürmer, S.L., 2010. Effects of arbuscular mycorrhizal fungi and phosphorus fertilization on *post vitro* growth of micropropagated *Zingiber officinale* Roscoe. Revista Brasileira de Ciência do Solo 34:765–771. http://dx.doi.org/10.1590/S0100-06832010000300018.

Hart, M.M., Reader, R.J., 2002. Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. The New Phytologist 153:335–344. http://dx.doi.org/10.1046/j.0028-646X.2001.00312.x.

Hetrick, B.A.D., Wilson, G.W.T., Cox, T.S., 1992. Mycorrhizal dependence of modern wheat varieties, landraces, and ancestors. Canadian Journal of Botany 70:2032–2040. http://dx.doi.org/10.1139/b92-253.

R CORE TEAM, 2015. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria Available at: URL. https://www.R-project.org/.

Javaid, A., Riaz, T., 2008a. Mycorrhizal colonization in different varieties of gladiolus and its relation with plant vegetative and reproductive growth. International Journal of Agriculture and Biology 10, 278–282.

Javaid, A., Riaz, T., 2008b. Effects of application of leaf green manure of allelopathic plants on growth and mycorrhizal colonization of maize. Allelopathy Journal 21, 339–348.

Jenkins, W.R., 1964. A rapid centrifugal-flotation technique for separating nematodes from soil. Plant Disease Report 48, 692.

Kapoor, R., Sharma, D., Bhatnagar, A.K., 2008. Arbuscular mycorrhizae in micropropagation systems and their potential applications. Scientia Horticulturae (Amsterdam) 116:227–239. http://dx.doi.org/10.1016/j.scienta.2008.02.002.

Kavoo-Mwangi, A.M., Kahangi, E.M., Ateka, E., Onguso, J., Mukhongo, R.W., Mwangi, E.K., Jefwa, J.M., 2013. Growth effects of microorganisms based commercial products inoculated to tissue cultured banana cultivated in three different soils in Kenya. Applied Soil Ecology 64:152–162. http://dx.doi.org/10.1016/j.apsoil.2012.12.002.

Kiers, E.T., Duhamel, M., Beesetty, Y., Mensah, J.A., Franken, O., Verbruggen, E., Fellbaum, C.R., Kowalchuk, G.A., Hart, M.M., Bago, A., Palmer, T.M., West, S.A., Vandenkoornhuyse, P., Jansa, J., Bücking, H., 2011. Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. Science 333:880–882. http://dx.doi.org/10.1126/science.1208473.

Klironomos, J.N., 2003. Variation in plant response to native and exotic arbuscular mycorrhizal fungi. Ecology 84:2292–2301. http://dx.doi.org/10.1890/02-0413.

Koffi, M.C., Declerck, S., 2015. In vitro mycorrhization of banana (*Musa acuminata*) plantlets improves their growth during acclimatization. In Vitro Cellular & Developmental Biology. Plant 51:265–273. http://dx.doi.org/10.1007/s11627-015-9666-0.

Koltai, H., 2010. Mycorrhiza in floriculture: difficulties and opportunities. Symbiosis 52:55–63. http://dx.doi.org/10.1007/s13199-010-0090-2.

Loges, V., da Costa, A.S., Guimarães, W.N.R., Teixeira, M.do.C.F., 2008. Potencial de mercado de bastão-do-imperador e sorvetão. Rev. Bras. Hortic. Ornam. 14, 15–22.

Mendiburu, F., 2015. Agricolae: Statistical Procedures for Agricultural Research. R Package Version 1.2-2. Available at. https://CRAN.R-project.org/package=agricolae.

Miachir, J.I., Romani, V.L.M., Amaral, A. de F.C., Mello, M.O., Crocomo, O.J., Melo, M., 2004. Micropropagation and callogenesis of *Curcuma zedoaria* Roscoe. Science in Agriculture 61:427–432. http://dx.doi.org/10.1590/S0103-90162004000400012.

Moreira, B.C., Mendes, F.C., Mendes, I.R., Paula, T.A., Prates Junior, P., Salomão, L.C.C., Stürmer, S.L., Otoni, W.C., Guarçoni, M.A., Kasuya, M.C.M., 2015. The interaction between arbuscular mycorrhizal fungi and *Piriformospora indica* improves the growth and nutrient uptake in micropropagation-derived pineapple plantlets. Scientia Horticulturae (Amsterdam) 197:183–192. http://dx.doi.org/10.1016/j.scienta.2015.09.032.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Crops and Products 15:473–497. http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x.

Newman, E.I., 1966. A method of estimating the total length of root in a sample. Journal of Applied Ecology 3:139–145. http://dx.doi.org/10.2307/2401670.

de Oliveira, J.R.G., Moraes, T.A. de L., de Melo, N.F., Yano-Melo, A.M., 2010. Fungos micorrízicos arbusculares e rizobactérias promotoras de crescimento na aclimatização de *Zingiber spectabile*. Bragantia 69:687–694. http://dx.doi.org/10.1590/S0006-87052010000300021.

de Oliveira, J.R.G., Morais, T.A. de L., de Melo, N.F., Yano-Melo, A.M., 2011. Acclimatization of *Tapeinochilos ananassae* plantlets in association with arbuscular mycorrhizal fungi. Pesquisa Agropecuária Brasileira 46, 1099–1104.

da Silva, M.F., Pescador, R., Rebelo, R.A., Stürmer, S.L., 2008. The effect of arbuscular mycorrhizal fungal isolates on the development and oleoresin production of micropropagated *Zingiber officinale*. Brazilian Journal of Plant Physiology 20: 119–130. http://dx.doi.org/10.1590/S1677-04202008000200004.

