

ENDOPHYTIC FUNGI AND FOLIAR EPIPHYTES OF *Peganum harmala* L. (ZYGOPHYLLACEAE) OF DAYATE AIAT (LAGHOuat, ALGERIA)

OUZID Yasmina^{1,2*}, SMAIL-SAADOUN Noria¹ et HOUALI Karim¹

⁽¹⁾Laboratory of Natural Resources, Mouloud Mammeri University of Tizi-Ouzou, Algeria

⁽²⁾Laboratory of Analytical Biochemistry and Biotechnologies, Mouloud Mammeri University of Tizi-Ouzou, Algeria

Email: *biochimie2009@hotmail.fr

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Abstract.- Whether epiphytes or endophytes, fungi live in complex interaction with the plants where both partners benefit. Our study was carried out on a spontaneous medicinal plant of the region Laghouat (Algeria): *Peganum harmala*. Our interest in this work has focused on the diversity of mycoendophytes and epiphytes in the leaves of this species. Ten healthy subjects were harvested in April 2015 at random. For the epiphytes, leaf suspensions were seeded on PDA and incubated at room temperature. As for mycoendophytes, surface sterilization of leaves was performed to remove epiphytic organisms that remain at the phylloplane. The fragments are seeded on PDA medium and incubated at room temperature. The microscopic identification revealed that the phyllosphere fungi of *Peganum harmala* belonged much more to the phylum Ascomycota. The genera listed indicate an abundance of the genus *Penicillium* and *Rhizopus* for the epiphytes, *Cladosporium* and *Alternaria* for mycoendophytes. This diversity is estimated by the global Shannon index with 1.78 for the epiphytes and 1.08 for the foliar endophytic fungi of this plant. Moreover, the overall ratio of similarity Sorensen is 0.62 between the fungal communities epiphytic and endophytic of these leaves. Significant interactions are described between different fungal taxa through a correlation matrix indicating the impact of these interactions on fungal biodiversity in leaves of *Peganum harmala*.

Key words: Foliar mycoendophytes, epiphytic fungi, diversity, *Peganum harmala*, Laghouat, Algeria.

CHAMPIGNONS ENDOPHYTES ET EPIPHYTES FOLIAIRES DE *Peganum harmala* L. (ZYGOPHYLLACEAE) DE DAYATE AIAT (LAGHOuat, ALGERIE)

Résumé.- Qu'ils soient épiphytes ou endophytes, les champignons vivent en interaction complexe avec les plantes où les deux partenaires tirent profit. L'étude a été réalisée sur une plante médicinale spontanée de la région de Laghouat (Algérie): *Peganum harmala*. L'intérêt dans ce travail s'est porté sur la diversité des mycoendophytes et des épiphytes au niveau des feuilles de cette essence. Dix sujets sains ont été récoltés durant le mois d'avril 2015 de manière aléatoire. Pour les épiphytes, des suspensions de feuilles ont été ensemencées sur PDA. Quant aux mycoendophytes, une stérilisation superficielle des feuilles a été réalisée afin d'éliminer les organismes épiphytes qui demeurent au niveau du phylloplan. Les fragments sont ensemencés sur milieu PDA. Le tout est incubé à température ambiante. L'identification microscopique a révélé que les champignons de la phyllosphère de *Peganum harmala* appartenaient pour la plupart au phylum des Ascomycota. Les genres recensés indiquent une abondance des genres *Penicillium* et *Rhizopus* pour les épiphytes, *Cladosporium* et *Alternaria* pour les mycoendophytes. Cette diversité est estimée par l'indice de Shannon avec 1.78 pour les épiphytes et 1.08 pour les champignons endophytes. Par ailleurs, le quotient global de similarité de Sorensen est de 0.62 entre les communautés fongiques épiphytiques et endophytiques de ces mêmes feuilles. Des interactions importantes sont décrites entre les différents taxons fongiques indiquant ainsi l'impact de ces interactions sur la biodiversité fongique au niveau des feuilles de *Peganum harmala*.

Mots clés: Mycoendophytes foliaires, champignons épiphytes, diversité, *Peganum harmala*, Laghouat, Algérie.

