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MICROBIAL BIOSTIMULANTS AS ALTERNATIVES FOR THE ROOTING OF OLIVE TREE CUTTINGS

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Abstract

Currently, southern Minas Gerais (MG) state is an important producer of different olive tree (*Olea europaea* L.) cultivars because, in this region, the plants can differentiate the buds to produce flowers and fruit. To stimulate the rooting of cuttings, the synthetic hormone indole-3-butyric acid (IBA) at a concentration of 3 g L^{-1} is used commercially. However, few studies have investigated arbuscular mycorrhizal fungi (AMF), isolated or combined with rhizobacteria, as a biotechnological tool to produce hormones that function in the rooting of olive tree cuttings. The aim of this study was to evaluate the capacity of different AMF species (*Rhizophagus clarus, Gigaspora rosea,* or *Acaulospora scrobiculata*), combined or not with IBA or rhizobacteria, to promote the rooting of three olive tree cuttings (Arbequina, Grappolo 541, and Maria da Fé) with potential for cultivation in this region. For this, three experiments were conducted at the Experimental Farm of EPAMIG in Maria da Fé (MG), and the rooting potential of the olive tree cuttings inoculated with I) AMF, II) AMF combined with increasing doses of IBA, and III) AMF combined with three isolates of rhizobacteria was evaluated. The inoculation of olive tree cuttings of cultivars Arbequina, Grappolo 541, and Maria da Fé with *Rhizophagus clarus, Gigaspora rosea,* or *Acaulospora scrobiculata* combined or not with IBA or rhizobacteria did not significantly promote rooting. Alternative forms of rooting olive tree cuttings are still a challenge, and further studies for standardizing methodologies and experimental conditions are required.

Keywords: Mycorrhiza. *Olea europaea* L. Rhizobacteria. Rhizogenic potential. Rooted cuttings.

1. Introduction

The cultivation of olive trees in agricultural areas in Minas Gerais (MG), Brazil, is an expanding economic activity. In 2018, approximately 58,000 L of olive oil was produced in Brazil. However, this production level is still not sufficient to meet the domestic demand, since 80,000 t of olive oil were imported to Brazil in 2018, with investment of approximately US$ 436,000,000 (Conab 2019).

Currently, the vegetative propagation technique most used for cultivating olive trees is cuttings, as this technique ensures important characteristics, such as uniformity, small size, and early production of descendants, and is less expensive than other propagation methods (Barazani et al. 2008). The root system of olive trees varies depending on the tree's origin, whether from a seed or cutting, and on soil attributes.

When originated from seeds, the root system is characterized by a taproot, while when originated from a cutting, a fibrous root system is formed. Most of these adventitious roots behave as main roots during tree development and growth (Rapaport et al. 2019).

According to Rosa et al. (2018), rooted cuttings have been produced using semi-hardwood cuttings with hormone treatment to promote root formation. This method, which has been adopted in many countries, has been implemented and is currently the most widely used in Brazil. The main advantage of this system is the ability to obtain a large number of rooted cuttings from the parent plant, ensuring better consistency and health of the material. During the rooting phase, adventitious roots are formed, and the proportion of these roots varies depending on the cultivar and availability and type of material used (Druge et al. 2016; Casarin et al. 2017).

In the production of olive rooted cuttings, Pio et al. (2005) demonstrated that the use of indole-3-butyric acid (IBA) at 3 g L^{-1} affects the root system characteristics, especially the number of roots and root length. Even with hormone treatment, olive tree cuttings are difficult to root, and the results vary according to the cultivar, time of year, plant phenological status (Silva et al. 2012), and possible differences between the actual and potential yields of the crops (GAPs) (Ruffo et al. 2015). Cultivar breeding over the years has not taken into account the interactions between the plants and biostimulants, generating GAPs from rooting to harvest (Yakhin et al. 2017).

In several plant species, the process of rooting from cuttings is directly related to hormone treatments. However, synthetic auxins, such as IBA and indole-3-acetic acid (IAA), are classified as biochemical pesticides by the U.S. EPA (USDA), thus, their use must be controlled and is prohibited in organic crops (Pretty 2008; Erturk et al. 2010; Montero-Calasanz et al. 2013).

