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Study of Arbuscular Mycorrhizal Fungi Diversity and Its Effect on Growth and Development of *Citrus aurantium* L.

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Citrus aurantium L. is the most used rootstock in Morocco. The use of arbuscular mycorrhizal fungi can favor the good growth of this species. A study was conducted under the greenhouse to assess its reaction to mycorrhizal colonization. *Citrus aurantium* L. plants were inoculated at the stage of 2 leaves, with a local composite inoculum of AMF, originating from the rhizosphere of many species of Citrus. The growth and mycorrhizal parameters were observed seven months after inoculation. The results obtained showed that the inoculated plants had a growth significantly higher than the control ones, with a gain of 90.1%. Inoculation of the plants was also effective on the leaves emission, branches formation and growth of collar diameter, gains were respectively 266, 300 and 300%. The fresh weight of root and vegetative masses also showed higher values on the inoculated plants, with a gain of 478% and 425% respectively. The average frequency and intensity of root colonization by mycorrhizal fungi were 93.33% and 50.6% respectively. The number of

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spores formed at the rhizosphere of plants inoculated was 132/100 g of soil and they were represented by 23 species belonging to the genera *Glomus* (8 species), *Acauolospora* (9 species), *Scutellospora* (3 species), *Pacispora* (2 species) and *Gigaspora* (1 species). The beneficial effect of mycorrhizae on *Citrus aurantium* L. was discussed in this study.

Keywords: Morocco; Citrus aurantium L.; rootstock; mycorrhizal fungi (AMF); growth; biomass.

1. INTRODUCTION

In Morocco, the citrus fruit sector has a great socio-economic importance. It represents a source of major currency estimated at 3 billion DH a year [1], contributes substantially to improving farmers' incomes and generates over than 21 million working days per year [1]. However, the Moroccan citrus industry knows several problems related to diseases, pests [2-7] and various abiotic environmental stresses, case of salinity, low temperatures and periods of drought [5,8].

To cope with these biotic and abiotic constraints, the use of door resistant grafts remains the most convenient and the most recommended way. The bitter orange is the most used rootstock in Morocco, due to its capacity to adapt to different types of soil, its resistance to limestone, its good affinity for grafting with almost all varieties of citrus and its good resistance to salinity and to *Phytophthora* sp. diseases, as well as the good quality of fruits [2,9,8,4,5,10,7].

Strengthening the resistance of the bitter orange is possible by the use of biotechnological techniques in the nursery which allows having *Citrus aurantium* L. seedlings more robust and also resistant to pathogenic organisms and water stress after transplantation. Several studies have indeed shown in other plant species that the mycorrhizal symbiosis allow more efficient uptake of water and nutrients, especially the phosphorus [11-14] and reduce the adverse effects of the biotic and abiotic environment constraints [15,16].

Growth of *Citrus aurantium* L. is dependent on mycorrhiza [17,18] The growth observed in *Citrus aurantium* L. could be explained by the ability of these fungi to colonize roots more rapidly and by improving the nutritional intake of plants, especially by increasing the minerals absorption [19-22] and the capacity of plants to explore more space in soil [23].

In this sense and to exploit the beneficial effects of endomycorrhizae, it is advisable to use in the citrus orchards vigorous rootstocks, previously mycorrhizal in nurseries. Thus, this work aims to study the effects of arbuscular mycorrhizal fungi (AMF) on growth parameters and root development of *Citrus aurantium* L. (bitter orange).

2. MATERIALS AND METHODS

2.1 Plant Material

Citrus aurantium L. seeds were disinfected with sodium hypochlorite; Seedlings are grown in plastic pots filled with a mixture of peat and sterile sand. Sterilization is performed in an oven at 250℃ for 2 hours to remove the soil micro flora. All pots were placed in a greenhouse until the stage of two true leaves, and regularly watered with tap water.