Introduction

Arid regions are characterized by a variety of hard environmental conditions, including low rainfall, high temperatures and UV irradiation [1]. All organisms that growth in these extreme environmental conditions adopt complex strategies to attenuate abiotic stresses [2-4]. Among these, we can mention the possible symbiosis with microorganisms in different parts of the plant, especially the leaves wich are exposed directly to these different stresses. Indeed the surface and inside the leaves provide habitat called phyllosphere which houses a wide range of microorganisms [5-7]. Fungi are a major component of the microbiome of this phyllosphere [7]. Many studies have shown that a single leaf can harbor a wide variety of fungal species [8]. These microorganisms live in complex interaction with these leaves, colonizing all the tissues of the latter. They play a role in improving the ecological performance of the aerial part, as well as the whole plant [9]. This fungus includes epiphytes that colonize the surface (phylloplan) and endophytes within the leaves, thus occupying two distinct microniches [5,6].

Among the plants found in these arid environments, we can cite *Peganum harmala* L. is a medicinal plant belonging to the family of Zygophyllaceae. This family is represented on all continents, mainly in the arid regions. It is a perennial plant with very rower's rods, up to 50 cm height and disappearing in winter. The leaves are divided into narrow strips [10]. It is known for its hallucinogenic and hypothermic properties since antiquity. Many alkaloids identified in this plant have shown numerous pharmacological actions such as antitumor effects [11,12], analgesic effects [13] as well as antimicrobial activities [14,15].

In Algeria, the areas occupied by this plant continue to increase, following the degradation of the pastoral steppes, due to recurring droughts and increasing anthropic pressure. It is a toxic plant rich in alkaloids, flavonoids and anthraquinones [16]. These repulsive compounds can be synthesized by the plant alone, or with the help of the various potential symbiotic fungi present in the arid lands in which it lives? No studies have been done on the microbiome associated with this plant.

Our work has focused on the epiphytic and endophytic fungi of the leaves of *Peganum harmala*. We were interested in (i) the phylum inventory and (ii) the genera present, the abundances of the various genres identified and the measurements of generic diversity, and (iii) the interactions between the fungi of the two microniches: epiphytes and endophytes.

1.- Material and methods

1.1.- Study zone

In the heart of the country of the dayas, in the southern part of the wilaya of Laghouat, is located our study area. Dayate Aiat is 50 km from the capital of the wilaya in the region of Timzerth. These GPS coordinates are 33 ° 31N for latitude, 2 ° 56E for longitude. It is at 871 m altitude (fig. 1). Bioclimatically, the study area is located in the arid zone with a dry season of 11 months a year [17].

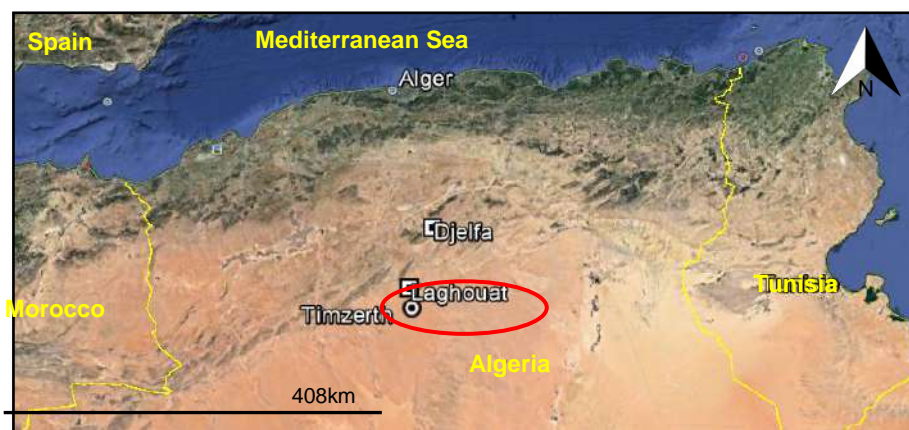


Figure 1.- Location of the study area (Laghouat, Timzerth, Algeria) (www.google.fr)

1.2.- Sampling

Peganum harmala leaves were randomly harvested from ten healthy subjects in April 2015. The samples were placed in paper bags, stored in a cooler, and then refrigerated until laboratory use in a relatively short time, or up to two days after collection.

