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Improved rooting capacity and hardening efficiency of carob (*Ceratonia siliqua* L.) cuttings using arbuscular mycorrhizal fungi

Abdellatif Essahibi[1,*], Laila Benhiba[1], Fouad Mohamed Oussouf[1], Mohamed Ait Babram[2], Cherki Ghoulam[1] and Ahmed Qaddoury[1]

[1] *Unit of Plant Biotechnology and Symbiosis Agrophysiology, Department of Biology, Faculty of Sciences and Techniques, Cadi Ayyad University*, Marrakech, Morocco

[2] *Department of Mathematics, Faculty of Sciences and Techniques, Cadi Ayyad University*, Marrakesh, Morocco

***Corresponding author**: abdellatif.essahibi@gmail.com

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Abstract: The present investigation was undertaken to improve the performance of carob cuttings in terms of adventitious roots formation and hardening using arbuscular mycorrhizal fungi (AMF). Softwood cuttings were treated with 5000 mg L^{-1} of indole-3-butyric acid (IBA) and kept non-inoculated (Non-AM) or inoculated with *Funneliformis mosseae* (*Fmo*) alone or combined with *Rhizophagus fasciculatus* (*Fmo+Rfa*) or *R. intraradices* (*Fmo+Rin*) or both (*Fmo+Rfa+Rin*) and then maintained under mist conditions. After two months, rooted cuttings were transplanted on sterilized substrate and transferred to a hardening greenhouse for five months. Obtained results showed that inoculation of the rooting substrate with AMF substantially improved the percentage of rooted cuttings and the number of roots per cutting. The highest rooting (63.33%) and number of roots per cutting (11.67) were recorded in the presence of the complex of the three AMF strains (*Fmo+Rfa+Rin*). Moreover, all mycorrhizal-rooted cuttings survived transplantation and hardening shocks and showed the highest growth and physiological performances. Indeed, in the *Fmo-Rfa-Rin*-plantlets the gains in plant height and shoot and root dry weights were 95.6%, 55.1% and 76.9% respectively. Furthermore, stomatal conductance, total chlorophyll content, photochemical efficiency of PSII (F_v/F_m) and nutrient concentrations were higher in mycorrhizal plantlets than in non-AM ones. Thus, AMF substantially improved carob cuttings' performance in terms of rooting capacity and hardening efficiency, thereby increasing the potential of carob propagation by cuttings.

Key words: *Ceratonia siliqua* L; arbuscular mycorrhizal fungi; cuttings; rooting; hardening.

INTRODUCTION

Carob tree (*Ceratonia siliqua* L.) is one of the most important Mediterranean species because of the high nutritional and economic value of its products and its tolerance to poor soils and drought [1-5]. This species is therefore suitable for the rehabilitation of marginal and sub-marginal areas of the Mediterranean basin. However, the large-scale cultivation of carob tree is limited by the traditional methods of propagation that fail to meet the growing demand for high-value carob plants. Carob propagation by cutting, which is the most appropriate technique for mass plant production, has not yet been fully achieved; carob has been described as one of the most difficult-to-root species [6]. Despite using exogenous auxin and intensive control of environmental factors, rooting is still the major bottleneck in such difficult-to-root species. Moreover, improper hardening is another hurdle that limits the potential of this technique; rooted cuttings produced under controlled conditions are fragile and most of them do not survive the transplantation and hardening shocks.

Currently, the management of arbuscular mycorrhizal fungi (AMF) as a plant growth enhancer, biofertilizer and bioprotectant is a key strategy for the sustainable production of plants with increased adaptation potential. AMF colonize the roots of over 90% of plant species to the mutual benefit of host plant and fungus [7]. In this mutual symbiosis, plants exchange photosynthates not only for mineral nutrients but also for increased tolerance to drastic environmental conditions [8-11]. These positive effects seem to be due to the enhanced growth and improved water relations

and nutrient acquisition in mycorrhizal plants [12-14]. Furthermore, AMF played a critical role in adventitious root formation when added to rooting substrate in many species [15-18]. This early inoculation provides the maximum benefits from AM colonization during a plant's acclimatization, thereby improving the survival and establishment of rooted cuttings [19-24]. However, the effectiveness of the mycorrhizal symbiosis varies according to mycorrhizal fungi strains and plant species rooting ability [18,25].

The aim of this work was to evaluate the effect of inoculation with individual or a complex of AMF strains on carob cutting performance in terms of rooting capacity under mist conditions and subsequent hardening efficiency.