2.2 Inoculum Production

Barley (*Hordeum vulgare*), mycotrophic plant, was chosen for the production of a composite arbuscular mycorrhizal fungi inoculum. Barley grains were disinfected with sodium hypochlorite at 5% for 2 minutes, and put to germinate in plastic bowls filled with a mixture of disinfected sand and soil from the rhizosphere of a large number of species of the genus Citrus. The rhizospheric soil used contains 70 number of endomycorrhizal species.

After four weeks of culture, the frequency and intensity of mycorrhizal barley roots were estimated using the method of Hyman and Philips [24] and these mycorrhizal roots were used as endomycorrhizal inoculum.

2.3 Inoculation with Mycorrhizae

The plants of *Citrus aurantium* L. were transplanted in pots filled with disinfected sand of Mamora and containing fragments of barley mycorrhizal roots.

The pots are then placed in the greenhouse; Control and mycorrhizal plants were regularly watered with distilled water.

2.4 Experimental Set

The experimental protocol is carried out randomly; two batches of plants were performed with five plants for each batch.

Lot 1: Control plants (T) have not undergone any treatment
Lot 2: Plants inoculated with mycorrhizal fungi (Myco).

The pots were then placed in a greenhouse for seven months, June to December, at a temperature varying from 21 to 27℃.

After seven months of culture, the plants of *Citrus aurantium* L. were cut at the root collar. The roots were washed with tap water and dried on paper towels overnight under ambient laboratory conditions. The height of the vegetative part was measured. The fresh weight of the aerial part and root biomass were measured using a numerical scale. The diameter of the rod was measured with caliper scales and the number of branches on the vegetative part was counted.

2.5 Mycorrhizal Rates inside Roots

The roots observation was prepared according to the method of Philips and Haymann [24]. They were first washed with water; the finest roots were then cut into a length of 1 cm then immersed in a solution of 10% KOH (potassium hydroxide) and placed in the water bath at 90℃ for one hour to eliminate cytoplasmic contents. At the end of this period, roots were rinsed and transferred in a solution of H_2O_2 (hydrogen peroxide) for 20 min at 90℃ in the water bath until the roots became white. Roots were then rinsed, after this; they were dyed with cresyl blue, at 90℃ for 15 min.

2.6 Evaluation of Mycorrhizal Rate

After eight months of culture, evaluation of mycorrhizal parameters was conducted by observing thirty root fragments of about 1 cm, randomly chosen to quantify mycorrhizae [25,26,27]. The mycorrhizal frequency (F%), the mycorrhizal intensity (M%), the arbuscular content (TA) and the vesicular content (TV) of AMF inside the root bark are measured by assigning a mycorrhizal index ranging from 0 to 5 [28].

2.7 Spore's Extraction

The wet sieving method described by Gerdemann and Nicholson [29] was adopted to extract the spores from soil of *Citrus aurantium L*., the samples of rhizospheric soil were taken at random from each pot. Species identification is performed basing on macroscopic and microscopic observation of morphological characters and referring to the key determination defined by Schenk and Perez [30] and the website of INVAM.

2.8 Statistical Analysis

Statistical analyzes were performed by analysis of variance by the ANOVA test at the 5% level using the STATISTICA software.

3. RESULTS

After seven months of growth, inoculation of seedlings of *Citrus aurantium L.* with endomycorrhizal composite inoculum marked a positive effect (Fig. 1) on all measured parameters. (Table 1) A clear improvement in the height of the inoculated plants was recorded with a gain of more than 90.1%. Similarly, inoculating the plants is also effective on the leaves show, the formation of branches and growth of the collar diameter, the gains were respectively 266%, 300% and 300% (Fig. 2).

Mycorrhiza was also beneficial for the development of the root system and the vegetative mass. After seven months of growth, the weight of the root and the aerial mass of inoculated plants were higher than those of the control ones, respectively with an increase of 478% and 425%.