1.3.- Isolation and cultivation of epiphytes

The epiphytic fungi were isolated according to the protocol of PUSZ *et al.* (2015) [18] as amended by us. Five leaves were removed from each subject and placed in 20 ml vials containing 10 ml of sterilized distilled water. The flasks are centrifuged for 10 min at 4250 per minute (6×550g). 1ml of the supernatant obtained was seeded in petri dishes in an amount of 3 repetitions for each subject. These dishes were previously cast with PDA containing chloramphenicol in order to avoid any bacterial contamination.

1.4. Isolation and cultivation of endophytic fungi

Surface sterilization was performed according to the protocol of SCHULZ *et al.* (1993) [19] to remove epiphytic organisms that remain at the phylloplane. To do this, leaves are washed with running water to remove dust and debris. Each sample is treated with 95% ethanol for 2 min, followed by a treatment with bleach water (99.99%) for 3 minutes, then a second treatment with 95% ethanol for 30 sec. Between these three treatments, we rinse with sterilized distilled water. Fifteen sheets per subject are seeded on PDA with Chloramphenicol, at the rate of five fragments per Petri dish, 5 to 7 mm each. Incubation is at room temperature for one week.

After incubation, we proceed to the isolation and identification of the fungal genera. The latter is made from macroscopic and microscopic observations, namely; The morphology of hyphae, the presence or absence of septa, the form and grouping of conidia and spores (solitary, chains, bouquets, etc.), as well as morpho-cultural characters on PDA (contours, color, relief, transparency, surface, and size) [20-23].

1.5. Statistic study

The abundances of different fungal genera identified in the ten subjects for epiphytic and endophytic fungi were calculated using this formula:

$$A(\%) = \frac{N_g}{N_t} \quad (1)$$

A: abundance of genera;
Ng: number of times the gender is recorded in a subject;
Nt: set of repetitions.

Fungic diversity is estimated by the Shannon index which is calculated by the following formula (Magurran, 1988) [24]:

$$H = - \sum \frac{x_i}{x_0} \ln \frac{x_i}{x_0} \quad (2)$$

x_i is the total number of specific isolates and x_0 is the total number of all isolates.

The Shannon index was calculated for each of the ten subjects concerning epiphytic and endophytic fungi as well as for each microniche.

Analyzes of variance (ANOVA) were performed to determine if there are significant differences between fungal diversity of different individuals for each microniche and between the two considered microniches and thanks to Box Stat 6.40 software.

To determine the similarity between the epiphytic and endophytic fungal communities for the different subjects harvested, the Sorensen similarity quotient (SQ) was used and calculated using the following formula (Krebs, 1999) [25]:

$$SQ = \frac{2a}{2a+b+c} \quad (3)$$

a: number of genera common to the surface (epiphyte) and inside the leaves (endophyte);
b: number of genera specific to the surface of the leaves;
c: number of unique genera within the leaves.

A correlation matrix (Pearson) was constructed between these endophytic and epiphytic fungal communities for the subjects sampled in order to demonstrate the interactions between the different fungal genes according to the subjects sampled through the software Stat Box 6.40. We were interested only in significant interactions ($p = 0.05$), which we summarized in a table.

2.- Results

2.1.- Abundances of epiphytic and endophytic fungi

The epiphytic fungi identified in the phyllosphere of *Peganum harmala* belong to different phyla, namely Ascomycota with 78%, Zygomycota with 21% and Basidiomycota with 1% (fig. 2). In addition, 97% of the endophytic fungi listed belong to the Ascomycota phylum, Basidiomycota only 2%, and Zygomycota 1% (fig. 3).

