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Diversity of endophytic fungal community associated to the roots of *Argania spinosa* (L.) Skeels growing in the arid and semi-arid regions of Algeria

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Diversity of endophytic fungal community associated to the roots of *Argania spinosa* (L.) Skeels growing in the arid and semi-arid regions of Algeria

Abstract: Current study identified endophytic fungi associated to *Argania spinosa* (argan) roots and revealed diverse haplotype diversity by the sequencing of internal transcribed spacer (ITS). 586 operational taxonomic units were identified and these operational taxonomic units (OTUs) could be assigned to fungal functional diversity such as endophytes, ectomycorrhiza and putative pathogens. Ascomycota phylum was abundant. Beside Ascomycota phylum, Basidiomycota members were also found in argan roots. *Geopora, Sebacina, Knufia, Tomentella, Penicillim* had high relative abundance. Our results highlighted a non-nested assemblage of fungi. Current non-nested findings also confirm that fungi have similar pattern found in other habitats. Pairwise analysis mirrored segregation pattern between same and different functional fungal group. Fungi in semi-arid conditions are non-randomly structured. Members of Ascomycota phylum had high Z-scores. This is the first molecular study conducted in arid and semi-arid habitats of Algeria aiming to identify fungi associated with roots in argan tree. Given the fact that deserts are among harsh environments and fungi associated to desert plants may have implications for biodiversity and ecosystem functioning.

Key words: *Argania spinose*; fungi, diversity; internal transcribed spacer; endophytes; ectomycorrhiza

Raznolikost endofitskih glivnih združb povezanih s koreninami argana (*Argania spinosa* (L.) Skeels), v sušnih in polsušnih območjih Alžirije

Izvleček: Namen raziskave je bil določiti endofitske glive, ki so povezane s koreninami argana (*Argania spinosa*) in odkriti raznolikost različnih haplotipov s sekvenciranjem ITS DNK. Določenih je bilo 586 operacijskih taksonomskih enot in te enote (Operational Taxonomic Unit, OTUs) lahko pripišemo funkcionalni raznolikosti gliv kot so endofiti, ektomikorizne glive in potencialni patogeni. V koreninah argana so bili najbolj pogosti predstavniki zaprtotrosnic (Ascomycota), poleg njih so bili najdeni tudi predstavniki prostotrosnic (Basidiomycota). Rodovi *Geopora, Sebacina, Knufia, Tomentella, Penicillim* so imeli veliko relativno pogostnost. Rezultati raziskave so osvetlili nepovezanost skupin gliv. Podobni vzorci nepovezanih skupin gliv so bili najdeni tudi v drugih habitatih. Analiza parov je pokaza vzorce segregacije med enakimi in različnimi funkcionalnimi skupinami gliv. Glive polsušnih območij niso naključno organizirane. Predstavniki zaprtotrosnic imajo velikokrat normalno porazdelitev. To je prva molekularna raziskava v sušnih in polsušnih habitatih Alžirije, katere namen je bil določiti glive, ki so povezane s koreninami argana. Puščave so med najbol ekstremnimi okolji in glive, ki so povezane s puščavskimi rastlinami so pomemben del raznolikosti in delovanja teh ekosistemov.

Ključne besede: *Argania spinose*; glive; raznolikost; ITS; endofiti; ektomikoriza

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1 INTRODUCTION

Fungi are important component of ecosystem and play pivotal role in ecosystem functioning such as carbon and nutrient cycling (Courty et al., 2010; Treseder, 2004). Among fungi there are diverse functional groups such as mycorrhizal, endophytic and pathogenic fungi. These fungi interact with diverse plant species (Smith & Read, 2008), and such interactions are vital for survival and growth of plant species (Finlay, 2008).