The need for alternatives to synthetic hormones for rooting, so-called plant biostimulants, such as plant growth-promoting rhizobacteria (PGPR), which have the ability to produce various plant growth regulators, such as auxins, gibberellins, cytokinins, ethylene, abscisic acid, nitric oxide, and polyamines (Cassan et al. 2014) is evident. In parallel, Barea and Azcón-Aguilar (1982) observed the production of gibberellin and cytokinin-like substances by *Glomus mosseae* arbuscular mycorrhizal fungi (AMF), opening the door for the evaluation of AMF in the rooting process given its plant cell growth and elongation potential (Pacifici et al. 2015). However, because AMF are obligate biotrophs (Buee et al. 2000), the relationship between the AMF propagules and the rooting of cuttings has been difficult to prove.

Due to the ability of soil microorganisms to alter the levels of hormone regulators in plants, Cosme and Wurst (2013), when analyzing the interaction between AMF and rhizobacteria in tobacco plants, observed that reduced cytokinin levels may be related to plant signaling for stimulating the growth of AMF hyphae. The presence of auxin can also stimulate mycorrhizal colonization mainly due to an increase in the lateral roots of plants (Kaldorf and Ludwig-Müller 2000).

Furthermore, there is growing evidence that the bacterium-fungus symbiosis has beneficial effects on plant growth. In this sense, AMF provide nutrients for the rhizobacteria that colonize fungal surfaces as well as protect against desiccation, radiation, predation, and salinity (Nadeem et al. 2014). Conversely, rhizobacteria associated with AMF spores can promote the growth of and rapid colonization by the fungus (Bhowmik and Singh 2004).

Although the biotechnological potential of rhizobacteria and AMF for promoting plant growth has been observed in several crops, such as corn, wheat, mint, strawberry, and winged-stem passion flower (Vitorazi Filho et al. 2012; Minaxi et al. 2013; Kumar et al. 2015; Koc et al. 2016), there have been few studies related to the effect of AMF and PGPR inoculation, isolated or combined, on the rooting of olive tree cuttings (Citernesi et al. 1998; Montero-Calasanz et al. 2013; Montero-Calasanz et al. 2014), especially in Brazil (Rosa et al. 2018).

Therefore, the present study aimed to evaluate the ability of different AMF species, combined or not with IBA or PGPR, to promote the rooting of olive tree cuttings of potentially important cultivars in the southeast region of Brazil.

2. Material and Methods

Microorganisms

The AMF Rhizophagus clarus, Gigaspora rosea, and Acaulospora scrobiculata were selected for this study given their broad distribution and interaction with the studied olive tree cultivars (Ferreira et al. 2015). The propagules were obtained from multiplication pots from the AMF collection of the "Luiz de Queiroz" College of Agriculture (ESALQ-USP). The wet-sieving method was used for the quantitative evaluation of the spores in the inoculate (Gerdemann and Nicolson, 1963).

The inoculant bacteria used in this study (PGPR) were isolated from the rhizosphere of olive trees and identified as isolates 32, 42, and 48, and exhibited the following in vitro IAA production values: 383.58 µg L−1, 374.69 µg L−1, and 301.50 µg L−1, respectively (Silva et al. 2017).

16s Sequencing rDNA

Genomic DNA from PGPR was extracted from pure cultures using the Quick-gDNA Zymo Research Quick-gDNA extraction kit according to the manufacturer's instructions. The 16s rDNA region was amplified using the primers 362F (5'-CTCCTACGGGAGGCAGCAGTGGGG-3') and 786F (5'-CGAAAGCGTGGGGAGCAAACAGG-3'), and the PCR conditions were as described by Menna et al. (2006). The PCR products were loaded onto an agarose gel (1.5%) and run at 70 V for 30 min in 1 × TAE buffer. After the confirmation of DNA amplification, the PCR products were sequenced. The sequences obtained were compared with those in the GenBank database using the BLAST tool of the National Center for Biotechnology Information (NCBI). Multiple-sequence alignment was performed in MEGA-X using the ClustalW package. The nucleotide substitution method used was the Tamura-Nei model, while the phylogenetic analysis was performed by using maximum likelihood with 500 bootstrap replications.

