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RESEARCH ARTICLE Agricultural Science Digest

Assessment of Mycorrhizal Fungal Diversity in Legumes (*Medicago sativa*, *Medicago truncatula* and *Trifolium rubens*) from Algeria and Their Influence on Soil Physicochemical and Microbiological Properties

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10.18805/ag.DF-586

ABSTRACT

Background: *Medicago sativa*, *Medicago truncatula* and *Trifolium rubens* are legumes widely distributed in Algeria. These species hold ecological and agricultural significance and serve as a natural resource for combating desertification and as livestock fodder.

Methods: Comparative investigations of arbuscular mycorrhizal fungi (AMF) colonization in the roots of these leguminous species were conducted. The physicochemical and microbiological attributes of AMF's infectious potential were explored for all three species. The presence or absence of endomycorrhizal structures was assessed in these species.

Result: The mycorrhizal infectious potential of the flora was significantly enhanced in the case of *Medicago truncatula* when compared to *Trifolium rubens*. Mycorrhization occurred at a frequency exceeding 80% in all three species. The impact of legume mycorrhizal fungi colonization on soil physicochemical properties was examined, revealing alterations in soil biological fertility, particularly in terms of phosphate and nitrogen content. *Medicago truncatula* exhibited a more pronounced positive influence on soil physical, chemical and microbiological characteristics when compared to *Medicago sativa* and *Trifolium rubens*. Consequently, these herbaceous species can be employed as nurse plants (facilitators) or as bio-fertilizers.

Key words: Fertilization, Legumes, Mycorrhizae, Mycorrhizal infectious potential, Soil.

INTRODUCTION

In terrestrial ecosystems, the majority of plants engage in symbiotic relationships with microorganisms, such as bacteria and fungi, with a particular emphasis on mycorrhizae, also referred to as "symbiosomes" (Brundrett, 2002; Chen *et al*., 2018; Wang *et al*., 2019; Milton *et al*., 2021). Mycorrhizae represent specialized organs formed through intricate interactions between higher plants and root-associated fungi (Fortin *et al*., 2016; Kaur and Garg, 2018; De León *et al*., 2018). These associations are prevalent in nature, with approximately 90% of terrestrial plants participating in mycorrhizal relationships (Smith and Read, 2008). These partnerships exhibit considerable diversity depending on the specific organisms involved, with arbuscular mycorrhizal fungi (AMFs) being the most common (Harrier and Watson, 2004). AMFs play a pivotal role, constituting a significant portion of soil microbial biomass while depending on the plant host for photosynthates and reciprocally providing essential nutrients, especially phosphorus (Cardoso and Kuyper, 2006; Strullu-Derrien, 2007; Ramasamy *et al*., 2020).

The Fabaceae family, commonly referred to as legumes, possess a distinctive ability to fix atmospheric nitrogen through symbiotic associations with rhizobium bacteria in specialized nodules on their roots (Giraud, 2007; Firoz *et al*., 2023). Assessing the mycorrhizal infectious potential (MIP) of soils is a crucial undertaking, as it represents the soil's capacity to initiate mycorrhizal associations from existing inoculum (Duponnois *et al*., 2001). In this study, we address

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How to cite this article: BENELHADJ DJELLOUL, S., NEHAL, F., M'HAMEDI, I., KADIRI, A. and BOUCHAKOUR, M. (2023). Assessment of Mycorrhizal Fungal Diversity in Legumes (*Medicago sativa*, *Medicago truncatula* and *Trifolium rubens*) from Algeria and Their Influence on Soil Physicochemical and Microbiological Properties. Agricultural Science Digest. DOI: 10.18805/ag.DF-586

Submitted: 07-10-2023 **Accepted:** 15-12-2023 **Online:26-12-2023**

this fundamental aspect by evaluating the soil's physicochemical attributes concerning three legume species: *Medicago sativa, Medicago truncatula and Trifolium rubens*, situated within the Chlef region of Algeria. By focusing on these three specific plant species, we aim to establish a comprehensive foundation for the identification of highly mycotrophic plant species that can be employed in soil rehabilitation and ecological restoration efforts. This investigation underscores the significance of these legumes in harnessing mycorrhizal associations for the enhancement of soil health and the promotion of sustainable land management practices in ecosystems where such improvements are critically needed.

