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First detection of *Arbuscular mycorrhizal* fungi (AMF) in the root system of plants growing under metallic trace elements stress in eastern Morocco at a polluted mining site and identification of spores in the soil

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Abstract: Soil pollution has become a major environmental concern, particularly in industrialized regions. The role of accumulator and hyperaccumulator plants in the remediation of soils contaminated by mining activities is now well recognized. Consequently, optimizing the growth of plant species capable of thriving in such environments is essential for enhancing the uptake of heavy metals. This study aims to investigate the presence of vesicular-arbuscular mycorrhizal (VAM) fungi in the roots of plants growing in a metal-stressed area, and to isolate and identify fungal spores from a former mining site located in eastern Morocco. This site, which operated from 1945 to 1980, was dedicated to the processing and refining of lead and silver for over 33 years. The mining site was closed due to the depletion of local raw material sources. Following the collection of plant species from the area, a root staining procedure using trypan blue was carried out to accurately assess the frequency and intensity of mycorrhizal colonization in each species. Subsequently, fungal spore identification was conducted. The results revealed that all seven plant species were mycorrhizal, exhibiting high colonization frequencies over 80% for *Diplotaxis erucoides* and an intensity of 86% for *Capsella bursa-pastoris*. The arbuscular mycorrhizal (AM) fungal spores present in the soil of Oued El Heimer were predominantly from the genus *Glomus*, whereas *Ambispora* was the least represented. Overall, this study demonstrates that the mining site harbors a high diversity of AM fungi, which contribute to enhanced plant tolerance to trace metal elements. Furthermore, these fungi aid in soil remediation by immobilizing metal ions on plant roots, indicating significant fungal colonization in the area.

Keywords: Endomycorrhiza; Arbuscular Mycorrhizal Fungi; Heavy metals; Fungal spores; Mining site; Oued El Heimer.

Introduction

Across the globe, a significant number of mines have been abandoned and their waste left in place, creating large amounts of heavy metal pollution without any decontamination processes in place (Remon, 2006). Various studies show that heavy metal contamination is irreversible in many cases (Smouni et al., 2015). Since heavy metals are not biodegradable, they can cause high toxicity even at low doses. Therefore, it is of great importance to find sustainable solutions to limit the risks they pose (Abd Elnabi et al., 2023). Also, it is worth noting that the presence of a diverse plant flora capable of tolerating high levels of these metals could serve as a significant tool for soil rehabilitation (Kumar et al., 2015). Heavy metals are known for their ability to interfere with the plant's vegetative system, disrupting essential functions such as photosynthesis, respiration, and water and mineral uptake, thereby inducing plant stress. This stress often leads to phytotoxicity, characterized by growth inhibition, chlorosis, or necrosis of aerial plant parts (Redon, 2009). The concentration of heavy metals in soils is influenced by the composition of each soil and by human activities in different countries around the world. Morocco is no exception to the environmental degradation caused by mining activities; in fact, many abandoned mining sites across the country reflect this issue.
The Oued El Heimer mine, located in the eastern region of Morocco, is primarily known for the extraction of lead (Pb) and zinc (Zn) over many years by the Zalija foundry. The mining activities in this area have led to significant soil contamination, with high concentrations of heavy metals persisting in the environment (Hasnaoui, 2020). In particular, lead (Pb), cadmium (Cd), zinc (Zn), and the critical metal antimony (Sb) pose serious environmental and health risks, highlighting the urgent need for remediation efforts.

