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Research Article

Isolation and characterization of endophytic fungi associated with *Ephedra alata* (macroscopic and microscopic characterization)

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Abstract

Ephedra alata, known in Algeria as *Alanda*, is a medicinal plant belonging to the *Ephedracea* family, it looks like a shrub without leaves. This species can grow in semi-arid and desert conditions thanks to its high tolerance to water shortage. It is used in traditional medicine for its therapeutic properties, in particular to treat the bronchi, the circular system, digestive system disorders, kidneys, oedema, fever, headaches, allergies, and to relieve asthma attacks as well as bacterial and fungal infections this is due to its production of secondary metabolites with a wide range of biological activities. On the other hand, This plant represents a host that allows endophytic fungi to feed, protect and spread; in return, this plant also benefits from certain advantages of endophytes. Despite its importance, no studies on its endophytic fungi has been conducted to date. This study aimed to isolate and identify the fungal endophyte community associated with the plant *Ephedra alata* growing in arid environments. the harvest of the *Ephedra alata* plant (aerial part) was made in the region of Bechar (southwest algeria). Isolation and purification of fungal strains were carried out on PDA medium supplemented with lactic acid. The identification of the isolated strains was made on the basis of morphological and cultural criteria. *Ephedra alata* presents a wide diversity of fungal species with a high load. A total of twelve fungal strains were isolated and the predominant genera were *Aspergillus*, then *Penicillium*, *Mucor*, and unidentified structures in small percentages. The most common phylum of fungal genera identified was *Ascomycota*, followed by *Zygomycota* and small percentages of unidentified structures (SNI).

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1. Introduction

In the mycological literature, the word endophyte is derived from the Greek meaning "In the plant" (endo = endon, means "in", phyte = phyton, means "plant") (Andrade-Linares et al., 2014). Endophytes are the group that colonizes the internal living tissues of plants without causing immediate negative effects (Tripathi et al., 2022). Fungi are the most frequently isolated microorganisms as endophytes (Caruso et al., 2022). They are present in all plant species studied and they can be found in the internal tissues of roots, stems, leaves, flowers, fruits or seeds (Tanapichatsakul et al., 2018). This unique habitat allows them to produce different types of natural products with significant bioactivity such as antimicrobial, antitumor, cytotoxic, anti-inflammatory, antiparasitic, antioxidant and neuroprotective activities (Liu et al., 2019). However, medicinal plant endophytes have attracted interest due to their wide range of bioactive metabolites. (Toghueo et al., 2017). Algeria is one of the richest Arab countries in medicinal plants with 3164 species (Benarba ,2016); among them *Ephedra alata* known as (*Alanda*) belongs to the *Ephedraceae* family (Jaradat et al., 2015). Phytochemical analyzes of *Ephedra alata* indicated the presence of substances such as: ephedrine, pseudoephedrine, cardiac glycoid, reducing sugars and flavonoids (Jaradat et al., 2015). This species, which is renowned for its high tolerance of water deficiency in the Saharan regions (Hadjadj, 2020). It is found in the northern and western Sahara at the level of sandy soils, ergs and the sandy beds of wadis. It is even found in the sand of the tropical floor and the Hamada of Tinghert (Hadjadj, 2020), *Ephedra alata* is widely used in traditional medicine. In Algeria, this plant is used to treat several diseases, it is applied in the form of herbal tea and by inhalation against influenza, whooping cough, general weakness (Bouafia, 2021) .In El ouede, *Alanda* is used against cancer, diabetes, cough, gastric ulcer, abortions intestinal gases , influenza, renal and cardiac insufficiency and against obesity (Hadjadj, 2020). Despite the importance of *Ephedra alata* in medicine and the potential bioactivity of related fungi, no studies on its endophytic fungi have been carried out to date. This study aimed to isolate and characterize endophytic fungal strains of *Ephedra alata*.

2. Materials and methods

Sampling of plant

The harvest of the *Ephedra alata* plant (aerial part) was made in the region of Bechar southwest Algeria (Latitude: 31° 37' 0.01" N, Longitude: -2° 13' 0.01" W), (Figure 1), during the month of April 2023. To ensure good isolation of endophytic microorganisms, healthy plants and fresh plant material must be chosen. Collected samples should be preserved at a temperature of 4°C until used, and should not exceed 24 hours. The sample was taken under aseptic conditions. So we cut off the aerial part with sterile scissors and place in sterile plastic bags then transported immediately to the laboratory where they were processed immediately.