The mycorrhizal frequency was about 93.3% and the mycorrhizal intensity 50.6%. (Fig. 3). Moreover, different structures characterizing arbuscular endomycorrhizae were observed in inoculated roots: Arbuscules, vesicles and intra and extracellular hyphae.

The arbuscular and vesicular contents of inoculated roots were about 40.83% and 3.36% respectively % (Fig. 3). However, the roots of non-inoculated plants were not mycorrhizal. The number of spores was in the order of 132 spores / 100 g of soil. (Fig. 4).

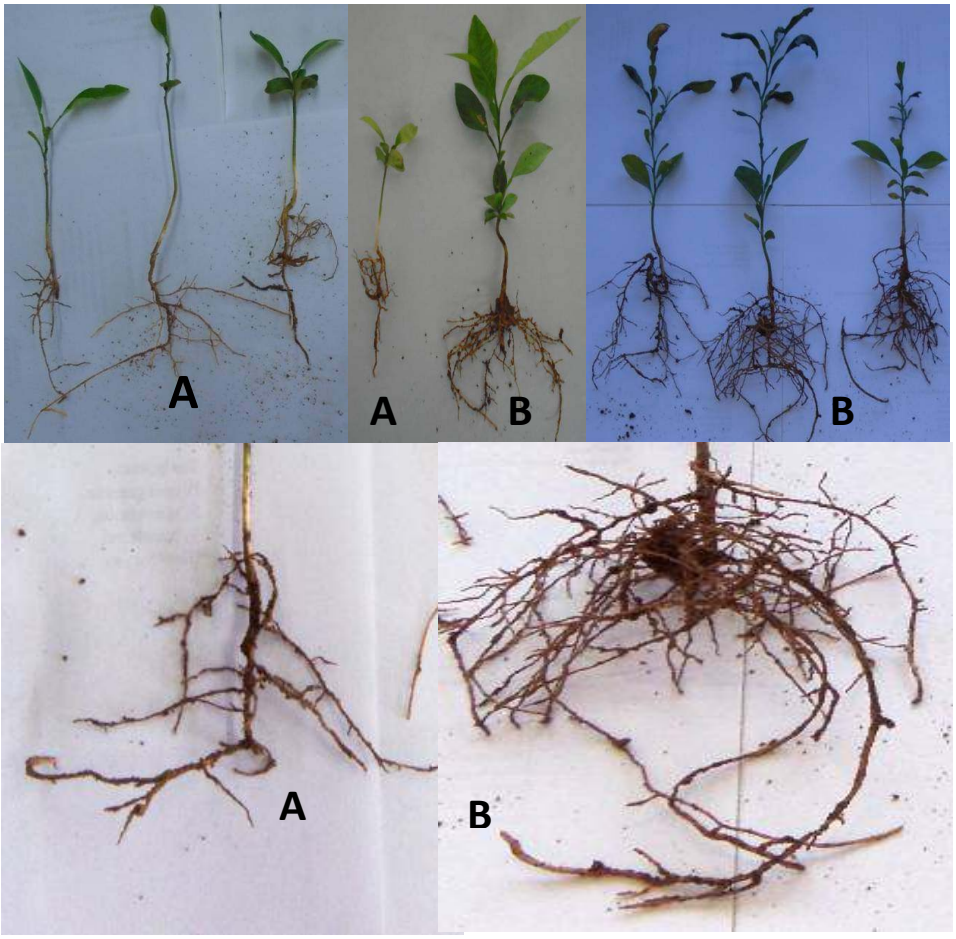

Fig. 1. Effect of endomycorrhizae on the growth of aerial and root parts of *Citrus aurantium* L. plants. Controls (A), Mycorrhizal plants (B)

Table 1. Agronomic parameters of *Citrus aurantium* L. after 7 months of inoculation with endomycorrhizal fungi