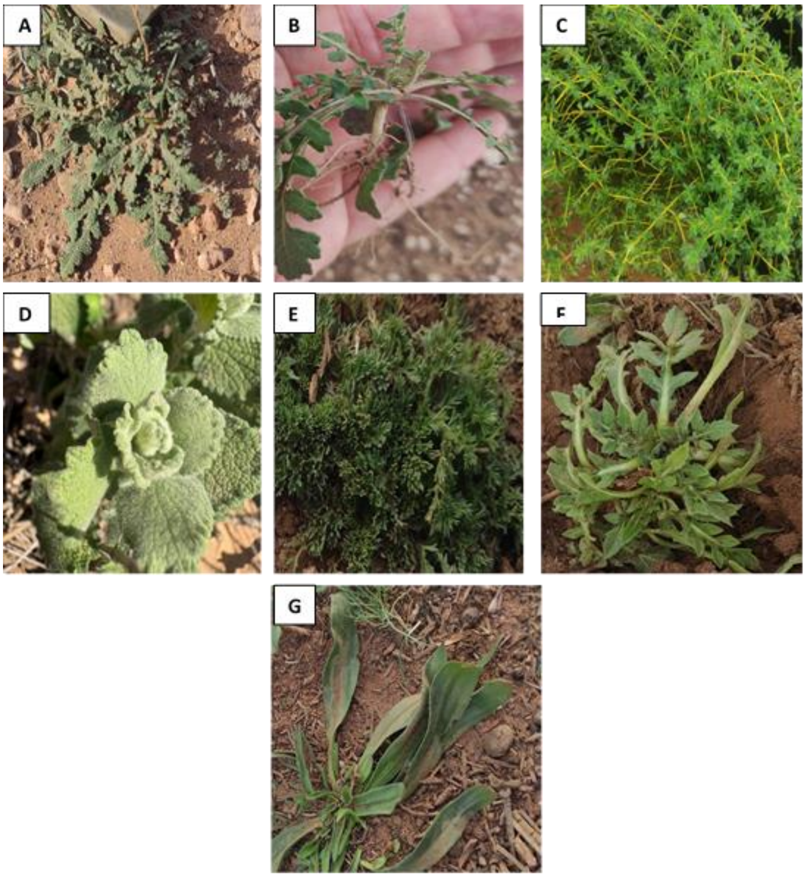

Figure 1. plants harvested from Oued El Heimer mining site (A: *Salvia verbenaca*; B: *Diplotaxis erucoides*; C: *Thymus vulgaris* D: *Marrubium vulgare*; E: *Artemisia Vulgaris*; F *Capsella-bursa-pastoris*; G: *Plantago lagopus*).

Arbuscular mycorrhizal fungi (AMF), present in all terrestrial ecosystems and found in almost all soil types across both arid and temperate zones (Parniske, 2008), establish symbiotic structures with the majority of plant species (Xiao et al., 2022). The benefits of arbuscular mycorrhizal fungi are widely recognized, as they play an important role in enhancing plant tolerance to abiotic stress (Augé, 2004; Augé, 2015; Amir, 2014; Porcel, 2012). Numerous studies have shown that arbuscular mycorrhizal fungi (AMF) effectively promote plant growth when introduced into environments similar to their native habitats (Calvente et al., 2004; Marulanda et al., 2007; Shen and Wang, 2011).
Moreover, Peñuelas (2008) demonstrated that strains of arbuscular mycorrhizal fungi isolated from specific environments, characterized by unique edaphic and climatic conditions, constitute adapted ecotypes endowed with physiological traits that enable them to thrive under the extreme conditions of their habitat It is therefore plausible to consider that mycorrhizal colonizing arbuscules (MCAs) from these specific environments possess unique characteristics and physiological properties adapted to such conditions. These arbuscular mycorrhizal fungi thus represent organisms of great interest as plant biostimulants, with the potential to promote the ecological restoration of degraded mining sites.
The primary objective of this investigation is to evaluate the frequency and intensity of root colonization of various harvested plants at the Oued El Heimer mining site, focusing on the naturally occurring arbuscular mycorrhizal fungi present there. This is followed by the identification of the arbuscular mycorrhizal fungi community.

