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ARTICLE

Diversity of arbuscular mycorrhizal fungi in the rhizosphere of saffron (*Crocus sativus*) plants along with age of plantation in Taliouine region in Morocco

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ABSTRACT Saffron cultivation is a viable alternative for marginal areas where low soil fertility and water availability severely limit the cultivation of other crops with higher water and input requirments. Under these conditions, arbuscular mycorrhizal fungi (AMF) are an essential alternative for maintaining fertility and water conservation, stimulating growth, and providing plant protection against soil-borne diseases. The aim of this work is to highlight the diversity of the arbuscular mycorrhizal fungi communities associated with saffron roots in plantations of different ages (two, four and ten years old) in the region of Taliouine (Morocco). The highest number of endomycorrhizal spores was recorded in the rhizosphere of saffron plants harvested at the level of plots that have carried saffron for two years (138.66/100 g soil), while the lowest number was observed in the rhizosphere of plants of plots that are occupied for 10 years by saffron. All collected spores from plots under study represent 17 morphotypes belonging to 5 genera: *Glomus* (7 species), *Acaulospora* (7 species), *Rhizophagus* (one species), *Densicitata* (one species), and *Funneliformis* (one species).

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Introduction

Saffron (*Crocus sativus* L.) is a very special crop that produces a valuable spice. It is the most expensive spice worldwide. In Morocco, the saffron sector represents a major issue for this local product in both social and economic terms. This sector is a promising way that may help to reduce inequalities in income in saffron areas as Taliouine and Taznakht. However, due to some ancestral cultural practices applied at the level of saffron fields, the lack of information about the behavior of the plant viz-a-viz the environment and the scarce knowledge of the potentialities of the local cultivated crop may constitute a handicap to the sector development.

Among many biotic and abiotic factors that disturb the growth and the yield of saffron plants, the ecological degradation of saffron fields related to plantation age turns out to be the most important (El Aymani et al. 2019). Generally, in Morocco, the cultivation of *C. sativus* L. is carried out in areas with low soil fertility, harboring rhizospheric microorganisms, such as endomycorrhizae that are supposed to be essential for plant growth and ecosystem functioning. Great saffron productivity would be achieved since the agricultural practices applied in field-grown saffron maintain the population of these microorganisms at a suitable level (Onguene 2000). Indeed, numerous studies have reported that soil fertility and productivity are highly correlated with the biological activity of soil microorganisms. The development of plants depends on the interactions they have with the surrounding environment, especially with soil microorganisms. Their importance in natural and semi-natural ecosystems (Sylvia et al. 1992) is to improve the absorption of water and nutrients such as phosphorus, nitrogen, and micronutrients, thereby improving plant growth and resistance to biotic and abiotic stresses (Goussous and Mohammad 2009; Lone et al. 2015).

