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ORIGINAL PAPER

Identification and salt tolerance evaluation of endophyte fungi isolates from halophyte plants

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Abstract

The harsh environments of desert areas lead to natural selection of resistant creatures with obvious characteristics. This experiment looked for salt-tolerant fungi from native halophyte plants. Forty fungi isolated from three halophyte plant families that were collected from desert areas of Yazd Province in Iran, and the most tolerant isolates were selected at concentrations of 1, 2, 3, 3.5 and 4 molar sodium chloride. Five selected superior isolates were assigned to the phylum *Ascomycota* based on internal transcribed spacers sequences and β-tubulin gene, as well as morphological characteristics of the genus and species. *Aspergillus terreus* showed superiority in terms of enzymes and antibacterial properties than other isolates. Other isolates were *Acremonium*, *Paecilomyces*, *Microascus* and *Monosorascus*. *Aspergillus terreus* also showed antifungal effects against *Aspergillus fumigatus*, a human pathogen.

Keywords Endophyte fungi · Halophyte plants · Halo-tolerant · Molecular approaches

Introduction

Salinity and drought are the most important abiotic stresses (Chen et al. 2009). It is estimated that more than 900 million hectares (more than 6%) of agricultural land and 30 percent of the irrigation water worldwide are affected by salt (http://www.unesco.org/water; Zhang et al. 2010). Iran has vast saline soils and about 15.2% of the country (approximately 33 million hectares) and 55% of the agricultural lands are affected by salinity (Martinez et al. 2004; Honarjoo et al. 2010). Salinity affects the growth of the plant directly through the toxicity of ions and indirectly by increasing osmotic stress (Bromham 2014). Halophytes are able to maintain high concentrations of electrolytes. In addition to other types of salts such as Na_2SO_4, $MgSO_4$, $CaSO_4$, $MgCl_2$, KCI and Na_2CO_3 (Flowers et al. 2010), NaCl is mostly present in saline environments. Over the past 30 years, the term endophytic fungi has been used in microbiological studies to describe fungi that live inside healthy plants (Stone et al. 2004; Soltani 2017). The association of endophytic fungi with plants improves plant growth, its tolerance to environmental stresses like dryness, salinity, temperature, heavy metals, etc., and resistance to pathogens (Kirch et al. 2000; Giri and Mukerji 2004; Waller et al. 2005; Soltani 2017; Golparyan et al. 2018). Endophytes include microorganisms that live inside tissues of higher plants without causing symptoms of intracellular or intercellular growth and are rich in bioactive compounds. Nearly all plant species host one or more endophytes (Li et al. 2008). Endophytes isolated from plants growing in warm soils and coastal saline soils indicate a high commercialization potential by proving increased yield in hot and salty water environments (Flowers and Yeo 1995; Lucero et al. 2008; Yuan et al. 2016).

The purpose of this study was to isolate and identify salt-tolerant, bioactive and enzyme-producing endophytic fungi associating halophyte plants of *Amaranthaceae*, *Rubiaceae* and *Asteraceae* families in Iran. The beneficial potentials of these fungi in dealing with salinity and drought stress would be valuable in salty and drought areas. This experiment started in 2016 by collecting samples from Yazd and screening on salty treatments at Bu-Ali Sina University.

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Materials and methods

Collecting halophyte plants

In September 2016, different plants including *Anabasis iranica*, *Seidlitzia rosmarinus*, *Salsola tomentos* and *Salsola yazdiana* in *Amaranthaceae* family; *Rubiatinctorum* in *Rubiaceae* and *Artemisia annua in Asteracea* family were collected from Chah Afzal, central district of Ardakan county in Yazd, a desert region in Iran, with the geographical coordinates of 32° 30′ 30″ N 53° 52′ 08″ E.

Isolation of endophytic fungi from halophyte plants

Root, stem and leaf of each plant specimen were first washed in tap water. The surface disinfection of the samples was performed with washing with 70% ethanol (volume/volume) for 5 min, disinfecting in sodium hypochlorite 2%(V/V) for 15 min, and ethanol 96% (V/V) for 2 min followed by three times washing with sterile water. Disinfected tissue segments were transferred to 8-cm-diameter plates, containing sterile potato dextrose agar (PDA) medium (QueLab), and incubated at 25 ± 1 °C for 4 weeks. Growing fungi around plant tissue segments were purified by hyphal tipping method and transferred to the new culture media for further identification.

