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Engineering Approach for Production of Arbuscular Mycorrhizal Inoculum Adapted to Saline Soil Management

Karima Bencherif [1,*], Frederic Laruelle [2], Benoit Tisserant [2], Yolande Dalpé [3] and Anissa Lounés-Hadj Sahraoui [2]

[1] Départements des Sciences Agrovétérinaires, Faculté des Sciences de la Nature et de la Vie, Université de Djelfa, Route de Moudjbara, Boite Postale 3117, Djelfa 17000, Algeria
[2] Unité de Chimie Environnementale et Interactions sur le Vivant (UCEIV), Université du Littoral Côte d'Opale, UR 4492, SFR Condorcet FR CNRS 3417, 50 Rue Ferdinand Buisson, 62228 Calais cedex, France
[3] Centre de Recherche et Développement d'Ottawa, Agriculture et Agroalimentaire Canada, 960 Carling Ave., Ottawa, ON K1A 0C6, Canada
* Correspondence: bencherif_karima@yahoo.fr

Abstract: (1) Background: Soil salinity impacts plant growth and productivity in steppic regions. Thus, the current study aims at producing, at low cost, an inoculum composed of native arbuscular mycorrhizal fungi (AMF) strains adapted to saline soil management. (2) Methods: The propagation of the native AMF strains was carried out in three natural saline soils presenting increasing salinity levels (4.5, 8.5 and 9.3 dS·m^{-1} in Boughzoul (BG), Salt Rocket (SR) and Zaafrane (ZA) sites, respectively). Three host plant species (alfalfa, clover and leek) were tested as trap cultures. AMF spore richness and diversity, the quantification of soil microbial biomass and the determination of the most probable number (MPN) were carried out before and after 24 months culture. Moreover, the mycorrhizal rates of the host plant species were assessed. (3) Results: The moderate saline soil (SR) planted with alfalfa was found to be the most suitable for AMF inoculum production with a maximum of 650 spores, 10 g^{-1} of soil, a mycorrhizal rate of 86%, 70 propagules·g^{-1} of soil and the highest microbial biomass content. (4) Conclusion: This study points out the best combination for the production of a more adapted AMF biofertilizer for saline soil management and proposes several indicators for biofertilizer quality evaluation.

Keywords: biofertilizer; AMF inoculum; salinity; host plant

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1. Introduction

Salinity is one of the most important and adverse agricultural and eco-environmental abiotic stresses, which impacts plant growth and productivity [1]. It has a severe negative effect on the international economy, with USD 27.3 billion annual global income loss [2]. Globally, the soil area affected by salinity reaches about 935 million ha, with an estimated annual increase of 1–2 million ha [3]. Up to 70% of this total area is distributed in arid and semi-arid regions [4]. The rate is expected to further increase over the coming decades with climate change paired with food demand resulting from growing populations [5,6]. There is a clear correlation between this increase in soil salinity and the loss of biodiversity, and a severe knowledge gap exists in understanding this interrelationship [1]. Therefore, policies establishing the interlinkage between the two facts should be adopted in order to counteract the loss of biodiversity due to salinity [4]. Saline soil management should be adopted for agriculture extension, and the thoughtful use of biofertilizers based on native microorganisms is part of this [5]. In this context, the use of arbuscular mycorrhizal fungi (AMF) is recognized as a valuable approach in sustainable agriculture practices, in particular in adverse conditions [6]. These Glomeromycota fungi are important components of soil microbiota that can be exploited to enhance the development of crops with more sustainable agriculture [5]. Indeed, AMF are essential soil beneficial microorganisms,

forming a mutualistic root symbiosis with more than 80% of terrestrial plants [7]. They are well known as helpful microorganisms that increase plant mineral and water nutrition and consequently host plant growth and tolerance to biotic and abiotic stresses such as salinity, drought and pollution [8–11].

Thus, the biofertilizer engineering approach could reduce the use of chemical inputs and pesticides in organic farming [6,12]. During the last decades, many biofertilizers have been developed and commercialized [6,13,14]. However, an important question remains unanswered: are all AMF strains efficient everywhere? It is important to mention that AMF distribution in soils depends on many criteria such as edaphic and climatic conditions [15]. Successful AMF propagation relies on different field conditions such as crop species, soil fertility, indigenous microbial communities, soil physical and chemical characteristics as well as management practices of the native soils [6]. Thus, the selection of AMF species should be appropriate for the desired application scenario [16]. The inoculant should contain enough viable propagules to achieve AMF root colonization [17]. Inoculum viability is highly variable between AMF isolates [7] and the host plant used for inoculum production [18]. Several important criteria should be taken into account in producing mycorrhizal inoculum including (i) the AMF propagules density within the inoculum, which can be assessed using the most probable number (MPN) [19]; and (ii) the diversity of the AMF species used [20,21]. Furthermore, the inoculum must improve soil health, which can be estimated by the soil microbial biomass [22] using, for example, phospholipid fatty acid (PLFA) analysis [23]. Indeed, the introduction of an exotic strain into an existing ecosystem might induce competition with native strains for available resources and compromise either the establishment of the conquering strain or the alteration of the potential and the composition of the indigenous microbial community [24,25]. It was reported that introducing alien strains in different geographical areas can have negative consequences on resident microorganism communities with decreasing natural soil fertility [16,26]. Native AMF species had better adaptability to local climatic and edaphic conditions in steppic areas than non-native species [27,28]. For example, in 2022, Jerbi et al. [29] showed that the use of a mycorrhizal inoculum made of a mixture of five native AMF species (*Pacispora franciscana*, *Funneliformis mosseae*, *Funneliformis geosporum*, *Rhizophagus irregularis*, *Glomus tenebrosum*) was more efficient than commercial inoculant containing six species of *Glomus* sp., under steppic regions. The native AMF inoculant was able to reduce drought damage by improving the physiological and biochemical responses of hulless barley [29].

Although several studies highlighted the potential role of AMF inoculation, in particular with native strains, as an innovative and eco-friendly technology for a sustainable crop growing system in arid and semi-arid areas, none of them provided an adequate approach for the production of suitable mycorrhizal inoculum for saline soil management. Thus, the present study aims at proposing the most appropriate combination between the physico-chemical (in particular the salinity level) and the microbial (in particular the richness and diversity of native AMF species) characteristics of the soil, and the host plant species to use for sustainable crop growing systems in arid and semi-arid saline soils. For that, the propagation of native AMF strains was carried out in different natural saline soils originating from three saline sites, namely, Boughzoul (BG), Salt Rocket (SR) and Zaafrane (ZA), presenting increasing salinity levels of 4.5, 8.5 and 9.3 $dS.m^{-1}$ respectively. Three host plant species, namely, alfalfa, clover and leek, were tested as trap cultures.

2. Results

2.1. Physico-Chemical and Meteorological Characteristics of the Studied Soils

The physico-chemical characteristics of the studied soils are presented in Table 1. The salinity of the studied soils varied from 4.15 $dS.m^{-1}$ to 9.93 $dS{\cdot}m^{-1}$. Thus, three levels of salinity could be distinguished: low saline soil BG (4.15 $dS{\cdot}m^{-1}$), moderate saline soil SR (8.5 $dS{\cdot}m^{-1}$) and high saline soil ZA (9.93 $dS{\cdot}m^{-1}$). Texture was defined as loamy–sandy for SR and sandy–loamy for the two other soils. Organic matter levels remained very low in the three soils, namely, 0.54, 1.43 and 1.53% in ZA, BG and SR soils, respectively. Nitrogen

levels were about 0.1, 0.2 and 0.4 mg·g^{-1} in ZA, SR and BG soils, respectively. The studied soils had low contents of phosphorus, namely, 0.027, 0.1 and 0.2 mg·g^{-1} in SR, ZA and BG soils, respectively (Table 1).

Table 1. Physico-chemical characteristics of the studied soils.

	SITES		
Soil Parameters	**Salt Rocket (SR)**	**Zaafrane (ZA)**	**Boughzoul (BG)**
M (°C)	35.5	36.3	34.5
m (°C)	1.2	1.8	0.86
R (mm/year)	279	245.23	950
Altitude (m)	1083	950	635
Emberger pluvio-thermic quotient (Q3)	27.9	24.37	21.43
Bioclimatic stage (Emberger classification)	Medium semi-arid with cold winter	Semi-arid lower with mild winter	High arid with mild winter
pH water	5.95	9.5	7.48
EC (dS·m^{-1})	8.5	9.93	4.15
Salinity (g·L^{-1})	4.94	5.75	3.24
Available phosphorus (mg·g^{-1})	0.027	0.1	0.21
Moisture (%)	13.85	23.8	4.94
Nitrogen (mg·g^{-1})	0.2	0.1	0.42
Carbone (mg·g^{-1})	8.9	3.18	8.3
C/N	44.5	31.8	19.76
Mg (meq 100 g^{-1})	31	15.2	38.4
K (mg·g^{-1})	0.12	0.14	30.01
Na (meq.100 g^{-1})	22	21	11
OM (%)	1.53	0.54	1.43
Total calcareous (%)	7.46	9.8	12.62
Clay (%)	2.90	13.5	9.5
Silt (%)	75.69	25.1	19.95
Sand	21.90	61.1	70.5
Texture	Loamy–sandy	Sandy–loamy	Sandy–loamy

M: maximum temperature; m: minimum temperature (M and m values represent means of annual temperatures); R: means of annual rainfall; Q_3: Emberger pluvio-thermic quotient (Q_3 = 3.43 P/M – m); OM: organic matter; C/N: carbon/nitrogen ratio.