Among harsh ecosystem, desert ecosystems represent one of the challenging habitats for microorganism, as water affects microbial activity, which in turn could play crucial role in ecosystem functioning (Austin et al., 2004; Collins et al., 2008). Argan tree (*Argania spinosa* (L.) Skeels) is a slow growing endemic plant species in distributed in north-west Africa (Díaz-Barradas et al., 2010). This tree species is known for its ecological importance because it creates a favourable microclimate for the development of other plant species and protects soils against erosion. In addition, it plays a socio-economic role in the regions where it grows. Each part of the plant including leaves and fruits are used as source of forage for cattle, whereas timber is widely used for fuel purpose, furthermore argan oils have therapeutic properties to cure scars and serve as an anti-ageing agent (Charrouf & Guillaume, 2009). In Algeria, there is decline in population of argan plant due to ecological and anthropogenic factors including climate change and grazing pressure (Charrouf & Guillaume, 2009; Díaz-Barradas et al., 2010).

Given the drastic effect of climate change and anthropogenic factors, it is important to explore the fungal community of desert plant species, such as argan tree. This plant has strong and deep root system (Kenny & De Zborowski, 2007), which harbours high fungal diversity (Sellal, 2016). Previous focus had been paid to study fungal endophytes in arid and semi-arid habitats. For instance, revealed high colonization of fungal endophytes in semi-arid. Martínez-García et al. (2011) also highlighted impact of shrubs on root associated fungi and highlighted importance of selective pressure in determining root associated fungi.

To our knowledge fungal diversity in arid and semi-arid is rarely explored and understood owing to correct identification and most of the fungal strains are non-cultivable in laboratory conditions and laboratory based culturing method may not capture the real fungal diversity (Zhang et al., 2016). Nonetheless, a development in a high throughput sequencing technology provides an excellent platform to explore below ground functional fungal diversity (Buee et al., 2009; Fortuna Miguel et al., 2010).

Nestedness or species-species interaction networks describing the interactions between species is important structural ecological property, and nestedness has revealed positive influence on diversity against catastrophic effect. Nestedness has been proposed to assess community assembly which can further push our understanding about community structure and interactions therein (Ulrich et al., 2009).

We hypothesize fungal community may lack nestedness pattern but could show other non-random pattern such as segregation and aggregation. We propose that limited resources, such as water paucity and nutrients availability generate the segregation pattern.

Key objectives of current study were to: I) identify root associated functional endophytic fungal diversity; II) assess how different fungal taxonomic genera are organize in roots? III) find potential fungal OTUs segregation and aggregation pattern.

2 MATERIAL AND METHODS

2.1 STUDY AREAS AND SAMPLING

The study was carried out in three different climatic regions of Algeria: Tindouf, Mostaganem and Chlef. The area of Tindouf is a desert region in the south-west of Algeria (28°29′56.47″N 8°07′09.72″W). Mostaganem (35°48’09.81″N 0°03’59.30″E) and Chlef (36°09′46.95″N 1°20′12.22″E) are situated in the north-west of Algeria (Table1)

At each region, five healthy specimens (20-30 m apart) of *Argania spinosa* were randomly selected. Four replications in cardinal directions of each tree were collected and homogenised to form a single sample in cardinal directions. In each direction the top litter (20-40 cm) was removed to eliminate part of the dry/not decomposed leaf litter, and samples (soils and argan roots) were collected at 0-30 cm depth and pooled. Fine roots were excavated and traced from the originating tree to ensure identity. Samples were kept in plastic bags and stored at 4 °C until processing.