Results

There should be a sub-heading explaining the results of plant species observed and collected from this site

Mycorrhizal status of the plants harvested at the site

All species harvested from the mining site showed strong root colonization by AMF, despite the high levels of lead and zinc present at the site. (Hasnaoui et al., 2020). The plants collected at Oued El Heimer are pictured in Figure 1.
Trypan blue staining of the root systems of plants collected in Oued El Heimer showed that all these plants are colonized by AMF. The presence of vesicles and arbuscules is remarkable (Fig. 2). Figure 3 shows a high mycorrhizal frequency of over 80% for *Diplotaxis erucoides*, *Salvia verbenaca*, *Thymus vulgaris*, *Marrubium vulgare*, *Artemisia vulgaris*, and *Plantago lanceolata*, while *Capsella bursa-pastoris* showed a frequency of 52%.
The diagram in Figure 4 shows a high mycorrhizal intensity for *Capsella bursa-pastoris* with a value of 86%, followed by *Marrubium vulgare* and *Plantago lanceolata* with values of 63% and 59%, respectively. Then comes *Salvia verbenaca* with 42%, and finally *Artemisia vulgaris*, *Diplotaxis erucoides*, and *Thymus vulgaris* with statistically non-significant values.

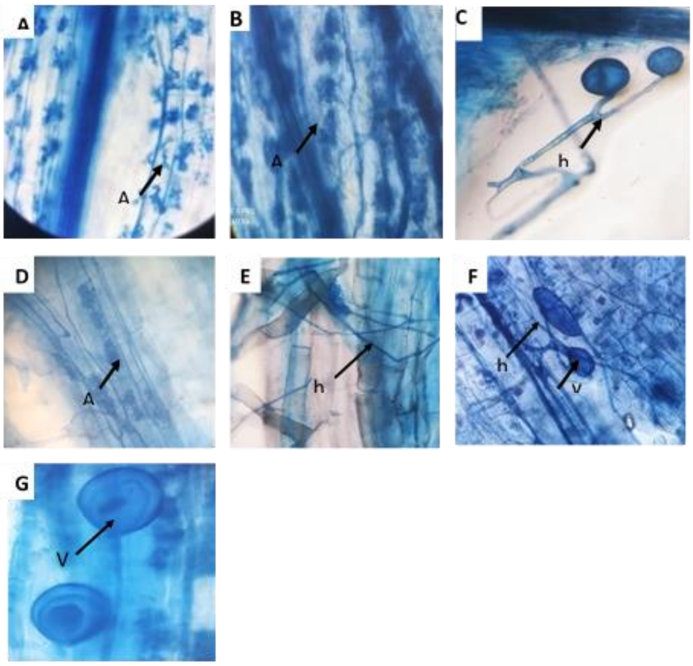

Figure 2. Mycorrhization status of plant species harvested from the mining site (A: *Salvia verbenaca*; B: *Diplotaxis erucoides*; C: *Thymus vulgaris;* D: *Marrubium vulgare*; E: *Artemisia Vulgaris*; F: *Capsella-bursa-pastoris*; G: *Plantago lagopus*; a :arbuscule; h: hyphae; v :vesicle.).

Identification of AMF spores from the mining soil

Figure 6 shows the frequency of occurrence of various AMF genera in the soil of the Oued El Heimer mine. The genus *Glomus* has a significantly high frequency, with 27 species. Other genera have different frequencies: *Scutellospora* (7 species), *Acaulospora* (9 species), and *Dentiscutata* (2 species). The genera *Funneliformis*, *Entrospora*, and *Ambispora* are each represented by only one species.

Discussion

Based on the root staining of plants collected at the Oued El Heimer mining site, we confirmed that all these plants are colonized by vesicular arbuscular mycorrhizal fungi (AMF), which occur naturally in the soil of the Oued El Heimer mine. This finding is consistent with the literature, which states that 80–90% of land plants form symbiotic associations with fungi. (Simon, 1993).