The meteorological characteristics of the studied soils are given in supplementary data (Table S1). The aridity index of DeMarton (IDM) according to DeMarton Abacus revealed that the low saline site BG is located in high arid regions with mild winters, the moderate saline site SR is located in medium semi-arid regions with cold winters and the high saline site ZA is located in semi-arid lower regions with mild winters. Precipitation was between 170 in the BG site and 260 mm in the SR site. The temperature mean was about 14 and 15 °C.

2.2. Evaluation of Produced Inoculum

In order to evaluate the quality of the three produced mycorrhizal inoculums, AMF spore species enumeration and identification were carried out before and after trap culture. In addition, microbial biomasses using specific lipid biomarkers, namely, neutral lipid fatty acids (NLFA), phospholipid fatty acids (PLFA), and the most probable number (MPN) were also determined.

2.2.1. AMF Spore Species Enumeration and Identification

AMF spore abundances per 10 g of soil sampled from each studied saline sites were determined before and after trap culture with three host plants (alfalfa, clover, leek) (Table 2).

Table 2. AMF spore species isolated from the saline soils of the three studied sites (BG, SR and ZA) before and after trap culture.

	Number of AMF Spore Species/10 g of Soil											
	Before Plantation			**After Plantation with**								
				Alfalfa			**Clover**			**Leek**		
	SR	**ZA**	**BG**	**SR**	**ZA**	**BG**	**SR**	**ZA**	**BG**	**SR**	**ZA**	**BG**
Claroideoglomus etunicatum	0	30	10	10	50	60	3	25	52	2	22	10
Dominikia sp.	80	10	74	120	8	98	95	54	90	90	50	88
Funnelifromis coronatus	16	0	47	30	8	52	25	0	36	10	0	0
Funneliformis mosseae	15	3	10	50	20	45	35	14	28	23	11	20
Glomus deseerticola (*Septoglomus deserticola*)	50	3	2	60	6	12	42	5	10	34	0	3
Gigaspora gigantea	2	0	0	8	0	0	2	0	0	0	0	0
Gigaspora margarita	3	0	2	20	0	7	4	0	1	1	0	0
Glomus macrocarpum	10	1	2	75	28	30	44	25	35	44	14	21
Innospora (*Paraglomus majewskii*)	13	8	7	60	28	70	23	24	32	22	12	15
Microkamienskia sp.	2	0	0	20	0	0	8	0	0	0	0	0
Rhizophagus fasciculatum	0	2	15	10	4	11	1	0	10	0	0	8
Rhizophagus irregularis	2	70	70	120	88	98	60	45	100	21	25	15
Septoglomus constrictum	2	20	60	52	50	80	2	20	60	2	20	60
Total spore number/10 g of soil	195	147	299	635	290	563	344	212	454	249	154	240
Species richness (S)	11	9	11	13	10	11	13	8	11	10	7	9
Shannon diversity index (H′)	1.70	1.54	1.86	2.28	1.94	2.17	2.04	1.91	2.11	1.81	1.80	1.78

Data are presented as means obtained from five replicates from each studied site. H'_{max}: maximum value of Shannon diversity index. SR: Salt Rocket, ZA: Zaafrane, BG: Boughzoul.

Before trap culture, the total spore numbers were higher in the lowest saline soil BG with 299 spores/10 g of soil. *Rhizophagus irregularis* was the most abundant species in BG soil. In total, 195 spores/10 g of soil was recorded in SR saline soil, with the dominance of *Dominikia* sp. and *Glomus deserticola.* In addition, *Gigaspora gigantea* and *Microkamienskia* sp. were recorded only in SR saline soil, and *Funneliformis coronatus* was recorded in SR and BG soils. The lowest total spore number was recorded in the most saline soil ZA (147 spores/10 g of soil). *R. irregularis* was the most dominant species followed by *Claroideoglomus etunicatum*. The AMF species richness (index S) and diversity (Shannon index H′) showed little fluctuation throughout soil salinity level in the three natural saline soils. AMF species richness was about 11 in the saline soils SR and BG, whereas it was about 9 in high saline soil ZA. Shannon diversity index H′ values were about 1.86 and 1.70, respectively, in BG and SR saline soils and about 1.54 in ZA saline soil (Table 2).

After trap culture, the AMF spore number increased in the three studied soils with the three host plants, except for ZA saline soil with leek host plants. In addition, the species richness increased from 11 to 13 in SR and BG saline soils with alfalfa host plants, while it decreased with leek host plants for the three studied soils (Table 2). Indeed, after trap culture, the highest spore number was recorded with alfalfa host plants in SR saline soil (635 spores/10 g of soil), and the lowest one was recorded in ZA, the most saline soil, with leek species (154 spores/10 g of soil). Additionally, AMF species richness and diversity varied according to the soil salinity and the host plant species (Table 2). The AMF species *C. etunicatum*, *F. mosseae*, *Innospora* (*Paraglomus majewskii*), *Glomus macrocarpum*,

R. irregularis, *Septoglomus constrictum*, *Dominikia* sp., *Microkamienskia* sp., *F. coronatus*, *G. deseerticola* (*Septoglomus deserticola*) and *Gigaspora margarita* were recorded in SR saline soil with the three studied plants, except for *Microkamienskia* sp. which was not recorded with leek species (Figure 1). AMF species richness increased in SR and BG saline soils after alfalfa plantation (13 and 11), respectively, with dominance of *R. irregularis* and *Dominikia* sp. In addition, *Rhizophagus fasciculatum* was isolated from alfalfa pot culture in the three studied saline soils. However, with the clover host plant, AMF species richness decreased in ZA saline soil (8), since *R. fasciculatum* was not isolated. With leek host plants, AMF species richness decreased for the three studied saline soils (10, 7 and 9, respectively, for SR, ZA and BG soils) (Table 2). *G. deserticlola*, *R. fasciculatum*, *Gi. margarita* and *Gi. gigantea* were not recorded after leek plantations.