Agronomic parameters	Inoculated plants	Control plants
Root fresh weight (g)	2.43^{b}	0.42^{c}
Fresh weight of the vegetative parts (g)	2.1^{b}	0.40^{c}
Height (cm)	21.3^{a}	11.2^{a}
Diameter of the rod (cm)	0.4^{c}	0.1^{d}
Number of leaves	22^{a}	6^{b}
Number of branches	3^{b}	0^{d}

Preliminary identifications (Table 2) have allowed to note that isolated spores belong to 23 species: *Pacispora boliviana, Acaulospora colossica, Acaulospora* sp1*, Acaulospora koskei, Acaulospora* sp2*, Acaulospora laevis, Pacispora robiginia, Glomus aggregatum, Acaulospora* sp3*, Acaulospora foveta, Scutellospora* sp1*, Scutellospora castanea, Glomus deserticola, Gigaspora* sp*, Glomus spinuliferum, Glomus fecundisporum, Glomus intraradices, Scutellospora* sp2*, Glomus boreale, Glomus ambisporum, Glomus fecundisporum, Acaulospora gedanensis, Acaulospora nicolsonii.*

This species identification was based solely on the morphological characteristics, complementary studies based on molecular analyzes will be considered in future studies for an exact identification of the spores.

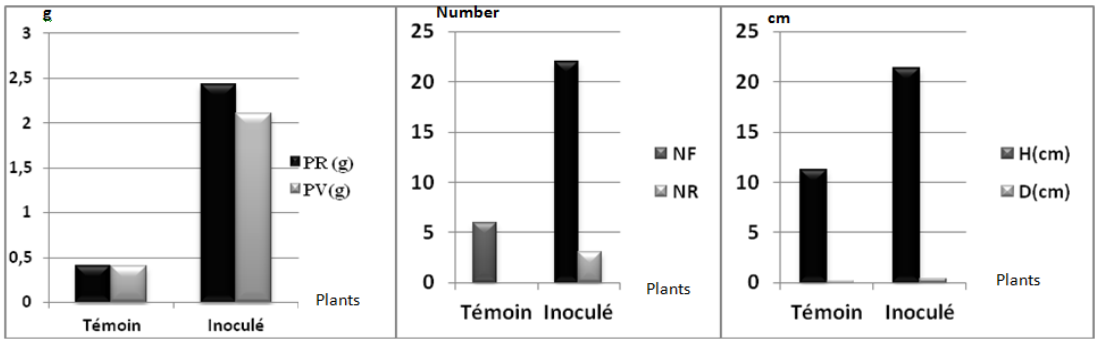

Fig. 2. Effect of mycorrhizal inoculation on RW: root weight, H: height, D: diameter, LN: leaf number, NB: number of branches, VW: vegetative weight

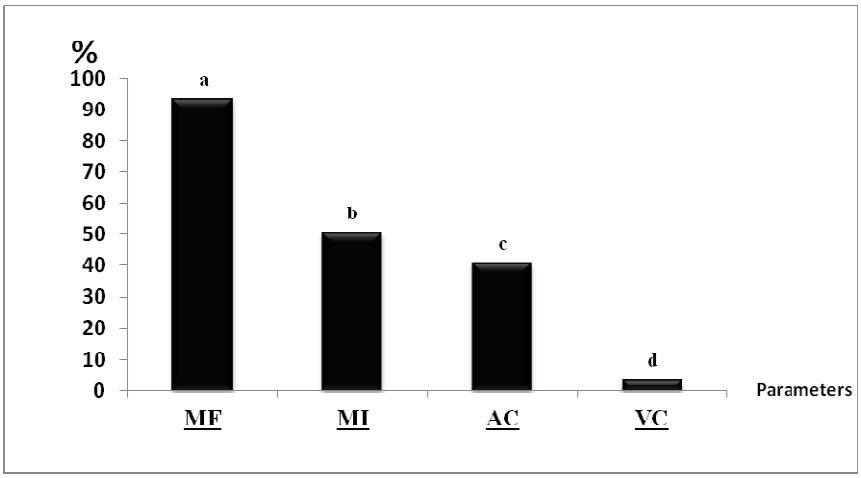

Fig. 3. Evaluation of the mycorrhizal rate of the inoculated roots of *Citrus aurantium* L. mycorrhizal frequency (MF), mycorhizal intensity (MI), arbuscular content (AC) and vesicular content (VC)