Figure 1. Morphology of Ephedra alata (El Maaiden et al., 2023).

Isolation of endophytic fungi

In order to get rid of any debris, the aerial part of the plants was rinsed with running water for 3 to 5 min.The surface was sterilized for 1 min with 70% ethanol, after that washed for 4 min with 3.8% sodium hypochlorite, followed by 30 sec of 70% ethanol .Finally rinsed three times with sterile distilled water to remove the hypochlorite. The disinfected aerial part (10 g) is macerated using a well disinfected mortar in 100 ml of sterile Phosphate Buffer Solution (PBS). A maceration time of 20 to 30 min at room temperature is then applied in order to let the endophytes diffuse in the solvent used. At the end of the maceration process, we obtain a solution considered to be the sample stock solution, which is then diluted decimally (from 10^{-1} to 10^{-3}). From the 3 dilutions, 0.1 ml of each was taken and spread on the surface on the PDA medium supplemented with lactic acid to inhibit the growth of endophytic bacteria. Then the dishes were incubated at 28 °C and checked all days until visible growth is detected (Mebarki, 2023).

Subculturing and purification

The purification is carried out after several subcultures by successive transplantations of the mycelial fragments of each fungus obtained in petri dishes containing the PDA medium and incubated at 25 °C (Talukdar, 2021). Subculturing was done by taking a colony fragment using a sterilized pipette while avoiding its contact with the other neighboring colonies of the same dish on the PDA medium.

Conservation of endophytic fungi

Each isolated and purified fungus was stored in sloped PDA tubes at a temperature of 4 °C (Fouda et al., 2015).

Enumeration of fungal colonies

The enumeration is carried out by counting the colonies. Countable plates are those whose number of colonies is between 30 and 300. The following formula was used to determine the microbial population (Mebarki, 2023):

$$N\ (UFC\ /ml) = \Sigma C/V[n1 + (0{,}1x\ n2)]\ d$$

Where:
N = colony counts per gram of sample (units = cfu. g^{-1}).
ΣC = total of all the colonies in all of the plates.
V = volume of inoculum applied to each disk (in ml).
n1= number of dishes retained at first dilution.
n2= number of dishes retained at second dilu-tion.
d = dilution factor corresponding to the first di- lution retained.

Identification of fungal strains

Identification

The identification of endophytic fungi is done like all microorganisms macroscopically and microscopically (Correia et al., 2018). We were able to observe 12 different isolates of endophytic fungi that live in the aerial part of *Ephedra alata.*

Macroscopic study

Macroscopic characterization is done with the naked eye, essentially based on the characteristics of the cultures such as the general appearance of the colony surface and its color, texture and pigmentation (Correia et al., 2018).

Microscopic study

The observation was made using a fragment of the pure strain taken with a sterile platinum loop. This fragment was then transferred to a slide on which cotton blue had been applied. The microscopic observation was made at x40 magnifications.

The determination of endophytic fungi is based on the morphological characters of the hyphae and reproductive structures. (Yua et al., 2018).

- Hyphae: color, presence or absence of septa, approximate diameter, special structures (cornecia).
- Reproductive organs (1 or more types): location (aerial part, etc.), color, size and shape of reproductive organs.
- Spore structure and arrangement: color, shape, partitions, ornamentation, size...

3. Results and discussion

Enumeration of fungal microflora

The count of the fungal microflora present in the aerial part of the plant studied was carried out by counting the number of colonies that appeared on the medium. PDA after 7 days of incubation. The results are shown in (Table 1).

