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YOUNES REZAEE DANESH

KHALIL KARIMAN

NURHAN KESKİN

SOLMAZ NAJAFI

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Characterization of arbuscular mycorrhizal fungal communities associated with vineyards in northwestern Iran

Younes REZAEE DANESH[1]*, Khalil KARIMAN[2], Nurhan KESKİN[3], Solmaz NAJAFI[4]

[1]Department of Plant Protection, Faculty of Agriculture, Urmia University, Urmia, Iran
[2]UWA School of Agriculture and Environment, The University of Western Australia, Perth, Australia
[3]Department of Horticulture, Faculty of Agriculture, Van Yüzüncü Yıl University, Van, Turkey
[4]Department of Field Crops, Faculty of Agriculture, Van Yüzüncü Yıl University, Van, Turkey

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Abstract: Arbuscular mycorrhizal fungi (AMF) establish beneficial symbiotic associations with plants, enhancing their nutrient uptake, growth, and stress tolerance. The present study was aimed to investigate AMF spore density, species composition, mycorrhizal colonization patterns, and correlation of these indices with soil physicochemical parameters at four vineyards located in one of the most important grape-producing regions of Iran (West Azerbaijan province). Forty-three soil samples were collected from four grape-producing regions during July-August 2019. The total spore abundance ranged from 39–647 spores per 100 g dry soil. Twelve AMF species, representing six genera (*Rhizophagus, Funneliformis, Glomus, Septoglomus, Claroideoglomus*, and *Scutellospora*) were identified. *Rhizophagus fasciculatus* was the most frequently found species, with a frequency of 75%. Two of the identified species, *G. glomerulatum* and *Scutellospora calospora* are new for the mycoflora of Iran. Significant negative correlations were detected between AMF attributes [spore density, AMF colonization frequency (F%) as well as AMF colonization intensity (M%)] and soil phosphorus content. The results provide insights into the AMF communities and dynamics in vineyards as influenced by soil parameters, improving our understanding of soil biological fertility in grape production systems.

Key words: Arbuscular mycorrhizal fungi, *Vitis vinifera* L, identification, spore density, root colonization

1. Introduction

Arbuscular mycorrhizal fungi (AMF) are obligate symbiotic soil-inhabiting fungi that form one of the Earth's most common symbiotic associations. These fungi form arbuscular mycorrhizal (AM) symbiosis with plants. They improve the availability of certain essential plant nutrients [e.g., phosphorus (P), zinc (Zn), iron (Fe), and copper (Cu)], which are considered to have low mobility in the soil (Ortas and Akpinar, 2006; Bucher, 2007, Schnepf et al., 2008). Approximately, 80% of higher plants have associations with AMF, which produce finely branched hyphal structures (arbuscules) inside the cortical cells of plant roots (Robinson-Boyer et al., 2009). Since the AMF hyphae are narrower, longer, and more versatile in their direction of growth, they are more efficient than root hairs in terms of nutrient/water acquisition (Goltapeh et al., 2008). Most vascular plants require mycorrhizal colonization to increase survival (Robinson-Boyer et al., 2009).

Grapevine (*Vitis vinifera* L. vitaceae), with about 60 species worldwide, are primarily distributed in the Northern Hemisphere. *Vitis vinifera* L., with around 6000 cultivars, originated from West Asia and has been cultivated for about 5000 years (Ercisli et al., 2008; Agar et al., 2012a). It is distributed between America and Asia, and its fruits have both nutritional and medicinal values (Agar et al., 2012b).

Currently, there is a widespread usage of synthetic pesticides and fertilizers to repress plant pathogens and stimulate plant growth in grapevine nurseries and vineyards. Such practices may reduce microbial complexity in the soil, declining the overall soil health and plant performance (Trouvelot et al., 2015). To minimize the adverse environmental effects of chemical inputs and increase soil fertility, numerous studies have been conducted to explore the impact of organic *versus* conventional systems on soil microbial dynamics (Ryan and Graham, 2002; Purin et al., 2006; Freitas et al., 2011).