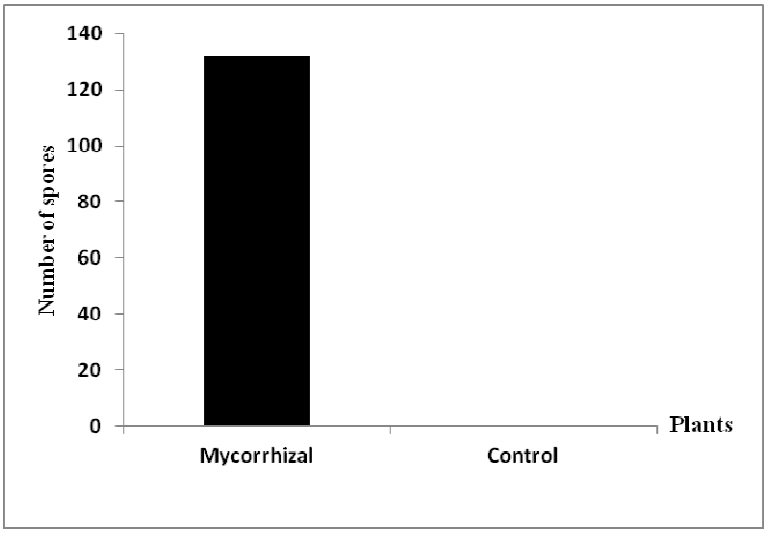

Fig. 4. Average spore density of AM fungi of mycorrhizal and control plants

Table 2. Identification of mycorrhizal fungi isolated from the rhizosphere of *Citrus aurantium* L.

Number	Name	Form	Color	Size	Wall thickness	Spore surface	Hyphe length
1	*P. boliviana*	globular	orange	70	2.5	granular	50
2	*A. colossica*	globular	mauve green	77.5	2.5	granular	-
3	*Acaulospora* sp1	globular	yellow	120	2.5	granular	-
4	*A. koskei*	globular	beige	107.5	7.5	smooth	-
5	*Acaulospora* sp2	globular	Yellow beige	105	7.5	smooth	-
6	*A. laevis*	globular	brown yellow	75	7.5	smooth	-
7	*P. robiginia*	globular	brown yellow	95	2.5	smooth	-
8	*G. aggregatum*	globular	brown yellow	80	7.5	smooth	-
9	*Acaulospora* sp 3	globular	yellow	72.5	2.5	smooth	-
10	*A. nicolsonii*	globular	yellow	112.5	7.5	smooth	83
11	*A. foveta*	globular	brown	50	12.5	granular	-
12	*Scutellospora* sp1	globular	beige	75	2.5	smooth	96.5
13	*G. deserticola*	globular	clear yellow	250	12.5	smooth	-
14	*S. castanea*	globular	Yellow brown	50	2.5	granular	-
15	*G. deserticola*	oval	clear yellow	112.5	7.5	smooth	50
16	*Gigaspora sp*	globular	clear Green	37.5	2.5	granular	-
17	*G. spinuliferum*	globular	Yellow darkened	112.5	7.5	smooth	50
18	*G.fecundisporum*	globular	brown yellow	200	10	smooth	-
19	*G. intraradices*	oval	Yellow darkened	60	7.5	smooth	-
20	*G. deserticola*	globular	Yellow orange	200	7.5	smooth	-
21	*G. intraradices*	globular	Yellow darkened	75	2.5	smooth	-
22	*Scutellospora* sp*2*	globular	brown	107.5	7.5	smooth	-
23	*G. boreale*	oval	brown yellow	62.8	2.5	smooth	23.6
24	*G. ambisporum*	globular	brown	70	7.5	smooth	83.2
25	*Glomus sp*	globular	clear yellow	75	7.5	smooth	-
26	*G. spinuliferum*	globular	Yellow darkened	112.5	7.5	smooth	-
27	*A. gedanensis*	globular	brown beige	115	7.5	smooth	-