Table 1. Results of enumeration on PDA agar

Dilution	Petri dish	Count results
10^{-1}	01	> 300
	02	> 300
10^{-2}	01	> 300
	02	206
10^{-3}	01	126
	02	57

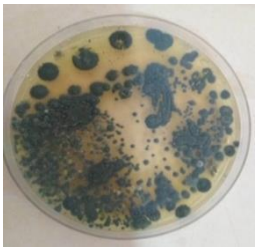
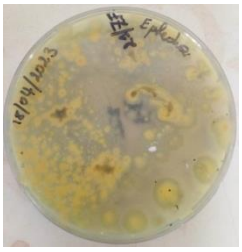
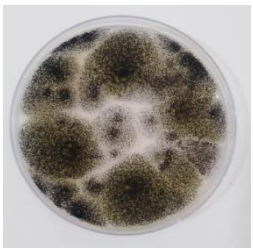
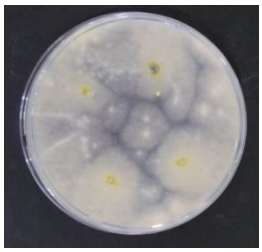
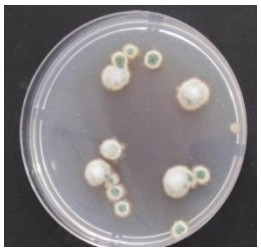
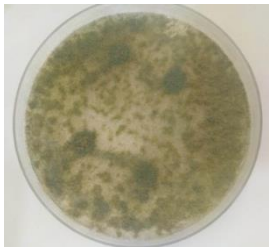
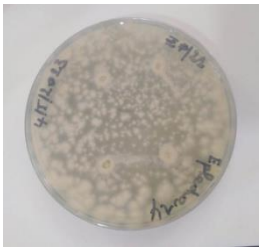
After application of the formula above. We find that culture on PDA agar indicates a load of $31.75.10^4$ CFU/g from the aerial part of *Ephedra alata.*

Identification of endophytic fungal strains

The isolation carried out from the aerial part of the plant studied made it possible to obtain 12 fungal strains. The macroscopic characteristics of representative strains are recorded in (Table 2). The cultural characteristics of the isolated fungal strains have also been described. These microscopic characteristics are summarized in (Table 3).

Table 2. Macroscopic characteristics of fungal strains isolated from the aerial part of Ephedra alata

Isolat	Front	Back	Macroscopic characters
Isolat 1			<u>Front:</u> Ochre then various colors (pink, yellow, ochre… <u>Back :</u> Colorless or yellow to reddish-brown

Isolat	Front	Back	Macroscopic characters
Isolat 2			Front: Fluffy to powdery, white then yellow to yellow-green Back : Colorless, pinkish to dark red-brown
Isolat 3			Front: Blue green Back : Dark yellow
Isolat 4			Front: White then yellow then grainy and blackish Back : Colorless to pale yellow
Isolat 5			Front: Dark green with a white outline Back : Orange
Isolat 6			Front: Olive green with a white outline Back : Colorless
Isolat 7			Front: Green Back : Colorless