AMF inoculation of grapevines has been proposed as the alternative to conventional methods to manage soil sickness problems and improve grapevine growth and health (Schreiner, 2003). In addition to enhanced plant

* Correspondence: y.rdanesh@yahoo.com

growth, association between AMF and grapevine roots can increase tolerance to drought (Nikolaou et al., 2003; Schreiner and Linderman, 2005; Schreiner et al., 2007; Belew et al., 2010). The dependency of plants on AMF for acquiring nutrients can be determined by a number of factors, particularly the type of their root system. Coarse-rooted plants, such as grapevines, are more dependent on AMF for nutrient uptake than fine-rooted species (Motosugi et al., 2002). It is well-established that AMF colonization of grapevine roots contributes to the optimum performance (Meyer et al., 2005). Van Rooyen et al. (2004) showed that inoculation of transplanted grapevines with AMF improves their photosynthetic performance. In contrast, it had no significant impact on the biomass and mineral nutrition of the transplanted grapevines.

Sustainable cropping systems, characterized by low inputs of synthetic fertilizers and biocides, strongly rely on the symbiotic benefits provided by AMF. Considering the well-documented growth/nutritional benefits of AMF for different agricultural and horticultural plants, it is crucial to determine the AMF composition and dynamics in the crop fields, fruit orchards, and vineyards. Our knowledge is quite limited on the AMF composition in vineyards of Iran (Sedaghati, 2002). Therefore, a comprehensive study was conducted to determine the AMF spore density, species composition, and mycorrhizal colonization patterns in four different vineyards located in one of the most important grape-producing regions of Iran (West Azerbaijan province) and determine whether these AMF attributes are correlated with soil chemical parameters.

2. Materials and methods

2.1. Study area, soil sampling and physicochemical analysis

During summer (July–August) 2019, 43 soil samples were collected from vineyards along the major grape-producing regions of West Azerbaijan province (northwestern Iran). The sampling areas were located in four different regions as the most important grape-producing areas including (Mahabad-Naghadeh), (Nazlou-Sero), (Oshnavieh-Shohada valley), and Salmas (Table 1, Figure 1). For each composite soil sample (3 kg), ten soil core samples with fine roots were randomly sampled at a depth of 35 cm (the mycorrhizal incidence is maximal) and mixed thoroughly. The soil samples were dried at room temperature (22 ± 1 °C) and stored at 4 °C until spore isolation. Different soil physico-chemical parameters such as pH, contents of K, P, and organic matter (OM) as well as total nitrogen (TN) were analyzed at laboratories of the Urmia University (Iran) as well as Van Yüzüncü Yıl University (Turkey).

2.2. Estimation of AMF spore density

The soil samples were directly used for the estimation of the AMF spore density. Three replicates (100 g each) were taken from all 43 soil samples and spores were isolated using the wet sieving method followed by centrifugation in 55% sucrose solution (Gerdemann and Nicolson, 1963). The total abundance of AMF spores per sample was calculated as the mean of the three replicates.

2.3. Establishment of trap culture and fungal species identification

Young and healthy AMF spores are required for microscopic slides preparation; hence, trap cultures with maize were established under greenhouse conditions for 6 months. As described above, the AMF spores were isolated from 250 g soil of each trap culture sample. Spores were selected and grouped according to their morphology (i.e. color, size, and structural characteristics) by using a dissecting microscope. Semipermanent slides were prepared using Melzer's reagent with polyvinyl-lacto-glycerol (PVLG). Two hundred and fifty slides were prepared for all trap culture soil samples. Fungal species were identified following Schenk and Perez (1998) and online taxonomic resources such as INVAM (http://www.invam.caf.wvu.edu) as well as collaboration with the School of Agriculture and Environment, University of Western Australia. AMF species frequency was also measured using the following formula (Rezaee Danesh, 2013):

$$\text{Species frequency (\%)} = \frac{A}{B} \times 100$$

A = Number of samples in which the species was observed
B = Total number of samples

2.4. Fungal colonization indices assay

Twenty short fine root fragments (about 1 cm in length) from each soil sample were cleared and stained (Philips and Hayman, 1970) and examined at 100×–400×

Table 1. Geographic details of the sampling areas used to study the arbuscular mycorrhizal fungal communities associated with grapevines in northwestern Iran.