MATERIALS AND METHODS

Plant and soil sampling

Plant samples were meticulously collected in February 2021 from the Ouled Fares region of Chlef, Algeria. The study focused on three plant species: *Medicago sativa*, *Medicago truncatula* and *Trifolium rubens*. For each plant species, five root samples were procured from the vicinity of the plants, specifically at a soil depth ranging from 20 cm to 30 cm. The careful extraction of these fine roots was executed. Concomitantly, five soil samples were extracted from the rhizospheres of each plant.

Physico-chemical characterization of soils

The physico-chemical analysis of rhizospheric soils associated with the three plant species, was conducted within the laboratory setting, employing automated techniques by the FERTIAL Laboratory of Oran.

Determination of soil mycorrhizal infectious potential

The Mycorrhizal Infectious Potential (MIP) of soil signifies its capability to initiate mycorrhizal associations from an inoculum consisting of spores, mycelium, or root debris bearing vesicles naturally present in the soil (Plenchette *et al*., 1989).

To assess the MIP, ten seeds of sorghum (*Sorghum bicolor* L.), a highly mycotrophic plant, were sown in 150 ml pots containing 100 g of soil of the three, species *Medicago sativa, Medicago truncatula and Trifolium rubens*, at 6 varying dilutions ranging from 3% to 100% prepared as a mixture of non-sterilized and autoclaved (120°C for 20 min) soil, as detailed in Table 1. Seeds were watered daily with sterilized distilled water.

Within a two-week period of cultivation, the entire root system of each plant was meticulously harvested for Mycorrhizal Infectious Potential (MIP) determination using the methodology described by (Philippe and Hayman, 1970).

A root system showing at least one infection point, indicating the penetration of hyphae into the root, was considered as mycorrhized. Linear regression models (Y = aX + b) were calculated for each soil sample and the percentage of mycorrhizal plants was plotted against the unsterilized soil quantity logarithm. The results were subsequently expressed as the percentage of mycorrhized plants per pot. Soil infectivity was expressed as Mycorrhizal Infectious Potential (MIP) units 100 g soil. An MIP unit is defined as the minimum dry weight (g) of soil required to infect 50% (MIP 5o) (Duponnois *et al*., 2001).

Estimation of natural root colonization degree

For each species (*Medicago sativa*, *Medicago trancatula* and *Trifolium rubens*), three randomly chosen plants root samples were harvested then colored as described previously (Phillips and Hayman 1970). They were washed with water, cut in about 1 cm fragments, cleared in 10% KOH solution for 45 min at 90°C then placed for 10 min in lactic acid at room temperature to eliminate the KOH. Root samples were then colored by Trypan Blue, during 20 min at 90°C. The excess of the coloring agent was removed by adding glycerin. The colonization frequency and intensity were calculated as previously described (Trouvelot *et al*. 1986).

Statistical analysis

The obtained results were analyzed using multivariate analysis of variance (ANOVA) at a significance level of 5% and post-hoc means comparison was executed using the Tukey test, using Statistica 7.1 software.

In addition, the Principal Component Analysis (PCA) method was applied to discern and elucidate the interrelationships among the various parameters under investigation.

RESULTS AND DISCUSSION

Physico-chemical characteristics of soils

Physicochemical characteristics of *Medicogo sativa*, *Medicago truncatula* and *Trifolium rubens* rhizospheric soil samples were studied (Table 2).

Particle size characterizations indicated that different soils showed a sandy texture (more than 82%) with very little clay (3.33-5.33%) and silt (9-12%). The three soils samples displayed an alkaline pH with values varying from 8.42 to 8.49 (Table 2). The studied soils expressed total limestone contents ranging from 7 to 12%. The highest content (11.58%) corresponded to *Medicogo sativa*, followed by *Trifolium rubens* (9.38%) and *Medicago truncatula* (7.73%) (Table 2).

Soil organic matter is made up of living and decaying organisms such as plants, animals and microorganisms. It represents generally 0.5 to 10% of the soil. The rhizospheric soils of the species *Medicago truncatula*, *Medicago sativa*

Table 1: Soil dilutions with varied proportions of sterilized and non-sterilized soils.