2.2 DNA EXTRACTION FROM ROOTS AND PCR

After roots surface sterilization by soaking in 70 % ethanol (7:3, v/v, 1 min), 3 % sodium hypochlorite (3 min) and 70 % ethanol (7:3, v/v, 1 min) and were then rinsed twice for 1 min in sterile water , genomic DNA was extracted from field roots, using the genomic DNA Kit (Nucleo Spin Soil). The manufacturer’s protocol was modified in that 250 mg of roots was ground by hand

Table 1: Sites characteristics

Location	Chlef	Mostaganem	Tindouf
Coordinates	36°09′46.95″N 1°20′12.22″E	35°48'09.81'N 0°03'59.30″E	28°29′56.47″N 8°07′09.72″W
Mean annual temperature	18.6° C	18.3° C	23.4° C
Mean annual precipitation	394 mm	436 mm	30 mm
Altitude	119 m	35 m	537 m
Associated plants	*Oxalis* sp. *Olea europea* L.	*Sonchus arvensis* L. *Malva sylvestris* L. *Avena sativa* L. *Echium vulgare* L. *Hordeum vulgare* L. *Bromus* sp. *Plantago laceolata* L. *Antenis coatula* L. *Chrysanthemum* sp. *Cenaurea napifolia* L.	*Acacia tortilis* Forssk. *Acacia raddiana* Forssk. *Anabasis articulata* Forssk., *Asphodelus* sp. *Aristida pungens* Desf., *Calotropis procera* Aiton *Chrysocomoides cassini* Desf. *Euphorbia guyoniana* Boiss. & Reut. *Faidherbia albida* Delile, *Genista saharae* Coss. & Durieu *Helianthemum lippii* L. *Moricandia arvensis* L., *Marrubium deserti* Noë *Nolletia. Retama monosperma* L. *Rhus tripartitus* L., *Zizyphus lotus* Lam., *Zilla spinosa* L.

with pestle in a mortar containing 700 µl of lysis solution before transfer to Eppendorf tube and incubated at 70 °C for 10 min. To study the effect of DNA dilution on PCR inhibitors, after extraction, DNA was diluted 1:1, 1:10, 1:20 and 1:100 with ultra-pure water, in order to obtain DNA containing less PCR inhibitors. Fungal sequences were amplified using the primers ITS1F-ITS4 (White et al., 1990) to target a complete rDNA ITS region. The PCRs were carried out in a final volume of 25 µl, containing 12.5 µl PPP Master Mix (Top-Bio, Prague, Czech Republic) 1 µl of each primer solution (10 µM), 1 µl of DNA template and 9.5 µl ultra-pure water. The PCR conditions were as follow: initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for 45 s and extension at 72 °C for 1min 30 s, with a final extension at 72 °C for 10 min. All PCR reactions were run in Eppendorf PCR cycler. PCR products were examined on a 1 % (w:v) agarose gel with an ethidium bromide staining in and compared to a 100 bp DNA ladder. PCR products (only DNA diluted 1:20 and 1:100 were used in this step) were purified using QIAquick® PCR purification Kit (Qiagen).

2.3 NESTED PCR

The products of the first PCR were diluted 1:1000 with ultra-pure water and used as template DNA for the second PCR amplification using various combinations between gITS7 (Ihrmark et al., 2012) and ITS4 (White et al., 1990) including Illumina adapters. Each PCR reactions (25 µl) contained 12,5 µl PPP Master Mix (Top-Bio, Prague, Czech Republic) 0.5 µl of each primer solution (10 µM), 1µl of DNA template and 10.5 µl ultra-pure water with the following cyclin conditions: initial denaturation at 94 °C for 4 min, followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min. PCR products were examined on a 1 % (w:v) agarose gel with ethidium bromide staining in the presence of 100 bp DNA ladder. PCR products were pooled and purified using QIA quick PCR purification Kit (Qiagen). DNA was quantified with Nano Drop (Thermo Scientific) and pooled before sequencing. Amplicons were sequenced on sequenced on Illumina MiSeq 2×250 bp platform in the laboratory of fungal biology, Institute of

microbiology, The Czech Academy of Sciences, Prague, Czech Republic. Species area curve was generated in PC-ORD version 5 (McCune and Mefford, 2006). Curves were generated based on samples.