This phenomenon of root colonization by AMF could be attributed to the crucial role of mycorrhization in the survival and maintenance of host plants under metallic stress conditions, which disrupt various cellular functions of the plant, particularly the uptake of water and mineral nutrients (Stress & Merr, 2020). In a similar study, 18 plant species growing near a Zn and Pb deposit were mycorrhized by two fungal species, *Glomus* spp. and *Acaulospora* spp., with colonization rates varying between 35% and 85%.(Wang, 2017).

Zarei et al. (2010) showed that mycorrhization of plants decreases significantly with increasing distance from the Anguran mining area, demonstrating a close relationship between proximity to heavy metal pollution and mycorrhization intensity. This finding is consistent with the results of our study. This effect may be due to the impact of heavy metal pollution on AMF community diversity, plant species development and growth, and certain soil properties such as pH, organic matter content, and phosphorus levels, which can directly or indirectly influence the rate of plant root colonization by AMFs.

Our results showed that *Thymus vulgaris* is mycorrhized with a frequency of 23%. The association of fungi with the roots of *Thymus vulgaris* revealed large vesicles and arbuscules clearly visible under the microscope after root staining. A study by Laffont-Schwob et al. (2020) in an abandoned mine site in Gard, France, confirmed that *Thymus vulgaris* was the only species from the Lamiaceae family identified, showing numerous vesicles and extensive mycelium. Members of the Lamiaceae family are well known as excellent candidates for phytostabilization, which supports our findings.

Due to the presence of heavy metals in mining sites, plants produce secondary metabolites to protect themselves against metal-induced stress, such as flavonoids that attract mycorrhizal fungi (Pham et al., 2017). This suggests that some mycorrhizal fungi can tolerate high concentrations of certain metals, such as Zn and Pb (Bano and Ashfaq, 2013). Numerous studies have shown that many heavy metals are immobilized by the ability of fungal hyphae to bind metals (Zn, Pb, Cu) without affecting the mobilization of beneficial macro- and micronutrients. This provides plants with protection in areas contaminated with heavy metals (Bano and Ashfaq, 2013).

After isolating the spores of AMFs present in the mining soil of Oued El Heimer, seven genera and 48 species were identified: *Glomus* (27 species), *Scutellospora* (7 species), *Acaulospora* (9 species), *Dentiscutata* (2 species), *Ambispora* (1 species), *Entrophospora* (1 species), and *Funneliformis* (1 species). The community was dominated by the genus *Glomus* and the species *Scutellospora nigra*. Another study conducted on an abandoned uranium site reported the significant abundance of the genus *Glomus* and showed that it is more tolerant and better adapted to metal stress and various environmental

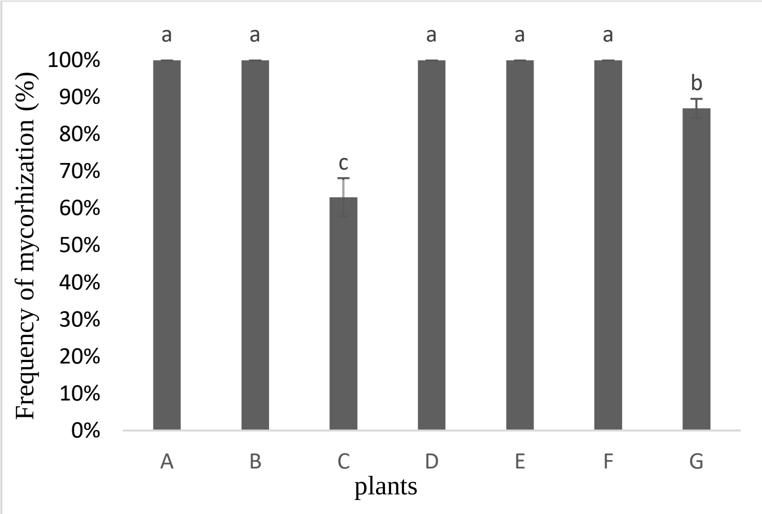

Figure 3. Mycorrhization frequencies of the plants collected at the mining site. *A:* Salvia verbenaca*; B:* Diplotaxis erucoides*; C:* Thymus vulgaris*; D:* Marrubium vulgare*; E:* Artemisia vulgaris*; F:* Capsella bursa-pastoris*; G:* Plantago lagopus*. Significant at (p < 0.05).*