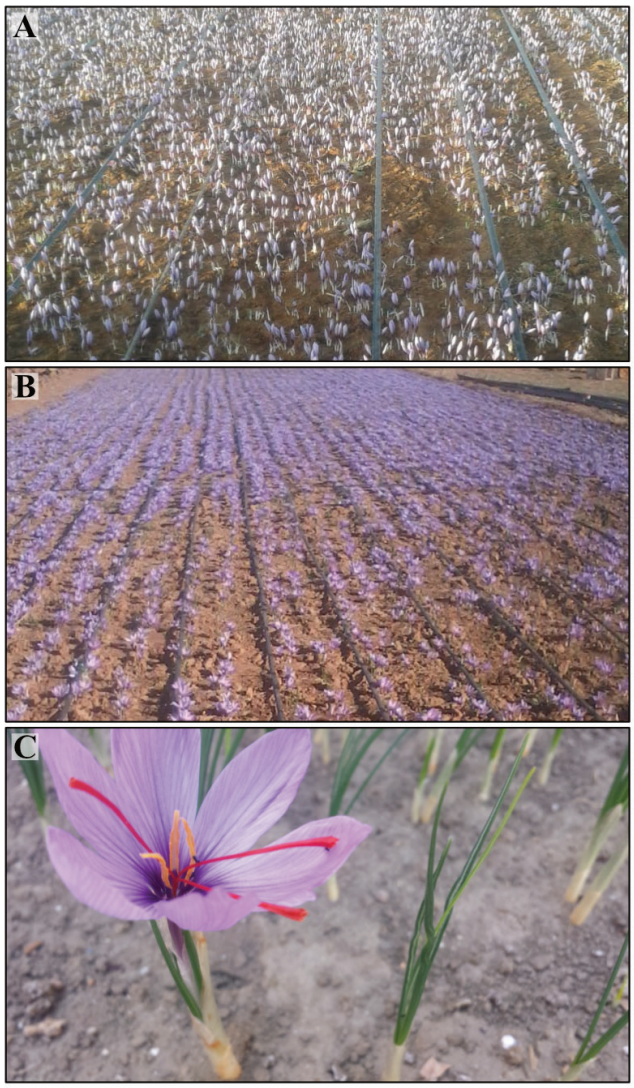

Figure 1. The fields of saffron in bloom. (A): Plot of 2 years of land use of saffron. (B): Plot of 4 years of land use of saffron. (C): Saffron plant at flowering stage or blooming and unflowering saffron plants.

Exploiting the beneficial effects of endomycorrhizae requires an appropriate knowledge regarding the diversity of arbuscular mycorrhizal fungi in areas of saffron crop in Morocco. The most exhaustive possible highlighting of this diversity needs the recruitment of more sites and samples of saffron plant rhizosphere for analysis. In this context, the present study ensures the continuation of the works already undertaken by El Aymani et al. (2019) and Chamkhi et al. (2018, 2019). Therefore, the aim was to assess the effect of the age of saffron plantations on the diversity of the populations of arbuscular mycorrhizal fungi. Such studies are expected to provide a broad overview of AMF communities existing at the rhizosphere level upon agronomic practices and should with time also lead to the selection of the best endomycorrhizal species colonizing the roots of saffron plants.

Materials and methods

Prospecting and sampling

The samples of rhizospheric soil and fine roots of saffron plants were collected in October 2020 from 15 different plots of saffron belonging to the Taliouine area (Morocco). Three lots of plots were selected, each one is characterized by the number of years (2, 4 or 10) of land use by saffron cultivation (Fig. 1). Soil samples were taken from the rhizosphere of plants from a depth of 0-25 cm. Very fine roots, likely to be mycorrhized and easily observable under the microscope, were also collected with the soil in sterilized polythene bags. Each lot of plots was represented by three composite samples after the samplings were homogenized.

Root clearing and staining

The roots were cleaned from soil particles by thorough rinsing with tap water in a sieve. Then only the smallest fine roots were selected. According to the lightening technique described by Philips and Hayman (1970), roots were cut into fragments of approximately 1 to 2 cm and placed in vials containing 10 ml of 10% potassium hydroxide solution. These flasks were then placed in a water bath at 90 °C for 15 min. The root fragments were then bleached by adding a few drops of H_2O_2 to the KOH solution. After 15 min, the fragments were rinsed with distilled water and then stained with a solution of cresyl blue (0.05%) for 15 min.

Assessment of AMF colonization

Evaluation of the mycorrhizal parameters was performed by observing thirty root fragments of about 1 cm, randomly chosen to quantify the mycorrhizae (Amir and Renard 2003; Kormanik and McGraw 1982). These fragments were mounted parallel in groups of 10 to 15 in a drop of glycerine water (8%) between blade and coverslip (Kormanik and McGraw 1982). Each fragment was thoroughly checked over its entire length, at magnifications of 100× and 400×.

The frequency and levels of arbuscules and vesicles of AMF within the root bark were measured by assigning a mycorrhizal index ranging from 0 to 5 (Derkowska et al. 2008): 0: absence; 1: traces; 2: less than 10%; 3: from 11 to 50%; 4: from 51 to 90%; 5: more than 91%.

Frequency of mycorrhization (F%):

$$F\% = 100 \times (N - n0) / N$$

Table 1. Parameters of mycorhization of saffron plant roots in studied sites.

Parameters of mycorhization	Site 2	Site 4	Site 10
Frequency (%)	95.20	88,23	60.40
Intensity of mycorrhization (%)	38.85	31.60	18.32
Arbuscules (%)	36.50	35.80	19.40
Number of spores/100 g soil	138.66	96, 00	71,00

where N is the number of fragments observed and n0 is the number of non-mycorrhizal fragments.