Rooting promotion with arbuscular mycorrhizal fungi propagules

To evaluate the rhizogenic potential of AMF propagules, a completely randomized experiment with a 4 × 3 factorial design was established with three species of fungi: Rhizophagus clarus, Gigaspora rosea, and Acaulospora scrobiculata. The control treatment consisted of a hydroalcoholic solution of IBA at 3 g L−1 in which 3 cm of the base of a cutting was submerged for 5 s. Three olive cultivars were used: Arbequina, Grappolo 541 (MGS GRAP541), and Maria da Fé (MGS MARIENSE). The whole experiment was performed with four replicates per treatment and four cuttings per experimental unit.

Rooting promotion with arbuscular mycorrhizal fungi propagules and varying concentrations of IBA

An experiment was carried out in a completely randomized design in a 3 × 4 × 5 three-factor structure with three olive cultivars: Arbequina, Grappolo 541 (MGS GRAP541), and Maria da Fé (MGS MARIENSE); three AMF species: R. clarus, G. rosea, and A. scrobiculata plus one control treatment (without fungus); and five IBA concentrations (0, 1, 2, 3, and 4 g L−1) with four cuttings per experimental unit. The cuttings were immersed for 5 s in the IBA solution, and those treated with 0 g L−1 were immersed in distilled water for the same period of time. The whole experiment was carried out in triplicate.

Rooting promotion using arbuscular mycorrhizal fungi propagules and rhizobacteria

This experiment followed a completely randomized design in a three-factor structure (4 × 3 × 3) with three AMF species: R. clarus, G. rosea, and A. scrobiculata, and a control treatment (no fungus + 3 g L−1 IBA); three PGPR isolates: 32, 42, and 48; and three olive cultivars: Arbequina, Maria da Fé, and Grappolo 541.

The isolated bacteria were reactivated in DYGS culture medium (2% glucose, 1.5% peptone, 2% yeast extract, 0.5% KH2PO4, 0.5% MgSO4.7H2O, and 1.5% glutamic acid at pH 6.8) until reaching a population of approximately 108 CFU/mL. Then, the base of the cuttings was immersed in this culture medium for 60 min. The whole experiment was performed with three replicates and 20 cuttings per experimental unit.

Experimental conditions

Each experimental unit consisted of a 300 mL flask filled with medium sand previously washed and autoclaved (1 h at 121°C) twice within 24 h. The AMF spores were inoculated in the substrate at a ratio of approximately 100 spores per experimental unit before adding the cuttings. The semi-hardwood olive tree cuttings were standardized to approximately 12 cm in length with two pairs of leaves in the apical region.

The experiments were conducted in a greenhouse with automated intermittent mist activated from 7:00 am to 7:00 pm for 10 s at 10 min intervals at the Maria da Fé Experimental Field (CEMF-EPAMIG/MG). The development of the cuttings was monitored for a period of 3 months. After this period, the following response variables were evaluated: number of roots formed, total length of roots, proportion of callused cuttings, and rooted cuttings.

The normality of the data was tested using the Kolmogorov–Smirnov test at 5% probability. Analysis of variance was then performed followed by the Scott–Knott test at 5% probability using the statistical software Sisvar®.

3. Results

Bacterial identification and phylogenetic analysis

The 16s rDNA gene sequence fragments from the bacterial isolates used as inoculants were analyzed by nucleotide BLAST analysis. The sequences of isolates 42 and 48 showed 100% similarity to that of *Paenibacillus polymyxa,* and the sequence of isolate 32 showed 100% similarity to that of *Pseudomonas protegens.* These sequences were aligned with bacterial sequences with the highest similarity according to the GenBank database, and this alignment was used to construct the phylogenetic tree (Figure 1).