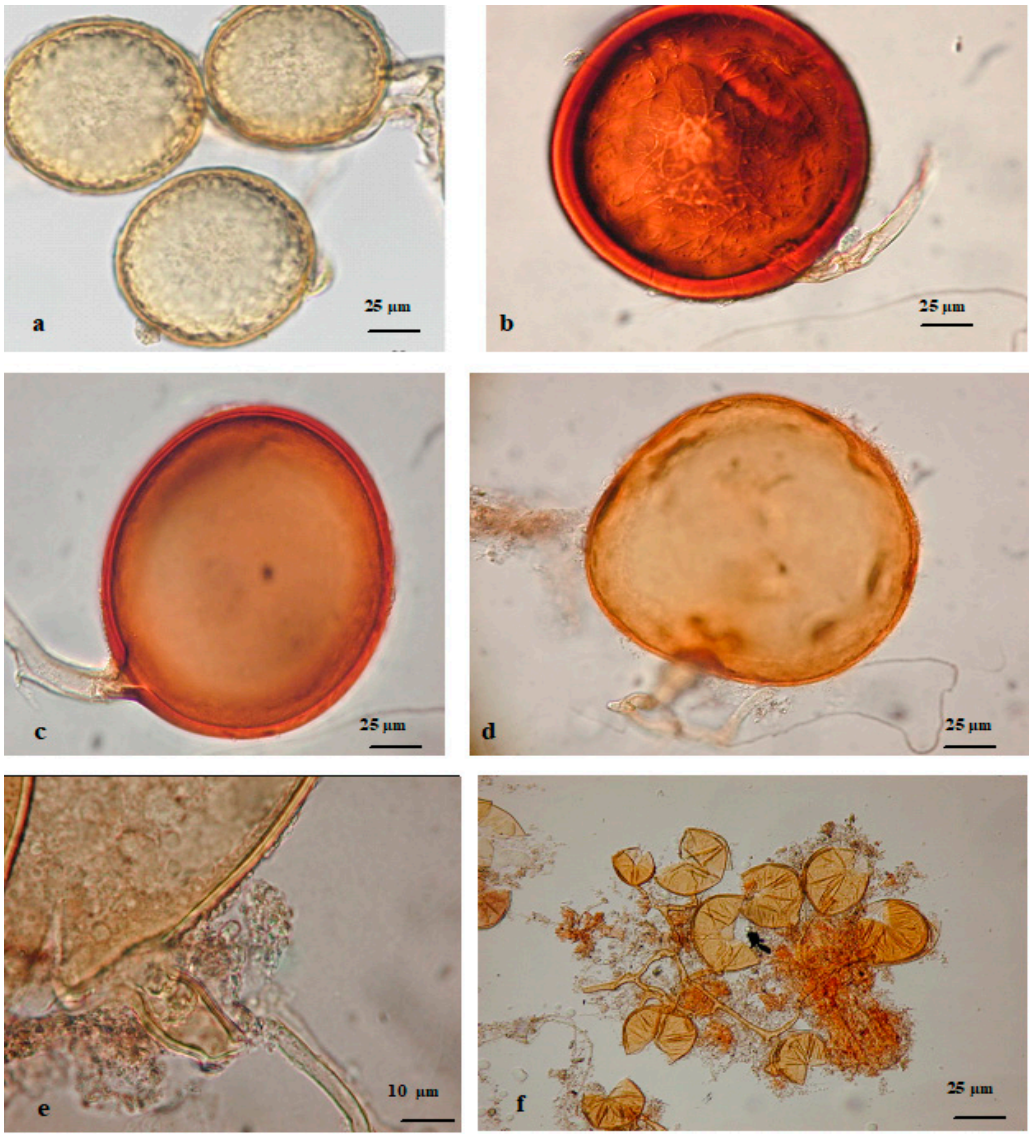


Figure 1. Arbuscular mycorrhizal fungi collected from trap culture. (**a**) *Rhizophagus irregularis* in BG soil, (**b**) *Septoglomus constrictum* in RS site, (**c**) *Glomus macrocarpom* in BG soil, (**d**) *Gigaspora margarita* in SR soil, (**e**) *Funneliformis mosseae* in SR soil, (**f**) *Dominikia* sp. in SR soil.

Shannon diversity indexes were higher under saline soils with alfalfa host plant (2.28, 2.17 and 1.94, respectively, for SR, BG and ZA saline soils) (Table 2). With clover host plant, Shannon diversity indexes were about 2.11, 2.04 and 1.91 for BG, SR and ZA saline soils, respectively. With leek host plant, the highest diversity index was recorded in SR saline

soil (1.81). For the remaining soils, H' were about 1.80 and 1.78, respectively, in ZA and BG saline soils.

2.2.2. Soil Microbial Biomass

In order to evaluate the impact of trap culture on the microbial biomass associated with the produced AMF inoculum, soil microbial biomasses (bacteria, saprotrophic fungi and AMF) were assessed before and after trap cultures.

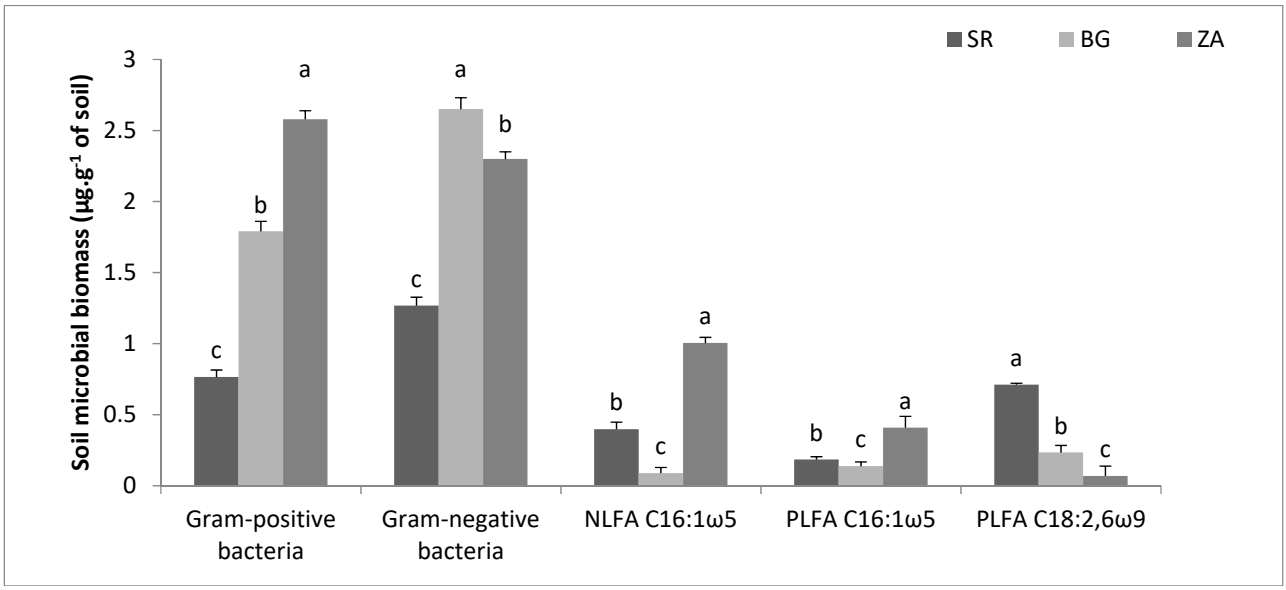
Soil Microbial Biomass before Trap Culture

In order to determine soil microbial biomass in the studied soils before plantation, specific lipid biomarkers were assessed and compared between the different saline sites. The amount of NLFA C16:1ω5 soil content, mainly representing the vesicle storage structures, was found to be 12 times higher in the most saline soil ZA than in low saline soil BG and 2.5 times higher than in the saline soil SR (Figure 2A). The amounts of PLFA C16:1ω5, a fatty acid used to quantify AMF in soils, were found to be 3-fold higher in the most saline soil ZA than in the low saline soil BG, and 2-fold higher than in SR saline soil (Figure 2A). The NLFA/PLFA C16:1ω5 ratio, used to distinguish between microbial biomass originating from AMF (ratio > 1) and bacteria (ratio < 1), were about 2.46 and 2.16 in ZA and SR saline soils, respectively, indicating mycorrhizal origins of the lipids. However, the ratio NLFA/PLFA was about 0.67 in BG low saline soil, indicating a bacterial origin of the lipids. Gram-negative bacterial biomass (estimated by the sum of the PLFA cy17:0, C18:1ω7 and cy19:0) was one-fold higher in BG saline soil than in ZA (the highest saline soil) and two-fold higher than in SR saline soil (Figure 2A). As well, Gram-positive bacterial biomass (quantified by the sum of the PLFA: i15:0, a15:0, i16:0, i17:0 and a17:0) was 1.5 times higher in the most saline soil ZA than in BG saline soil, and 3 times higher than in the saline soil SR (Figure 2A). Quantification of C18:2,6ω9, the lipid biomarker of saprotrophic fungi, was found to be highest in SR saline soil, followed by the BG soil (the lowest saline site) and then ZA soil (the highest saline site) at 0.7, 0.23 and 0.06 $\mu g \cdot g^{-1}$ of soil, respectively (Figure 2A).

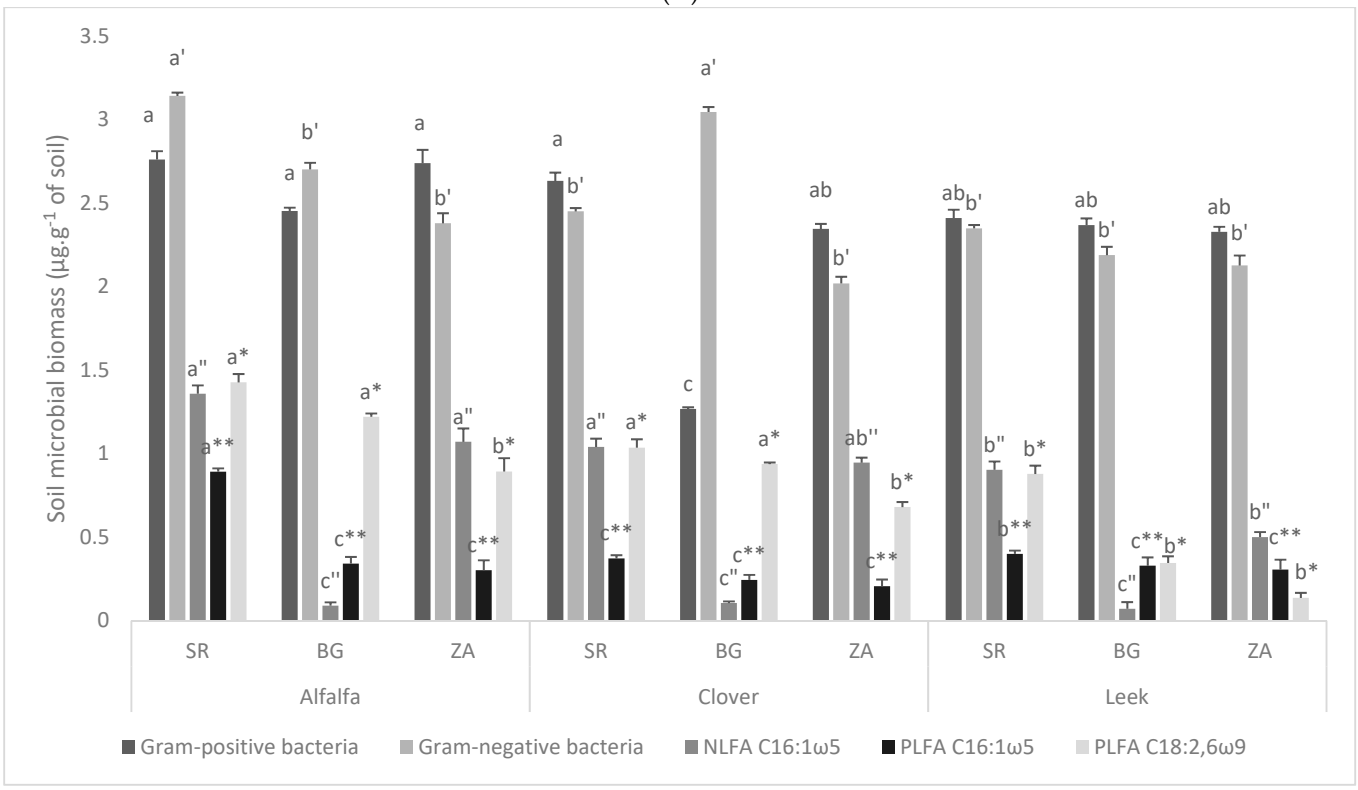
Soil Microbial Biomass after Trap Culture