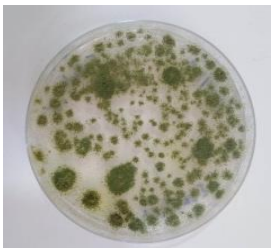
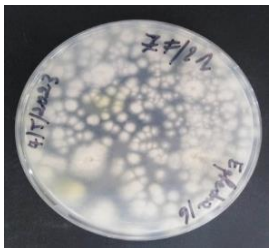
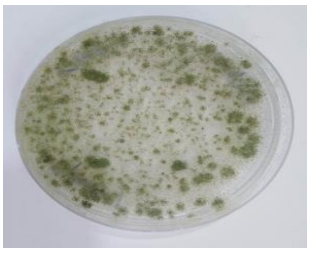
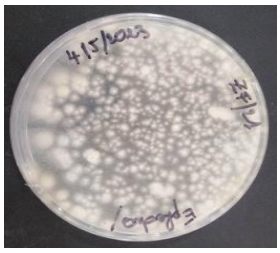
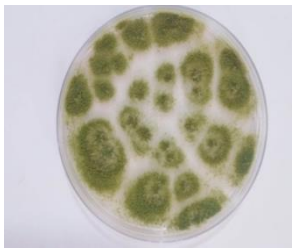
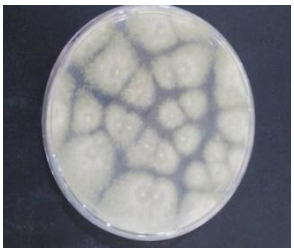
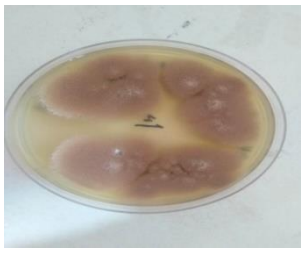
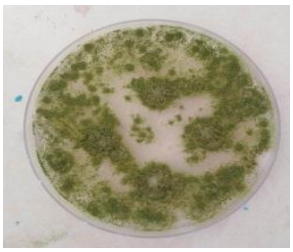
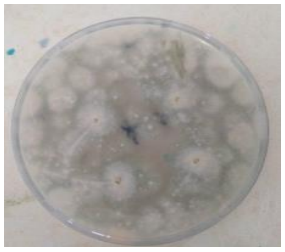
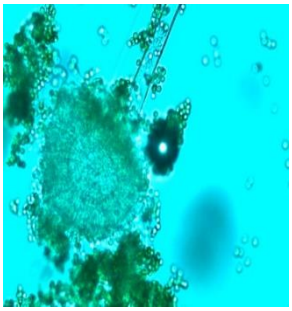
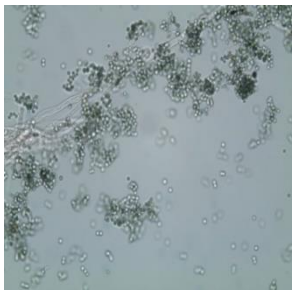
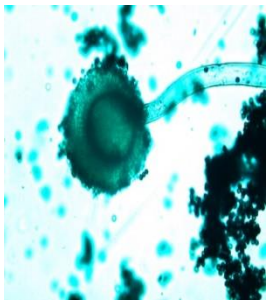
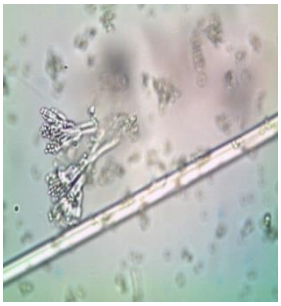
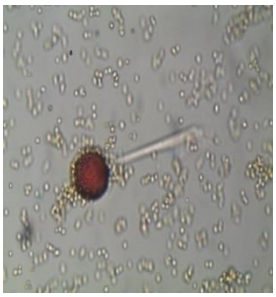
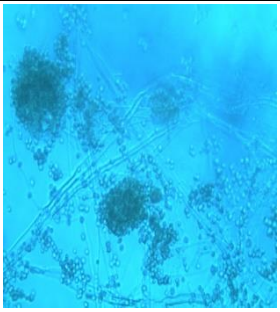
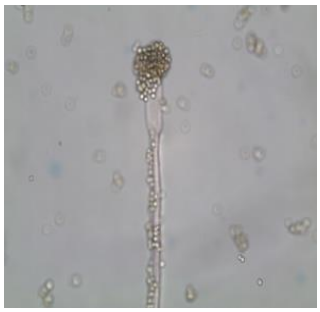
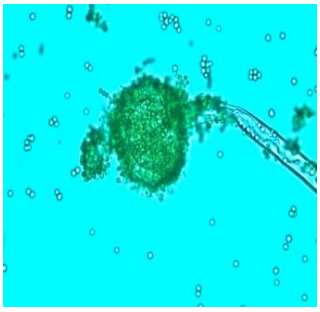
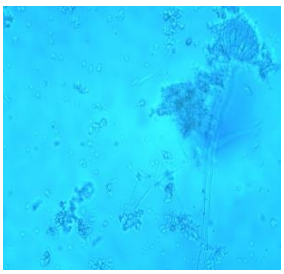
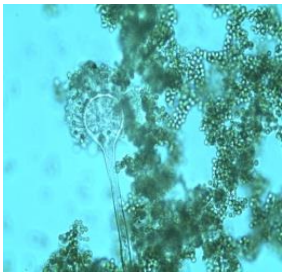
Isolat	Front	Back	Macroscopic characters
Isolat 8			<u>Front:</u> Green <u>Back :</u> Colorless
Isolat 9			<u>Front:</u> Small green colonies with a powdery texture <u>Back :</u> Colorless
Isolat10			<u>Front:</u> Large, light green colo-nies <u>Back :</u> Colorless
Isolat 11			<u>Front:</u> Fluffy to powdery, beige to cinnamon <u>Back :</u> yellow to orange-brown
Isolat 12			<u>Front:</u> Fluffy to powdery, white then yellow to yellow-green <u>Back :</u> Colorless

Table 3. Microscopic characteristics of fungal strains isolated from the aerial part of Ephedra alata.

