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ISOLATION AND IDENTIFICATION OF ENDOPHYTIC FUNGI ON PLANTS GROWING IN SALT ENVIRONMENTS, USING ITS AND 18S MOLECULAR METHODS

Rabiaa KOUADRIA[a,b]*, Mohammed BOUZOUINA[a] and Brahim LOTMANI[a]

[a]Plant Protection Laboratory, Abdelhamid Ibn Badis University, Mostaganem, BP300, 27000, Algeria.
[b]Molecular Genetic Laboratory, Ain Shams University, Cairo, Egypt.

Abstract

Endophytic fungi seem to play particle roles for the survival of plants inhabiting stressful habitats. This study focused on the identification of fungal endophytic community, associated with roots plants growing in salt environments by sequencing ITS and 18S rDNA regions. Based on the culture characteristics and growth morphology of colonies, 6 fungi species obtained from roots were collected from three plots with different salinities (< 4 dS/m, 4 to 8 dS/m and 8 to 16 dS/m). ITS sequences and 18S rDNA gene were compared with those available in the GenBank databases, to identify the following species: *Alternaria chlamydospora* and *Chaetomium coarctatum* (salinity < 4dS/m), *Alternaria chlamydospora*, *Embellisia phragmospora*, *Phoma betae*, *Fusarium equseti*, *Chaetomium coarctatum* and *Fusarium graminearum* (4 to 8dS/m); and *Chaetomium coarctatum* (8 to 16dS/m). Results indicate that *Chaetomium coarctatum* was considered as the most dominant fungus in studied plots. The fungal root endophytic community in natural vegetation under abiotic salt stress opens up possibilities for further investigations on the role of endophytes.

Key words : Endophytic fungi, salinity, rDNA, molecular identification.

Introduction

Soil salinity has become a global problem. In Algeria, a large part of agricultural regions characterized by an arid and semi-arid climate is affected by salinity process. Nearly 3.2 million hectares are threatened by salinity problem (Benmahioul et *al*., 2009); two million square kilometers are desert (arid) over the total area of the country (2.381.740 km^2), while the rest (381.740 km^2) is semi-arid (semi-desert) and sub-humid (Nedjraoui, 2001). Salt stress affects physiological processes of plants, causing a nutrient imbalance, altering levels of growth regulators, inhibiting photosynthesis and protein synthesis, all of which lead to reduced plant growth (Abeer et *al*., 2015).

Search for biological means such as endophytic fungi is a cheaper alternative and certainly more effective than the use of conventional means of desalination such as drainage for the tolerance of cultivated plants to salt stress.

***Author for correspondence :** E-mail: rabia-rk@hotmail.com

Fungal endophytes can colonize plants and help their partner to survive under extreme environmental conditions by secreting beneficial secondary metabolites. Among the >300.000 plants on Earth, almost all the vegetal species had diverse endophytes within their tissues (Strobel and Strobel, 2007). Endophytes are microorganisms that live within the intracellular or intercellular spaces of host plants (Strobel, 2003). They play crucial roles as decomposers, mutualists and parasites in ecological processes on earth (Liu et *al*., 2015). Endophytes live and grow in roots, stems and/or leaves, without causing any apparent disease symptoms (Petrini, 1991). Plant roots can be associated with a large variety of endophyte microorganisms (Dighton et *al*., 2005).

Identification of filamentous fungi, from plants is very complex (Souza et *al*., 2004). Because of their difficult morphological identification, genetic methods can be used for classifying microbial strains in diverse hierarchical taxonomic levels. Information contained in the 18S and 28S ribosomal genes and the internal transcribed spacer

(ITS) and intergenic spacer (IGS) sequences have been extensively used in characterization of fungal community diversity (Sterflinger and Prillinger, 2001). 18S rDNA and ITS PCR primers are useful for assessing fungal diversity (Anderson *et al*., 2003).

The goal of this study was to investigate the fungal endophyte community associated with roots of plants growing in saline soils. We isolated and identified root-associated endophytic fungi of plants by amplification of the internal transcribed spacer (ITS) region and the 18S gene of fungus genomic DNA, using polymerase chain reaction (PCR) amplification with specific primers.