Dilution	1	2	3	4	5	6
Non-sterilized soil (g)	3	6	12	24	48	100
Sterilized soil (g)	97	94	88	76	52	0

and *Trifolium rubens* organic matter contents were 5.71%, 8.03% and 9.52%, respectively. Carbon and nitrogen are a major product of various organic compounds, such as bacteria and fungi decaying organisms, mineralization. Interestingly, *Medicogo sativa* rhizospheric soil carbone content was higher (5.54%), compared to *Trifolium rubens* (4.67%) and *Medicago truncatula* (3.32%). However, soil with the lowest carbon content (*Medicago truncatula*) displayed the highest nitrogen rate (0.36%) and the other soils contained less nitrogen (0.18-0.2%) (Table 2). Phosphorus is one of the major elements essential for the growth and development of plants. It plays an critical role in the establishment of the root system, photosynthesis and plant reproduction. Studied soils phosphorus contents were similar for *Medicago sativa* and *Trifolium rubens* and higher (0.98 ppm) for *Medicago truncatula* soil (Table 2).

Soil cationic exchange capacity represents the reservoir size enabling the reversible storage of certain cationic fertilizing elements such as potassium, magnesium and calcium.These cations can be weak acids or strong acids, depending on the pH. For the three rhizospheric soils, calcium (Ca) content was greater than 20 mg/l, potassium (K) between 18 and 22 mg/l, magnesium (Mg) between 24 and 31 mg/l and natrium (Na) varies from 10 to 12 mg/l (Table 2).

Medicago trucatula significantly improves soil tenure in terms of total carbon, organic matter, total nitrogen and available phosphoore compared to *Medicago sativa* and *Trifolium rubens.*

Soil physicochemical characteristics were plant depending suggesting *Medicogo sativa*, *Medicago trucatula* and *Trifolium rubens* growth influence on soil parameters. Likewise, changes in the edaphic parameters were observed following a symbiotic associations plant-microorganisms (Hodge *et al*., 2001; Chen *et al*., 2018).

Our results agreed with Baize and Jabiol (1995) pedological reference, indicating that the plant species *Medicogo sativa*, *Medicago trucatula* and *Trifolium rubens* rhizospheric soils had an alkaline pH. This parameter is one of the most important indicators of soil quality (Liu *et al*., 2006). In arid and semi-arid environments like that of the region of our study (the Chlef ecosystem, Algeria), pH can be strongly influenced by climate and vegetation (Smith *et al*., 2000). It increase is mainly due to low leaching given the low rainfall characterizing these regions (Wezel *et al*., 2000). The studied plants were growing in a sandy soil. Such a sandy texture is the sign of a well aerated soil while too much clay is indicative of an impermeable and poorly ventilated environment, thus forming an obstacle to the penetration of roots. The texture greatly influences the chemical composition of sandy soils (Koske and Halvorson, 1981). These soils are generally poor in nitrogen and available phosphorus (Brundrett, 1991). mycorrhizal plants can however improve soil fertility (Fall *et al*., 2022).

Soil organic matter content is usually influenced by climatic factors, vegetation, soil texture, topographic

Table 2: Physico-chemical analysis of soil samples from *Medicago sativa*, *Medicago trancatula* and *Trifolium rubens* species.

Soil	Particle size (%)			Conductivity (1/5 mS/cm)	pH	Total carbone (%)	Total limestone (%)	Organic matter (%)	Total nitrogen (%)	Available phosphorus (ppm)	Exchangeable cations (mg/l)			
	Clay	Silt	Sand								Ca	K	Mg	Na
MT	4±0^{a}	9.33±2.06^{a}	84.6±2.73^{a}	0. 18±0.02^{a}	8.49±0.04^{a}	3.32±0.24^{b}	7.73±0.84^{a}	5.71±0.41^{b}	0.365±0.01^{b}	0.98±0.01^{b}	21.57±2.16^{a}	22.48±2.28^{a}	24.83±5.85	11.14±0.98^{a}
MS	3.33±1.03^{a}	9.33±2.06^{a}	185.33±4.13^{a}	0.18±0.02^{a}	8.46±0.08^{a}	5.54±0.15^{a}	11.52±0.21^{a}	9.52±0.25^{a}	0.2±0.04^{a}	0.70±0.07^{a}	22.72±0.33^{a}	18.57±1.02^{a}	30.52±0.95^{a}	11.89±0.41^{a}
TR	5.33±2.06^{a}	12±0^{a}	82.66±5.46^{a}	0.14±0.03^{a}	8.42±0.05^{a}	4,67±0.72^{a}	9.38±2.97^{a}	8,03±1.23^{a}	0.18±0.02^{a}	0.698±0.12^{a}	20.57±0.66^{a}	20.94±3.39^{a}	26.28±1.59^{a}	10.98±0.03^{a}
SEm±	0.471	0.94	1.50	0.009	0.021	0.16	0.63	0.25	0.011	0.03	0.47	0.86	1.25	0.22
CD (p≤0.05)	1.39	2.78	2.12	0.028	0.063	0.467	1.86	0.76	0.03	0.087	1.38	2.54	3.70	0.66