2.4 BIOINFORMATICS

Sequencing data was analysed in Seed software (Větrovský & Baldrian, 2013), version 2.0.4. Following parameters were carried out: after rarefying the reads and demultiplexing of the samples based on their unique barcodes, removing low-quality sequences and sequences shorter than 40 bp, ITS sequences were extracted from the sequences, the contigs were chimaera-cleaned/clustered by the Usearch tool, version 8.1.1861, (Edgar & Flyvbjerg, 2015) at the similarity level 97 % and the most abundant sequences were compared while following blast tool version 2.2.26 + (Altschul et al., 2008). Sequencing similarity was matched against the sequences in GenBank database (environmental sequences, metagenomes and unidentified organisms excluded).

2.5 STATISTICAL ANALYSIS

To assess fungal community assemblage in argan plant species we carried out a nestedness analysis based on Nestedness Metric Based On Overlap And Decreasing Fill (NODF metric) (Almeida-Neto et al., 2008) implemented in Aninhado software (Guimaraesjr & Guimaraes, 2006). We used present and absent data to compute NODF metric in Aninhado. Fungal OTUs present and absent data was permuted 1000 times and significance was assessed while following null models in Aninhado software. NODF values were inferred according to *p*-values of null models.

To discern pair wise fungal OTUs association in argan plant roots collected from sampling regions, non-random association between fungal OTUs was assessed. In order to remove a rare species bias, relationship between fungi having relative frequency of 7 was calculated. Unclassified/unidentified fungal species were also not kept in pairwise analysis as this can lead to false conclusion on OTU community. Presence/absence matrix was randomized and computed to get C-scores and *p*-values respectively. Values were inferred while following fixed-fixed null model. All the calculations were carried out in the Pairs software (Ulrich, 2008).

3 RESULTS

3.1 FUNGAL COMMUNITY COMPOSITION

Fungal community was diverse, as 1220 fungal OTUs including singletons were recorded. We removed singletons from our analysis, none the less it resulted in 586 OTUs belonging to 65 different genera. Ten most relatively frequent genera belonged to all three functional groups - endophytic, pathogenic and ectomycorrhizal fungi (Fig.1).

Some genera, such as *Geopora*, *Sebacina*, *Knufia*, *Tomentella* and *Penicillim* had high relative abundance in terms of sequence abundance reads (Fig.2).

Ascomycota was the most abundant phylum in all regions (461 reads) comparing to Basidiomycota (125 reads) ($p = 0.0001$).