Materials and Methods

Study site and biological material

Sampling areas are located in Relizane; Algerian West. Spontaneous plant species were collected from three plots with different degrees of salinity (Plot1: < 4dS/m, Plot2: from 4 to 8dS/m and Plot3: from 8 to 16dS/m), to isolate endophytic fungi (Lat. 35° 47' 46"N, Long. 0° 33' 11", Alt. 50m). In this study, as the soil is plowed, only one plant species was collected from the low-salt plot (< 4dS/m).

Plant sterilization and endophytes isolation

Endophytes were isolated from plant roots using Huang *et al*. (2007) method. Surface sterilization of roots followed the procedure of Larran *et al*. (2007). Root segments (5mm) were washed thoroughly in running tap water, sterilized with sodium hypochlorite (NaOCl, 5%) for 3min, rinsed three times in sterile distilled water, and then dried on a sterile filter paper. Segments were placed in a 90mm diameter Petri dish containing a mixture of potato dextrose agar (PDA) medium and amoxiallin (15mL/l), and incubated at 25° C with darkness. After 7 days, hyphae emerging were transferred to fresh PDA for purification and identification.

Molecular identification

DNA extraction

DNA was extracted using the acid washed beads extraction method (Moller *et al*., 1992). Fungal mycelia (2-3mm) was immersed in 2mL tube with glass beads, containing 500µL of CTAB extraction buffer (20mM EDTA, 0.1M Tris-HCL pH 8, 1.4M NaCl, 2% CTAB and 0.1% β-mercaptoethanol) and 20µL of solution of Proteinase K. Solution was incubated at 56°C. After centrifugation (3min on 12000 rpm), supernatant was transferred to a fresh tube following the addition of 400µL of chloroform-isoamyl alcohol (4:1). Samples were gently mixed and centrifuged at 12000 rpm for 5min. After transferring supernatant to a fresh Eppendorf tube, 1mL of absolute cold Ethanol (-20°C) was added. Samples were kept on -80° C for an hour then centrifuged at 4°C for 10min. Liquid solution was released and DNA pellet was washed with 1mL of Ethanol 70% at -20°C and centrifuged at 4°C for 10min. Supernatant was discarded. Pellet was resuspended in 50µL of TE buffer (Ultrapure water) and stored at -20°C. Quality of DNA was evaluated by electrophoresis on 1% Agarose gel.

PCR amplification

PCR with primers ITS1/ITS2 and NS1/NS7 was applied to amplify the internal transcribed spacers ITS region and 18S rDNA, respectively. PCR mixture (50µL) consisted of 50-100ng genomic DNA, 25µL of 2x PCR buffer: MyTaq DNA polymerase, 1 µL (10mM) of primer forward (ITS1/NS1) and 1 µL of primer reverse (ITS2/NS7) and 22µL H_2O. All amplification products were electrophoresed in Agarose gel 1.8%.

PCR products were purified using the DNA fragment purification kit and sequenced in forward and reverse directions by Macrogen (Holland).

Sequence analysis

Sequence analysis of the ITS and 18S sequences was carried out using Bio Edit Sequence Alignment Editor v.7.2.3 (Hall, 1999). Phylogenetic trees were built using the neighbor-joining (NJ) methods and bootstrap test (Kumar *et al*., 2004). Bootstrap tests were performed using 1000 replicates. The search for homologous sequences was done using Basic Local Alignment Search Tools (BLAST) at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST). All fungal sequences considered were at least 98% identical to the best hit in the NCBI database (Varanda *et al*., 2016).

Results

Plants species and isolated endophytic fungi

Based on 63 root segments of plant species seeded, 31 showed infection with endophytic fungi (table 1), representing an infection rate of 49.21%. A total of 06 fungi were isolated from plant roots collected from salt environments.

Molecular identification

PCR amplification

Amplification of ITS and 18S regions were successfully carried out with the primers ITS1/ITS2 and NS1/NS7 respectively, amplification by PCR showed a single band of about 500 bp for all isolates (fig. 1). Sequences were trimmed and assembled to its pair (forward+reverse of each sample/locus).