Screening of salt-tolerant endophytic fungi

In order to evaluate salt tolerance of fungal strains, PDA medium with three different concentrations of 1, 2 and 3 molar sodium chloride is used. For each concentration of NaCl, three replications were used. Fungi that were able to grow on 1 M NaCl were tested for growing in 2 M and so on in 3 M NaCl concentrations. Fungi were incubated at 25 ± 2 °C for 7 days, and the ability of fungal isolates to grow in the presence of NaCl was monitored daily.

Enzyme activity of fungal isolates

To evaluate extracellular enzymatic activity of fungal isolates, amylase (Hankin and Anagnostakis 1975; Simair et al. 2017), protease (Hankin and Anagnostakis 1975; Razzaq et al. 2019), cellulose (Samanta et al. 1989; El-Said et al. 2014), keratinase (Joshi et al. 2007), and pectinase (Khairnar et al. 2009) production assays were applied in the medium base containing appropriate substrate. The treatments were incubated for 72 h at 25 °C, and the enzymatic activity was measured by eye estimation of diameter of the zones with an opalescent halo (Hankin and Anagnostakis 1975; Legodi et al. 2019).

Antimicrobial properties of fungi

The superior fungi were grown in potato dextrose broth (PDB) on a shaker at 120 revolutions per minute (rpm) for 1 week; then, they were placed in dark for two more weeks. PDB media containing fungal mycelia were filtered (Hosseini Moghaddam et al. 2013). Chloroform was added to the resultant solution (1:1; V:V), and the mixture was placed on a shaker with 120 rpm for 24 h. Two formed phases were separated by separator funnel. The medium including the chloroform solvent and metabolites was poured into the glass and dried in an oven at 45 °C. In total, 100 mg of each extracts dissolved in 1 ml of 100% dimethyl sulfoxide (DMSO) solvent. Five human pathogenic microbes including *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhimurum* and *Candida albicans* were selected to evaluate antimicrobial effect of isolated fungi. Antibiotic disks containing 100 µg of gentamicin, kanamycin and erythromycin were used as positive controls of inhibition. A volume of 15 microliters of microbial suspension (optical density: 0.8) was poured into each petri dishes containing Mueller–Hinton medium. An aliquot of 20 µl of each fungal extract added to the wells was made in each petri. An aliquot of 20 µl of DMSO was used as the control. The plates were placed in a refrigerator for 2 h and then incubated at 37 °C for 48 h (Smania et al. 1999).

Isolate 1 was tested in a dual culture assay against *Aspergillus fumigates*. For this test, a disk in 4 mm diameter from the colony margin of actively growing cultures of each fungus was placed on PDA. Disks were placed 5 cm far from each other in the same petri. Petri dishes were incubated in dark at 25 °C for 7 days according to the growth rate of fungi (Demirci et al. 2011). Amount of growth was compared and evaluated with control.

Identification of fungal isolates

A combination of morphological, physiological and molecular characteristics was used to identify fungal isolates. Isolates were classified based on morphological characteristics including color, shape, texture or hyphae and spore characteristics like size, shape and reproductive structures. Genomic DNA of spores was extracted by cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1987; Healey et al. 2014). The internal transcribed spacer (ITS) of the nuclear ribosomal DNA (rDNA) was amplified using the universal ITS1 and ITS4 primers (White et al. 1990; Martin and Rygiewicz 2005). In addition, the β-tubulin gene was amplified using βt2a and βt2b primer pairs (Glass and Donaldson 1994). The ITS1 and ITS4 primers amplify

Table 1 The PCR program used to amplify fungal ITS and β-tubulin gene (White et al. 1990; Glass and Donaldson 1994)

Reaction stage	Temperature (°C)	Time
Initial denaturation	95	5 min
Denaturation	95	1 min
Annealing	ITS: 58 βt: 55.5	45 s
Extension	72	40 s
Final extension	72	7 min

550 to 750 base pair of the gene, and βt2a and βt2b primers amplify the 500–600-bp region through the PCR (Table 1). PCR products were sequenced by Bioneer Company, and data were obtained in FASTA format, edited by Chromas software 2.6.6. and compared to GenBank sequences at the National Center for Biotechnology Information (NCBI).

Results and discussion

Results

In total, 40 fungi were isolated and colony purified using hyphal tipping culture. Out of 40 isolates, 23 recovered from roots, 15 from stems and 2 from leaves. Thus, about 57% of the isolates were isolated from root tissues. Eleven fungi were isolated from *A. iranica,* whereas only two fungi were isolated from *S. rosmarinus* and *A. annua* (Table 2).