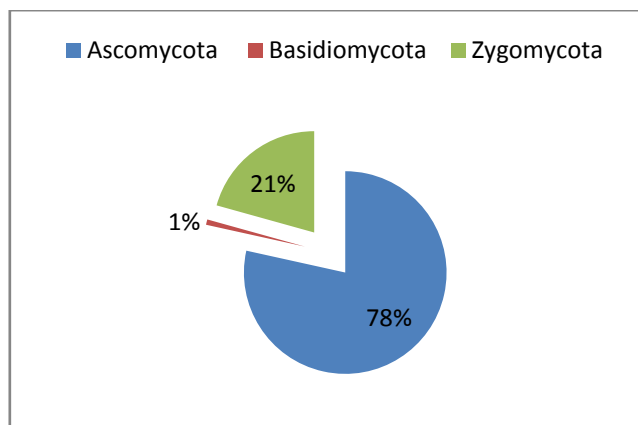


Figure 2.- Phyla of the epiphytic fungi of the leaves of *Peganum harmala*

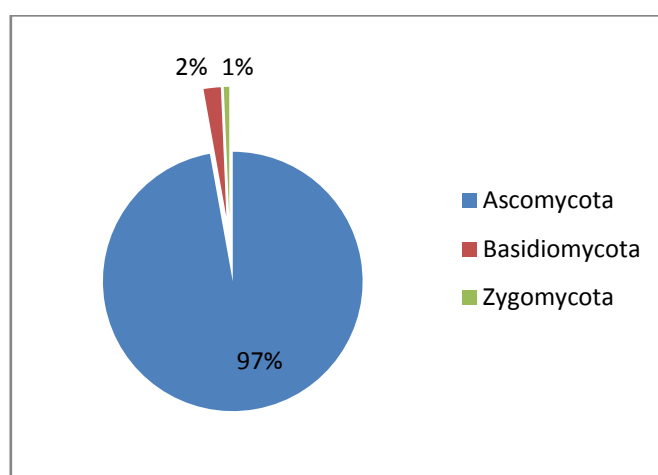


Figure 3.- Phyla of the mycoendophytes of the leaves of *Peganum harmala*

15 genus of epiphytic fungi and 14 genus of mycoendophytes were identified in the leaves of *Peganum harmala*. The results obtained show an abundance of the genus *Penicillium* and *Rhizopus* for the epiphytes, *Cladosporium* and *Alternaria* for the endophytes. For the genus *Mycocladius*, *Mucor*, *Scopulariopsis*, *Phoma*, *Colletotricum* and *Epicoccum*, they exist only at the level of the epiphytes. The genera *Exophiala*, *Aureobasidium*, *Verticillium*, *Paraphaephaeria* and *Absidia* are present only on the level of the endophytes.

For the epiphytic fungi, the genus *Penicillium* dominates with 14.66%, followed by *Rhizopus* (13.33%) and *Alternaria* (11.99%). *Mycocladius*, *Phoma*, *Rhizoctonia* and *Colletotricum* (0.66%) are the least abundant of the foliar epiphytes of *Peganum harmala* (fig. 4). As for mycoendophytes, the genus *Cladosporium* (34.63%) is the most abundant, followed by *Alternaria* (21.89%) and *Aspergillus* (14.74%). The genera *Absidia*, *Paraphaephaeria* (0.76%), *Bipolaris* (1.25%) and *Trichophyton* (1.66%) are the least abundant in foliar mycoendophytes. Some strains could not be morphologically determined. We called them unidentified strains (SNI) (fig. 5).