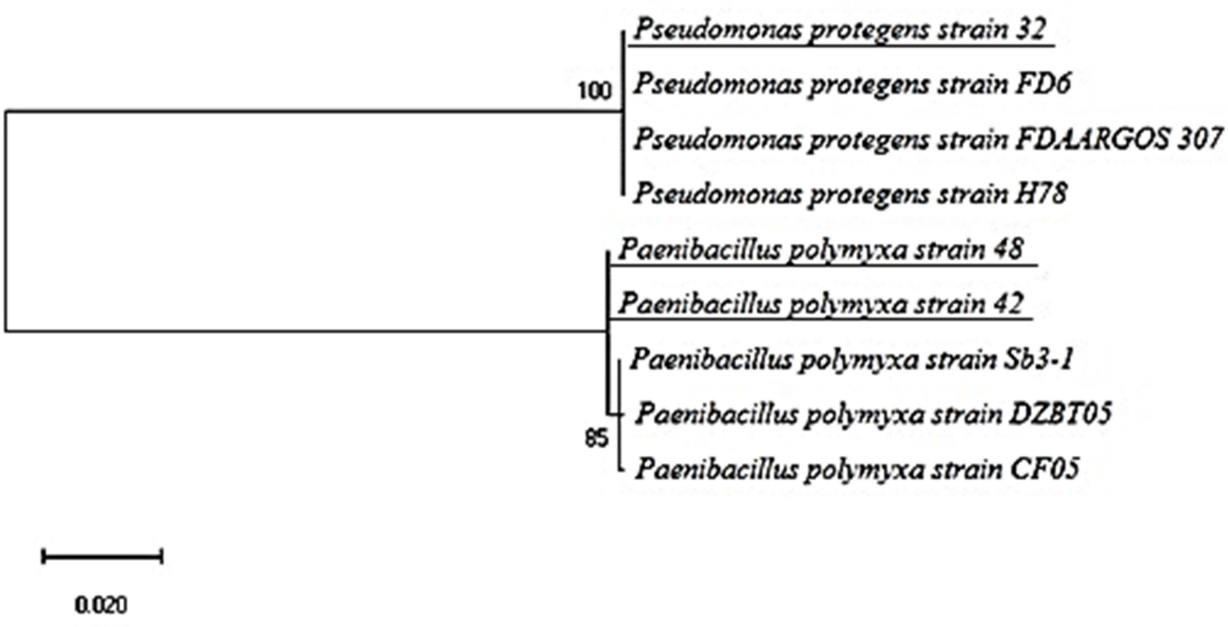

Figure 1. Phylogenetic tree of 16s rDNA gene sequences showing the relationship among the isolates from the olive tree rhizosphere (*P. protegens* strain 32; *P. polymyxa* strain 48, and *P. polymyxa* strain 42).

Rooting promotion with arbuscular mycorrhizal fungi propagules

The effect of treatment with AMF propagules on the rooting of olive tree cultivars is presented in Table 1 and was found to be significant ($p < 0.05$). However, no significant interaction ($p > 0.05$) was observed between treatments (cultivars × AMF propagules). In the different olive tree cultivars, the numbers and mean lengths of the roots did not differ by the Scott–Knott test at 5% significance. The

proportion of rooting differed among the olive tree cultivars and was the highest for cultivar Maria da Fé, with 5.46% rooting, while the greatest callus formation (24.1%) was observed for Grappolo 541.

Among the treatments with inoculation and AMF propagules, no significant differences were observed relative to the treatment with IBA at 3 g L^{-1} (Table 1). However, even in the control treatment, there was a low proportion of rooting (5.55%).

Table 1. Mean percentage of rooting (PR), number of roots (NR), root length (RL), and number of cuttings with callus (NC).