The number of reads of OTUs varies between the

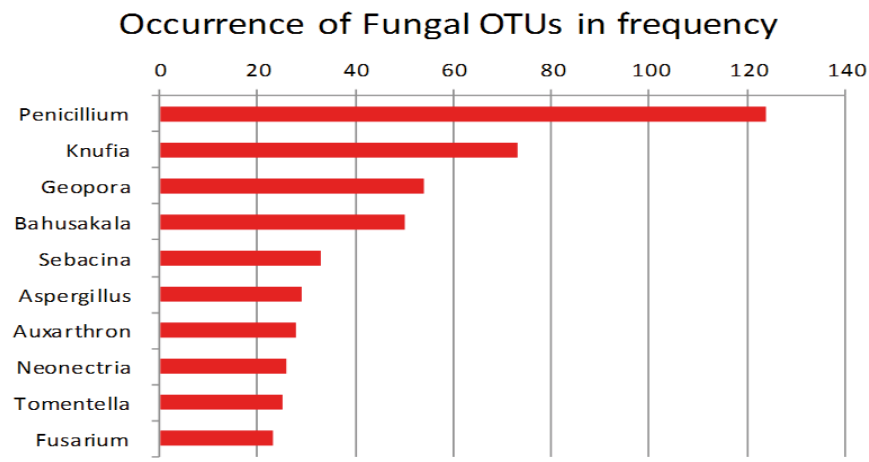

Figure 1: Relative frequency of different functional fungal genus

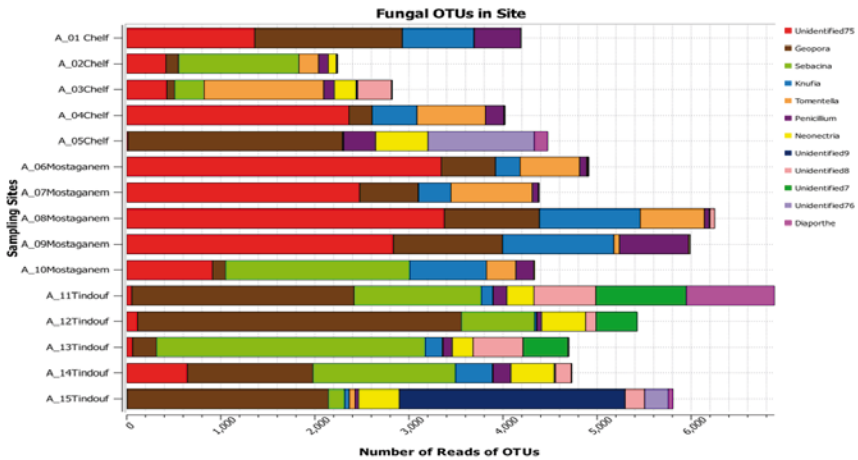

Figure 2: Relative abundance of fungal genus

three regions and from one tree to another in the same region. In fact, Mostaganem was the richest region with fungal community. The genus *Neonectria* was absent in all samples of Mostaganem region. (Figure 2). *Sebacina* was the most abundant genus in Tindouf and absent in all samples of Mostagenem, *Geopora* came in the second position in the same region. However the undefined fungi were the most important fungi in Chlef and Mostaganem ($p = 0.001$).

Sample based curve captured maximum diversity for common fungal OTUs (Fig. 3a), and rarefaction revealed increase in OTU richness, whereas we did not obtain a plateau curve for rare fungal taxa (Figure 3a, Figure 3b).