Sampling areas	Sample codes	Geographic location
Mahabad-Naghade	G1-G10	(36°45'51"N, 45°43'26"E)–(36°57'21"N, 45°23'39"E)
Nazlou-Sero	G11-G19	(37°37'23"N, 45°1'7"E) – (37°43'4"N, 44°45'22"E)
Oshnavie-Piranshahr	G20-G31	(37°2'23"N, 45°5'54"E) – (37°17'21"N, 45°8'5"E)
Salmas	G32-G43	(38°11'50"N, 44°45'55"E)

Figure 1. Location of different sampling areas used to study the arbuscular mycorrhizal fungal communities in vineyards in West Azerbaijan province, Iran.

magnification under a compound microscope (Nikon Eclipse E200, Japan) to study different AMF structures in roots. The AMF colonization patterns, including colonization frequency (F%) and colonization intensity (M%), were also determined on the randomly selected fine roots (Trouvelot et al., 1986). Roots were considered mycorrhizal if they contained vesicles, arbuscules, coils, or nonseptate hyphae within the cortex at the point of intersection between the root and eyepiece micrometer.

2.5. Statistical analysis

Spore density values were compared with the colonization indices in each sampling area to find any correlation between the fungal spore density and root colonization patterns. All recorded data were subjected to one-way analysis of variance (ANOVA) in R Studio Desktop 1.2.5042 software by using Duncan multiple range test (DMRT) ($p < 0.01$) to detect significant differences in variable means. The mean spore numbers of four sampling areas were subjected to hierarchical cluster analysis by using the UPGMA method and Euclidean distance using the software package SPSS 26 for Windows. Pearson's correlation coefficients determined the relationship between soil chemical parameters and AMF spore density and AM colonization indices. According to Guilford's rule-of-thumb, the magnitude of correlations was interpreted (Guilford, 1973).

3. Results

3.1. Soil physicochemical analysis

The analysis of the soil physico-chemical parameters (Table 2) showed that the total nitrogen (TN) values were significantly higher in Nazlou-Sero and Oshnavie-Piranshahr areas than Mahabad-Naghade and Salmas ($p < 0.01$). These values were in the same range in Nazlou-Sero and Oshnavie-Piranshahr areas and Mahabad-naghade and Salmas, with no significant differences. The soil P contents were not significantly different between Mahabad-Naghade and Salmas regions but showed difference with the Nazlou-Sero region ($p < 0.01$). The highest p value (13.5 ppm) belonged to the Salmas area. The soil exchangeable K ranged between 322.42 $mgkg^{-1}$ soil (Salmas) and 375.51 $mgkg^{-1}$ soil (Nazlou-Sero) with no significant differences among the four sampling areas. The soil pH ranged from 7.57 (Nazlou-Sero) to 7.92 (Salmas) but it did not show significant differences among the sampling regions. Salmas had the lowest soil OM content among the regions, whereas the soil OM content did not differ significantly among Nazlou-Sero, Oshnavie-Piranshahr, and Mahabad-Naghade regions.

3.2. Estimation of AMF spore density

The AMF spores were found in all soil samples collected from four different sampling areas. Total spore density ranged from 39 (G3 in Mahabad-Naghade area) to 647 spores/100 g dry soil (G26 in Oshnavie-Piranshahr area);

Table 2. The mean values of soil physicochemical parameters in four different sampling areas.

Area	TN (%)	P (ppm)	K ($mgkg^{-1}$)	pH	OM (%)
Mahabad-Naghade	0.38^{b}	12.3^{a}	330.32^{a}	7.87^{a}	9.45^{ab}
Nazlou-Sero	0.57^{a}	7.17^{b}	375.51^{a}	7.57^{a}	12.85^{a}
Oshnavie-Piranshahr	0.52^{a}	10.07^{ab}	352.35^{a}	7.63^{a}	12.63^{a}
Salmas	0.30^{b}	13.5^{a}	322.42^{a}	7.92^{a}	6.54^{b}

(TN): total nitrogen; (P): phosphorous; (K): potassium; (OM): organic matter

Means within columns followed by the same letters are not significantly different according to the DMRT test ($p < 0.01$)

with a mean of 213.91 spores/100 g dry soil. Analysis of variance (ANOVA) for the density of AMF spores of various sampling areas revealed no significant differences among the different sampling areas (df 3, MS 3123.82, F 0.07). Still, the highest number of spores (224 spores/100 g dry soil) were observed in the Nazlou-Sero area. In contrast, there was a significant ($p < 0.01$) difference for the mean number of AMF spores among samples collected within each area (df 39, MS 48013.25, F 29.72). The cluster analysis grouped different sampling areas in three major clusters based on the AMF spore density. Mahabad-Naghade and Oshnavie-Piranshahr areas were grouped together, showing a high degree of similarity. Nazlou-Sero and Salmas areas were separately grouped at levels of distance below 5 (Figure 2).