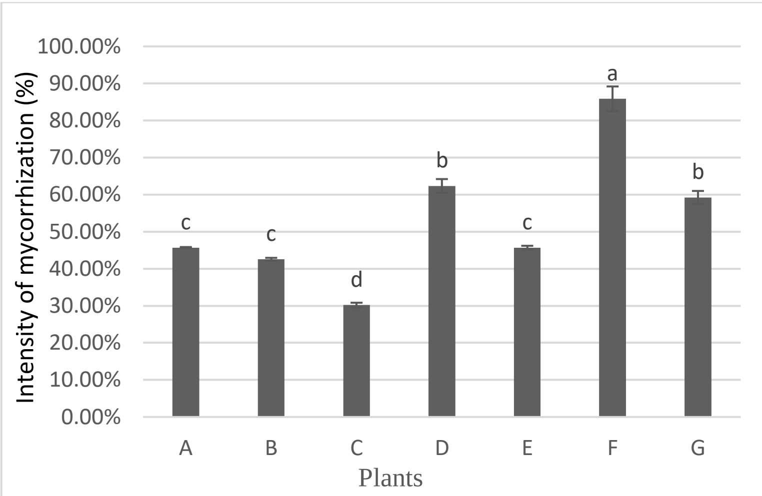

Figure 4. Intensity of mycorrhization of plants collected at the mining site. *A: Salvia verbenaca*; B: *Diplotaxis erucoides*; C: *Thymus vulgaris*; D: *Marrubium vulgare*; E: *Artemisia vulgaris*; F: *Capsella bursa-pastoris*; G: *Plantago lagopus*. The difference is statistically significant (p < 0.05). Values followed by different letters are significantly different (p < 0.05). Values with the same letters indicate no significant difference (p < 0.05).

Table 1. Morphological description of spores of various AMF species from the Oued El Heimer mine soil.

Number	species name	Color	Diametre (µm)	wall (µm)	shape	Aspect	Hyphae lenght
1	*Glomus. rubiformis*	Yellow	45	1	Circular	Mooth	30
2	*Glomus hoi*	Brown	27	2	Circular	Smooth	12
3	*Glomus corymbiforme*	Yellow	43	2	Circular	Smooth	____
4	*Glomus mosseae*	Orange	15	____	Circular	Rough	____
5	*Glomus macrocarpum*	Brown	16	3	Oval	Smooth	____
6	*Acaulospora Laevis*	Orange	55	6	Circular	Smooth	____
7	*Scutellospora nigra*	Black	32	____	Circular	Smooth	____
8	*Acaulospora sp3*	Yellow	45	1	Circular	Smooth	____
9	*Aculospora scrobiculata*	Mustard yellow	74	1	Circular	Smooth	____
10	*Scutellospora fulgida*	Yellow	50	4	Circular	Smooth	____