Salt tolerance screening

From 40 fungal isolates, 32 grew on 1 M, 20 on 2 M, 11 on 3 M, 5 on 3.5 M and 1 on 4 M NaCl. As isolates no. 1, 2, 3, 7 and 31 were all morphologically the same, then isolate 1 was used for further experiments. Isolates no. 1, 6, 11, 14 and 19 were able to grow on 3.5 M NaCl. However, only isolate 1 was capable of growing on 4 M NaCl (Fig. 1).

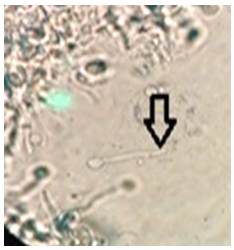

Fig. 1 Spore germination of isolate no. 1 at concentration of 4 M NaCl under microscope (magnification ×100)

The identification of fungi

The proper fragment in the range of 500 to 750 bp was amplified by PCR using ITS1 and ITS4 primers. Also sequence of beta-tubulin gene using primers βT2a and βT2b amplified the fragment of 500 bp by PCR (Fig. 2).

Antimicrobial properties of fungal extract

Antimicrobial properties of extracts of *Aspergillus terreus* (isolate 1), *Acremonium sclerotigenum* (isolate 6), *Paecilomyces formosus* (isolate 11), *Monosporascus ibericus* (isolate 14) and *Microascus pyramidus* (isolate 19) were assessed on 5 pathogenic microbes including *B. cereus*, *S. aureus*, *P. aeruginosa*, *S. typhimurum* and *C. albicans* by agar diffusion assay. Inhibitory zone data of the fungi extracts were analyzed by a one-way ANOVA and compared with Tukey's test in SPSS software (Fig. 4). The inhibition zone diameter was very different between fungal extracts. Based on inhibitory zone data *A. terreus* and *A. sclerotigenum* had the most antimicrobial effect compared with other

Table 2 Classification of isolated endophytic fungi from four halophyte plant families

Assigned code to fungal isolate	Plant source	Superior isolates	Closest relative based on sequence homology	GenBank accessions of fungi	Similarity (%)	
					ITS	β-Tubulin
1, 2, 3, 5, 6, 7, 30, 31, 32	*Rubia tinctorum*	1	*Aspergillus terreus*	MK811126	100	99
		6	*Acremonium sclerotigenum* strain CCTU1171	MK811127	100	–
9, 10, 11, 12, 13, 14, 35, 36, 37, 38, 39	*Anabasis iranica*	11	*Paecilomyces formosus*	MK811128	100	97 SUM 3339
		14	*Monosporascus ibericus*	MK811129	100	91 CBS 110550
15, 17, 18, 21, 22, 23, 24, 40	*Salsola yazdiana*	–	–		–	–
16, 19, 20, 25, 26, 27, 28, 29	*Salsola tomentosa*	19	*Microascus pyramidus*	MK811130	100	99 CBS 668.71
33, 34	*Seidlitzia rosmarinus*	–	–		–	–
4, 8	*Artemisia*	–	–		–	–

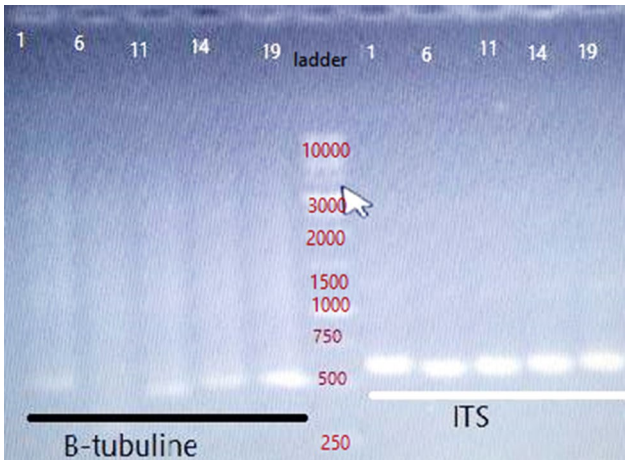

Fig. 2 ITS and β-tubulin amplification of five superior fungal isolates growing on high salt concentration

isolates and antibiotics, whereas the extracts from *M. ibericus* and *M. pyramidus* had no inhibitory effect (Fig. 4).