MATERIALS AND METHODS

Biological materials and experimental design

Softwood cuttings, 8-10 mm in diameter and 10-15 cm in length, were collected in March 2014 from productive vigorous trees of a carob population cultivated in the Tamellalet region of Morocco (31.47° N, 7.33°E; altitude 843 m a.s.l.). The cuttings' ends were dipped or not in 5000 mg L^{-1} of Indole-3-butyric acid (IBA) solution. They were then planted in alveolus (4 cm × 4 cm × 4.5 cm) containing sterilized peat, supplemented with 10 g of inoculum of *Funneliformis mosseae* (*Fmo*) alone or combined with *Rhizophagus fasciculatus* (*Fmo+Rfa*), or *R. intraradices* (*Fmo+Rin*) or both (*Fmo+Rfa+Rin*). The inoculum consisted of soil containing infected root fragments of *Hordeum vulgare* L. and spores of the respective fungus. The inocula from each AM fungus possessed similar infective characteristics (75% of infected roots and approximately 20 spores g^{-1} of inoculum). The control (non-AM) received the same amount of autoclaved inoculum. Each treatment was repeated three times, with 50 cuttings per replicate (i.e., n=150 cuttings per treatment). Cuttings were randomly kept under intermittent mist in a polyethylene greenhouse under natural light/dark conditions, 80-90% relative humidity and at 30±2°C and 20±2°C day and night temperatures, respectively. After two months, the rooting percentage was determined as the ratio of the number of rooted cuttings to the total number of cuttings. A sample of three cuttings per replicate was taken to determine mycorrhizal status and number of roots per cutting.

Rooted cuttings were then transplanted into plastic bags (15 cm × 20 cm) containing 1.5 kg of a sterilized substrate consisting of a mixture of sand and soil (2:1). The substrate had the following characteristics: pH 8.23, organic matter 1.54%, total P 0.375 mg g^{-1}, K 2.16 mg g^{-1}, Ca 4.66 mg g^{-1} and Na 12.21 mg g^{-1}. Cultures were then transferred to the hardening greenhouse under the same conditions as the rooting phase during the first four weeks. During the following weeks, relative humidity was progressively decreased to the normal conditions of the greenhouse.

Mycorrhizal colonization and plant growth parameters

After five months of hardening, survival rate, plant height, root length, leaf area and mycorrhizal colonization were determined. Shoot and root fresh materials were weighed and oven-dried at 80°C for 48 h to determine the shoot and root dry weights.

Leaf area was assessed by image analysis using Image J software available as a free download from National Institute of Health (http://rsb.info.nih.gov/ij/index.html).

To evaluate fungal colonization, roots were washed with tap water, cleared in 10% KOH at 90°C for 2 h, treated with 7.5% H_2O_2 for 5 min and acidified in 1% HCl for 5 min. The cleared roots were stained with 0.05% Trypan blue in lactoglycerol at 90°C for 20 min [26]. The intensity of root colonization was determined according to Giovannetti and Mosse [27] using the MYCOCALC computer program available at: www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html

Stomatal conductance

Stomatal conductance was measured at a temperature of 25±1°C and a relative humidity of 60±3% using a leaf porometer (model SC1, DECAGON DEVICES, Version 2012, Inc., USA).

Chlorophyll fluorescence measurement

Chlorophyll fluorescence was measured using a portable fluorometer (Chlorophyll Fluorometer OS-30p,

Opti-Sciences, Hudson, New Hampshire, USA) after 30 min of dark adaptation. The chlorophyll fluorescence ratio F_v/F_m ($F_v=F_m-F_0$) was used to evaluate the maximum quantum yield of PSII in samples, where F_0 and F_m are the minimum and maximum fluorescence yields in dark adapted leaves, respectively [28].

Total chlorophyll content

The total chlorophyll content was evaluated as described by Arnon [29]. Fresh leaves (200 mg) were homogenized in a cold mortar with 5 mL of acetone (80%, v/v) and centrifuged at 5000×g for 10 min. The absorbance of the supernatant was determined at 663 and 645 nm and the concentration of total chlorophyll was calculated using the following formula:

$$Chl\ (a+b)=8.02\ OD_{663}+20.20\ OD_{645}.$$

Mineral nutrient content

For phosphorus (P), potassium (K), sodium (Na) and calcium (Ca) analysis, fresh leaves were dried in an oven at 80°C for 48 h, incinerated at 500°C for 5 h in a furnace and then digested in 2 M HCl. The P content was determined according to AFNOR [30], while K, Na and Ca concentrations were measured with a flame spectrophotometer (AFP100) according to Brown and Lilleland [31].