According to the classification of Oehl and Sieverding [31], these species are distributed in 5 genera (*Glomus, Gigaspora, Acaulospora, Pacispora, Scutellospora*) and 5 families (*Glomaceae, Gigasporaceae,* et *Acaulosporaceae, Pacisporaceae, Scutellosporaceae,*) and 2 orders (*Glomerales, Diversisporales*).

Glomus intraradices, *Glomus deserticola*, *Acaulospora foveta*, *Glomus aggregatum* were respectively the most dominant species in the rhizosphere of the *Citrus aurantium* L. inoculated plants, their frequency of occurrence (Fig. 5) varied between 6.81% and 11.36%.

Similarly, the isolated species were represented by 38% of *Glomus*, 37% of *Acaulospora*, 13% of

Scutellospora, 8% and 4% of *Pacispora* and *Gigospora* (Fig. 6).

4. DISCUSSION

The surveys carried out in the rhizosphere of the inoculated *Citrus aurantium* L. plants showed that these roots contained endomycorrhizal structures: Vesicles, arbuscules, internal and external hyphae and endophytes. The presence of these structures characterizing endomycorrhizae allows to classify *Citrus aurantium* L. as a mycotrophic species [32-34].

The plants inoculated with the composite endomycorrhizal inoculum were clearly better compared to the control plants in terms of leave's number, stem diameter and plant heights which confirm the results of Lahlali [35], Syversten et Graham [36] and Menge J.A [37] The ability of AMF to stimulate growth was also confirmed by the positive influence on aerial and root biomasses, in studies of [18,11,12,13,38,39].

The individual AM fungal hyphae have much smaller diameters than roots, allowing access to narrower soil pores and hence increasing the soil volume explored [40] of a greater number of rootlets, which confirms that the AM fungi increase the rooting zone [41], then, increasing absorption of phosphorus that may perhaps cause proliferation and cell elongation [42] and thus, Phosphorus is translocated rapidly to the roots (probably as polyphosphate), overcoming the slow diffusion that occurs in the soil solution, these factors are the major cause of increased P uptake and positive AM growth responses [43]. However, new researches, using a combination of molecular and physiological approaches are required to find out this complex interplay between the two symbionts.