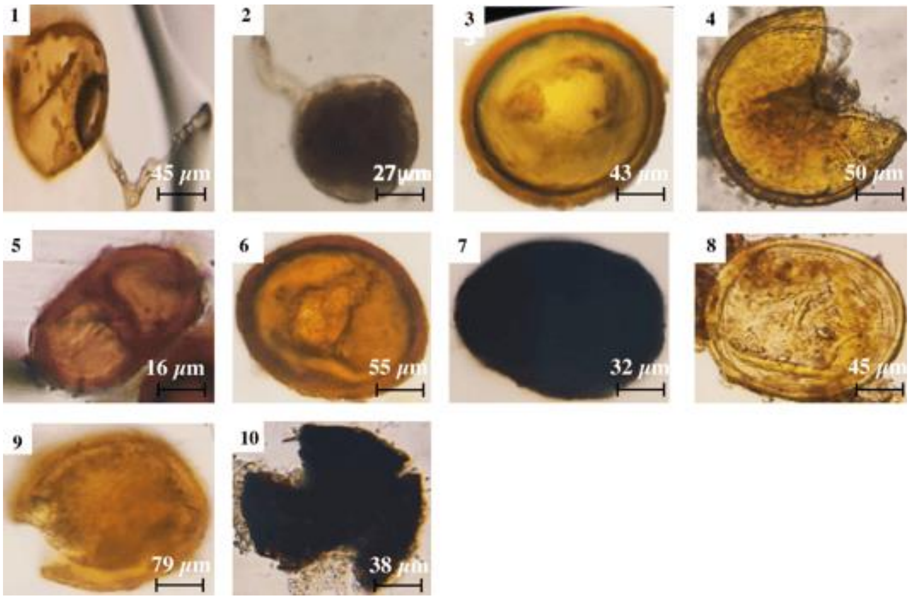

Figure 5. AMF spores isolated from Oued El Heimer soil. 1- *Glomus. Rubiformis ; 2- Glomus hoi ; 3- Glomus corymbiforme ; 4- Glomus mosseae ; 5- Glomus macrocarpum; 6- AcaulosporaLaevis; 7- Scutellospora nigra; 8- Acaulospora sp3; 9- Aculospora scrobiculata; 10- Scutellospora fulgida.*

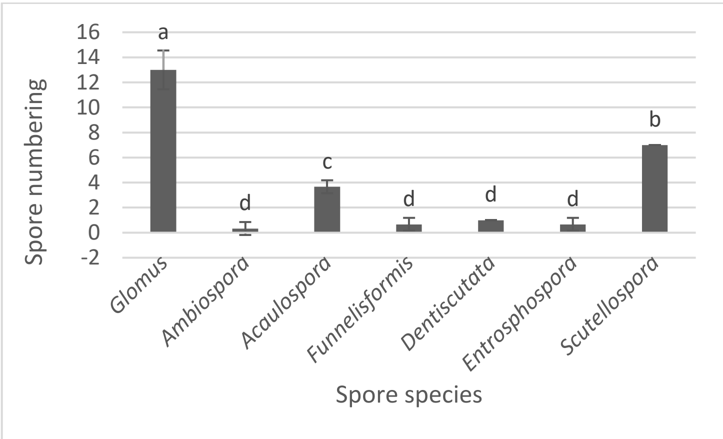

Figure 6. Frequency of occurrence of mycorrhizal fungi genera in Oued El Heimer soil. The difference is statistically significant p<0.05. *Values followed by different letters are significantly different (P<0.05). Values with the same letters indicate no significant difference (P<0.05).*

conditions compared to the genus *Scutellospora*. This is attributed to the small size of its spores and the ability of the Glomeraceae family to sporulate easily and rapidly (Rosas-Moreno et al., 2023). These findings are consistent with our results, which confirm the dominance of the genus *Glomus*, accounting for 80% of the AMF population found in Oued El Heimer. However, the number of spores found per 100 g of soil in our study exceeds 18 morphotypes, which differs from the results reported by Rosas-Moreno et al. (2023).

In another study conducted at the mining site of Mawsmai village in India, 77 species were identified: *Acaulospora* (13 species), *Glomus* (21 species), *Funneliformis* (7 species), *Entrophospora* (2 species), and *Scutellospora* (5 species) (Gary et al., 2021). The spores of *Acaulospora* and *Glomus* were predominant due to their tolerance to heavy metals, as well as their small size and short sporulation and reproduction times(Gary et al., 2021). According to the same study, the spore diversity of AMF in an abandoned mine site older than 14 years is higher than that in forest soils. This could be attributed to the increase in vegetative cover over the years, changes in the physico-chemical properties of the mining soil, and the fact that forests have a denser canopy and grow in shaded environments with high light requirements. Consequently, low light availability can reduce photosynthesis and the rate of carbon translocation to the roots, thus limiting carbon availability for AMFs (Gary et al., 2021).