3.3. Root AM colonization indices assay and correlation analysis

Different AMF structures such as arbuscules, vesicles, hyphal coils, and in some cases, spore masses were observed in all root samples (Figure 3). Vesicles and hyphae were the most common structures observed. AMF spores were also observed in relatively low numbers in or around the roots in a few cases.

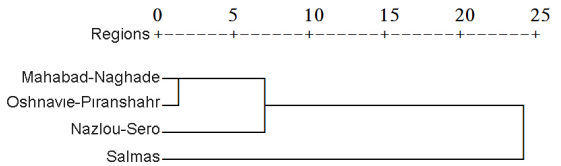

Figure 2. Dendrogram resulting from the UPGMA clustering for the different sampling areas based on the mean number of AMF spores. The scale shows the distance among the sampling areas.

The mean values of AMF spore density and colonization indices in four sampling areas are presented in Table 3. Observations of semipermanent slides showed that AMF colonized all examined plant roots over all sampling areas with colonization frequency (F%) ranging from 83.33% to 94%, with an average of 88.71%. The colonization intensity (M%) ranged between 29.3% to 61.06%, with an average of 43.96%. There were no significant differences in root AM colonization indices ($p < 0.01$) among the four sampling areas. The spore density (SD) did not differ significantly among sampling areas. In addition, the results showed that the spore density was not significantly correlated with colonization indices.

Pearson's correlation coefficients (r) for different soil chemical parameters, AMF spore density, and root colonization indices are presented in Tables 4 and 5, respectively. A significant negative correlation (with moderate magnitude) was observed between AMF spore density and soil P ($r = -0.42$, Table 4). Except for soil P, no significant correlations were noted between AMF spore density and other soil parameters. The results showed that there were significant negative correlations between AMF colonization frequency (F%) and AMF colonization intensity (M%) with soil P (with weak magnitude; $r = -0.232$ and $r = -0.175$, respectively, Table 5). Other than soil P, no significant correlation was detected between

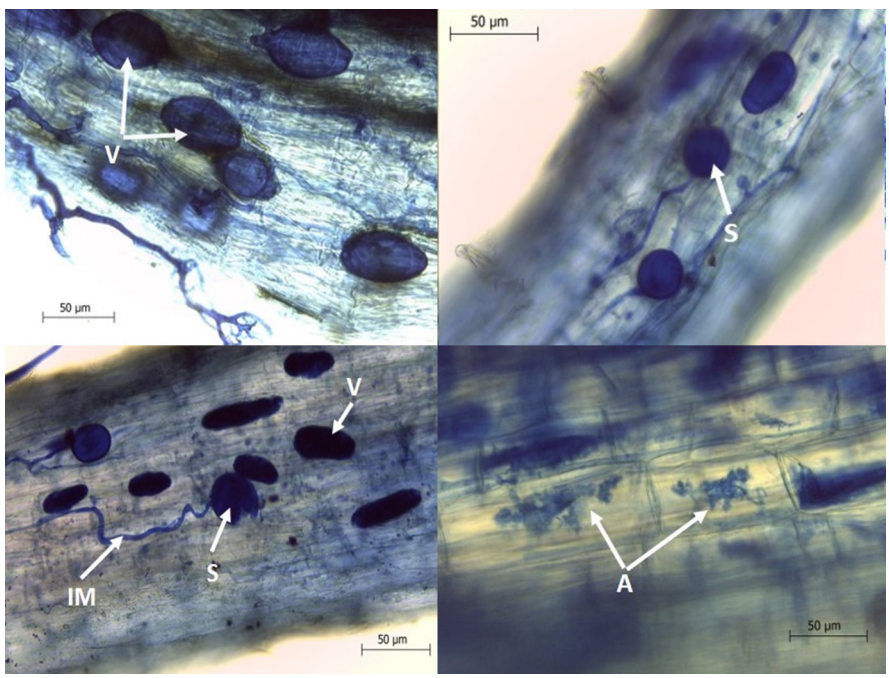

Figure 3. Semipermanent slides showing grapevine roots colonized by AMF. (V) vesicles, (IM) intraradical mycelium, (S) spores, and (A) arbuscules.