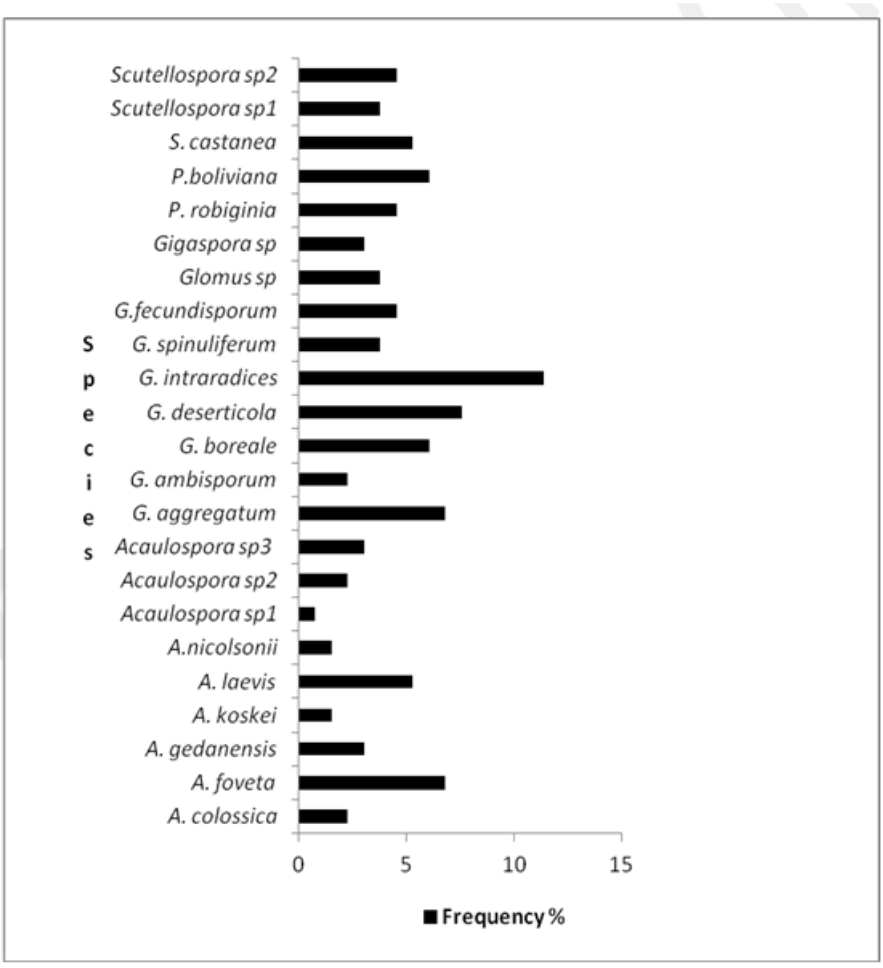

Fig. 5. Isolation frequency of mycorrhizal species from the rhizosphere of *Citrus aurantium*

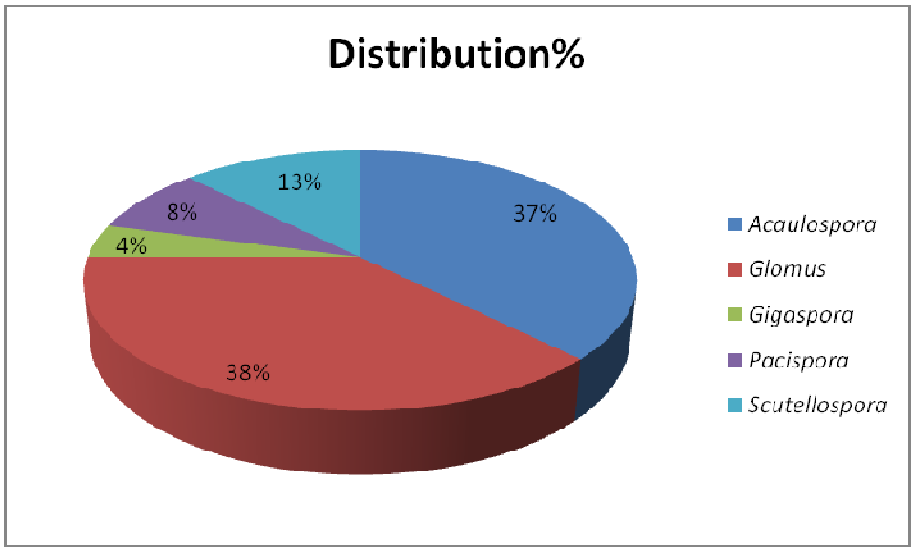

Fig. 6. Distribution of the isolated genera from the rhizosphere of *Citrus aurantium* L.

Indeed, endomycorrhizae allow the transport of the little mobile nutrients in the soil to the plant, especially, phosphorus [44,45] and improve drought tolerance [46], and induce in them morphological and physiological transformations to tolerate the environmental constraints [16].

The beneficial effect of endomycorrhizal fungi on the growth of *Citrus aurantium* L. has been demonstrated; this is in agreement with the works of Mwangi et al. [47] and Chliyeh et al. [48] they also demonstrated that the inoculation of tomato plants with AM fungi stimulated the weight and length of the aerial and root parts of these plants.