3.2. FUNGAL COMMUNITY ASSEMBLAGE/NESTEDNESS ANALYSIS

According to our expectations, we detected low lev-

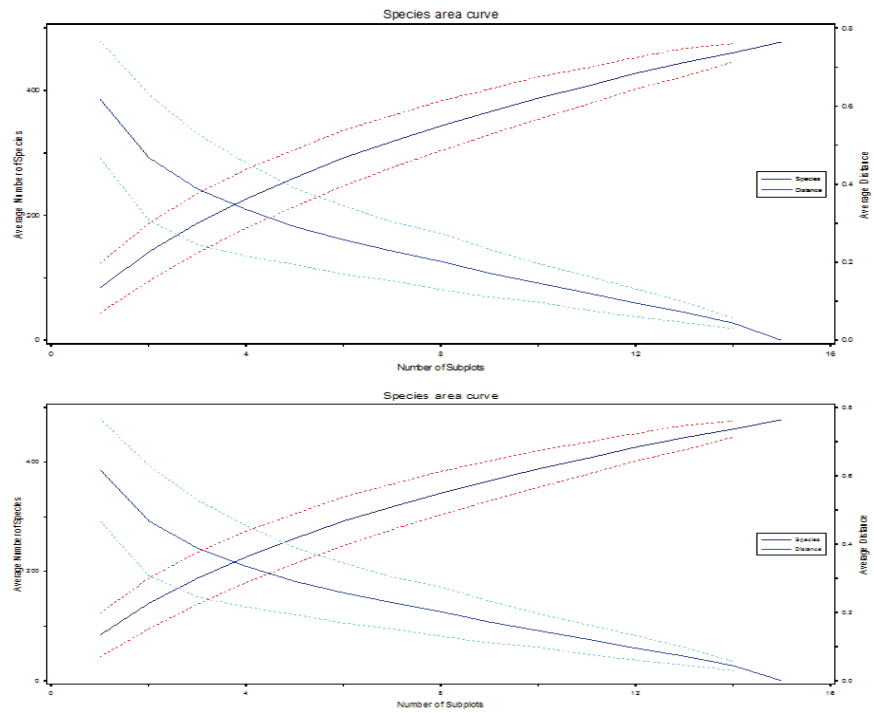

Figure 3a, b: Rarefaction curve for all fungal OTUs. a) Species area curve without singletons. b) Species area curve with singletons. X-axis = number of subplots and Y-axis = OTUs and Sorenson distance of OTUs

Table 2: Species pair results based on Z and P values

Species 1	Species 2	Z -score	P-value
Phomopsis sp.	*Tricholoma* sp.	4.9	6×10^{-7}
Tricholoma sp.	*Pseudogymnoascus* sp.	4.9	6×10^{-7}
Knufia sp.4	*Phomopsis* sp.	4.6	3.5×10^{-6}
Tomentella sp.	*Phomopsis* sp.	4.5	4.2×10^{-6}
Pseudogymnoascus sp	*Bahusakala* sp.2	4.4	8.7×10^{-6}
Knufia sp.4	*Pseudogymnoascus* sp.	3.7	1.4×10^{-4}
Knufia sp.4	*Diaporthe* sp.	3.7	2.0×10^{-4}
Knufia sp.3	*Phomopsis* sp.	3.3	8.8×10^{-4}
Tomentella sp.	*Pseudogymnoascus* sp.	3.1	1.6×10^{-3}
Tricholoma sp.	*Embellisia* sp.	3.1	1.7×10^{-3}
Pyrenochaeta sp.	*Penicillium* sp.6	2.9	3.2×10^{-3}
Tricholoma sp.	*Diaporthe* sp.	2.8	3.8×10^{-3}
Knufia sp.3	*Diaporthe* sp.	2.8	4.8×10^{-3}
Knufia sp.3	*Embellisia* sp.	2.7	5.9×10^{-3}
Phomopsis sp.	*Pseudogymnoascus* sp.	-1.9	4.7×10^{-2}

el of nestedness as revealed by analysis. NODF values for whole fungal community were significantly not higher than expected by chance (NODF = 16, $p = 0.7$).

3.3 GENUS PAIR ANALYSIS

19 species depicted significant non-random association and majority of species showed segregation pattern, while 5 species revealed positive co-occurrence (Table 2). Competitive interactions were predominant at phylum and subphylum level. Positive interactions between the fungi at same and different genera were observed (Table 2).

4 DISCUSSION

Using high throughput sequencing, we explored fungal communities in semi-arid region. Majority of fungal OTUs belonged to Ascomycota phylum. Culturing based study also revealed dominancy of Ascomycota fungi in desert covered by *Artemisia herba-alba* Asso and *Zygophyllum dumosum* Boiss. (Grishkan & Nevo, 2010). Current results are also in line with study conducted in semi-arid areas. Based on high throughput sequencing technology (Wehner et al., 2014), highlighted abundance of Ascomycota phylum in semi-arid. Our results support notions that desert plant *Argania spinosa* harbour diverse fungal communities. We reported fungal OTUs representing different functional diversity such as mycorrhizal, endophytic and pathogens. It is not uncommon to report and document such functional diversity in semi-arid habitat. It has been well documented occurrence of mycorrhizal, endophytic and pathogens diversity in semi-arid (Porras-Alfaro et al., 2008). We highlighted dominancy of Ascomycota phylum and fungal OTUs such as *Penicillium* and *Fusarium*. Indeed, semi-arid supports high endophytic fungal diversity, as strong evidences suggest prevalence and dominancy of Ascomycota, *Penicillium* and *Fusarium* (Gonzalez-Teuber et al., 2017) Fungal OTUs i.e. *Geopora* which are identified and characterized in present research could be compared with other habitats, as *Geopora* formed mycorrhizal association with *Pinus* species (Flores-Renteria et al., 2014), and samples collected in dry season revealed dominancy of *Geopora* genus fungi (Gordon & Gehring, 2011). This shows adaptability and occurrence of *Geopora* in various environmental conditions argan plant supports high fungal diversity, as Basidiomycota members were also recorded. Usually Basidiomycetes fungi are found in relative moist habitat (Buee et al., 2009), therefore fungal diversity explored could be linked other habitats. Research carried out in secondary temperate forest documented occurrence of endophytic and ectomycorrhizal fungi in single host plant species (Frossard et al., 2015). *Sebacina* genus was among abundant fungal genus detected in argan root samples from Tindouf. There is strong evidence which supports ubiquitous nature of Sebacinales and such emerging evidences indicate ubiquitous occurrence