Intensity of mycorrhization (M%):

$$M\% = (95\ n5+70\ n4+30\ n3+5\ n2+n1) / N$$

Where n is the number of affected fragments of the index 0, 1, 2, 3, 4 or 5

Arbuscular content (A%):

$$A\% = (100\ mA3 + 50\ mA2 + 10\ mA1) / 100$$

where mA3, mA2 and mA1 are assigned with the notes A3, A2 and A1, respectively, with mA3 = (95 n5 A3 + 70 n4 A3 + 30 n3 A3 + 5 n2 A3+ n1 A3) / N, as the same for A1, A2.

In this formula, n5A3 represents the number of fragments noted with A3; n4A3 the number of fragments rated 4 with A3; etc.

A0: no arbuscules; A1: few arbuscules (10%); A2: moderately abundant arbuscules (50%); A3: very abundant arbuscules (100%).

Extraction of spores

The spores were extracted from 100 g of each rhizospheric soil sample using the humid sifting technique (Gerdemann and Nicolson 1963), then centrifuged in a sucrose solution (Daniels and Skipper 1982, as modified by Brundrett et al. 1996). After centrifugation (2000 rpm for 4 min), the supernatant was discarded, and a viscosity gradient was created by adding a solution of 50% sucrose into each centrifuge tube. After centrifugation at 5000 rpm for 10 min, the supernatant was poured onto sieve of 2 mm- 50 µm mesh screen. The resulting substrate was rinsed with distilled water to remove sucrose and recovered in an Erlenmeyer flask.

This content was observed with a binocular magnifying glass, by successive samples of small quantities. These aliquots were reversed on filter paper placed in a Petri dish and then observed using a binocular loupe. Microscopic observations of spores were made in a few drops of polyvinyl-lactic acid-glycerol (PVLG) and checked at magnifications of 100x and 400x. A preliminary identification at the genus level was made based on the criteria proposed by Ferrer and Herrera (1981), Berch and Koske (1986), Schenck and Smith (1982), Hall (1987), Schenck and Perez (1987), Morton and Benny (1990), Walker et al. (1982), Dalpé (1995), Pérez and Peroza (2013), Pérez et al. (2012), Monroy et al. (2013), Rodríguez-Morelos et al. (2014), Rajeshkumar et al. (2015), Błaszkowski et al. (2018), and information available in different databases.

Statistical analysis

The statistical treatment of results focused on the analysis of variance to a single criterion of classification (ANOVA).

Results

Study of the diversity of arbuscular mycorrhizal fungi (AMF) in the rhizosphere of *C. sativus* showed that overall roots of saffron plants were mycorrhized and showed the existence of typical structures of mycorrhizae including arbuscules, intra- and extracellular hyphae and spores (Fig. 1, 2, 3 and 4).

As shown in Table 1, the average frequencies of mycorrhization rate, which reflect the inoculum pressure or propagule rate infecting surrounding medium, varied among sites. Thus, the maximum frequency value was 95.20% at site 2 occupied during 2 years by saffron, while the lowest frequency value was 60.40% noted at the site exploited for 10 years. The highest mean percent mycorrhizal root colonization intensity expressing the percent of mycorrhizal root cortex attained 38.85% in roots of saffron plants growing in 2-year-old plantation, while in those from the 10-year-old plantation, the value was 18.32%. The arbuscular content of root at aged plantation site (10 years) was 19.4% and increased to 35.50% and 36.50% at plantation sites operated from four years and six years, respectively, whereas the vesicles were not found (Table 1).

The maximum spore richness (138.66/100 g soil) was observed at plantation site exploited for 2 years, followed by the 4-year-old plantation (96 spores/100 g soil) while the site operated for 10 years contained only 71 spores/100 g soil (Table 1).

Based on morphological criteria of spores (shape, colour, mean size, wall, surface) and hyphal size, 12, 10 and 7 morphotypes of arbuscular mycorrhizal fungi were distinguished in plots occupied along 2, 4 and 10 years by saffron, respectively (Tables 2 and 3; Figs. 1 and 2). The species *Acaulospora mellea*, *Glomus macrocarpum*, *Glomus versiforme* and *Rhizophagus intraradices* were common in three plots. *Acaulospora laevis* and *Densicitata nigra*

Table 2. Occurrence of mycorrhizal species in saffron plantation sites.