Table 3. The mean values of AMF spore density and colonization indices in four different sampling areas.

Areas	SD (N/100 g)	F (%)	M (%)	FSD (%)
Mahabad-Naghade	215[a]	94[a]	61.06[a]	83.33[a]
Nazlou-Sero	224[a]	83.33[a]	29.3[a]	33.33[a]
Oshnavie-Piranshahr	216[a]	87.5[a]	39.4[a]	50[a]
Salmas	201[a]	90[a]	46.09[a]	66.67[a]

(SD): spore density; (F): colonization frequency; (M): colonization intensity; (FSD): fungal species diversity.
Means within columns followed by the same letters are not significantly different according to the DMRT test ($p < 0.01$).

Table 4. Correlation coefficients between soil parameters and AMF spores density.

Parameter	pH	P	OM (%)	TN (%)	K
Spore density (SD)	–0.122	–0.420**	0.210	0.225	0.125

(TN): total nitrogen; (P): soil phosphorous; (K): soil potassium; (OM): organic matter.
**: Significant at $p < 0.01$

Table 5. Correlation coefficients between AMF root colonization indices and soil parameters.

Colonization indices	pH	P	OM (%)	TN (%)	K	SD
Colonization frequency (F%)	–0.454	–0.232**	0.152	0.178	0.182	–0.081
Colonization intensity (M%)	–0.375	–0.175**	0.127	0.153	0.134	–0.032

(TN): total nitrogen; (P): soil phosphorous; (K): soil potassium; (OM): organic matter; (SD): spore density
**: Significant at $p < 0.01$.

AMF colonization indices and other soil parameters. Root colonization indices were not significantly correlated with the AMF spore density (Table 5).

3.4. AMF species identification and their frequencies

A total of 12 AMF species were identified in all samples, which belonged to six genera (*Rhizophagus, Funneliformis, Glomus, Septoglomus, Claroideoglomus*, and *Scutellospora*). Three species belonged to each *Rhizophagus, Funneliformis*, and *Glomus* genera. Also, the *Septoglomus, Claroideoglomus*, and *Scutellospora* genera each had one species (Table 6 and Figure 4). Two species, *G. glomerulatum* and *S. calospora*, were new records for the mycoflora of Iran. Moreover, *G. versiforme*, *Claroideoglomus etunicatum*, and *Funneliformis caledonius* were recorded for the first time

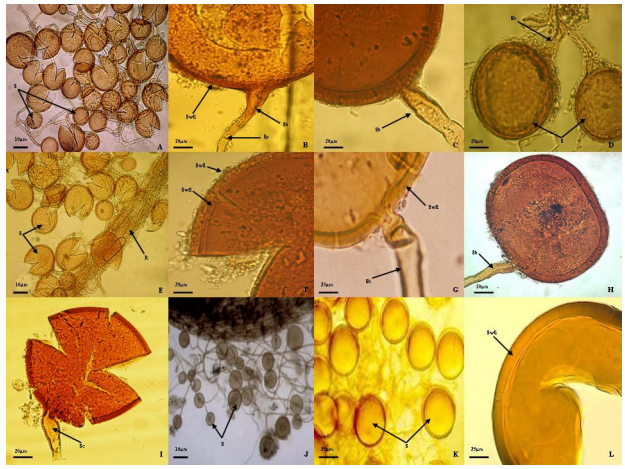

Figure 4. Microscopic images of the AMF species associated with grapevines, A: *Rhizophagus fasciculatus*, B: *Funneliformis mosseae*, C: *Glomus macrocarpum*, D: *Funneliformis geosporus*, E: *Rhizophagus intraradices,* F: *Funneliformis caledonius*, G: *Septoglomus constrictum*, H: *Claroideoglomus etunicatum*, I: *Scutellospora calospora*, J: *Rhizophagus aggregatus*, K: *Glomus glomerulatum*, L: *Glomus versiforme*. AM fungal structures, Spores (s), Attached hypha (Sh), Septum (Sp), Cell wall layers (swl1, swl2) and Sporogenous cell (Sc).