of fungi in various habitats. Our results did not capture rare fungal diversity (Figure 3b). The plausible explanation for such absence of rare fungal diversity is overexploitation of argan plant species and overexploitation may stem local extinction of rare fungal taxa.

Fungal community depicted non-nested assemblage and on the contrary fungi revealed competitive interactions (Table 2). Current non nested findings also confirm that fungi have similar pattern as compare to fungi in forests. We found support for our hypothesis that fungi may lack nested pattern and it could also lead to conclusion that argan root do not support facilitative interactions. Similar results were documented by some other authors (Bahram et al., 2014; Roy-Bolduc et al., 2016). Since, argan plants are cultivated and thus under human management and grazing pressure (Charrouf & Guillaume, 2009; Díaz-Barradas et al., 2010), we cannot exclude anthropogenic factors causing a non-nested assemblage of fungi. Another explanation is that host plants were under abiotic stress, which in turn may had generated non nested pattern.

Pair wise analysis mirrored segregation pattern between same and different functional fungal group. Several mechanisms are proposed to reveal such non-random occurrence pattern and competitive interactions between fungal OTUs could generate segregation pattern (Chen et al., 2000; Chilvers et al., 1987).

Competitive interactions between fungal OTUs in semi-arid have revealed that fungal communities were non-randomly structured in semi-arid. Our results are in line with (Wehner et al., 2014) who highlighted the abundance of Ascomycota phylum in semi-arid. We also showed that majority of fungal OTUs belonging to Ascomycota phylum had Z- scores -2 above 2 (Table2), showing statistical significance (Ulrich, 2008). Fungal pairs having endophytic and mycorrhizal mode of life style had high Z-scores, which indicated strong signal of competition between them. It is important to mention that previous studies focused on fungal groups and highlighted the prevalence of segregation patterns between ectomycorrhizal and endophytic fungi (Pickles et al., 2012; Saunders et al., 2010).

Positive interactions between same and different fungal OTUs suggested facilitative interactions between fungi. Facilitative interactions between fungi could be due to fact that fungi having similar functional requirements may occupy the same habitat which in turn could result in facilitative interactions. Species may sort according to shared requirements (Leibold et al., 2004), this may generate positive interactions between species. Given the fact, different functional genus in root occur due to different ecological and physiological requirements; henceforth there is high probability of positive interactions between fungi and facilitative interactions are well documented between different functional fungal groups (Wagg et al., 2008).

5 CONCLUSIONS

The present study is the first study to assess fungi associated with *Argania spinosa* roots growing in the arid and semi-arid climate. We revealed non nested assemblage pattern at community level, whereas pair wise association showed non-random pattern. Quite significant numbers of fungal OTUs were explored.

Future studies may carry out abundance-based data to assess pair wise association between fungi. We provide framework and initial study while using present-absent data to reveal non-random association of fungi in argan roots. Furthermore, inoculation experiments may be conducted to confirm facilitative and competitive interactions. Perhaps most striking feature of our research was high fungal diversity associated to single desert plant species.

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