Phillips, J.M., Hayman, D.S., 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular fungi for rapid assessment of infection. Transactions of the British Mycological Society 55:158–161. http://dx.doi.org/10.1016/S0007-1536(70)80110-3.

Sato, A.Y., Nannetti, D. de C., Pinto, J.E.B.P., Siqueira, O., Blank, M. de F.A., 1999. Fungos micorrízicos-arbusculares no desenvolvimento de mudas de helicônia e gérbera micropropagadas. Horticultura Brasileira 17:25–28. http://dx.doi.org/10.1590/S0102-05361999000100007.

Silva, M.A., Silva, F.S.B., Yano-Melo, A.M., Melo, N.F., Maia, L.C., 2006. Fungos micorrízicos arbusculares e vermicomposto na aclimatação de *Alpinia purpurata* (Viell.) Schum e *Zingiber spectabile* Griff. (Zingiberaceae). Acta Botânica Brasílica 20: 249–256. http://dx.doi.org/10.1590/S0102-33062006000200001.

Silva, J.A.T., Dobránszki, J., Winarto, B., Zeng, S., 2015. *Anthurium in vitro*: a review. Scientia Horticulturae (Amsterdam) 186:266–298. http://dx.doi.org/10.1016/j.scienta.2014.11.024.

Smith, S.E., Read, D.J., 2008. Mycorrhizal symbiosis. Mycorrhizal Symbiosis, 3rd ed Academic Press, New York, London.

Smith, F.A., Smith, S.E., 2011a. What is the significance of the arbuscular mycorrhizal colonisation of many economically important crop plants? Plant and Soil 348: 63–79. http://dx.doi.org/10.1007/s11104-011-0865-0.

Smith, S.E., Smith, F.A., 2011b. Roles of arbuscular mycorrhizas in plant nutrition and growth: new paradigms from cellular to ecosystem scales. Annual Review of Plant Biology 62:227–250. http://dx.doi.org/10.1146/annurev-arplant-042110-103846.

Smith, F.A., Grace, E.J., Smith, S.E., 2009. More than a carbon economy: nutrient trade and ecological sustainability in facultative arbuscular mycorrhizal symbioses. The New Phytologist 182:347–358. http://dx.doi.org/10.1111/j.1469-8137.2008.02753.x.

Tarraf, W., Ruta, C., Tagarelli, A., Cillis, F. De, Mastro, G. De, 2017. Influence of arbuscular mycorrhizae on plant growth, essential oil production and phosphorus uptake of *Salvia officinalis* L. Industrial Crops and Products 102:144–153. http://dx.doi.org/10.1016/j.indcrop.2017.03.010.

Tiwari, P., Adholeya, A., 2002. *In vitro* co-culture of two AMF isolates *Gigaspora margarita* and *Glomus intraradices* on Ri T-DNA transformed roots. FEMS Microbiology Letters 206, 39–43.

Uma, E., Muthukumar, T., Sathiyadash, K., Muniappan, V., 2010. Mycorrhizal and dark septate fungal associations in gingers and spiral gingers. Botany 88:500–511. http://dx.doi.org/10.1139/B10-021.

van Tuinen, D., Jacquot, E., Zhao, B., Gollotte, A., Gianinazzi-Pearson, V., 1998. Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. Molecular Ecology 7:879–887. http://dx.doi.org/10.1046/j.1365-294x.1998.00410.x.

Wen, S.-S., Cheng, F.-Y., Zhong, Y., Wang, X., Li, L.-Z., Zhang, Y.-X., Qiu, J.-M., 2016. Efficient protocols for the micropropagation of tree peony (*Paeonia suffruticosa* "Jin Pao Hong", *P. suffruticosa* "Wu Long Peng Sheng", and P.×lemoinei "High Noon") and application of arbuscular mycorrhizal fungi to improve plantlet establishment. Scientia Horticulturae (Amsterdam) 201:10–17. http://dx.doi.org/10.1016/j.scienta.2016.01.022.

Wong, W., 2008. Light up Your Garden With a Torch Ginger [WWW Document]. URL http://www.greenculturesg.com/archives.html accessed. 3.11.2015.

Xavier, L.J.C., Germida, J.J., 2002. Response of lentil under controlled conditions to co-inoculation with arbuscular mycorrhizal fungi and rhizobia varying in efficacy. Soil Biology and Biochemistry 34, 181–188.

Yadav, K., Aggarwal, A., Singh, N., 2013. Arbuscular mycorrhizal fungi (AMF) induced acclimatization, growth enhancement and colchicine content of micropropagated *Gloriosa superba* L. plantlets. Ind. Crops and Products 45:88–93. http://dx.doi.org/10.1016/j.indcrop.2012.12.001.

Yano-Melo, A.M., Saggin Júnior, O.J., Lima-Filho, J.M., de Melo, N.F., Maia, L.C., 1999. Effect of arbuscular mycorrhizal fungi on the acclimatization of micropropagated banana plantlets. Mycorrhiza 9:119–123. http://dx.doi.org/10.1007/s005720050009.

Yunus, M.F., Aziz, M.A., Kadir, M.A., Rashid, A.A., 2012. *In vitro* propagation of *Etlingera elatior* (Jack) (torch ginger). Sci. Hortic. (Amsterdam) 135:145–150. http://dx.doi.org/10.1016/j.scienta.2011.12.016.

Zeng, Y., Guo, L., Chen, B., Hao, Z.-P., Wang, J.-Y., Huang, L.-Q., Yang, G., Cui, X.-M., Yang, L., Wu, Z.-X., Chen, M.-L., Zhang, Y., 2013. Arbuscular mycorrhizal symbiosis and active ingredients of medicinal plants: current research status and prospectives. Mycorrhiza 23:253–265. http://dx.doi.org/10.1007/s00572-013-0484-0.