Figure 3 shows the antagonistic interactions between two species of aspergillus, one is plant endophyte and the other is a human pathogen. Dual culture assay for *A. terreus* showed inhibition of pathogen growth. Evaluation of inhibition zone was difficult due to spreading of *A. terreus* spores, but finally *A. terreus* covered whole plate.

Enzymatic activities of fungi

The enzymatic activity of 5 fungi *A. terreus*, *A. sclerotigenum*, *P. formosus*, *M. ibericus* and *M. pyramidus* with a higher salt tolerance ability than other screened isolates, was evaluated (Table 2). Diameter of the zones with an opalescent halo was comparatively measured.

Discussion

As halophyte plants can survive in high salt conditions, the study and evaluation of their symbiotic fungi would be very beneficial for crop production in salty areas. In the present study, 40 fungal isolates were purified from domestic halophyte plants growing in one of the saltiest regions of Iran, Chah Afzal village in Yazd Province. Obtained data showed that more than half of isolates were inhabited on the roots. Young et al. (2012) focused on roots of six halophyte plants, looking for endophytic fungi with plant growth ability. In addition, the involvement of root microbiome of *Suaeda salsa*, a halo-tolerant plant, has been studied (Yuan et al. 2016). The association of endophytic microorganisms with halophyte plants may enhance plant salt tolerance. Out of forty collected fungal isolates in this research, half of them could survive on 2 M NaCl and about 12% of them on 3.5 NaCl. There was variation among isolates for salt tolerance screening. For example, isolate 34, *Penicillium,* had ability to grow on 1 and 2 M but stopped at 3 M, while *Aspergillus* (isolate no. 1) survived on 4 M NaCl. The five halotolerant isolates growing on 3.5 M NaCl were identified as *A. terreus*, *A. sclerotigenum*, *P. formosus*, *M. ibericus* and *M. pyramidus*, all in *Ascomycota* phylum based on morphological, physiological and molecular data (Tables 2, 3 and Fig. 2). To determine the genus and species of the fungus, ITS and β-tubulin amplification were used. The isolated

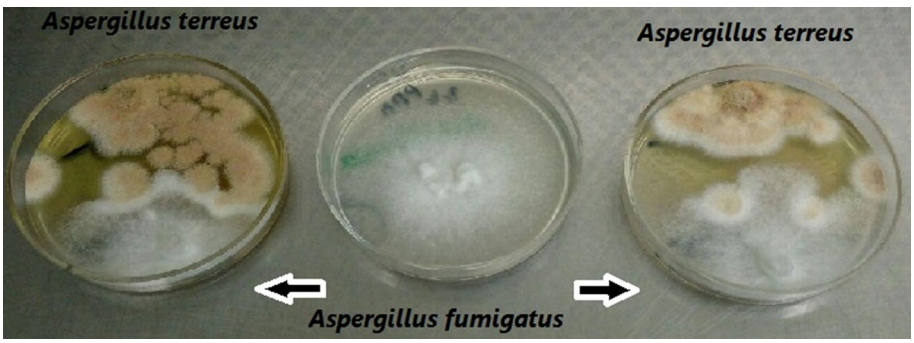

Fig. 3 Antimicrobial activity of five fungal extracts on five pathogenic microbes

Table 3 Enzymatic activity of five superior fungal isolates

Fungi	Keratinase	Gelatinase	Pectinase	Cellulase	Amylase
Aspergillus terreus	+	++	+	+	+
Acremonium sclerotigenum	++	+++	+++	+	+
Paecilomyces formosus	–	+	+	–	–
Monosporascus ibericus	–	+	+	–	–
Microascus pyramidus	–	++	+	+	–