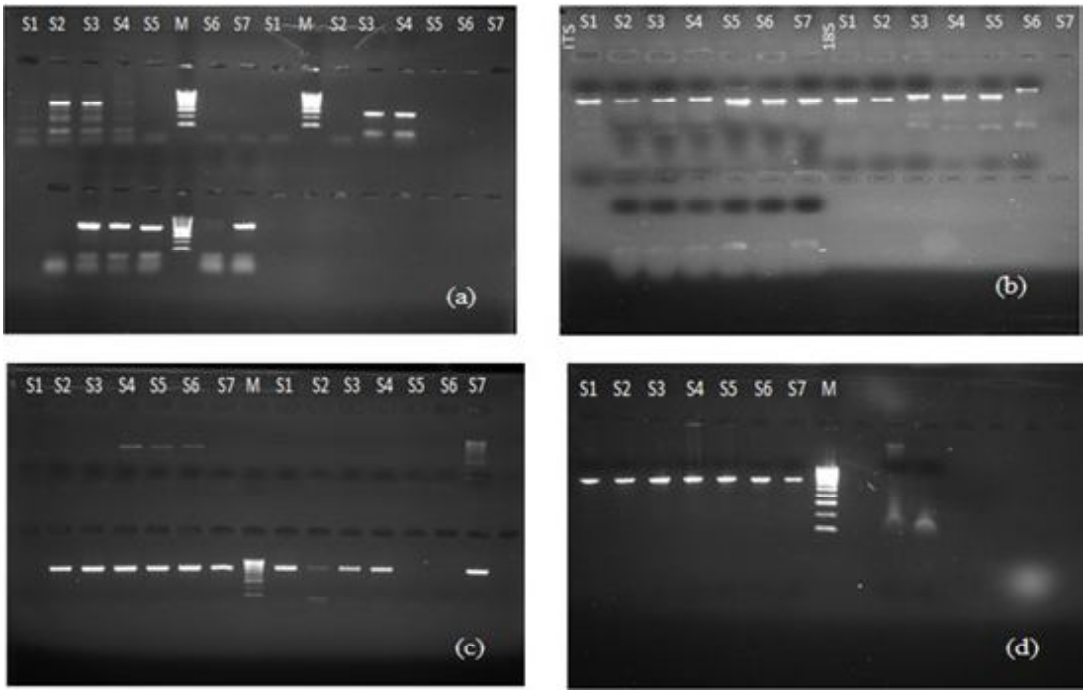

Fig. 1 : Electrophoresis gel photos for (a) DNA extraction, (b) PCR amplification of ITS and 18S sequences, (c) purification of the PCR product for ITS sequences and (d) purification of the PCR product for 18S sequences. S1-S7: samples (endophytic fungi). M: DNA Marker.

Table 1 : Plants species and isolated endophytic fungi.

Plots	Number of collected species	Name of plant species	Number of isolated endophytic fungi
Plot 01	01	*Avena fatua*	02
Plot 02	05	*Beta macrocarpa*	01
		Anabasis prostraba	02
		Avena fatua	01
		Sueda fructosa	01
		Lolium perenne	01
Plot 03	01	*Sueda fructosa*	01

Similarity of the sequences

Sequences were compared with GenBank database, and results are shown in table 2. According to molecular data, strains S1 and S2 were identified to *Alternaria* genus with Pairwise identity 99.80%. Results of BLAST showed that ITS rDNA sequences of isolate S5 shared high homology with *Fusarium equiseti* with high bootstrap support value of 100%. Sequence identity ranged from 99 to 100% in ITS rDNA sequences and from 98 to 100% in 18S rDNA sequence.

Phylogenetic analysis using NJ method and bootstrap

Phylogenetic tree is shown in fig. 2. Use of ITS and 18S sequencing and phylogenetic analysis identified 6 of endophytic fungi strains belonging to Dothidiomycetes and Sordariomycetes: *Alternaria chlamydospora* and *Chaetomium coarctatum* (salinity<4dS/m), *Alternaria chlamydospora*, *Embellisia phragmospora*, *Phoma betae*, *Fusarium equseti*, *Chaetomium coarctatum* and *Fusarium graminearum* (4 to 8dS/m) and *Chaetomium coarctatum* (8 to 16dS/m).

Discussion

Fungal endophytes have been found within all plants from diverse habitats (Rodriguez *et al*., 2009). Isolation results showed that spontaneous collected plants hosted one or two endophytic fungi. Debbab et *al*. (2012) explained that all plant species host at least one or more type of endophyte.