Table 6. List of the identified AMF species and their frequencies in vineyard soils from four different sampling areas (West Azarbaijan province, Iran).

No.	Species	Frequency (%)
1	*Rhizophagus fasciculatus*	75
2	*Funneliformis mosseae*	43
3	*Rhizophagus aggregatus*	38
4	*Glomus macrocarpum*	30
5	*Funneliformis geosporus*	22
6	*Septoglomus constrictum*	18
7	*Rhizophagus intraradices*	13
8	*Funneliformis caledonius*	9
9	*Claroideoglomus etunicatum*	5
10	*Glomus versiforme*	5
11	*Glomus glomerulatum**	2
12	*Scutellospora calospora**	1

* New species for mycoflora of Iran.

from the vineyards in Iran. The AMF species frequency estimated from different grapevines soil samples showed that the most frequent species belonged to the genus *Funneliformis,* particularly *F. fasciculatus* (75%). On the other hand, *G. glomerulatum* and *S. calospora* had the lowest frequencies (2% and 1%, respectively, Table 6).

The AMF species diversity varied among the regions. The number of AMF species per sample ranged from 4 to 10 (average of 7). The highest AMF species diversity was observed in Mahabad-Naghade area (83.33%) followed by Salmas (66.67%), Oshnavie-Piranshahr (50%), and Nazlou-Sero (33.33%) areas, respectively which correlated with AMF colonization indices (Table 3). *Funneliformis fasciculatus* and *F. mosseae* were the most frequently found AMF species in all four sampling areas.