Treatments	PR (%)	NR	RL (cm)	NC
Cultivars				
Arbequina	0.9[b]	0.08[a]	0.10[a]	14.84[c]
Maria da Fé	5.46[a]	0.95[a]	1.81[a]	18.6[b]
Grappolo 541	1.95[b]	0.35[a]	1.01[a]	24.21[a]
FMAs				
G. rosea	1.21[a]	0.20[a]	0.38[a]	19.09[a]
R. clarus	1.56[a]	0.39[a]	0.70[a]	20.31[a]
A. scrobiculata	2.77[a]	0.27[a]	0.42[a]	19.27[a]
IBA (3 g L^{-1})	5.55[a]	1.00[a]	2.40[a]	18.22[a]
VI%	43.44	55.6	63.32	6.69

Variation index (VI%) = variation coefficient/repetition numbers. Means followed by the same letter in the same column do not differ from each other by the Scott–Knott test at 5% significance.

Rooting promotion with arbuscular mycorrhizal fungi propagules and varying concentrations of IBA

The second experiment was carried out using varying concentrations of the synthetic hormone IBA combined with AMF propagules in order to analyze their effect on the rooting of cuttings. The interaction between treatments was not significant ($p > 0.05$). However, each isolated treatment was significant ($p < 0.05$). The results of the experiment are presented in Table 2 along with the complementary test of means (Skott–Knott).

The olive tree cultivars did not exhibit significant differences in the analyzed variables according to the Skott–Knott test at 5% probability (Table 2). Likewise, there were no differences between the different AMF propagules, even in the control treatment without fungus. The IBA concentrations showed differences relative to the zero concentration, which did not promote rooting. The highest proportion of cuttings with calluses was observed at 1 g L^{-1} IBA, regardless of the olive tree cultivar.

Table 2. Mean percentage of rooting (PR), number of roots (NR), root length (RL), percentage of rooting, and number of cuttings with calluses (NC).

Treatments	PR (%)	NR	RL (cm)	NC
Cultivars				
Arbequina	3.33[a]	0.31[a]	0.48[a]	2.75[a]
Maria da Fé	5.00[a]	0.31[a]	0.37[a]	5.75[a]
Grappolo 541	6.66[a]	0.65[a]	0.72[a]	3.75[a]
AMFs				
Control	4.44[a]	0.82[a]	0.93[a]	4.25[a]
G. rosea	7.14[a]	0.52[a]	0.86[a]	7.5[a]
R. clarus	4.16[a]	0.08[a]	0.05[a]	1.00[a]
A. scrobiculata	4.44[a]	0.31[a]	0.31[a]	3.75[a]
IBA concentration (g L^{-1})				
0	0[b]	0[b]	0[b]	0.5[b]
1	8.33[a]	1.02[a]	1.31[a]	9.00[a]
2	5.55[a]	0.16[a]	0.26[a]	4.00[b]
3	8.33[a]	0.88[a]	0.99[a]	4.75[b]
4	2.77[a]	0.05[a]	0.04[a]	2.00[b]
VI%	147.32	188.92	198.75	90.24

Variation index (VI%) = variation coefficient/repetition numbers. Means followed by the same letter in the same column do not differ from each other by the Scott–Knott test at 5% significance.

Rooting promotion using arbuscular mycorrhizal fungi propagules and rhizobacteria

Similar to the previous experiments, no significant interaction ($p > 0.05$) was observed between the analyzed treatments (cultivars x AMF x PGPR). The results in Table 3 show the low rooting of the cultivar Arbequina compared to that of the cultivars Maria da Fé and Grappolo 541. Cultivar Grappolo 541 had the best results for mean number of roots and mean root length. Cultivar Maria da Fé exhibited a higher percentage of cuttings with callus. However, inoculation with AMF did not promote the rooting of the cuttings, in contrast to treatment with the synthetic hormone IBA, which produced the highest proportion of rooting. Both in the previous experiments (Table 1 and 2) and in this experiment (Table 3), inoculation with AMF alone or in combination with PGPR did not present rhizogenic potential in the production of olive cuttings.

Table 3. Mean percentage of rooting (PR), number of roots (NR), root length (RL), and number of cuttings with callus (NC).