In order to determine the quality of the mycorrhizal inoculum produced in terms of associated microbiota, the microbial biomasses were quantified through assessment of specific lipid biomarkers (PLFA and NLFA) after the trap culture period (24 months) and compared according to the different studied soils combined with the three studied host plants. The amount of the different soil microbial groups (AMF, Gram-positive bacteria, Gram-negative bacteria and saprotrophic fungi) increased after trap culture, in particular with alfalfa host plant.

The amount of NLFA C16:1ω5 varied according to the soil salinity level and the host plant species. With the alfalfa host plant, the highest amount was recorded in SR medium saline soil (1.35 $\mu g \cdot g^{-1}$ of soil), followed by ZA the highest saline soil (1.07 $\mu g \cdot g^{-1}$ of soil) and then by the lowest saline soil BG (0.08 $\mu g \cdot g^{-1}$ of soil). Again, the NLFA C16:1ω5 amount recorded with the clover host plant was the highest in SR saline soil and the lowest in BG soil (1.03 and 0.2 $\mu g \cdot g^{-1}$ of soil respectively), with 0.9 $\mu g \cdot g^{-1}$ of soil recorded in the ZA high saline soil. With leek host plant, the NLFA C16:1ω5 amount was about 0.9 and 0.07 $\mu g \cdot g^{-1}$ of soil, respectively, in SR and BG saline soils, and about 0.5 $\mu g \cdot g^{-1}$ of soil in ZA, the high saline soil.



(**A**)



(**B**)

Figure 2. Soil microbial biomass associated with the AMF-produced inoculum in the three studied saline soils (SR: Salt Rocket, BG: Boughzoul, ZA: Zaafrane). (**A**) Before trap culture and (**B**) after trap culture with alfalfa, clover and leek. Data are presented as means obtained from three replicates from each studied site. Different letters indicate significant differences between the studied soils combined with the three host plants according to the LSD test ($p < 0.05$). The symbols: ', '', *, ** correspond to comparisons between Gram-positive bacterial biomass, Gram-negative bacterial biomass, NLFA C16:1 ω 5 and PLFA C16: 1ω5 respectively.

Globally, the amount of PLFA C16:1ω5 varied with the host plant species. The amounts were about 0.8, 0.34 and 0.3 µg·g^{-1} of soil with the alfalfa host plant, respectively, in SR, BG and ZA saline soils. With the clover host plant, the amounts of PLFA C16:1ω5 were

about 0.34, 0.24 and 0.20 $\mu g \cdot g^{-1}$ of soil, respectively, in SR, BG and ZA saline soils. The PLFA C16:1ω5 amount recorded with the leek host plant was the highest in SR saline soil (0.39 $\mu g \cdot g^{-1}$ of soil) and the lowest in ZA, the high saline soil (0.30 $\mu g \cdot g^{-1}$ of soil).

In the same context, the ratio NLFA/PLFA C16:1ω5 were found to be higher in the high saline soil ZA for the three studied host plants (3.5, 4.5 and 1.6, respectively) for alfalfa, clover and leek host plants. The NLFA/PLFA C16:1ω5 ratio was higher than 1 for SR saline soil, while it was about 1.5, 2.5 and 1.2, respectively, for alfalfa, clover and leek host plants (Figure S1). For BG saline soil, the ratio was lower than 1 in the three studied host plants (Figure S1).

Gram-positive bacterial biomasses were about 2.75, 2.73 and 2.45 $\mu g \cdot g^{-1}$ of soil, respectively, in SR, ZA and BG saline soils with alfalfa host plants. Likewise, with clover host plant, Gram-positive bacterial biomasses were about 2.63, 2.08 and 1.26 $\mu g \cdot g^{-1}$ of soil, respectively, in SR, ZA and BG sites. Gram-positive bacterial biomasses with leek host plant were the highest in the saline soil SR (2.40 $\mu g \cdot g^{-1}$ of soil) and the lowest in the most saline soil ZA (2.36 $\mu g \cdot g^{-1}$ of soil). Similarly, Gram-negative bacterial biomasses varied according to the host plant and soil salinity level. They were about, 3.14, 2.37 and 2.70 $\mu g \cdot g^{-1}$ of soil, respectively, in SR, ZA and BG saline sites with the alfalfa host plant. With the clover host plant, the highest amount of Gram-negative bacterial biomass was recorded in the lowest saline soil BG (3.04 $\mu g \cdot g^{-1}$ of soil), and the lowest amount was recorded in the highest saline soil ZA (2.01 $\mu g \cdot g^{-1}$ of soil). For the leek host plant, the Gram-negative bacterial amount was the highest in SR saline soil (2.34 $\mu g \cdot g^{-1}$ of soil), and no significant difference was recorded between the two remaining BG and ZA studied soils (2.1 $\mu g \cdot g^{-1}$ of soil).

As well, the saprotrophic fungal biomasses were the highest in SR saline soil. They were about 1.42, 1.03 and 0.9 $\mu g \cdot g^{-1}$ of soil, respectively, with alfalfa clover and leek host plants. The lowest amounts of saprotrophic fungal biomass were recorded in ZA saline site, namely, 0.9, 0.6 and 0.13 $\mu g \cdot g^{-1}$ of soil, respectively, with alfalfa, clover and leek host plants.

2.3. Determination of Mycorrhizal Propagules Using the Most Probable Number (MPN)

The number of mycorrhizal propagules was evaluated by MPN before and after trap culture in order to assess the effectiveness of the native AMF-produced inoculum (Figure 3). Before trap culture, the mycorrhizal potential was higher in the saline soil SR with an average of 27 propagules.g^{-1} of soil, followed by ZA high saline soil with 18.5 propagules$\cdot g^{-1}$ of soil. The lowest saline soil BG recorded the lowest MPN value (14 propagules$\cdot g^{-1}$ of soil).

After trap culture, the MPN of mycorrhizal propagules increased in the three studied saline soils combined with the host plants. In addition, MPN varied according to soil salinity and host plant species (Figure S3). The mycorrhizal potential was higher following alfalfa plantation with an average of 70, 53 and 46 propagules per gram of soil, respectively, in SR, BG and ZA saline soils (Figure 3). Likewise, with the clover host plant, the MPN propagule numbers were about 52, 32 and 24 propagules, respectively, in SR, BG and ZA saline sites. The leek host plant revealed the lowest mycorrhizal potential with 46, 28 and 21 propagules$\cdot g^{-1}$ of soil in SR, BG and ZA saline sites, respectively (Figure 3).

2.4. Mycorrhizal Rates

Total mycorrhizal rates were evaluated for the three studied plant species after 24 months of trap culture in order to determine the richness in mycorrhizal propagules, since fragments of the roots will also serve for the production of the AMF inoculum. The comparison of the total mycorrhizal rate (MR) between the three studied host plants indicated that the highest mycorrhizal rates were recorded with alfalfa host plant for the three studied saline sites (Figure 4). They were about 86.7, 61.1 and 51.8% respectively for SR, BG and ZA saline soils.