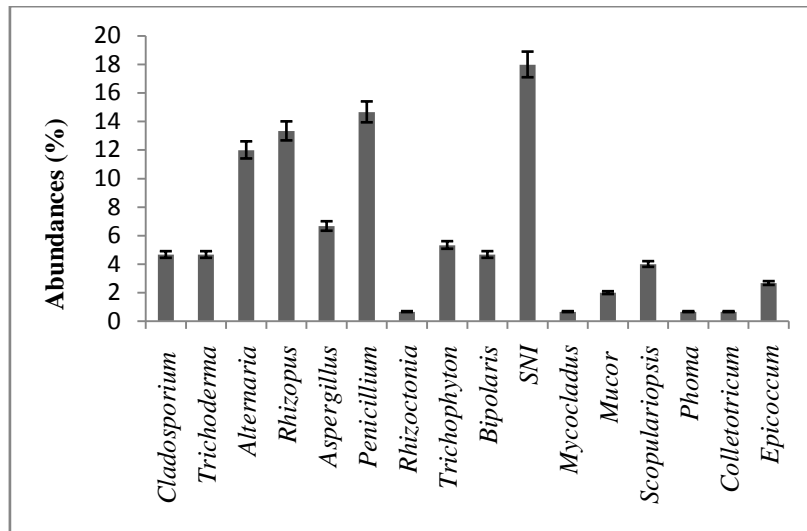


Figure 4.- Abundances of the epiphytic genera of the leaves of *Peganum harmala*

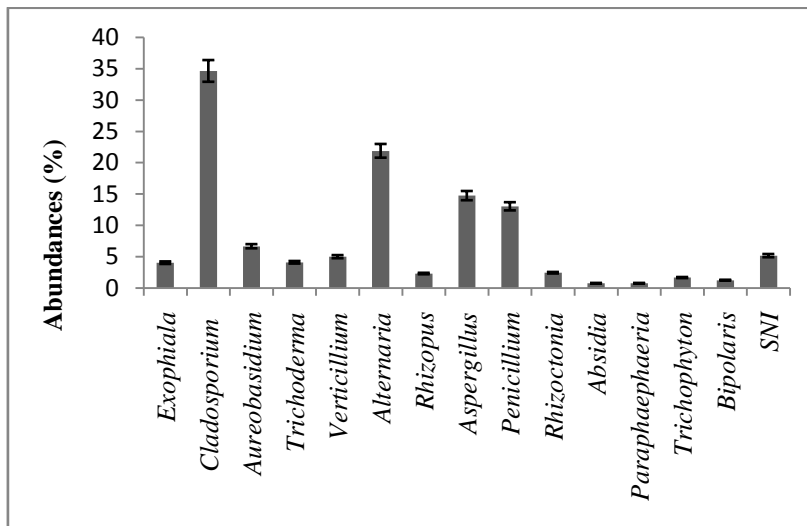


Figure 5.- Abundances of the genus of mycoendophytes of the leaves of *Peganum harmala*

2.2.- Diversity of fungal phyllosphere

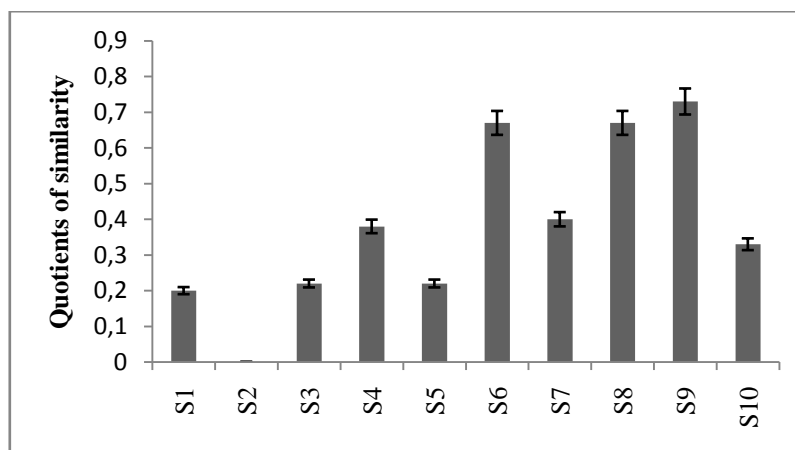
The diversity of fungal phyllosphere sheets *Peganum harmala* in subjects identified is estimated by Shannon index (tab. I). The indices vary from one subject to another. The highest index for epiphytes is that of subject 10 (2.118), and for endophytes that of subject 4 (2.177). For the lowest index, for epiphytes it is noted in subject 8 (0.995), for endophytes in subject 2 (0). In addition, the global Shannon index for epiphytes is 1.78 and that for endophytes is 1.08.

Analysis of variance (ANOVA) between the Shannon indices of the leaves of the different *Peganum harmala* subjects did not indicate any significant difference between the epiphytic communities of the leaves ($p = 0.21$). On the other hand, this difference is significant between the endophytic communities ($p = 0.05$). For the ANOVA performed between the global Shannon indices, the difference is highly significant between the two communities: epiphytes and foliar endophytes ($p = 0.00$).