Isolat	Species	Description	Microscopic appearance At x40 magnification
Isolat 1	*Aspergillus versicolor*	The conidial heads are radiated. The conidiophores are colorless to yellow. They are wide and not partitioned and end in a pear-shaped to spatulate vesicle, half or totally covered by metulae themselves bearing phialides. The conidia are globose.	
Isolat 2	*Aspergills Flavus*	The conidiophores are formed of rough stipes and end in a spherical vesicle, fertile over more than three quarters of its surface; bearing both metulae and phialides The conidia are finely rough and spherical.	
Isolat 3	*unidentified structures* (SNI)	The unseptate mycelium, no cognate head, many chain-like Spores arising from the undifferentiated, branch-like conidiophore.	
Isolat 4	*Aspergillus Niger*	Dark brown to black conidial heads The Conidiophores: smooth with non-chambered stipes, hyaline or brownish in their upper half. Vesicles: globular the Phialides formed on usually brown, frequently septate mutulae. Conidia: are asexual, unicellular spores produced by phialides.	
Isola 5	*Aspergills SP 1*	The conidiophore has a swollen end. The phialides are arranged all around the vesicle and are carried by a sterile cell (or metula) directly inserted on the vesicle. The conidiophores are very long. The aspergillus head is globose.	

Isolat	Species	Description	Microscopic appearance At x40 magnification
Isolat 6	*Penicillium SP*	The branched conidiophore has a brush-like shape. Conidia are arranged in long chains.	
Isolat 7	*Mucor SP*	Non-septate mycelium, the head of the conidium is round	
Isolat 8	*Aspergillus SP2*	The conidiophore has a swollen end. The phialides are arranged all around the vesicle and are carried by a sterile cell directly inserted on the vesicle. The conidiophores are very long. The aspergillus head is globose.	
Isolat 9	*Aspergillus SP3*	The conidiophore is very long and has a swollen end. The spores are arranged at the top of the elongated vesicle	
Isolat1 0	*Aspergillus SP4*	The conidiophore has a swollen end. The phialides are arranged all around the vesicle and are carried by a sterile cell (or metula) directly inserted on the vesicle. The conidiophores are very long. The aspergillus head is globose.	

Isolat	Species	Description	Microscopic appearance At x40 magnification
Isolat 11	*Aspergillus Terreus*	The Conidiophore smooth, colorless, the phialides are arranged on the dry rot on the upper surface of the vesicle, the conidia are globose with a homogeneous size.	
Isolat 12	*Aspergillus Flavus*	the conidiophores are formed of rough stipes and end in a spherical vesicle, fertile over more than three quarters of its surface, bearing both metulae and phialides The conidia are finely rough and spherical	

The identification of fungal strains was made mainly from cultural characters (macroscopic identification) and morphological (microscopic identification). These characteristics made it possible to identify certain fungi by using the specific determination keys of Compaore et al., (2016). The fungi identified from the aerial part of *Ephedra alata* belong to the phylum of Ascomycetes (83.33%), Zygomycetes (8.33%) and we also have the unidentified strain (SNI) (8.33%) (Figure 2). Endophytic fungi are mainly from the phylum Ascomycota (Vincent ,2018).