Mycorrhizal species	Ages of saffron plantation		
	10 years	4 years	2 years
Acaulospora mellea	+	+	+
Acaulospora foveta	+	+	-
Acaulospora laevis	-	+	+
Acaulospora scorbiculata	-	-	+
Acaulospora sp. 1	+	-	+
Acaulospora sp. 2		-	+
Acaulospora sp. 3	-	-	-
Glomus macrocarpum	+	+	+
Glomus microcarpum	+	-	+
Glomus versiforme	+	+	+
Glomus lamellosum	-	+	-
Glomus deserticola	-	+	-
Glomus sp. 1	-	-	+
Glomus sp. 2	-	+	-
Rhizophagus intraradices	+	+	+
Funneliformis geosporum	-	-	+
Densicitata nigra	-	+	+

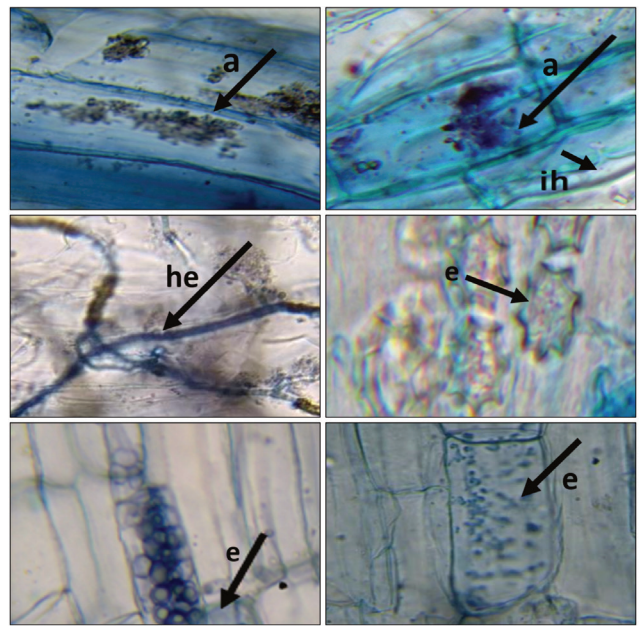

Figure 2. Crocus sativus roots with arbuscular mycorrhizal structures: arbuscles (a); intraductal hyphae (ih); external hyphae (he) and endophytes (e). (G. x 400).

were frequent in 2- and 4-year-old saffron plantations. *Glomus microcarpum* was reported in 2- and 10-year-old saffron plots.

These results show that the optimum of all mycorrhization parameters such as mycorrhization frequency and intensity, number of arbuscules and spores, as well as species richness of arbuscular mycorrhizal fungi was found at the site exploited by saffron for 2 years.

Discussion

Mophological examination of saffron under light microscope revealed the presence of AMF structures among the examined roots which showed different colonization levels. This suggests that *C. sativus* is a mycrotrophic species. In addition, the values of mycorrhization frequency and intensity and the arbuscular contents strongly differ versus age of the saffron plantation. The same observation was fulfilled for spore richness which appeared to be dependent on the age of the plantation. In agreement with our results, El Aymani et al. (2019) reported a smallest richness and mean number of spores in the rhizosphere of saffron plants from 10-year-old plantation with 27 spores/100 g soil and the highest density level at a 4-year-old site (45 spores/100 g soil). Conversely, in the same region, Chamkhi et al. (2019) signaled that the density of AMF spores in Taliouine saffron soils is significantly affected by the plantation age, it is higher in the soil of the oldest saffron plantation (169.33 spores/100 g soil). But these authors did not mention any significant effect of planting or fertilization methods on the density of AMF spores.

The increase of spore density with field age has been mentioned in the literature (Burni et al. 2011; Birhane et al. 2017). These authors related this to increasing soil organic matter and water capacity retention over time. Johnson et al. (1991) found a positive correlation between increased organic matter fraction including elements like carbon and azote and diversity of Glomales spores. Talbi et al. (2016) pointed out that each mycotrophic species may favor the proliferation and dominance of one or many species of endomycorrhizal fungi. The variation often observed can be related to the physicochemical and microbiological properties of the soil (Anderson et al. 1984; Johnson et al. 1991; Houngnandan et al. 2009), to the microclimate fluctuation (Koske 1987; Dalpé et al. 1989), to the vegetation coverage; (Benjamin et al. 1989) and the sampling season (Gemma et al.1989; Bouamri et al. 2006).