Table I.- Diversity of the fungal phyllosphere of the leaves of *Peganum harmala*: diversity index Shannon (H)

H	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
H		0	1,03	2,17	1,27	1,31	1,16	0,92	1,55	0,73
Endophytes	0,562		9	7	7	3	2	6	9	5
H Epiphytes	2,059	2,06	1,58	2,04	1,52	1,58	1,87	0,99	1,97	2,11
		2	9	6	2	5	6	5	9	8

Sorensen similarity quotients for the ten subjects sampled vary between 0 - 0.73 (fig. 6). The subject 9 shows the highest similarity quotient (0.73), followed by 8 and 6 subjects (0.67). On the other hand, subject 2 (0) shows no similarity between these fungal communities. In terms of the overall Sorensen similarity quotient, it is 0.62 between the epiphytic and endophytic communities of the leaves of *Peganum harmala*.

**Figure 6.-** Sorensen Similarity Quotients of different *Peganum harmala* subjects

2.3.- Interaction between epiphytic and endophytic fungi

Significant interactions are described between the different fungal taxa of the phyllosphere of *Peganum harmala* of dayate Aiat. For this purpose, a correlation matrix is made to identify and describe the nature of these interactions (tab. II). From the correlation matrix, we find a single negative correlation between *Cladosporium* and unidentified strains (SNI) (-0.62). Highly positive correlations were observed between two exclusively endophytic fungal genera: *Absidia* and *Paraphaephyria* (1), *Aureobasidium* and *Verticillium* (0.89), *Exophiala* and *Absidia* (0.49). It should be noted that these positive correlations between two fungal genera, where one is exclusively epiphyte: *Mucor* and the other both endophyte and epiphyte: *Rhizopus* (0.88), likewise for *Aureobasidium* only epiphyte and *Trichoderma* endophyte and epiphyte (0.83), between *Verticillium* only endophyte and *Trichoderma* both (0.70), *Epicoccum* exclusively epiphyte and *Bipolaris* endophyte and epiphyte (0.62), *Mycocladius* exclusively epiphyte and *Rhizopus* endophyte and epiphyte (0.56). *Aspergillus* and *Rhizoctonia* are both endophytes and epiphytes (0.63). Exclusively epiphytic genera are also positively correlated with each other: *Mucor* and *Mycocladius* (0.55), *Epicoccum* and *Mycocladius* (0.46) and finally *Phoma* and *Epicoccum* (0.46).

Table II.- Significant interactions between the phyllosphere fungi of *Peganum harmala* (SNI: unidentified strains)

Genus	Significant value of the interaction (p=0,05)
<i>Cladosporium</i> – SNI	-0,62
<i>Absidia</i> – <i>Paraphaephaeria</i>	1,00
<i>Aureobasidium</i> – <i>Verticillium</i>	0,89
<i>Rhizopus</i> – <i>Mucor</i>	0,88
<i>Trichoderma</i> - <i>Aureobasidium</i>	0,83
<i>Trichoderma</i> – <i>Verticillium</i>	0,70
<i>Aspergillus</i> – <i>Rhizoctonia</i>	0,63
<i>Bipolaris</i> – <i>Epicoccum</i>	0,62
<i>Rhizopus</i> – <i>Mycocladius</i>	0,56
<i>Mycocladius</i> – <i>Mucor</i>	0,55
<i>Scopulariopsis</i> – <i>Mucor</i>	0,53
<i>Bipolaris</i> – SNI	0,53
<i>Exophiala</i> – <i>Absidia</i>	0,49
<i>Exophiala</i> - <i>Paraphaephaeria</i>	0,49
<i>Epicoccum</i> – <i>Mycocladius</i>	0,46
<i>Phoma</i> – <i>Epicoccum</i>	0,46
<i>Epicoccum</i> – SNI	0,45