4. Discussion

Understanding the factors that drive the diversity and community composition of AMF in vineyards is important due to the role that AMF play in soil health and the functionality of vineyard ecosystems. Numerous studies have shown that grapevines are dependent on AMF for normal growth and development as well as nutrient uptake (Linderman and Davis, 2001; Schreiner, 2007). Grapevines preinoculated with different *Glomus* species grew considerably faster than noninoculated plants (Karagiannidis et al., 2007; Almaliotis et al., 2008). Ozdemir et al. (2010) showed that *G. mosseae* had a more significant effect on shoot growth parameters, whereas *G. intraradices* had a more significant effect on root growth parameters and uptake of P and Zn. They suggested that these species aid grapevines overcome nutrient deficiencies, especially in soils with low P and Zn contents. Kara et al. (2011) reported positive effects of *Glomus* spp. applications on vegetative development of grapevine cuttings; they used two commercial AMF preparations as Mycosym® (*G. intraradices*) and MycoApply® (consortium of *G. mosseae*, *G. intraradices*, *G. aggregatum*, and *G. etunicatum*) on young plants of table grape varieties and rootstock 41B under greenhouse conditions. Van Rooyen et al. (2004) reported higher stomatal conductance, transpiration rate, and midday xylem water potential in young grapevine plants inoculated with *G. etunicatum.* Spore analysis is an established assay to investigate the viable AMF communities in the soil. Direct extraction of AMF spores from the soil is inadequate for AMF community studies, and sequential trap cultures must be established to record the full range of available species and encourage the initially nonsporulating AMF species. In other words, trap culture techniques might better reflect the AMF community make-up (Robinson-Boyer et al., 2009). Different environmental and soil physicochemical properties such as topography, K, N (Treseder, 2004), soil compaction (Waltert et al., 2002), as well as climatic conditions and host plant (Kivlin et al., 2011) might affect the number of AMF spores and AMF species diversity among different regions. Soil acidity or alkalinity was also demonstrated to be a detrimental factor to AMF sporulation in field soils (Isobe et al., 2007; Balestrini et al., 2010; Sas-Paszt et al., 2020; Betancur-Agudelo et al., 2021). In the present study, spore densities and AM fungal colonization indices were negatively correlated with soil P contents. These results confirmed other studies (Karaarslan and Uyanöz, 2011; Halder et al., 2015; Hindumathi and Reddy, 2016; Mirzaei and Moradi, 2017) but in contrast with others (Aytok et al., 2013; Palta et al., 2018). Therefore, it could be supposed that the correlation depends on AM fungal species, environmental conditions as well as host plant species. The number of spores in soils may indicate the ability of those soils to support infection of plant roots by AMF (Schubert and Cravero, 1985). Despite differences in soil properties in four sampling areas, this study showed that no significant differences in spore density and root colonization indices were observed. Also, there was no correlation between the spore density and grapevine root colonization attributes. These findings are in contrast with some previous studies (Zahka et al., 1995; Brundrett et al., 1996), but they are consistent with those of Becerra et al. (2009) and Moradi et al. (2015). The efficiency of both spore formation and root colonization (from spores or extra radical mycelia) are important factors for maintaining and expanding AMF occupation in ecosystems (Wu et al., 2007). An effective colonization rate is usually about 40% (Thonar et al., 2011), although it is context-dependent. In the present study, based on observation of AMF structures such as vesicles, arbuscules, and mycelia in roots, the average AMF colonization frequency (F%) and colonization intensity (M%) was 88.71% and 43.96%, respectively; indicating a potentially-effective symbiosis between grapevine and AMF. The AM colonization rate can be affected by environmental conditions and host plants. The observed differences in grapevine root colonization by AMF in the present study agree with previous studies on grapevines (Bavaresco and Fogher, 1996; Linderman and Davis, 2001). The percentage of root colonization and the degree of growth response and nutritional benefits provided by AMF may vary according to the AMF species and the rootstock cultivars involved (Linderman and Davis, 2001). We identified 12 AMF species associated with grapevines using the morphology-based approach. The AMF species which are associated with grapevine has been addressed in several studies, initially using surveys based on spore morphology (Schubert and Cravero, 1985; Oehl et al., 2005). These studies highlighted the dominance of species of the former genus *Glomus* which now is classified in different genera such as *Glomus, Rhizophagus, Funneliformis, Claroideoglomus*, and *Paraglomus*. Recent studies have used DNA-based detection and identification methods to analyze AMF communities in colonized roots. Schreiner and Mihara (2009) detected AMF species in a set of ten vineyards in Oregon (USA) that belonged to Glomeraceae, Claroideoglomeraceae, Gigasporaceae, Paraglomeraceae, and Archaeosporaceae families. The family Acaulosporaceae was not found in this study.

Among the identified species, *Rhizophagus irregularis* and *Scutellospora callospora* were the most dominant and rare species. In our study, also the *S. callospora* was found as the rarest fungal species. Balestrini et al. (2010) was also studied the AMF community structures in two vineyards in Piedmont (Italy). They reported the Glomeraceae family as the dominant AMF communities in roots using DNA markers, whereas Acaulosporaceae were completely absent. Some phylotypes were in common with the Oregon study, such as certain Glomeraceae, and rare *S. calospora*. Also, the communities in the two different sites differed strongly, which were attributed to the different soil conditions. Lumini et al. (2010) used a pyrosequencing approach in vineyards in Sardinia to characterize local AMF communities. Again, the Glomerales order was dominant. Using similar sequencing technology, Holland et al. (2014) studied the AMF communities in vineyards of British Columbia (Canada). The identified fungal species in this study belonged to the genera *Rhizophagus, Funneliformis,* and *Claroideoglomus*, together with many unidentified Glomeraceae. In summary, there is currently not much conclusive evidence to set AMF communities in vineyards apart from communities in similar habitats. Dominance of the Glomeraceae family has also been demonstrated in our study which was in agreement with other studies in many other ecosystems.

5. Conclusion

In the present study, we identified 12 AMF species associated with grapevine roots in vineyards located in Northwestern Iran, reporting two new AMF species for the mycoflora of Iran (i.e. *G. glomerulatum* and *Scutellospora calospor*). Results showed that the AMF spore density, as well as AMF colonization frequency and intensity parameters, were negatively correlated with soil P contents. These observations are in line with the existing knowledge on the adverse effects of high soil P on AMF communities and dynamics. Hence, appropriate soil management practices can be considered to enhance the AMF diversity and ensure maximal benefits from AMF in vineyards.

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