The composite endomycorrhizal inoculum containing 26 species monitored in the rhizosphere of olive plants by Semane et al. (2018) for 30 and 42 months revealed a distinct evolution of the initial inoculum numbers of species which were not able to sporulate versus time, while others, viz. four species of *Glomus* (*G. clarum*, *G. intraradices*, *G. mossea* and *G. versiforme*) have been able to maintain a stable sporulation rate in the rhizosphere

Table3. Morphological characteristics of endomycorrhizal species isolated from rhizospheric soil of different aged saffron plantations (2, 4 and 10 years).

Species	Shape of spores	Color of spores	Mean spore size (µm)	Wall surface	Hyphal length (µm)
Glomus microcarpum	Globular	brown	102.36	Granular	-
Acaulospora foveata	Globular	brown	112.36	Granular	-
Acaulospora sp. 1	Ovale	brown	104.2	Smooth	-
Acaulospora mellea	Ovale	yellow brown	115.36	Granular	-
Acaulospora laevis	Globular	brown	102.7	Granular	-
Glomus versiforme	Ovale	brown	104.2	Granular	-
Rhizophagus intraradices	Globular	yellow	91.1	Granular	-
Glomus macrocarpum	Ovale	brown	102.37	Smooth	-
Glomus deserticola	Globular	black	113.51	Granular	-
Densicitata nigra	Globular	brown	86.37	Smooth	-
Glomus sp. 1	Globular	brown	122.37	Granular	-
Glomus lamellosum	Globular	brown	114.14	Granular	-
Funneliformis geosporum	Globular	yellow brown	102.36	Smooth	-
Acaulospora scorbiculata	Globular	yellow	106.18	Smooth	-
Glomus sp. 2	Globular	brown	107.9	Smooth	25.6
Acaulospora sp. 2	Globular	yellow brown	128.36	Granular	-
Acaulospora sp. 3	Globular	brown	124.2	Granular	-

of olive plants. Yang et al. (2010) reported that AMF soil community evolved in the function of environmental conditions and better-adapted AM fungi appeared to replace less-adapted AM fungi as environmental conditions changed.

Studies on AMF linked to the roots and rhizosphere of *C. sativus* plants in saffron-producing countries are still rare. Lone et al. (2016) signaled that the early studies on the AM-saffron fungal association focused on the seasonal variation of spore density in the rhizosphere of saffron plants in Iran (Kianmehr 1981). Zare Maivan and Nakhaei (2000) noted the dominance of *Acaulospora morrowiae* and *Glomus coronatum* among endomycorrhizal species associated with the rhizosphere of three saffron cultivars that grow in the Irano-Turani climatic region. Mohebi-Anabat et al. (2015) found three species belonging to the *Glomus* genus in northeastern Iran (*G. aggregatum*, *G. mosseae* and *G. etunicatum*). In the present paper, the AMF richness of saffron plant rhizosphere in the Taliouine zone was reflected by a total of 17 species making up *Acaulospora mellea*, *A. laevis*, *A. scorbiculata*, *A.* sp. 1, *A.* sp. 2, *Glomus macrocarpum*, *G. microcarpum*, *G.* sp. 1., *G. versiforme*, *Rhizophagus intraradices*, *Funneliformis geosporum*, *A. foveata*, *G.* sp. 2, *G. deserticola*, *G. lamellosum*, *Densicitata nigra* and *Acaulospora* sp. 2. The highest number was 12 species recovered from the rhizosphere of saffron plants originating from 2-year-occupied plots against 7 species in 10-year-occupied plots. The species *A. mellea*, *G. macrocarpum*, *G. versiforme* and *Rhizophagus intraradices* were most common at three lots of plots exploited by saffron for 2, 4 and 10 years, while the most predominant species in saffron-grown fields of 2 and 4 years were *A. laevis* and *Densicitata nigra*. Saffron plots operated for 2 and 10 years shared *G. microcarpum*. There is a difference in the composition and the species richness of the AM fungal community of saffron plants within the same sampling region in comparison with the research work of Chamhki et al. (2019), who reported the occurrence of 10 AMF species in the soil of saffron grown fiels in Taliouine, viz *Glomus tenebrosum*, *G. reticulatum*, *Septoglomus deserticola*, *Sclerocystis taiwanensis*, *Rhizoglomus aggregatum*, *R. intraradices*, *Funneliformis coronatus, Enterophospora infrequens*, *Acaulospora* sp. and *Funneliformis* sp. with the predominance of Glomeraceae family species.