3.- Discussion

3.1.- Phyla and genera of phyllosphere

In this study, the Ascomycota phylum dominates at the two microniches: epiphytes and endophytes, followed by Zygomycota for epiphytes and Basidiomycota for the leaf endophytes of *Peganum harmala* of dayate Aiat (Laghout, Algeria). This is consistent with the work of KHAN *et al.* (2010) on *Withania somnifera* from semi-arid regions (Karachi, Pakistan) [26]. Indeed, the mycoendophytes of the phyla of Zygomycota and Basidiomycota are found much more in herbaceous plants [27]. Ascomycota and Basidiomycota are the most commonly encountered endophytic fungi in the phyllosphere [28]. In hot deserts characterized by very high temperatures and nutrient-poor soils, plants adapt by developing morphological and biochemical changes that include microbial symbioses, most often represented by fungal species belonging to the richest phylum species: Ascomycota [29].

The presence of all these fungi in the phyllosphere may be explained by the sampling period (April). Many fungi develop during the spring season, which is related to precipitation, which can increase the diffusion of fungal spores, thus promoting the colonization of plant tissues [30,31]. Although many species of fungi are generally described as endophytes, others may occasionally be found colonizing the host tissue [32, 33]. Thus, the presence of epiphytic and endophytic fungi is influenced by various factors, such as nutrient availability, moisture and leaf age. The growth of filamentous fungi at phylloplan is generally considerable when humidity is high and temperatures are moderate [34,7].

Our results indicate that the *Cladosporium* and *Alternaria* genera are the most abundant as endophytes in the leaves of *Peganum harmala*. These two fungal genera are the most

frequently recorded at the phyllosphere level. KITA (1988) and KUTRZEBA (1993) confirmed that these fungi are ubiquitous in leaf blades of many plant species [35,36]. For PUSZ *et al.* (2015), these two genera dominate as epiphytes in the leaves of *Amaranthus spp.* Moreover, our study revealed the dominance of the *Penicillium* and *Rhizopus* genus as epiphytes in the leaves of *Peganum harmala* [18]. The study of THAKUR (2017) indicated that *Penicillium sublateralium* and *Cladosporium cladosporioides* are common fungi in the phylloplane of all terrestrial plants [7].

The genus *Alternaria* is abundant as an endophytic fungus (21.89%), but also as an epiphyte (11.99%). GRBIC *et al.* (2015) demonstrated the genus *Alternaria* (endophyte and epiphyte) with a frequency of 75% at *Nepeta rtanjensis* [6]. It is also dominant in *Withania somnifera* [26]. This genus has been reported common to other plant species such as *Triticum aestivum* and *Zea mays* [37-39]. Most *Alternaria* species are saprophytes commonly found in soil or in plant tissues. Some species are pathogenic (opportunistic) plants causing a range of diseases. The two main characteristics of *Alternaria* species are: melanin production in particular in spores and production of host-specific toxins in pathogenic species [40]. The strong pigmentation of their spores can provide good UV resistance and contribute to the colonization of many plant surfaces. However, they also have an effective tolerance to abiotic factors; they produce hydrolytic enzymes necessary for competitiveness in these ecological niches [41].

3.2.- Diversity and interaction between epiphytic and endophytic fungi

The Shannon index revealed a high diversity in the epiphytes compared to the foliar endophytes of *Peganum harmala*. It is unclear to what extent plants control endophytes capable of entering the leaf and how epiphytes can affect this process [42]. While the inside of the leaves undergoes changes due to temperatures and other conditions, the outer surfaces for their part, can be considered much more extreme in terms of abiotic stress. PETRINI (1991) [28] suggested that endophytic communities often contain a variety of classical epiphytic species including *Alternaria alternata*, *Cladosporium cladosporioides*, *Epicoccum purpurascens*. *Alternaria*, *Epicoccum* and *Cladosporium* are considered common primary plant colonizers and are essentially epiphytic when the leaf is fresh [43].