Statistical analysis

Data were statistically analyzed with ANOVA (IBM SPSS 20.0). The Tukey test (α=0.05) was performed to determine the differences between the considered means treatments. The results were expressed as means±standard error, and P≤0.05 was considered as statistically significant.

RESULTS

Effect of AMF and exogenous IBA on rooting percentage and number of roots of carob cuttings

The rooting capacity of carob cuttings was very sensitive to exogenous IBA application, while 43.80% of rooting was induced by IBA treatment; no root induction was recorded in untreated cuttings (Table 1). Indeed, adventitious root formation in the carob cuttings was significantly improved (P<0.01) by inoculation with individual or mixed AMF strains (Table 1). The highest rooting percentage (63.33%) was recorded in cuttings inoculated with *Fmo+Rfa+Rin* followed by *Fmo+Rin* (56.67%), representing an increase of 44.6% and 29.4%, respectively, compared to non-inoculated cuttings. AMF inoculation also significantly (P<0.01) enhanced the number of roots per cutting (Table 1). Multiple-root-formation capacity was about two times higher in the presence of *Fmo+Rfa+Rin* or *Fmo+Rin* (11.7 and 11.3, respectively) than in non-AM cuttings (6.67).

Effect of AMF on hardening of rooted cuttings

The hardening of rooted cuttings was strongly dependent on mycorrhizal symbiosis. The performances of rooted cuttings in terms of survival rate and growth capacity were significantly (P<0.01) improved in mycorrhizal-rooted cuttings compared to their non-mycorrhizal counterparts (Table 2). In fact, all plantlets colonized with *Fmo+Rfa+Rin* or *Fmo* survived to the hardening shock (100% of survival). Moreover, mycorrhizal plantlets showed greater development than non-mycorrhizal ones, regardless of the AMF inoculum used. The most enhanced growth and biomass production were recorded in plantlets inoculated with the complex *Fmo+Rfa+Rin*. The gain in plant height and shoot and root dry weights provided by

Table 1. Effect of exogenous IBA and AMF inoculation on rooting percentage and number of roots in cuttings of carob.

Mycorrhizal treatments	IBA treatments	Rooting (%)	Number of roots per cutting
Non-AM	Non-IBA	0.00±0.00 d	0.00±0.00 c
Non-AM	**+IBA**	43.80±1.98 c	6.67±0.67 b
Fmo	**+IBA**	51.33±2.91 bc	8.67±0.66 ab
Fmo+Rfa	**+IBA**	50.67±1.76 bc	8.67±0.33 ab
Fmo+Rin	**+IBA**	56.67±2.90 ab	11.33±0.88 a
Fmo+Rfa+Rin	**+IBA**	63.33±2.40 a	11.67±0.88 a
	IBA	133.17***	52.28***
ANOVA	**AMF**	8.96**	8.52**

NS: not significant; * p < 0.05; ** p < 0.01; *** p <0.001. Means±SE in the same column followed by the same letters are not significantly different at (P ≤0.05). Rooting percentage and number of roots in cuttings of carob non-treated (Non-IBA) or treated with IBA (+IBA) and non-inoculated (Non-AM) or inoculated with *Funneliformis mosseae* (*Fmo*) alone or combined with *Rhizophagus fasciculatus* (*Fmo+Rfa*) or *R. intraradices* (*Fmo+Rin*) or both (*Fmo+Rfa+Rin*) conducted under mist conditions.

Table 2. Effect of AMF inoculation on survival and growth of carob plantlets after five months of hardening.