In 5 sites of the same zone, El Aymani et al. (2019) noted the presence of diversified AMF community making up 36 identified species. According to these authors, this diversity varied among sites and increased in the site in four successive years of exploitation by saffron (24 species), which registered the highest Shannon diversity index (H' = 2.82). A regression Shannon index was noted at the sites of six or ten years of occupation by saffron.

According to Fuji et al. (1991), saffron is among the plants that are recognized as strong allelopathic plants. Variations in the AMF population structure among studied plots are likely related to the accumulation of allelopathic substances in the soil. Indeed, Pellissier and Trosset (1989) have reported the negative effect of *Molinia caerulea* on AMF. Afzal et al. (2000) demonstrated that aqueous shoot extracts of *Imperata cylindrica*, an allelopathic herb, reduced

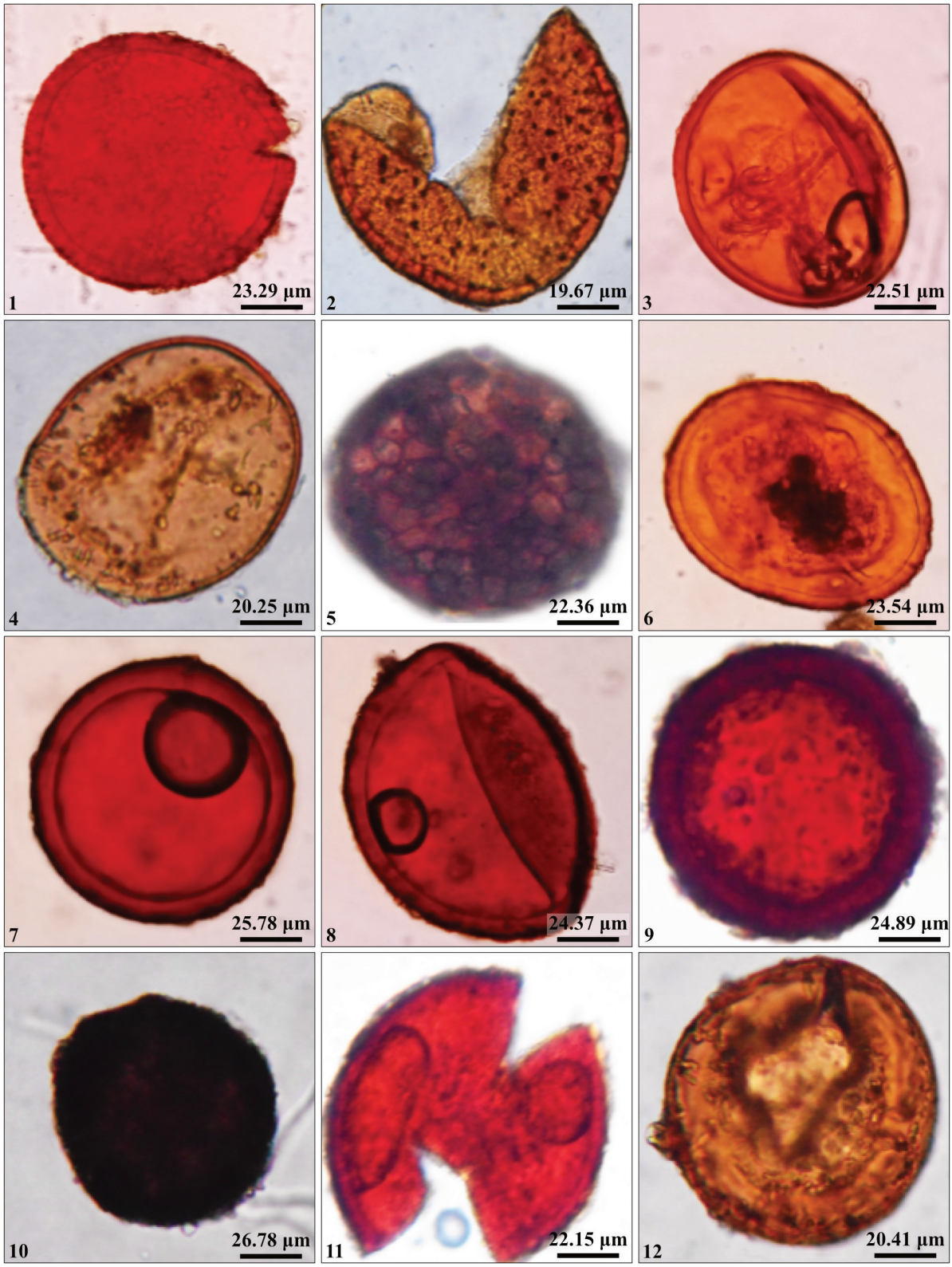

Figure 3. Morphology of some AMF species recovered from the rhizospheric soil of saffron plantations. (1): *Glomus microcarpum*; (2): *Acaulospora foveata*; (3): *Acaulospora* sp. 1; (4): *Acaulospora mellea*; (5): *Acaulospora laevis*; (6): *Glomus versiforme*; (7): *Rhizophagus intraradices*; (8): *Glomus macrocarpum*; (9): *Glomus deserticola*; (10): *Densicitata nigra*; (11): *Glomus* sp. 1; (12): *Glomus lamellosum*.