MT- *Medicago trucatula;* MS- *Medicogo sativa;* TR- *Trifolium rubens*; Ca- calcium; K- potassium; Mg- magnesium; Na- sodium;

Data in the same column followed by the same letter are not significantly different according to Tukey test $p<0.05$.

conditions, drainage and cultivation practices (Drouet, 2010). Studies legumes' soils can be considered as very rich in organic matter with contents higher than 5.7%. The richness of the soil in organic matter is increased by the renewal of roots and leaves and by litter decomposition in addition to organic nitrogen decomposition enhanced by arbuscular mycorrhizal fungi (Hamel and Plenchette, 2017).

Mycorrhizal infectious potential (MIP)

The MIP50, is non-sterilized soil quantity of required to mycorrhize 50% of plants, varied among the three soils. *Medicago truncatula* significantly improved soil MIP50 with a value of (23.80) compared to *Trifolium rubens* and *Medicago sativa* which had (21.75) (20.28) respectively (Table 3).

Terrestrial microbial biodiversity is recognized as a potential biofertilizer with the capacity to enhance soil fertility and support plant performance under environmental stresses (Gentili and Jumpponen, 2006). Mycorrhizal fungi, in particular, are regarded as vital biological agents for soil restoration, promoting plant growth, aiding in water and mineral nutrient uptake and contributing to plant protection (Duponnois *et al*., 2005; Cardoso and Kuyper, 2006).

Plants with a high mycorrhizal dependency play a pivotal role in fostering fungal proliferation, consequently elevating the mycorrhizal infectious potential of the soil (Sellal *et al*., 2021). Leguminous plants are generally classified as hyper-mycotrophic species capable of stimulating the multiplication of fungal symbionts and enhancing the MIP of the soil (Duponnois *et al*., 2013).