Mycorrhizal treatments	M (%)	Survival (%)	SH (cm)	RL (cm)	LN	LA (cm^2)	SDW (g)	RDW (g)
Non-AM	0.00±0.00 c	81.4±1.68 b	10.63±0.26 c	14.67±0.84 c	5.67±0.33 b	34.13±1.91 d	2.87±0.13 b	0.78±0.06 b
Fmo	78.83±0.82 b	100.00±0.00 a	18.03±0.58 b	21.83±0.64 a	10.33±0.33 a	42.08±1.78 bc	3.83±0.18 a	1.47±0.12 a
Fmo+Rfa	83.33±2.61 ab	94.47±2.76 ab	19.07±0.72 ab	17.27±0.64 bc	7.00±0.58 b	67.68±1.65 a	3.96±0.16 a	1.30±0.11 ab
Fmo+Rin	81.11±2.33 ab	90.00±5.77 ab	20.67±0.50 a	20.83±0.89 ab	9.33±0.33 a	56.65±2.37 b	3.99±0.67 a	1.23±0.07 ab
Fmo+Rfa+Rin	87.39±0.89 a	100.00±0.00 a	20.83±0.96 a	17.30±1.10 bc	9.67±0.33 a	43.51±1.30 c	4.45±0.2 a	1.38±0.09 ab
ANOVA	491.46**	6.95**	43.88***	12.13**	25.14***	52.30***	10.03**	3.97*

Means±SE in the same column followed by the same letters are not significantly different (P≤0.05). M – Mycorrhizal colonization (M); SH – shoot height: RL – root length: LN – leaf number; LA – leaf area: SDW – shoot dry weight, RDW – root dry weight, both after hardening of carob plantlets; Non-AM – non-inoculated or inoculated with *Funneliformis mosseae* (*Fmo*), alone or in combination with *Rhizophagus fasciculatus* (*Fmo+Rfa*) or *R. intraradices* (*Fmo+Rin*) or both (*Fmo+Rfa+Rin*). NS – not significant; * p<0.05; ** p<0.01; *** p<0.001.

Table 3. Effect of AMF on nutrient contents of carob plantlets after hardening phase.

Mycorrhizal treatments	P (mg g^{-1} DW)	K (mg g^{-1} DW)	Na (mg g^{-1} DW)	Ca (mg g^{-1} DW)
Non-AM	1.60±0.13 d	1.88±0.14 b	1.10±0.09 c	4.19±0.17 c
Fmo	4.39±0.17 ab	2.39±0.02 a	6.89±0.20 a	10.02±0.27 a
Fmo+Rfa	3.40±0.25 bc	2.01±0.07 b	1.74±0.10 b	5.37±0.16 b
Fmo+Rin	2.94±0.19 c	2.46±0.02 a	1.21±0.12 bc	9.29±0.20 a
Fmo+Rfa+Rin	4.85±0.30 a	2.68±0.09 a	1.74±0.12 b	5.70±0.18 b
ANOVA	34.90***	16.67***	342.14***	166.97***

NS: not significant; * p<0.05; ** p<0.01; *** p<0.001. Means±SE in the same column followed by the same lower-case letters are not significantly different at P≤0.05 by Tukey's test. Phosphorus (P), potassium (K), sodium (Na) and calcium (Ca) contents in non-mycorrhizal (Non-AM) and mycorrhizal (*Fmo, Fmo+Rfa, Fmo+Rin, Fmo+Rfa+Rin*) hardened carob plantlets.

this AMF complex was 95.6% and 55.1% and 76.9%, respectively (Table 2).

Effect of AMF on nutrient acquisition

Mycorrhizal colonization of carob plants with any of the AMF species significantly (P<0.001) increased nutrient acquisition. Analyses of leaf nutrients showed that P, K, Na and Ca contents were higher in mycorrhizal plants than in the control. However, plants colonized with *Fmo+Rfa+Rin* showed the highest levels of P (4.85 mg g^{-1} DW) and K (2.68 mg g^{-1} DW), while the highest concentrations of Na (6.89 mg g^{-1} DW) and Ca (10.02 mg g^{-1} DW) were noticed in *Fmo*-plants (Table 3).

Effect of AMF on total chlorophyll content, photochemical efficiency of PSII and stomatal conductance

The data presented in Fig. 1 demonstrate the positive effect of mycorrhizae on the photosynthetic activity of carob plantlets. The total chlorophyll content and photochemical efficiency of PSII were remarkably higher in AMF-colonized plantlets compared to the non-colonized ones. The highest chlorophyll contents, 721 μg g^{-1} FW and 714 μg g^{-1} FW, were observed in plantlets inoculated with *Fmo+Rfa+Rin* and *Fmo*, respectively (Fig. 1A). These plantlets also displayed the following values for F_v/F_m: 0.867 and 0.844, respectively (Fig. 1B). Moreover, analysis of variance (ANOVA) revealed that stomatal conductance (SC) was significantly (P<0.001) increased in the mycorrhizal carob plantlets during the hardening phase. The highest SC values were recorded in plantlets colonized with *Fmo+Rin* followed by those inoculated with *Fmo+Rfa+Rin* (59.57 mmol m^{-2} s^{-1} and 52.80 mmol m^{-2} s^{-1}, respectively) (Fig. 1C).