Treatments	PR (%)	NR	RL (cm)	NC
Cultivars				
Arbequina	1.94[c]	0.58[c]	6.0[c]	0.03[b]
Maria da Fé	16.25[b]	17.76[b]	77.0[a]	0.56[b]
Grappolo 541	24.72[a]	31.29[a]	23.0 b	1.59[a]
AMF				
G. rosea	5.18[b]	2.19[b]	34.0[a]	0.12[b]
R. clarus	6.85[b]	2.32[b]	38.0[a]	0.14[b]
A. scrobiculata	3.51[b]	1.12[b]	31.0[a]	0.07[b]
AIB 3 g L^{-1}	41.66[a]	60.54[a]	39.0[a]	2.57[a]
PGPR				
P. protegens 32	13.61[a]	16.56[a]	37.0[a]	0.73[a]
P. polymyxa 42	14.30[a]	16.35[a]	32.0[a]	0.72[a]
P. polymyxa 48	15.00[a]	16.72[a]	38.0[a]	0.72[a]
VI%	35.41	47.57	13.25	52.46

Variation index (VI%) = variation coefficient/repetition numbers. Means followed by the same letter in the same column do not differ from each other by the Scott–Knott test at 5% significance.

4. Discussion

The influence of microorganisms, such as AMF and PGPR, on plant growth is already widely recognized and studied (Erturk et al. 2010; Koc et al. 2016; Wang et al. 2017). The same is true for the symbiotic relationship of AMF and rhizobacteria with olive tree crops produced in southern MG, Brazil, as demonstrated previously (Vieira et al. 2011; Ferreira et al. 2015; Silva et al. 2017; Mariosa et al. 2018).

Despite the well-known relationships between AMF and plants, few studies have investigated the application of AMF for vegetative propagation from cuttings, especially in difficult-to-root species. Amri (2015) demonstrated that the application of AMF propagules could improve rooting in *Dalbergia melanoxylon* cuttings. When evaluating the capacity of AMF to promote the rooting of Kinnikinnick (*Arctostaphylos uva*) cuttings, Scagel (2004) reported an increase in rooting when using propagules from roots of the same plant species compared to the rooting promoted by inoculation with *Glomus intraradices* propagules obtained from pure cultures. In the same experiment, inoculation with these propagules showed increased rooting when combined with synthetic hormones.

Hypotheses of this nature are considered difficult to prove since AMF, because they are obligate biotrophs, have limited growth in culture medium, making it difficult to isolate and identify substances with rhizogenic potential (Buee et al. 2000; Wang et al. 2017). However, AMF are considered biofertilizers with high efficiency, low cost, and easy access by farmers, especially in developing countries that need a more sustainable cultivation system or even in organic crop systems where the use of chemical fertilizers is prohibited (Erturk et al. 2010; Berruti et al. 2016).

The present study did not produce sufficient evidence of the direct effect of AMF on the rooting of cuttings, as also observed by Souza et al. (1995). However, studies indicate that these fungi can produce substances with hormone activity similar to that of gibberellins, cytokinins, IAA, and indole-3-ethanol

(Barea and Azcon-Aguilar 1982; Ek et al. 1983; Barroso et al. 1986), which may influence the rooting of cuttings.

The relationship between AMF and the production of plant hormones is still controversial, with Fernández et al. (2014) suggesting that mainly abscisic acid, salicylic acid, and jasmonic acid are affected. This relationship confers resistance to plants and is dependent on the genetic characteristics of both mycorrhizal plants and fungi.

Although not directly related to the rooting of cuttings, inoculation with AMF propagules of interest at the rooting stage may help promote the mycorrhization of cuttings, which may lead to a decrease in the length of the nursery stage and greater resistance of plants when transplanted to the field (Amri, 2015). Ferreira et al. (2015) demonstrated mycorrhizal dependence in 60% of the Grappolo 541 (MGS GRAPP 541) olive trees when inoculated with *Acaulospora scrobiculata* or *Gigaspora rosea*, which may justify the use of these propagules in the rooting substrate.

Although the PGPR isolates exhibited auxin production (Silva et al. 2017), they did not have a direct effect on the rooting of the cuttings. However, in the same way that the presence of AMF can produce higher yields at the nursery stage, the presence of rhizobacteria may aid in plant development.