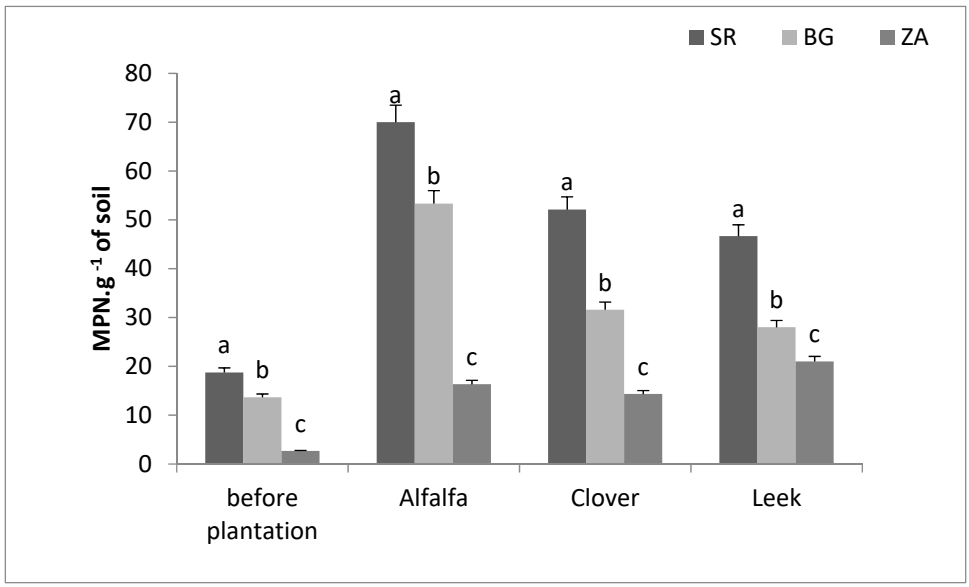


Figure 3. Evaluation of the most probable number (MPN) before and after trap culture of alfalfa, clover and leek in SR: Salt Rocket, BG: Boughzoul, ZA: Zaafrane. Data are presented as means obtained from five replicates from each studied sites. Different letters indicate significant differences between studied soils for the three studied host plants according to the LSD test ($p < 0.05$).

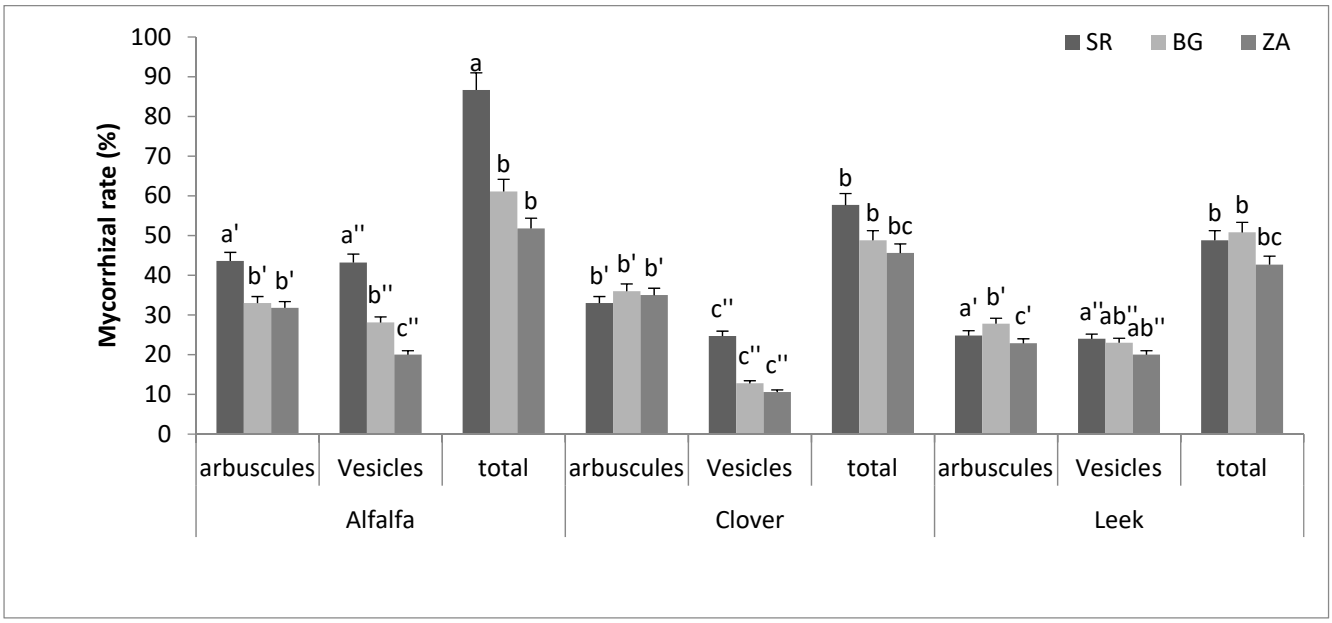


Figure 4. Mycorrhizal rates of alfalfa, clover and leek roots after trap culture in SR, BG and ZA sites. SR: Salt Rocket, BG: Boughzoul, ZA: Zaafrane. Data are presented as means obtained from five replicates from each studied site. Different letters indicate significant differences between the studied sites combined with the three host plants according to Tukey's test ($p < 0.05$).The symbols: ', " correspond to comparisons of arbuscules and vesicles respectively.

2.5. Global Statistical Analyses

Total mycorrhizal rates were negatively correlated with the soil salinity levels. Significant positive correlations were recorded with Shannon diversity indexes, AMF species richness, NLFA C16:1ω5, PLFA C16:1ω5 as well as Gram-positive and Gram-negative bacterial biomasses under alfalfa plantation for SR and BG saline soils. No significant correlation was recorded between total mycorrhizal rate and Gram-positive and Gram-negative

bacterial biomasses and soil humidity for clover host plant. No significant correlation was recorded between total mycorrhizal rate for leek host plant and MPN, while a positive correlation was recorded with Shannon diversity indexes, species richness, NLFA C16:1ω5 and PLFA C16:1ω5 (Table 3).

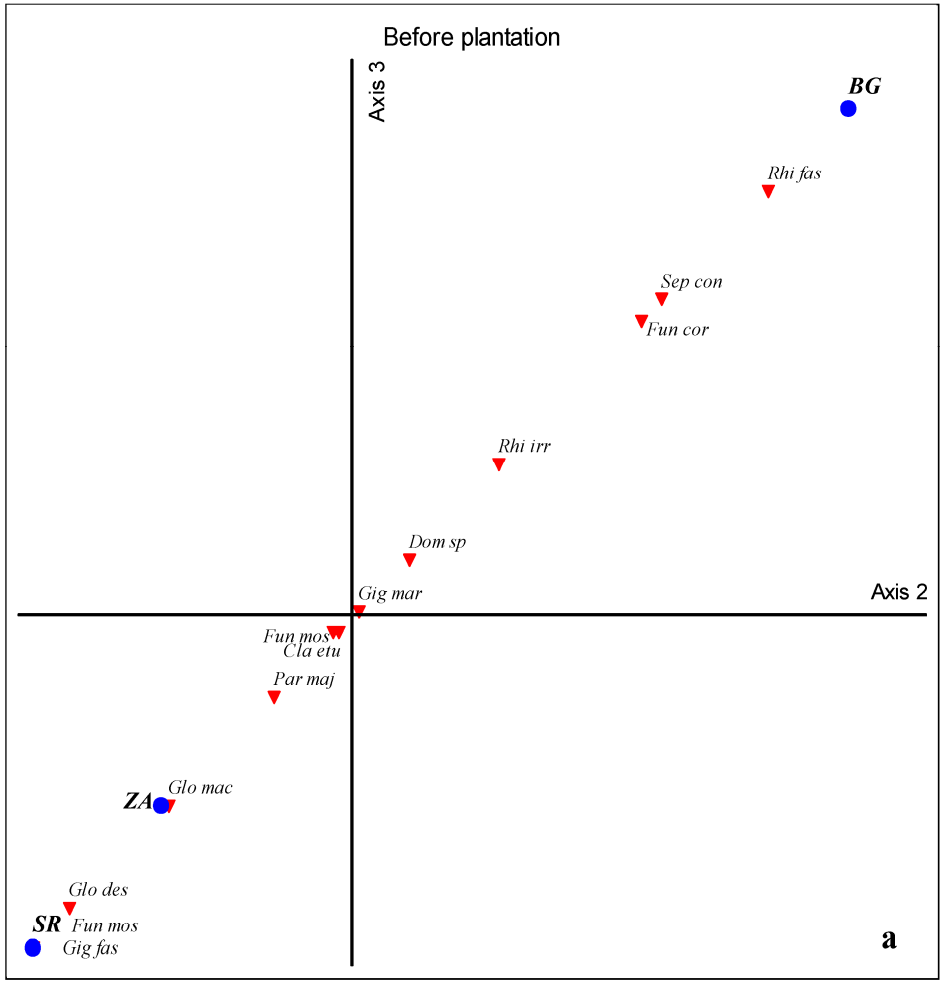
Table 3. Pearson multiple variable analysis correlation between mycorrhizal rates and the studied variables for the three studied host plants.