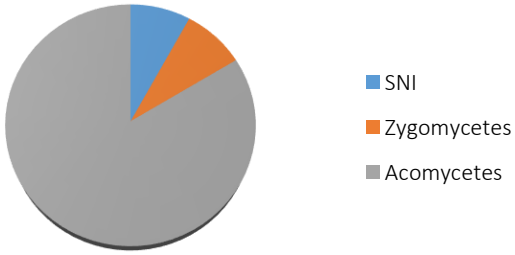


Figure 2. Dominance of the phylum of Endophytic fungi isolated from the aerial part of Ephedra alata

We were able to isolate 12 different strains of fungi from the aerial part of *Ephedra alata*: *Aspergillus sp*, *Penicillium sp*, *Mucor sp* and an unidentified species. *Aspergillus sp* was the most frequent with 09 isolates, followed by *Penicillium sp* with 1 isolate, *Mucor sp* with 1 isolate and an unidentified species with 1 isolate, with a percentage of (75%, 8.33%, 8.33% and 8.33%) respectively (Figure 3).

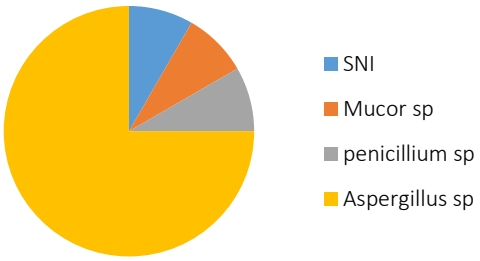


Figure 3. Dominance of genus of Endophytic fungi isolated from the aerial part of Ephedra alata

The genus *Aspergillus* is often associated with the *Penicillium* and is distinguished from the latter by the appearance of the conidiophores which are terminated by a swollen head (Meghazi, 2015). *Penicillium* is a ubiquitous fungus whose development takes place from organic substances or decaying plants. From its microcolonies are born multiple spores which are dispersed in the ambient air (Kadhem et al., 2019). This genus contains about 300 species (Visagie et al., 2014). *Aspergillus* are very common contaminants, this genus includes 180 to 250 species according to the authors, of which only *Aspergillus fumigatus*, *A.flavus, A.nidulans, A.terreus*, and *A.niger* are considered thermotolerant (Meghazi, 2015). Species of this genus (*Ephedra*) can grow in semi-arid and desert conditions, making all six continents suitable for the growth of this genus. The latter usually grows in sandy soils, dry slopes and dry sides of mountains (Limberger et al., 2013). Host plants allow endophytic fungi to feed, protect and spread; in return the plants also benefit from certain advantages of the endophytes. Several studies have demonstrated that plants associated with endophytic fungi were more tolerant of drought, heat, metal toxicity and high salinity (Li et al.,2021). Endophytic fungi represent a very diverse group, with an estimate of 1.5 million species and an average of about 50 species of endophytes per plant species (Kouadria,2019). The diversity of species, frequency and abundance of endophytes dependent on climatic and edaphic conditions and heterogeneity of habitats and niches occupied by their hosts (Mohamed Mahmoud, 2017). Geographical variations are the factors that most often contribute to the diversity of endophytic fungi. *Aspergillus* is a fungal genus that has a wide geographical distribution, but it is often associated with hot climate regions (Zareb and Smail-Saadoun, 2020). Most species of endophytic fungi of the genus Aspergillus are known to produce several types of siderophores. These molecules can directly stimulate plant growth, by increasing the availability of soluble iron around the roots or indirectly by inhibiting the growth of pathogens with regard to the phenomenon of competition for iron (Le Govic et al., 2017). *Aspergillus niger*, as one of the most important species of filamentous fungi, is used in biotechnology and is also one of the most common natural contamination fungi in animal feed and foodstuffs (Cairns et al., 2018).

4. Conclusion

The results from the present study point out that the aerial part of *Ephedra alata* (Bechar, Algeria) contains a wide diversity of fungal species with a high load of $31.75.10^4$ CFU/g. 3 genera of fungi were identified: *Aspergillus sp, Penicillium sp, Mucor sp*, SNI (unidentified structure). Our study revealed that the most abundant fungal genera are represented by *Aspergillus* (75%) followed by *Penicillium* (8.33%) and *Mucor* (8.33%) and unidentified structures (8.33%). The phylum of the genera of fungi identified are the Ascomycota (83.33%), the Zygomycota (8.33%) We also identified unidentified structures (SNI) (8.33%). However, further studies are needed to explore their potential in the plant protection sector.

Author's declaration and contribution

The authors show no conflict of interest.

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