The phytohormones produced by bacteria are important for the bacterium–plant relationship; the production of IAA by microorganisms stimulates the development of the host plant root system (Cheryl and Patten 2002). Beyond the capacity of phytohormone production, the capacity to establish and proliferate in the tissue of the stem base and in the callus is essential for efficiency to promote rooting (Peralta et al. 2012).

Strains of *P. protegens* and *P. polymyxa* can be considered plant growth promoters due to their capacity for auxin production, phosphate solubilization, protease production, nitrogen fixation, and antagonistic action against pathogens (Erturk et al. 2010; Bensidhoum et al. 2016; Du et al. 2017; Rai et al. 2017; Etminani and Harighi 2018). Thus, there is potential for the use of the microorganisms in the present study as biostimulants for olive tree cultivars, even if these microorganisms have not shown a direct effect, either alone or in combination with AMF, on the rooting of cuttings.

Abdel-rahman and El-Naggar (2014) reported the potential use of AMF, IBA, and PGPR in ornamental plant rooting. These practices led to a reduction in the price of production by vegetative propagation due to the shorter preparation time, reduced use of chemical compounds, and greater resistance to soil pathogens. Ercisli et al. (2003) also reported a positive effect of inoculation with *Agrobacterium rubi* on kiwi tree rooting, with a higher rooting rate when IBA was combined with inoculation.

In the rooting of olive tree cuttings, Montero-Calasanz et al. (2013) demonstrated that different olive tree cultivars may have different responses to bacterial inoculation. When testing inoculation with different microorganisms, such as *Pantoea* sp., *Chryseobacterium* sp., *Pseudomonas* sp., and *Azospirillum brasilense,* it was verified that the best rooting response was obtained with the bacterium *Pantoea* sp.; in turn, Rosa et al. (2018) demonstrated that *A. brasilense* in combination with IBA produced an increase in the proportion of rooting. However, in the present study, no statistically significant interaction was observed between the IBA concentration and inoculation with AMF propagules (Table 2).

Callus formation is directly related to the rooting of cuttings, acting as a precursor to rooting since it represents a dedifferentiation of the plant tissue. However, a low rooting rate was observed for the three cultivars, both in the control treatments (IBA) and in those with the AMF and rhizobacteria, which may indicate a need for a longer redifferentiation time of the tissue in the roots.

AMF and PGPR have the potential to promote rooting due to their intrinsic characteristics related to plant physiology and biochemistry, such as the dedifferentiation of tissues in the callus. However, a weak or absent response in this process for olive tree cuttings may be linked not only to these aspects but also to the physiological conditions of the plants, which are difficult to root (Porfírio et al. 2016), and the time for redifferentiation into new roots. The standardization of microorganism inoculation techniques is essential to reduce data variation and assess the potential of these groups for rooting, thus, produce olive tree rooted cuttings.

Therefore, the use of microorganisms for olive tree rooting is not yet an alternative to currently used propagation methods. However, further studies are needed to ensure the optimization of these

practices in order to enable rooted cutting production in commercial nurseries to decrease the variation of the rooted cuttings and allow their use, mainly in organic farming systems (Montero-Calasanz et al. 2013). Even though microbial inoculation in this phase of vegetative propagation was not efficient, the presence of microbial stimulants could allow the colonization of rooted cuttings with microorganisms of interest and, consequently, improve acclimatization and filed transplantation conditions (Teixeira et al. 2007; Ferreira et al. 2015; Mariosa et al. 2018).

5. Conclusions

The inoculation of olive cuttings of the cultivars Arbequina, Maria da Fé, and Grappolo 541 with the AMF *Rhizophagus clarus, Gigaspora rosea*, and *Acaulospora scrobiculata* combined or not with IBA and with the *Paenibacillus polymyxa* rhizobacteria isolates 42 and 48 and *Pseudomonas protegens* isolate 32 did not promote the rooting of semi-hardwood cuttings of olive trees. Alternative forms of rooting olive tree cuttings are still a challenge because of the low natural rooting rates for this crop; thus, further studies for standardizing methodologies and experimental conditions are required.

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Ethics Approval: Not applicable.

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