Studied Parameters	Alfalfa		Clover		Leek	
	Pearson r	***p* Value**	**Pearson r**	***p* Value**	**Pearson r**	***p* Value**
Mycorrhizal rate × soil salinity	−0.5 *	0.05	−0.7 *	0.05	−0.9 **	0.01
Shannon index × Mycorrhizal rate	0.9 *	<0.0001	0.9 ***	<0.02	0.8 *	0.01
AMF species richness × Mycorrhizal rate	0.7 *	<0.05	0.7 *	0.05	0.9 *	0.01
MPN × Mycorrhizal rate	0.9 *	<0.001	0.9 **	0.2	0.3 ns	0.01
NLFA C16:1ω5 × Mycorrhizal rate	0.2 ns	0.7222	0.9 **	0.5	0.4 *	0.01
PLFA C16:1ω5 × Mycorrhizal rate	0.6	<0.0001	0.7 *	0.5	0.3 *	0.01
Gram-positive bacterial biomass × Mycorrhizal rate	0.7 *	0.6	0.3 ns	0.0	0.6 *	0.01
Gram-negative bacterial biomass × Mycorrhizal rate	0.9 *	0.0	0.2 ns	0.1	0.5 *	0.01
Soil organic matter × Mycorrhizal rate	0.38 *	0.05	0.52 *	0.05	0.5 *	0.05
Soil nitrogen × Mycorrhizal rate	0.5 *	0.04	0.41 *	0.05	0.48 *	0.05
Soil phosphorus × Mycorrhizal rate	0.51 **	0.01	0.51 *	0.05	0.2 ns	0.654
Soil humidity × Mycorrhizal rate	−0.04 ns	0.878	0.21 ns	0.78	0.6 ns	0.542

Pearson r: coefficient of parametric correlation. * Correlation significant at the 0.05 level. ** Correlation significant at the 0.01 level. *** Correlation significant at the 0.0001 level. ns: not significant. MPN: most probable number; NLFA: neutral lipid fatty acids; PLFA: phospholipid fatty acids.

Analysis of variance (ANOVA) revealed that AMF species diversity was affected by soil salinity. A significant impact of plant species on AMF diversity was also recorded. A significant effect of the combined impact of plant species and soil salinity was evidenced (Table 4). Soil microbial biomass was affected by soil salinity level (F = 4.54 ***), by the host plant species (F = 2.33 **), by the combination of both host plant species with soil salinity and by the combination of host plant species, soil salinity and climate (F = 2.3 **). Additionally, ANOVA test revealed no significant effect of the combination between climate and soil salinity on mycorrhizal potential (F = 1.31, $p < 0.302$). However, a significant effect was recorded with the combination between the soil salinity and the host plant species on mycorrhizal potential (F = 6.39, $p < 0.05$) (Table 4).

In order to determine the relationship between the three studied saline sites and AMF species communities before and after trap culture as well as the relationship between AMF species and the trap culture host plants (alfalfa, clover and leek) based on two factorial axes, the Detrended Correspondence Analysis (DCA) was used (Figures 5 and S2). The proximity of the AMF species to a saline soil indicates that the AMF species are specifically associated with the site (Figure 5a). Before trap culture, *G. derticola*, *F. mosseae* and *Gi. gigantea* were associated with SR saline soil, while *R. fasiculatum* was associated with BG soil and *G. macrocarpum* with ZA high saline soil. After trap culture, one cluster indicated that SR saline soil was associated with the totality of the AMF species under alfalfa plantation (Figure 5b) and with 80% of the AMF species under both leek and clover (Figure 5c,d). ZA soil was far from the center without any additional AMF species under the three studied host plants (Figure 5b–d). However, BG soil was associated with *R. fasciculatum* with both leek and clover host plants (Figure 5c,d).