Internal transcribed spacer (ITS) region and 18S rRNA genes are applied in fungi molecular studies

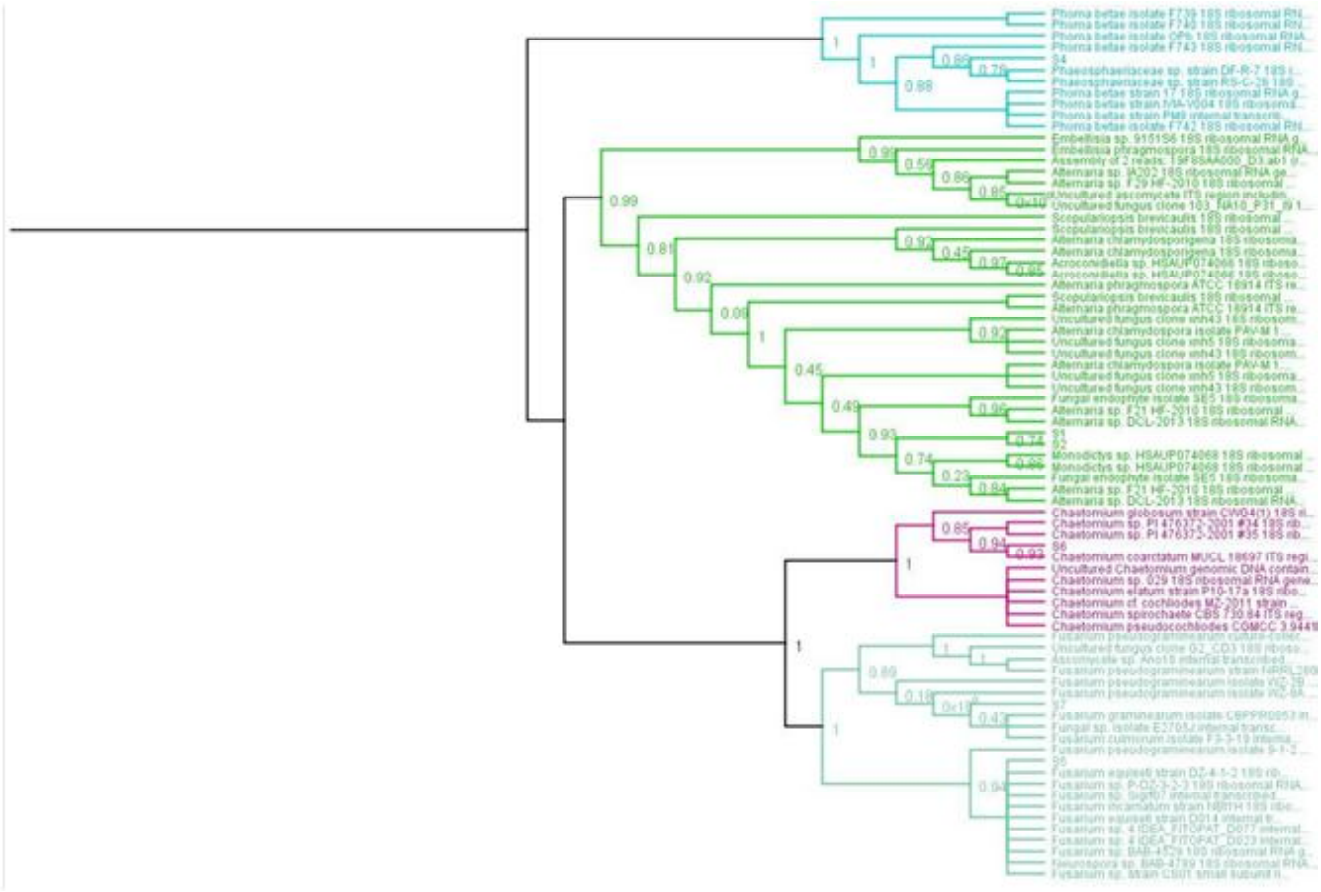

Fig. 2 : Phylogenetic relationships among 7 endophytic fungi derived from Dothidiomycetes and Sordariomycetes (Ascomycota).

Table 2 : Summary of the fungal endophytic community associated with roots plants growing in salt environments.