Materials and methods

Sampling

Soil samples and plant species were collected from the Oued El Heimer mining site (34.44829 N; 1.450 W), a small mining village in the eastern region of Morocco, about 28 km south of Oujda city (Fig 7). In the area of the lead (Pb) and zinc (Zn)

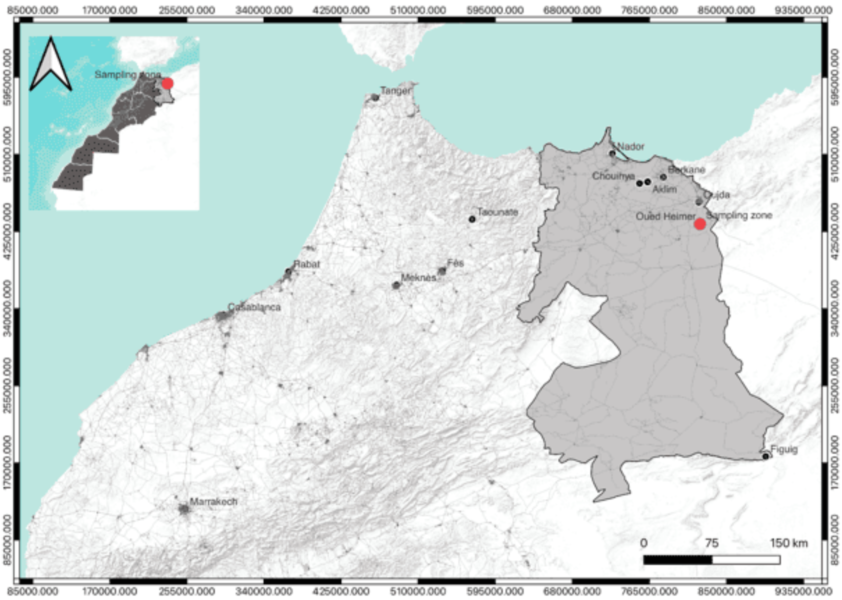

Figure 7. Sampling site of mycorrhizal plants.

dikes, seven plant species were harvested along with their root systems and identified based on the monographic characteristics of each plant. Soil samples were collected from the rhizosphere of each species at a depth of 0 to 20 cm

Climatic characteristics

The climatic data were determined by Abatzoglou et al. (2018). The Oued El Heimer mining site is characterized by low precipitation levels and moderate average temperatures (Table 2).(Abatzoglou et al., 2018).

Mycorrhizal status of the plant species harvested at the Oued El Heimer site

Root staining

The roots of each plant were cleaned with tap water to remove any soil impurities adhering to the roots after harvesting. The roots were then cut into 1 cm pieces and placed in tubes containing a 10% KOH solution. Next, the roots were incubated in a water bath at 90°C for 20 minutes to clear them. This procedure was repeated twice for 30 minutes each to free the cells from their cytoplasmic content. After the incubation process, the samples were cooled for 15 minutes and rinsed with distilled water to remove all traces of KOH. The root fragments were then immersed in distilled water with a few drops of lactic acid for five minutes to acidify the medium, ensuring deeper penetration of the trypan blue stain. Afterwards, the root fragments were stained with a 1% trypan blue solution. Finally, the root samples were thoroughly rinsed with distilled water until the rinse water ran clear. (Second and Notiser, 1970).

Frequency and intensity of mycorrhizal plants

The frequency and intensity of mycorrhization were evaluated following the protocol described by Trouvelot et al. (1986), where approximately 10 stained root fragments were observed under a light microscope. The mycorrhization rate for each plant species was estimated using the following formula:

<u>Equation 1: Calculation of the mycorrhization frequency</u>

$$F(\%) = \frac{(N - N0)}{N} * 100$$

Mycorrhizal frequency (F) : Extent of infection of the root system
N = number of fragments observed
N0 = number of fragments without traces of mycorrhiza.