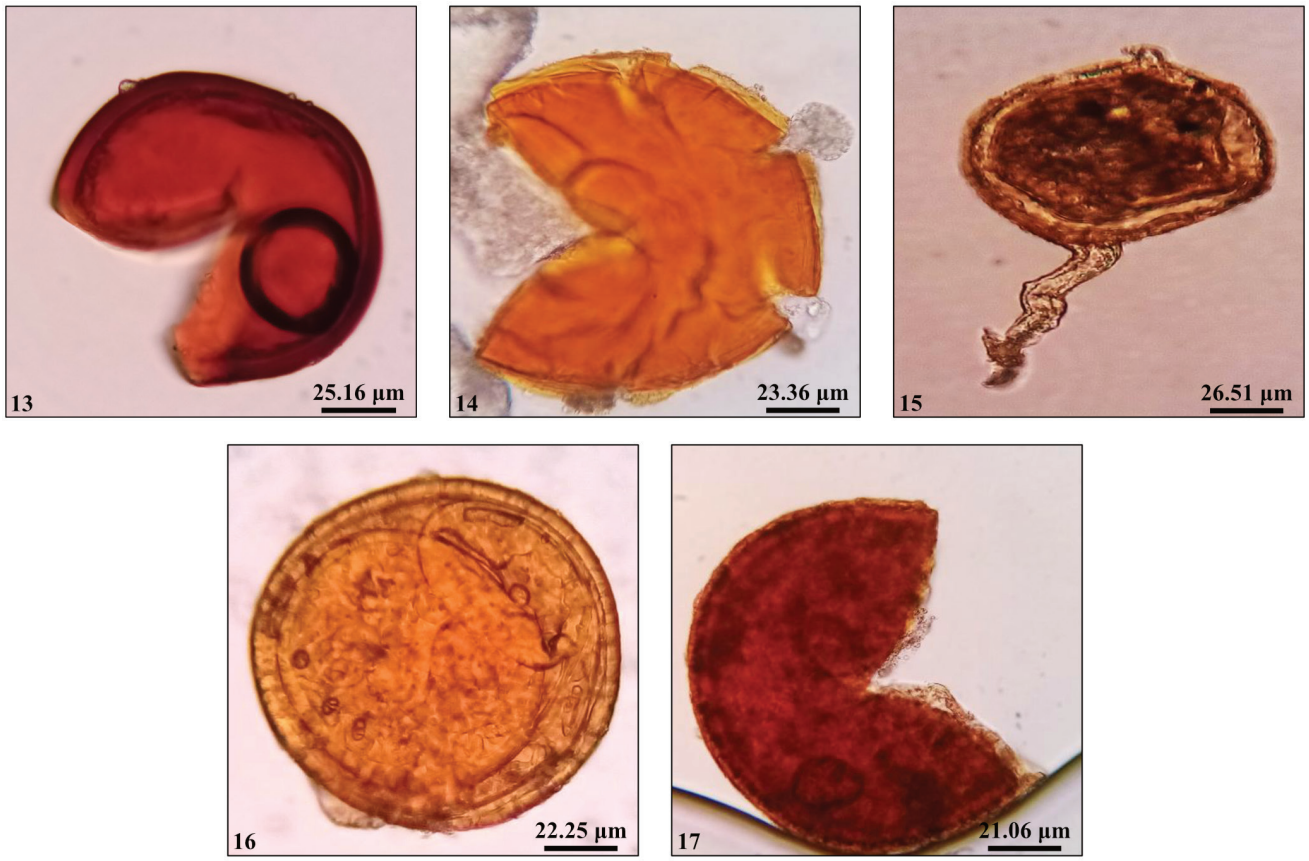

Figure 4: Morphology of some AMF species recovered from the rhizospheric soil of saffron plantations. (13): *Funneliformis geosporum*; (14): *Acaulospora scorbiculata*; (15): *Glomus* sp. 2; (16): *Acaulospora* sp. 2; (17): *Acaulospora* sp. 3.

root endomycorrhizal colonization in *Vigna radiata* (L.) Wlczek and *Phaseolus vulgaris*.

The influence of cultural practices on the populations of rhizospheric microorganisms of saffron plants is not yet well known. Jalali (1962) stated that after one period of cultivation, saffron cannot be cultivated in the same soil. Other researchers found that the continuous saffron cultivation induces some undesirable changes in soil chemical and physical properties (Khozaei et al. 2015). These changes can play an important role in decreasing the saffron yields even after 6 years of cultivation (Khozaei et al. 2015). Azizi Zohan and Sepaskhah (2002) affirmed that the unsuccessful saffron cultivation after one cultivation period can be due to allelopathic effects or accumulation of special salts in the root zone. Qarai and Beiji (1995) noted that the yield decreases with the age of the saffron fields, afterwards the plot becomes unsuitable for cultivation. Following these authors, the probable reason for this diminution can be a change in physicochemical and biochemical properties of soil or the major change in the population of soil microorganisms. Sharif and Moawad (2006) noted that the diversity of AMF species in agricultural systems is highly affected by the types of input. For instance, the effect of the tissue extract of one plant species on the growth or reproduction of another species has been observed in numerous cases (Hoseini and Rizvi 2003; Jadhav et al. 1997; Kobayashi 2004). Jansa et al. (2014) have found that soil properties such as pH, soil fertility and texture, as well as site geography, especially the altitude strongly affected AMF community profiles.