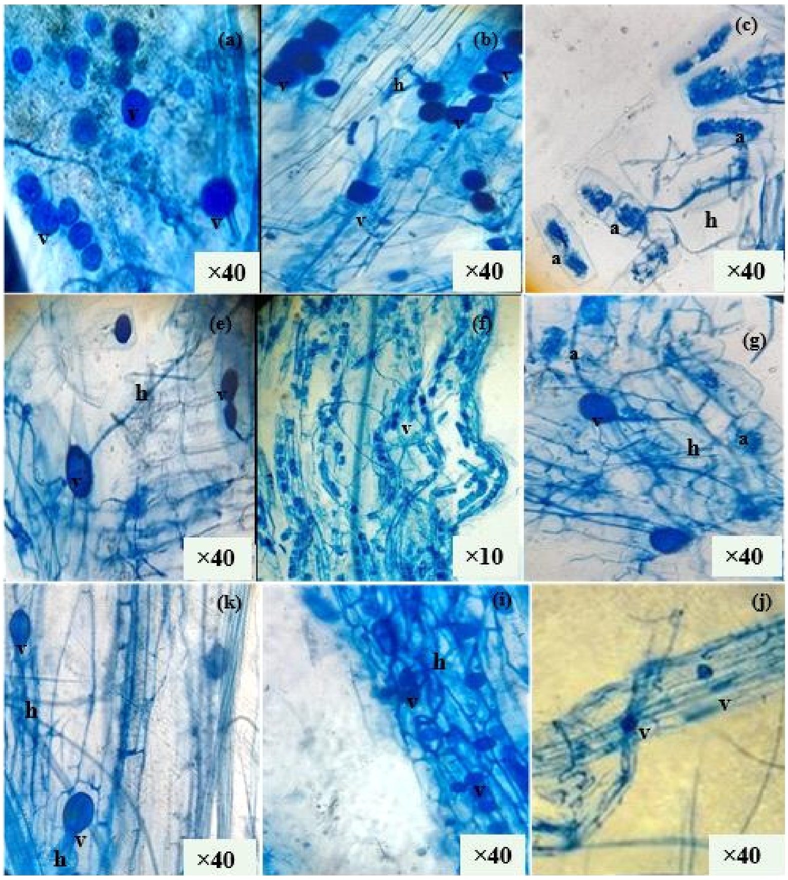
Root colonization

Microscopic observation of mycorrhizal forms

The roots of *Medicogo sativa*, *Medicago atrucatula* and *Trifolium rubens* underwent treatment following the procedure outlined by Phillips and Hayman (1970). Microscopic examinations unveiled the presence of diverse endomycorrhizal structures, including hyphae, vesicles and arbuscules (Fig 1).

Estimation of mycorrhization rates

The frequency of mycorrhization exceeded 90% in these plants, indicating their suitability as valuable mycorrhizal inoculum material or nurse plants. Furthermore, the cortex colonization intensity and mycorrhization intensity of the



a- Arbuscule; h- Hyphae; v- Vesicle.

Fig 1: Endomycorrhizal structures in the roots of *Medicago sativa* (a, b and c), *Medicago trucatula* (e, f and g) and *Trifolium rubens* (k,i and j) Magnification ×40 and Magnification ×10.

mycorrhizal fragments exceeded 70% for all plant species. The arbuscular percentage of the mycorrhizal fragments also exceeded 50% (Fig 2).

According to Read (1989), sandy soils are known to host diverse arbuscular mycorrhizal fungi and a rich, varied and ecologically beneficial microflora (bacteria and fungi) (Hu *et al*., 2010). The presence of arbuscules, considered the primary site of nutrient exchange, signifies that plants have established functional symbiosis (Mishra *et al*., 2018). Moreover, plant colonization is contingent upon the affinity between plant and fungi species, which can influence the abundance and composition of mycorrhizal fungi (Lovelock *et al*., 2003). Several experimental studies have shown that soil characteristics influence the diversity and community composition of AMF colonizing plant roots in various sites or habitats (Alguacil *et al*., 2016; Casazza *et al*., 2017; Sarkodee-Addo *et al*., 2020).

Table 3: Determination of MIP50 for rhizospheric soils of *Medicago sativa*, *Medicago trucatula* and *Trifolium rubens* plants.

Soil	Linear regression (Y=Ax+B)	R^2	MIP50
Medicago trucatula	Y=16.72x+103.7	0.986	23.80±0.1^a
Medicogo sativa	Y=14.95x+95.10	0.992	20.28±0.161^b
Trifolium rubens	Y=17.42x+103.7	0.978	21.75±0.62^c
			SEm±0;0982
			CD=0.2898
			α=0.05

Y- Linear regression of the percentage of mycorrhizal plants as a function of the logarithm of the quantity of non-sterilized soil; MIP50- Quantity of non-sterilized soil corresponding to 50% mycorrhizal infection; R^2- Correlation coefficient.

Data sharing the same letter within the same column are not significantly different according to Tukey test p<0.05.

Principal component analysis (PCA)

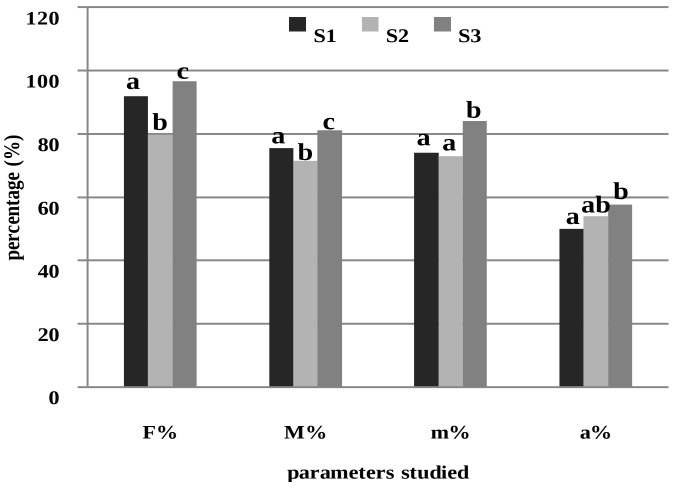
Projection onto the factorial plane (F1 × F2) of data corresponding to the two factors: physicochemical and microbiological characteristics, respectively, for the diverse soils associated with the plant species has been given in Fig 3.