<u>Equation 2: Calculation of the intensity of CMA root colonization</u>

$$M(\%) = [(95 * n5) + (70 * n4) + (30 * n3) + (5 * \mathrm{n}^2 + \mathrm{n}^1)/\mathrm{N}$$

Mycorrhizal intensity (M) : Expresses the proportion of colonized bark in relation to the entire root system.
n= Number of infected fragments with an index from 0 to 5 n5, n4,...nl are the number of fragments that were rated 5, 4,..., 1 respectively
N: number of fragments observed
Class 5: more than 91%; Class 4: from 51 to 90%; Class 3: from 11 to 50% ;Class 2: less than 10% Class 1: traces; Class 0: no mycorrhiza (Meddich et al., 2017).

Spore isolation

The AMF spores were isolated by wet sieving according to the technique described by Gerdemann and Nicolson (1963). Soils from the rhizospheres of various plant species at the Oued El Heimer mining site were mixed in a

Table 2. Climatic caracteristics of Oued El Heimer mining site.

Year	TM (°C)	Tm(°C)	T Average(°C)	Precipitation(mm)	Bioclimate
2023	24.09	10.93	17.51	139,5	Arid

TM: Annual Maximal Temperature; Tm: Annual minimal Temperature.

beaker. A 100 g sample of this mixture was combined with tap water and then passed through two sieves with mesh sizes of 200 µm and 50 µm, respectively, under a continuous flow of water until the discoloration disappeared. All material retained on the 200 µm sieve was discarded, while the contents on the 50 µm sieve were further washed until the brown coloration at the bottom disappeared. The material collected was transferred into distilled water using a wash bottle. The suspension was then centrifuged at 3,000 rpm for 10 minutes. The resulting pellet was resuspended in a volume of 60% sucrose solution, to which an equal volume of distilled water was added. The mixture was centrifuged again at 3,000 rpm for 10 minutes. The sucrose solution creates a density gradient that allows the spores to float to the surface. The spore suspension collected is then poured onto the 50 µm sieve and rinsed with distilled water to remove all traces of sucrose. The material retained on the 50 µm sieve is transferred to a Petri dish filled with distilled water for observation under a stereomicroscope. At this stage, the spores can be stored at 4°C for several weeks.

Spore identification

Conventional methods for identifying fungi are based on spore morphology. This approach is considered time-consuming and complex. It relies on specific spore characteristics—particularly wall structure, color, size, shape, and hyphal attachments—which are considered the most important features for the identification of vesicular-arbuscular mycorrhizal fungi (Morton and Bentivenga, 1994). Additionally, the International Collection of Arbuscular and Vesicular Mycorrhizal Fungi (INVAM) provides a valuable reference for morphological identification and classification (INVAM, 2017).

Statistical analysis

The collected data were subjected to descriptive statistical analysis and analysis of variance (ANOVA) using SPSS software (version 23 for Windows). Mean comparisons were performed using Tukey's post hoc test at a significance level of 5% ($p \leq 0.05$).

Conclusion

In conclusion, our study at the Oued El Heimer mining site in eastern Morocco revealed a significant presence and diversity of arbuscular mycorrhizal fungi (AMF) in both plant roots and soil, underscoring the mycorrhizal richness of this environment. The high mycorrhization levels observed in native plant species highlight the potential of AMF to enhance heavy metal resistance in other plant species. Future research should focus on rehabilitation strategies that involve inoculating plants with native AMF strains to promote ecological restoration and improve plant tolerance to heavy metal stress.

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Authors Contribution

El yeznasni hanae: conception idea, literature review, data analysis, methodology and article write up. Chafai wissame: methodology, data analysis, literature review. Bouchentouf Halima: data analysis. Smouni abdelaziz: article editing, methodology. Khalid Ahmed: conception idea, article editing, article write up and approval of article.

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