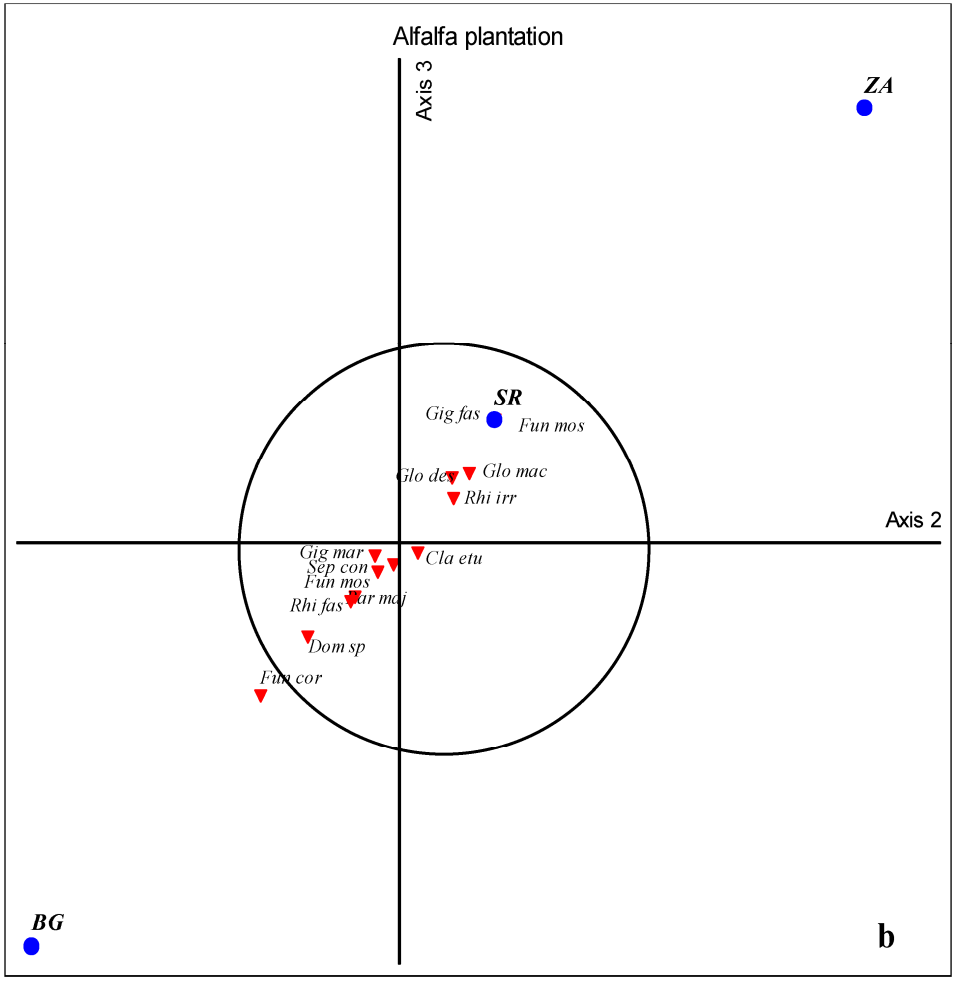


Figure 5. *Cont.*

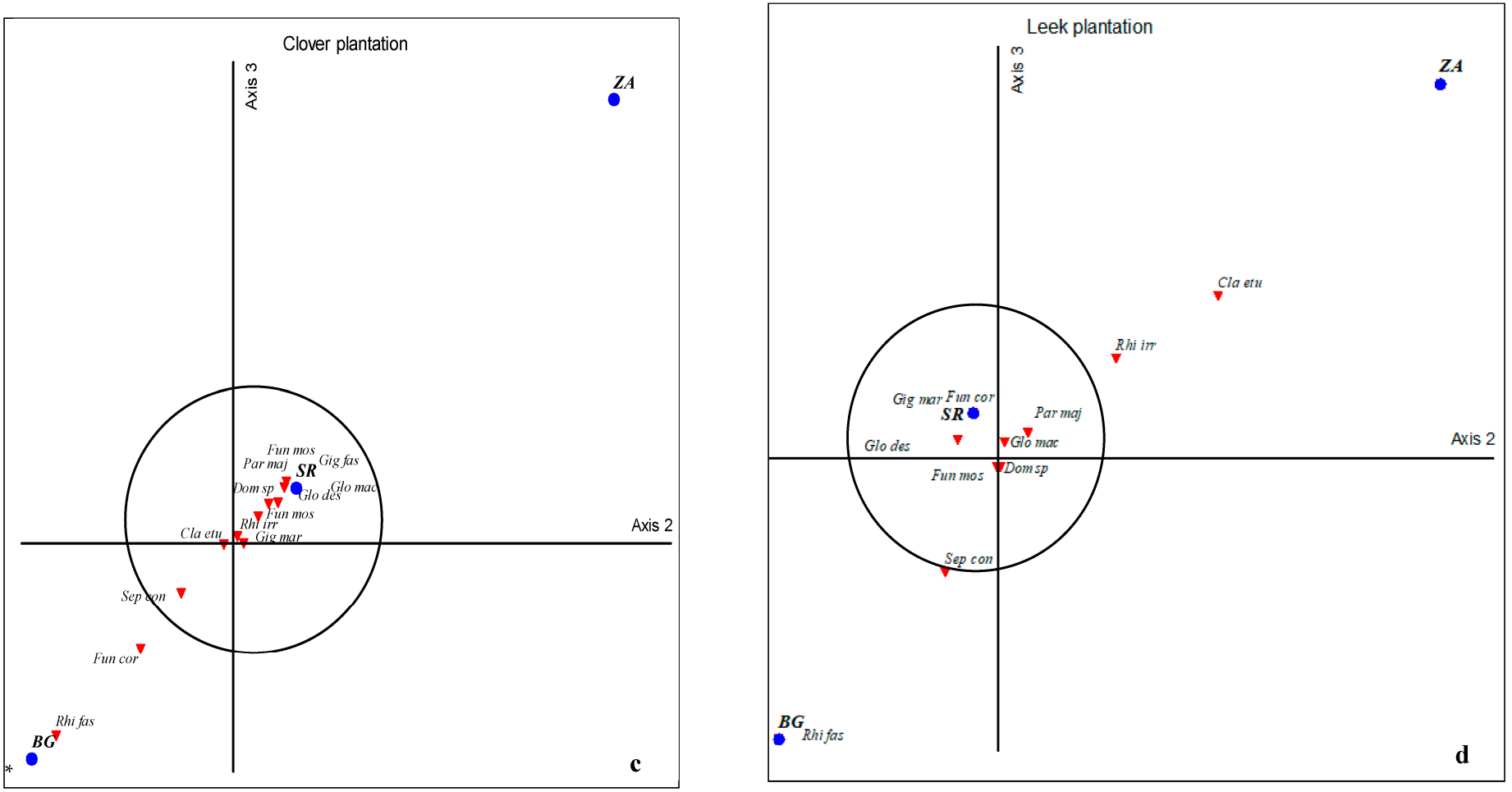


Figure 5. Detrended Correspondence Analysis (DCA) ordination between AMF species distribution before and after trap culture with the different host plants (alfalfa, clover and leek) in the three studied saline sites, namely, SR: Salt Rocket, BG: Boughzoul, ZA: Zaafrane. Data are based on means of three replicates. Red triangles indicate the identified AMF species. Blue dots represent the studied sites. Red triangles close to the center indicate that the AMF species are common to the studied site under the indicated host plant species. (**a**) DCA ordination before trap culture; (**b**) DCA ordination for alfalfa host plant; (**c**) DCA ordination for clover host plant; (**d**) DCA ordination for leek host plant.

Table 4. Influence of host plant species, soil salinity and climate on root mycorrhizal rates, soil microbial biomasses and AMF diversity according to three-way ANOVA for independent variables.

	Host Plant Species	Soil Salinity	Climate	Host Plant × Soil Salinity	Host Plant × Climate	Soil Salinity × Climate	Host Plant × Soil Salinity × Climate
F (value) Mycorrhizal colonization	9.14 *	7.06 **	1.31 *	6.39 *	2.85 ns	3.61 *	8.54 *
F (Value) Soil microbial biomass	2.33 **	4.54 ***	1.32 ns	2.25 **	5.36 *	1.52 ns	2.3 **
F (Value) AMF diversity index	8.69 ***	5.64 **	6.5 *	1.36 *	2.36 *	2.52 *	8.45 *
F (Value) Mycorrhizal potential	3.17 *	2.15 **	1.31 ns	6.39 *	1.23 ns	1.15 ns	1.14 ns

Note: Significant differences: *: 0.05, **: 0.01; ***: 0.001; ns: not significant.

3. Discussion

To the best of our knowledge, the present study is the first one that aims to determine the best combination between native AMF strains, a natural saline substrate and a host plant species in order to produce a more adapted mycorrhizal inoculum for the management of saline soils of Algerian arid and semi-arid regions.

Our findings showed that the host plant species and the soil salinity level significantly influenced the quality of the produced mycorrhizal inoculum. The use of alfalfa as host plant for the trap culture on Salt Rocket (SR) saline soil (8.5 $dS{\cdot}m^{-1}$) was found to be the best combination to produce mycorrhizal inoculum by comparison to leek and clover on SR soil. Indeed, the choice of the host plant is known to be one of the most important criteria for the inoculum production [30]. The host plant must have a high mycorrhizal potential (its capacity to be colonized by the AMF strain and to promote its growth and sporulation), a good ability to grow under growth chamber and greenhouse conditions and an extensive root system made of solid but non-lignified roots [31]. The most frequent host plants employed in AMF inoculum propagation are leek (*Allium porrum* L.), sudan grass (*Sorghum bicolor* (L.) Moench), corn (*Zea mays* L.) and bahia grass (*Paspalum notatum Flugge*) [32]. Our results suggest that alfalfa is suitable for the propagation of native AMF to produce adapted inoculum for arid and semi-arid saline conditions. Alfalfa seems to be more attractive to native AMF strains than leek and clover, as demonstrated by the DCA analysis, where the totality of AMF species was associated with alfalfa in the center of the axis. These results are in agreement with those of dos Santos et al. [33], who suggested that using the appropriate host plant species for trap culture allows for better AMF species diversity. The high attractive power of alfalfa may be attributed to the high secretion of flavonoids [34]. It was reported by Wang et al. [34] that when confronted with external adverse factors such as high salinity, alfalfa increased its excretion of isoflavonoids to ensure a balance of its rhizospheric microbiota. Likewise, Catford et al. [35] reported that alfalfa inoculated with *F. mosseae* released a high quantity of isoflavonoids, compounds known to increase AMF root colonization. Other studies have demonstrated that flavonoids can induce AMF spore germination and hyphal branching, potentially increasing the root colonization rate [36]. In addition to the host plant species, it was previously reported that the substrate used for AMF cultivation can directly influence the production and the infectivity of the produced inoculum [28–37]. The substrate should contain minimal nutrients to guarantee the survival of the host plant so that the fungus can sporulate and propagate [37]. The soil originating from SR site presented the highest level of organic matter and C/N ratio compared to BG and ZA soils. Indeed, Moreira et al. [38] showed that soil chemical composition influences AMF diversity with regard to organic matter, pH, Ca and Mg contents. In addition, SR site has a special substrate composition; it is considered as diapers with evaporitic facies containing lamellar gypsum of anhydrite, marl and schist

with inclusion of conglomerate [39]. Previous studies reported interesting AMF diversity in soils presenting marl and gypsum composition [40,41].

Moreover, it was reported that the inoculum viability is highly variable according the AMF isolates [7] and the host plant species used for inoculum production [18]. In the present study, the greatest total spore number (635 spores per 10 g of soil) and the highest value of Shannon AMF diversity index, with the dominance of *R. irregularis* and *Dominikia* sp., were recorded in SR saline soil under alfalfa plantation. These results may be attributed to the low phosphorus content in SR soil, which makes it more suitable for developing AMF symbiosis. In addition, it was reported that *R. irregularis* colonization operates earlier than many other fungi belonging to the *Glomus* genus, and the sporulation operates densely in the rhizosphere of the host plant [42]. *Dominikia* is a new genus described in soil with a high proportion of sand [43], which is the case with SR soil texture (loamy–sandy). *G. macrocarpum* is another interesting species isolated with alfalfa trap culture on SR saline soil. This AMF species is delicate, and its germination and sporulation depend on the host plant [44]. It is also interesting to note that some AMF species were not found after trap culture with the leek host plant (*G. deserticlola*, *R. fasciculatum*, *Gi. margarita* and *Gi. gigantea)*, while they were isolated with alfalfa in SR soil.

On the other hand, the mycorrhizal rates, indicating the AMF propagule abundance in the host plant, were highest in SR saline soil with the alfalfa host plant. Indeed, structures formed by AMF (arbuscules, vesicles, intraradical hyphae, intra-and extraradical spores) are important germinating structures useful for long-term preservation of AMF species in the produced inoculum and their propagation [32]. The mycorrhizal roots are mixed with the substrate and used as infective propagules in the inoculum to improve its infectivity [45,46]. That is why the root mycorrhizal rate is a suitable criterion for AMF inoculum quality evaluation.

In addition to direct indicators (AMF species composition, spore density, mycorrhizal rates of the host plant roots), indirect indicators such as the MPN and the soil microbial biomass (bacteria and fungi) should be assessed to complete the evaluation of the produced mycorrhizal inoculum quality. Indeed, the inoculum should contain enough viable propagules to achieve AMF root colonization. High concentrations of viable propagules are particularly important to ensure the mycorrhizal potential of the produced inoculum for a long period [16]. Chantelot [47] explained that MPN, giving information on the mycorrhizal potential of the studied soils, is acceptable around 1500 propagules per kg of soil. In the present study, the highest MPN value was recorded in SR saline soil with alfalfa, suggesting the capacity of this host plant to generate more propagules in this saline soil.

In the same context, the amounts of PLFA and NLFA C16:1ω5 (indicators of the AMF biomass), PLFA C18:2,6ω9 (indicator of saprotrophic fungal biomass) and the sum of PLFA i15:0, a15:0, i16:0, i17:0, a17:0, cy17:0, C18:1ω7 and cy19:0 (indicators of bacterial biomass) were found to be the highest with SR saline soil under alfalfa host plant. This result suggested higher microbial activity associated with the produced mycorrhizal inoculum and also showed that soil microbial biomasses evaluated by specific lipid biomarkers could be used as indicators to evaluate the quality and the potential of the produced mycorrhizal inoculum in terms of associated microbiota and improvement of the microbial soil health [23]. Indeed, the global statistical analysis of the entire results indicated positive correlations between root mycorrhizal rates, rhizospheric microbial biomass content and AMF richness. These correlations were particularly strong in the presence of alfalfa and soils with moderate salinity levels.