The utilization of Principal Component Analysis (PCA) enabled the graphical representation of the interrelationships among various physicochemical parameters and the Mycorrhizal Infectious Potential (MIP50) of the soils under investigation. The two principal axes account for a cumulative variance of 70.56% in total.

The first axis regroups parameters such as available phosphorus, total nitrogen, conductivity, pH and MIP50, which were positively correlated with ranging from 0.86 to 0.89. This axis accounts for the largest proportion of variance (43.27%). Conversely, negative correlations were observed between carbon and both phosphorus and MIP50, with correlation coefficients of -0.72 and -0.91, respectively.

The second axis, responsible for 27.30% of the variance, exhibits a positive correlation between nitrogen and available phosphorus, with a correlation coefficient of 0.84.

MIP50 corresponds to the amount of unsterilized soil (which contains mycorrhizal microorganisms) needed to



S1- *Medicago truncatula;* S2- *Trifolium rubens;* S3- *Medicogo sativa;* F%- Frequency of mycorrhization; M%- Intensity of colonization in the root system; m%- Mycorrhizal intensity of the mycorrhizal fragments; a%- Arbuscular richness of the mycorrhizal fragments; Data in the same means followed by the same letter are not significantly different according to Tukey test at p<0.05.

Fig 2: Percentage of root infection for three legume species.

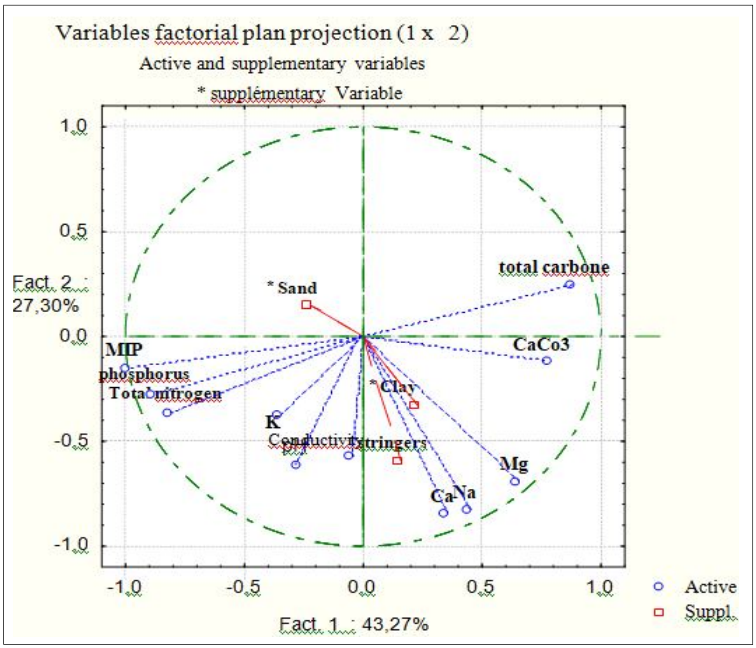


Fig 3: Principal component analysis of physicochemical and microbiological parameters of soil across three plant species: *Medicogo sativa*, *Medicago trucatula* and *Trifolium rubens*.

mycorrhize 50% of plants, which explains the correlation of PIM50 with nitrogen and phosphorus, mycorrhizal symbiosis improves the amount of these two elements in the soil (Vanek and Lehmann, 2014; Dejana *el al*., 2022). He *et al.* (2021) found that *Medicago sativa* improves significantly the nitrogen and phosphorus amount in soil rich in mycorrhizal propagules and rhizobia.

CONCLUSION

This study explored mycorrhizal symbiotic associations' impact on the physicochemical and microbiological characteristics of three plant species: *Medicago Truncatula, Medicago Sativa and Trifolium rubens* in the Ouled-fares region, Algeria. Soil analysis indicated alkaline pH, sandy texture and significant nitrogen and organic matter content. Microscopic assessment revealed robust mycorrhizal fungal colonization (90%-95%) in root systems, characterized by arbuscules and vesicles. These findings highlight the mycorrhizal potential of diverse soils for these native plant species in the Ouled-fares region, offering promise for ecosystem restoration and controlled mycorrhization in semi-arid regions.

Conflict of interest

The authors declare no competing of interests.

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