DISCUSSION

Arbuscular mycorrhizal fungi (AMF) form mutual associations with over 90% of plant species. Mycorrhizal plants show not only high nutrient- and water-acquisition efficiency, but are also less susceptible to disease and more productive under difficult conditions than non-mycorrhizal plants [11,14,32,33]. The benefits from AMF are thought to be highest when

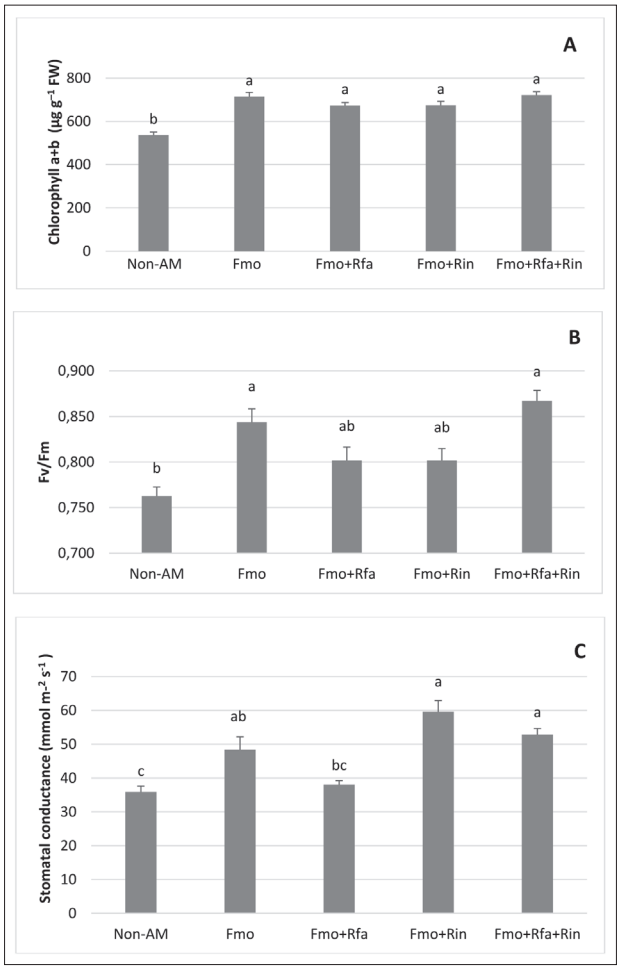

Fig. 1. Effect of AMF on chlorophyll content, chlorophyll fluorescence and stomatal conductance of carob plantlets after hardening phase. **A** – chlorophyll content; **B** – chlorophyll fluorescence; **C** – stomatal conductance in plantlets of carob, non-inoculated (Non-AM) or inoculated with *Funneliformis mosseae* (*Fmo*) alone or combined with *Rhizophagus fasciculatus* (*Fmo+Rfa*) or *R. intraradices* (*Fmo+Rin*) or both (*Fmo+Rfa+Rin*).

colonization occurs as early as possible during vegetative growth [20]. Thus, in plant propagation using cuttings, the maximum benefit from AMF association is obtained when inoculation is carried out during adventitious root formation [15-18].

In the present study, there was a clear difference in rooting response between the control (no IBA) and IBA-pretreated cuttings of carob. In the absence of IBA, cuttings were not able to take root, while all IBA treatments induced the initiation of numerous roots per cutting. These data showed that exogenous IBA is substantially required to induce adventitious root formation in cuttings of carob as was observed in other plant species [34-39]. Auxin may i) enhance cell division and differentiation in the vascular cambium, leading to the formation of roots [37], ii) antagonize the effects of other hormones that can inhibit rooting, such as gibberellin and cytokinin [6], or iii) stimulate the redistribution and mobilization of some auxin cofactors and carbohydrates towards the base of cuttings [34,40].