The VA mycorhizae receive increasing attention during the last decades for their potential role in improving agricultural yield of economic crops (Mosse and Hayman 1971; Powell and Daniel 1978; Powell 1979; Kormanik et al. 1982; Fontana 1985; Strullu 1990). Indeed, these fungi are essential components of soil-plant systems (Smith and Read 2008; Van Der Heijden et al. (1998), they improved phosphorus assimilation, micronutrients (Bürkert and Robson 1994) and azote (Barea et al. 1991). They improved water nutrition and provided a greater tolerance to abiotic constraints to different host plants (Smith and Read 2008; Campagnac et al. 2010; Miransari 2010), like drought (Dalpé 2005; Gianinazzi et al. 2010; Pozo et al. 2013), organic pollutants and heavy metals (Joner

and Leyval 2001). The potential of AMF as biocontrol agents has also been proved for various root infections (Khaosaad et al. 2007; Fiorilli et al. 2011). The optimization of the symbiotic potential of the endomycorrhizal species taking place in the studied sites deems necessary to improve the productivity of the saffron culture and reduce the damage caused by fungal telluric pathogens. These symbionts will be able to increase the agronomic efficiency of phosphates and the resistance of the plants to different types of biotic and abiotic stresses. In this sense, it is very important to give a great importance to the multiplication and the production of a composite endomycorrhizal inoculum based on all the met species that will serve for the treatment of the corms of saffron destined to the culture. Indeed, according to Deirdre et al. (2009), arbuscular mycorrhizal fungi represent one of the key groups to ensure sustainable productivity of this agricultural system.

Through this study we can confirm the diversity of indigenous AMF community associated with saffron plants grown in Taliouine, the primary area of saffron production in Morocco and to underline the variation of AMF community structure versus age of saffron plantation. Therefore, it is time to explore the efficacy of different AMF species and identify the most effective association for developing a composite endomycorrhizal inoculum as a more suitable and environmentally acceptable alternative enabling to preserve, improve yield and protect saffron plants from telluric bio-agressors. It also may help to formulate, based on this inoculum, a biostimulant and biofungicide used in new agricultural practices for a sustainable agriculture less dependent on chemical inputs.

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