The similarity quotients (SQ) obtained in our study range from 0 to 0.73, the overall SQ is 0.62. The highest SQ is that of subject 9 (0.73), which has a strong similarity between epiphytic and endophytic communities. Moreover, the lowest SQ is observed in subject 2 where similarity is absent. This difference is due to the environmental conditions and location of the different subjects at day level. According to Osono and Mori (2004), the assemblage of epiphytic and endophytic phyllospheric fungi is intermediate in the SQ range between 0.12 and 0.79. The results of the SQ obtained by GRBIC *et al.* (2015) [6] are between 0.24 and 0.32 and suggest intermediate species overlap between the phylloplan and the inside of the leaves. All fungi determined as phylloplan species cannot penetrate the host tissue and colonize the inside of the leaves. Indeed, only certain microbial species can actively penetrate the tissues of the leaf to become endophytes [44, 45]. Since climate conditions on leaf surfaces vary rapidly with day / night cycles and rainfall events, climate change can be expected to have an effect on microbial communities in the phyllosphere. Temperature and surface moisture are environmental parameters that determine growth rate, germination, and colonization parameters. However, temperature and humidity levels often exhibit strong changes even within the leaf, thus limiting the frequency of appropriate conditions for plant tissue infection [46]. According to

MASSIMO *et al.* (2015), fungal diversity differs among host species, but not by tissue type. On the other hand, horizontal transmission and frequent sexual reproduction also increases genetic diversity and the number of non-systemic mycoendophyte species, even in a single leaf [47].

The genus identified in our study indicate that *Mycocladius*, *Mucor*, *Scopulariopsis*, *Phoma*, *Colletotricum* and *Epicoccum* exist only as epiphytes in the leaves of *Peganum harmala*. *Exophiala*, *Aureobasidium*, *Verticillium*, *Paraphaephaeria* and *Absidia* exist only as endophytes in this plant. The genus *Cladosporium*, *Alternaria*, *Rhizopus*, *Aspergillus*, *Trichoderma*, *Rhizoctonia*, *Penicillium*, *Trichophyton* and *Bipolaris* are both endophytic and epiphytic in the leaves of *Peganum harmala*. This distribution can be explained by the interactions between these different fungal genera. Indeed, epiphytic and endophytic fungi can have distinctive biological properties that demonstrate functional or ecological differences between these associations. Thus, ARNOLD (2007) also considers that interactions are created between the mycoendophytes on the one hand and between the mycoendophytes and the host plant on the other hand. The leaf surfaces of plants contain fungal communities [48]. These interact with each other, affecting foliar traits, influencing the physical conditions of plants, and contributing to several ecosystem functions, including the nutrient and water cycle [49]. The species richness, their assemblage and the diversity of microbial communities that make up the plant microbiome, as well as the factors that affect it and their performance, are mostly unknown. It is likely that a single plant species has thousands of epiphytic and endophytic microbial species. In addition, the interactions between these microorganisms could regulate several physiological processes in the host [50].

The positive relationship between biodiversity and the functioning of ecosystems is due mainly to complementarity between species. Most of the BEF (Biodiversity Ecosystem Functioning) studies focus on interactions between plants. However, plants are integrated into a dense network of multitrophic interactions above and below the soil, which are likely to play a crucial role in BEF relationships [51]. According to LOREAU and HECTOR (2001), biodiversity is divided into selection and complementarity. Unlike the selection effect, when species with special features dominate the ecosystem processes, complementarity consists of differentiation and facilitation of species, but untangling of these two components is often impossible [52].

Conclusion

Our work has concerned the diversity in Mycetes at the level of the phyllosphere of *Peganum harmala* of dayate Aiat (Laghouat, Algeria). 14 genus of endophytic fungi and 15 genera of epiphytes were identified, with the existence of only endophytic genera: *Exophiala*, *Aureobasidium*, *Verticillium*, *Paraphaephaeria* and *Absidia*. For the genus *Mycocladius*, *Mucor*, *Scopulariopsis*, *Phoma*, *Colletotricum* and *Epicoccum*, they exist only as epiphytes in the same leaves. The Shannon index revealed a high diversity in the epiphytes compared to the foliar endophytes of *Peganum harmala*. The similarity quotients (SQ) obtained indicate a strong similarity between the epiphytic and endophytic communities of the leaves of this species. This is due to environmental conditions and climate that undergo the surface and inside the leaves.

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