The AMF have a positive effect on the growth and development of the roots of *Lycium europaeum* plants [49], the olive tree [48,50], date palm tree [51-53] *Retama monosperma* [54], *Argania spinosa* L. [55], and Carob tree [56].

In this work, 23 species of (*Glomerales, Gigasporales, Acaulosporales, Diversisporales*) were detected in the rhizosphere of *Citrus aurantium* L. treated with endomycorrhizal composite inoculum constituted by these genera, however, 2 species of *Glomerales* have been reported in the rhizosphere of *Citrus aurantium* L. grown in the field, Artib et al. [32] and Wang [33] reported only 12 species in the citrus rhizosphere in southern China. In the rhizosphere of other plant species, Abbas et al. [57] reported 6 species of arbuscular mycorrhizal fungi (AMF) in seven Moroccan Tetraclines. Tellal et al. [58] noted 10 species in the rhizosphere of *Casuarina cunninghamiana* and *Casuarina. glauca* developing in 15 sites and two nurseries in Morocco. Bouamri et al. [59] and Sghir et al. [51] revealed 15 and 9 species in the Tafilalt and Zagora palm rhizosphere. In Central Europe, Oehl et al. [31] identified 12 species in the rhizosphere of the vine. In Jordan, Mohammad et al. [60], Kachkouch et al. [61] isolated 6 species and 5 genera respectively in the rhizosphere of the olive tree. Talbi et al. [62] reported 31 species in the Carob tree rhizosphere.

The count of the spores of the mycorrhizal fungi showed a dominance of the genus *Glomus*. This dominance was also found in the citrus rhizosphere [33] and in the rhizosphere of *Citrus aurantium* L. Artib et al. [32], the olive-tree rhizosphere [62] Oleaster [63], date palm [64].

Plants that develop AM symbioses can in most cases be colonized by AM fungi from different taxa [43], However, plant species can have preferences for individual AM fungi, resulting in different densities of colonization [65].

Colonization by different AM fungi does not result in the same growth responses in a single AM plant species [65] It is clear, therefore, that there is considerable functional diversity among plant-AM fungal symbioses in terms of benefits [43], thus, it is necessary, to obtain citrus plants more robust and resistant to different pathogens and stresses after transplantation, to select certain

species or a complex of native fungi, composed of several species, exhibiting high infectivity and good adaptation to different climatic and soil [32].

The root mycorrhizal frequency is very high in inoculated *Citrus aurantium* L. (93.33%) with an important spore number of 132/100 g of soil. However, previous results showed a mycorrhization frequency of (73%) and a number of spores of 48 per 100 g of soil [32].

Plants products in nurseries are provided with a few vigorous root systems and slightly structured and cannot, therefore, withstand biotic and abiotic stresses they face after transplantation. From this study, and after the production of a complex of native fungi, composed of several species, it can be concluded that the plants of *Citrus aurantium* L. inoculated with AMF present another way of promoting the growth of this plant which can be used as biological means to tolerate stresses. Indeed, its establishment is interesting for nurseries, since in the USA, inoculation with AMF of Citrus grown in nurseries has become a common practice [37].

5. CONCLUSION

From the whole of this study, it is clear that the endomycorrhization (AMF) has a beneficial effect on the growth parameters of *Citrus aurantium* L. plants. The positive effects obtained with the rootstock following the inoculation can therefore be explained by the great exploitation of the soil by the mycelium.

The rootstock inoculation by endomycorrhizal fungi has several advantages: Substantially shortens the development time of the plants, significantly reduces the application of chemical fertilizers, and has the advantages of providing quality products.

The increased performance of mycorrhizal plants in digesting food from the soil and resisting environmental stresses gives fungi symbiotes a role as bio fertilizers and crop protection agents. At the same time, adequate management of mycorrhizae in agricultural environments would extend the quality and sustainability of soils, protect the environment in the long term and reduce the production costs.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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