In light of the present study, the most appropriate inoculum for the management of sustainable agroecosystems in saline steppic regions should consist of the tripartite combination of native AMF strains, a natural substrate (such a soil with moderate salinity level) and alfalfa as the host plant species. In addition, the conventional method is recommended as an engineering process that is affordable at a reasonable cost for native AMF inoculum production.

4. Materials and Methods

4.1. Experimental Sites and Soil Samples

The soils used in the experiment were collected from three localities in steppic arid and semi-arid Algerian areas: Salt Rocket (SR) (34°23′04″ N; 2°,50′17″ E), Zaafrane (ZA) (34°52′40″ N; 2°50′41″ E) and Boughzoul (BG) (35°42′03″ N, 2°50′17″ E). Soil descriptions are presented in Table 1. Soil samples were harvested at the end of the rainy season during March–April 2014 at a depth of 0–40 cm and wet-sieved to <5 mm. Physicochemical analyses of the soil are presented in Table 1. Soil texture was estimated using a textural triangle according to the United States Department of Agriculture (USDA) method. The triangle represents percent of sand, percent of clay and percent of silt. By these percentages, the soil texture classification was determined [48], and organic matter and organic carbon were determined by the Walkley–Black method according to CEEAEQ [49] using soil moisture; total calcareous content was quantified by the calcimeter method according to Petard (1993) [50]. Soil samples were analyzed for pH on (1:5) soil; water suspension and suspension soluble salt were determined by measuring the electrical conductivity at 25 °C [51]. Soil salinity was established according to the USDA [48] description. Available phosphorus (P) was measured according to the Olsen method [52]. Total soil nitrogen (N) was determined using the Kjeldahl method followed by titration of the distillates after Kjeldahl sample preparation and analysis [48]. Available potassium (K), magnesium (Mg) and sodium (Na) were determined according to Nathan et al. [53]. These soil samples served as substrates for AMF inoculum production.

4.2. Description of AMF Native Community

The AMF spores contained in the natural saline soils were extracted by wet-sieving method from 100 g of air-dried sampled soils according to the Gerdmann and Nicolsson [54], quantified and identified morphologically (Blaskowski, 2012) [55] in order to determine the natural AMF biodiversity.

4.3. Experimental Design for AMF Inoculum Production

The experiment was carried out in the soils previously sampled from the three different saline sites (SR, ZA and BG) to produce an AMF inoculum. Soils were used without sterilization in order to propagate native AMF strains. The trap plants used were *Medicago sativa*, *Trifolium repens* and *Allium porum*. Plant seeds were previously sterilized in ethanol 90° for 5 min and rinsed with sterile distilled water. Ten seeds were sowed per pot (500 mL). For each plant species, the experimental design was completely randomized with the three substrate treatments and five replicates (30 experimental units); the experimental design is represented in the supplementary material (Figure S3). Plants were grown under greenhouse conditions: photoperiod 16 h, day/night relative humidity 60/70%, day/night temperature 25/16°C. All pots were irrigated with distilled water every two days. After 4 months, aerial parts were cut, roots were mixed with soil and new sterilized seeds were placed for another 4 months. This operation was repeated 6 times, until 24 months.

4.4. Host Plant Total Mycorrhizal Rates

After 24 months of trap culture, AMF spores were extracted from the substrates using the wet-sieving method [54]. Spores were identified on the basis of their morphological characteristics according to Balskowski [55]. Host plant mycorrhizal rates were determined according to McGonigle et al. [56]. From each pot culture, root fragments were extracted and stained according to the Philipps and Haymann method [57]. Fifteen root fragments of 1 cm were deposited on glass slides in drop of glycerol. Observations with a light microscope (×40) were conducted to annotate each fragment based on the presence or absence of AMF structures (arbuscules, vesicles and hyphae).

4.4.1. Most Probable Number (MPN)

The number of mycorrhizal propagules was estimated using the technique of the MPN, adapted for AMF propagules by Poter (1979) [27] based on the use of a series of successive soil dilutions at the rate of 10 (1/10, 1/100, 1/1000, 1/10,000 and 1/100,000) to gradually deplete the soil and thus identify the limiting dilution in which the existing propagules are no longer able to establish mycorrhiza. The dilutions were prepared by mixing the original soil with the same soil autoclaved at 120 °C for an hour [58]. The soil mixture was divided into five replicates of 50 g per pot. Pre-germinated clover seeds were planted with one seedling per pot, transferred under controlled conditions in a greenhouse (average daily temperature 18–22 °C, with 60–70% relative humidity). The seedlings were watered daily with distilled water. After six weeks, the entire root system was stained according to the aforementioned technique. Using mycorrhizal and non-mycorrhizal roots obtained for each level of dilution and for the five repetitions, the number of propagules present in soil was evaluated using a Cochran table [59].

4.4.2. Soil Microbial Biomass Quantification

Microbial biomass was determined on soil pot culture after 24 months of culture by the use of the fatty acid methyl ester (FAME) method [60]. The amounts of the phospholipid fatty acids (PLFAs) C16:1ω5 and the neutral lipid fatty acids (NLFA) C16:1ω5 were determined and used as indicators of AMF biomass [61]. The NLFA/PLFA ratio of C16:1ω5 fatty acid was calculated; when it was superior to 1, it indicated that the C16:1ω5 fatty acid was originating from AMF and not from bacteria. Gram-positive bacterial biomass was quantified by the sum of the PLFA: i15:0, a15:0, i16:0, i17:0, a17:0, and Gram-negative bacteria biomass was quantified by the sum of the PLFA: cy17:0, C18:1ω7 and the cy19:0 amounts and saprotrophic fungal biomass were evaluated using PLFA C18:2ω6,9 [60].

4.5. Meteorological Data

In order to investigate the effect of climate conditions on mycorrhizal intensity and spore biodiversity and the possible impact of these factors on biofertilizer production, daily mean temperatures (t) and daily precipitation data from the meteorological studies (SR, ZA, BG) were used. Raw daily data for three months (90 days) prior to the date of soil sampling were used to calculate the following seasonal parameters: T: average of mean air temperatures (°C); PP: cumulated daily precipitation (mm); PP/T: average of hygrometry calculated as the ratio between precipitation (PP) and mean temperatures (T) [62]; K: hydrothermal coefficient of Sielianinov ($K = 10 \times PP/(\sum t)$), where PP is the total precipitation of the season, and t is the average daily temperature [63]; and IDM: aridity index of De Martonne ($IDM = 12\ PP/(T + 10)$) [64].

4.6. Statistical Analysis

Data were analyzed statistically by means of comparison by multivariate analysis with Statistica 12 using the Tukey test (HSD b 5%). A Pearson correlation test was carried out between the mycorrhizal colonization rate as the independent quantitative variable and different measured parameters, namely, AMF species richness, Shannon Biodiversity index, Gram-positive and Gram-negative bacteria, PLFA and NLFA C16:1ω5 content, as well as pedological parameters of the three studied saline soils under different host plants. All measurements were performed five times for each treatment, and the calculated means and standard deviations were mentioned. The species richness (S) and the Shannon diversity index (H′) were used to evaluate the diversity of AMF in the studied soils through different trap culture plantations after 24 months of culture using (PC-Ord 5.0). Detrended Correspondence Analysis (DCA) using the DECORANA program of PC-Ord 5.0 [65] with means of AMF data samples was applied to determine the relationship between studied soils and AMF species before and after trap culture as well as the relationship between AMF species and the three studied host plants based on two factorial axes. Three-way ANOVA with defining specific contrast for salinity/host plant/climatic factors with three levels was

performed on root mycorrhizal rate, soil microbial biomasses and biodiversity indices using STATISTICA 12. The F value indicated equality of variances. It was calculated as F = (sum of squares between group/degree of freedom)/(sum of squares within group/degree of freedom).

5. Conclusions

In the context of soil salinity and arid conditions, the most appropriate inoculum should be composed of native AMF strains/natural substrate (soil with a moderate level of salinity)/alfalfa as host plant species. Moreover, the tested combination allows the improvement of the AMF spore richness and diversity as well as the rhizospheric microbial biomass and the root colonization. Thus, our findings open new perspectives for the production of AMF biofertilizers adapted to arid and semi-arid saline soils. Knowing that the mycorrhizal potential of inoculum can be affected by a diversity of parameters such as climate, vegetation and soil composition, the proposed tripartite approach for obtaining inoculum adapted to natural environments is feasible, realistic and promising. The worldwide huge problem of soil salinity can benefit from the results obtained in this study.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/stresses3020030/s1, Table S1: Meteorological data analysis in the sampling period; Figure S1: NLFA/PLFA C16:1ω5 ratio for the studied soils under the three host plant species (alfalfa, clover and leek); Figure S2: Detrended correspondence analysis (DCA) ordination between AMF species and the three studied host plants (alfalfa, clover and leek); Figure S3: Experimental design of trap cultures with the three studied saline soils (ZA: Zaafrane, SR: Salt Rocket; BG: Boughzoul) with the three host plants: alfalfa, clover and leek. (PA: alfalfa plant; PC: clover plant, PLE: leek plant).

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