On the other hand, cuttings of carob, which is one of the most difficult-to-root species, were induced to enhance rooting and to form more adventitious roots using AMF in the rooting substrate. In fact, inoculated cuttings rooted more frequently and produced considerably more roots than non-inoculated ones. Several explanations were suggested to clarify the positive effects of AMF on adventitious root formation before colonization. Scagel [16] suggested the existence of a pre-colonization signal between cuttings and propagules of AMF similar to those existing in the presence of host plant roots. This signal is triggered in the basal ends of cuttings by the liberation of CO_2 or other metabolites able to activate AMF propagules [41,42]. AMF release exudates may induce changes in the cuttings' metabolism, thereby enhancing adventitious root initiation [43]. Moreover, after colonizing the root, AMF induced the formation of new roots by increasing the water and nutrient acquisition through the extraradical mycelia and increasing the accumulation of phenolic compounds involved in resistance against soil-borne pathogens [43].

The positive effect of AMF is more evident in the acclimatization of rooted cuttings. AMF treatments significantly increased plantlet survival to the transplantation and hardening shocks as has been reported in many investigations [18,19,21-24]. In this study, all mycorrhizal-rooted cuttings of carob completed the hardening-off process. Moreover, mycorrhizal plants showed higher performance in terms of growth, physiology and biomass production compared to non-AM plantlets. Nutrient contents, stomatal conductance, F_v/F_m that is directly proportional to the maximum yield of primary photochemistry of PSII and chlorophyll content were significantly higher in mycorrhizal plants than in the control. It is well known that mycorrhization positively affected a plant's gas exchange by increasing stomatal conductance [44,45], consequently providing better CO_2 assimilation to the host plant and thereby enhancing photosynthesis. The high Fv/Fm,

high chlorophyll content and high stomatal conductance observed in mycorrhizal carob plants are proof of their improved light harvesting and photosynthetic performance. The increase in photosynthesis is often mediated by an increase in nutrient uptake, especially P acquisition [46]. Mineral nutrients are essential for plant growth, metabolism and tolerance to environmental stresses. They can function as constituents of organic structures, as activators of enzymatic reactions, or as charge carriers and osmoregulators [47]. Phosphorus is a constituent of many essential components, including nucleic acids, phospholipids, phosphoproteins, dinucleotides and adenosine triphosphate. It is necessary for energy storage and transfer, photosynthesis and the regulation of many enzymes [48]. Potassium is an essential activator of many enzymes involved in photosynthesis, protein synthesis and carbohydrate metabolism. It mediates cell expansion and stomatal movement adjustment [47]. Calcium is essential for cell division and cell-wall synthesis and stability [49,50]. It plays a direct or signaling role in systems involved in plant defense against biotic and abiotic stresses [48]. In this study the concentrations of P, K, Ca and Na were higher in mycorrhizal carob plantlets than in non-mycorrhizal ones. The high nutrient content in mycorrhizal plants is proof of their high nutrient-acquisition efficiency due to the beneficial effect of AMF. AMF, through the extraradical mycelium, which penetrates deeper and wider in the soil thereby increasing the rhizosphere exploring area, consequently improves nutrient acquisition and water uptake [51]. Indeed, AMF can provide to the host plant other forms of nutrients that are unavailable to non-AM plants by enhancing the decomposition process [18,52] and by increasing the dissolution of minerals that contain P [53]. The performance of mycorrhizal plants may also be explained by their higher tissue hydration due to the efficiency of the extraradical hyphal network to extract soil water, resulting in higher stomatal conductance and transpiration fluxes as compared to non-AM plants. This is accompanied by improved CO_2 fixation in mesophyll, which contributes to increased plant photosynthesis [54].

The positive effect of mycorrhizal fungi on carob cuttings significantly varied depending on the inoculum used. Generally, the inoculation with all strains of AMF showed the best results in terms of improving the rooting capacity of cuttings and the acclimatization and development of rooted cuttings. According to Van der Heijden et al. [55], the diversity of AMF increases the productivity of host plants through a better use of resources.

CONCLUSIONS

The obtained results highlight the importance of exogenous IBA in the induction of adventitious root formation in carob cuttings. Early mycorrhizal inoculation, mainly with mixed strains, increased the rooting capacity of cuttings and substantially improved the survival, growth and physiology of rooted cuttings during the hardening phase. This finding provides evidence of the benefit of using mycorrhizal technology with IBA to promote successful propagation of carob (a rooting-recalcitrant species) by cutting.

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Authors' contribution: AE and AQ designed the experiments, developed the methodology and prepared the manuscript. AE, LB and FMO collected the data and carried out analysis. MAB provided the plant material and the nursery and participated in the data analysis. CG assisted with data analysis and manuscript preparation.

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