Sample	Loci	Accession n°	Description	Similarity
S1	ITS	KF993329	*Alternaria chlamydospora*	99.5%
S2	ITS	KF993329	*Alternaria chlamydospora*	99.5%
S3	ITS	JQ796758	*Embellisia phragmospora*	99.6%
S4	ITS	KM249077	*Phoma betae*	99.6%
S5	ITS	KU377478	*Fusarium equiseti*	100.0%
S6	ITS	NR_144822	*Chaetomium coarctatum*	99.4%
S7	ITS	JN862234	*Fusarium pseudograminearum*	99.6%

(Curlevski *et al*., 2010). In this study, various primer were used for examining the fungal community on roots by isolating, culturing, and molecularly identifying in ITS region (Mello *et al*., 2011) and 18S rRNA genes (Hoshino and Morimoto, 2010). ITS locus was successfully assembled for all the 7 isolates. ITS sequences are hypervariable and used particularly for fungi identification at species or lower levels (Toju *et al*., 2012). While in 18S, only Reverse direction passed the HQ% (high quality) for samples 2 to 7 except for sample 1. 18S rRNA sequences may not always allow fungi identification on genus or species level (Anderson and Parkin, 2007). ITS region revealed higher richness, diversity, and more dynamic than 18S rRNA. Comparing with GenBank, results showed that sequences had more than 97% similarity. Comparison between ITS and 18S-rRNA sequences with those available in GeneBank databases allowed us to analyze the phylogenetic affiliation of these fungi. Sequence identity of ITS rDNA of strains S1 to S7 ranged from 99 to 100%, strains S2 to S7 were located with high bootstrap support ≥ 98% in their 18S rRNA sequence. All isolates described belong to Ascomycota phylum (Porras-Alfaro and Bayman, 2011), where the seven isolated fungi represented two classes: Dothidiomycetes (S1 and S2: *Alternaria*, S3: *Embellisia*, S4: *Phoma*) and Sordariomycetes (S5 and S7: *Fusarium*,

S6: *Chaetomium*).

Endophytic fungi associated with plant roots can provide potential benefits to their host plants (Urcelay *et al*., 2011). It is assumed that most plant–fungus mutualistic interactions take place in roots (Rodriguez *et al*., 2009). Data showed a low diversity of endophytic fungi, there are evidences that mycorrhizal populations become reduced or absent in habitats subjected to certain environmental stresses (Porras-Alfaro *et al*., 2008). *Fusarium* and *Phoma* were the most frequent root endophytic genera, followed by *Alternaria*, *Embellisia* and *Chaetomium* (Jose *et al*., 2008). *Phoma* and *Fusarium* might play potential roles on plant host tolerance to high-stress environmental conditions (González-Teuber *et al*., 2017).

Only a few halophytic plants were studied (Anita and Sridhar, 2009), *Alternaria* and *Phoma* isolated from *Beta macrocarpa* and *Salicornia* sp., respectively, are considered to play an important ecological role for halophytes stress resistance (Barrow *et al*., 2007). *Alternaria chlamydospora* and *Fusarium equiseti* were more common in salt environments (Jose *et al*., 2008). Fungi belonging to *Alternaria* genera were clearly predominant under desert and salty environments (Smolyanyuk and Bilanenko, 2011). *Alternaria*, *Embellisia*, *Phoma* and *Fusarium*, pigmented endophytes and common in halophytes, play an important ecological role for plant survival and stress resistance (Sun *et al*., 2012). *Alternaria* spp. was noted for its resistance to UV radiation through its dark pigmentation (English and Gerhardt, 1946) by producing high levels of pigments, which protect fungi inhabiting plants growing in high-salinity. *Embellisia* and *Phoma*, showing pigmented spores, are likely predominant on *Salicornia* (Wong and Hyde, 2001).

Conclusion

This study provided information that all studied species harbored fungal endophytes, which seem to play potential roles on plant host tolerance to stressful conditions. The five fungal genera detected: *Alternaria*, *Embellisia*, *Phoma*, *Fusarium* and *Chaetomium*, dominated by Dothideomycetes and Sordariomycetes classes were belonging to Ascomycetes. *Chaetomium coarctatum* was common endophyte all plots.

The fungal root endophytic community in natural vegetation under abiotic salt stress opens up possibilities for further investigations on the role of endophytes. Moreover, it is imperative to conserve the endophytes of these plants *ex situ*, to discover their roles on plant host tolerance to stressful conditions.

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