No halo zone: –, small halo zone: +, medium halo zone: ++, large halo zone: +++

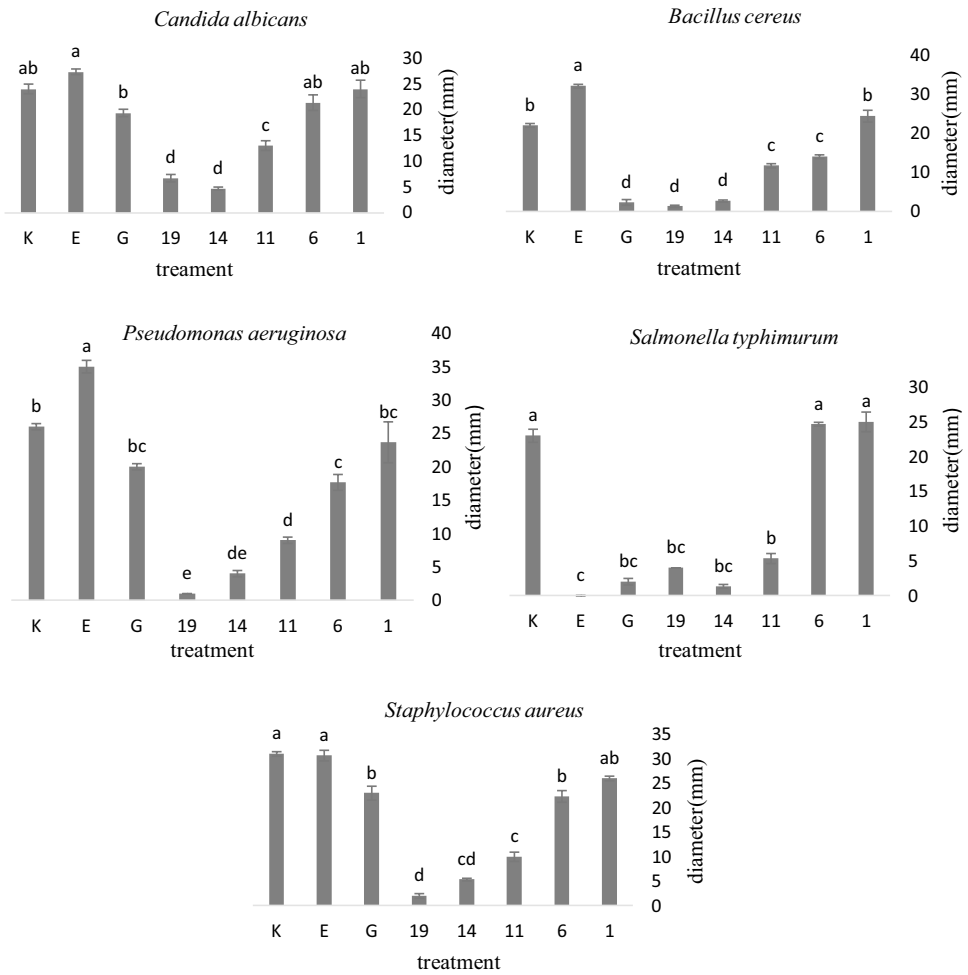

Fig. 4 Antagonistic interaction of two *Aspergillus* isolates: *A. terreus* (brown) and *A. fumigates* (white). *Aspergillus terreus* (isolate 1), *Acremonium sclerotigenum* (isolate 6), *Paecilomyces formosus* (isolate 11), *Monosporascus ibericus* (isolate 14) and *Microascus pyramidus* (isolate 19). E = erythromycin, K = kanamycin, G = gentamicin. The different letters (a, b, c, d and e) indicate statistically significant difference between the groups ($p < 0.01$)

A. terreus superior halo-tolerant fungus from *R. tinctorum* roots was reported here for the first time. It was also isolated before from plant species at sandy soils and marshes at southeast Spain (Maciá-Vicente et al. 2008). The superior halo-tolerant fungus *A. terreus* (isolate no. 1) was capable of decomposing cellulose. The commercial application of this species in production of organic acids and enzymes has been reported (Okabe et al. 2009). Also soil-isolated *A. terreus* MS105 has been introduced as a cellulase production strain by Sohail et al. (2016). Fungal isolates like *Alibertia macrophylla* and *Uncaria gambier Roxb* (Rubiaceae) with a broad range of biologically active compounds were introduced by Oliveira et al. (2009). In this study *A. terreus* also showed keratinase, protease, pectinase and amylase activities. In addition *A. terreus* and *Acremonium* isolates exhibited in vitro activity against five human pathogens and almost were placed in one group with antibiotics based on Tukey's test (Fig. 4). The antibacterial effect of the fungal extracts in case of Salmonella was particularly interesting. This pathogen showed resistance to erythromycin and gentamicin but was sensitive to extract of isolates 1, 6 and kanamycin. The antimicrobial-resistant strains of *Salmonella* are a great threat to public health (Liao et al. 2019); therefore, finding new antibiotics seems necessary. Marine *A. terreus* var. africanus showed antimicrobial activity against virulent fish pathogens (Barakat and Gohar 2012). *Aspergillus fumigatus* is an airborne fungal pathogen and shows resistance to environmental invasion (Valsecchi et al. 2019). Here the endophytic fungus *A. tereus* was able to inhibit the growth of this pathogen.

Secondary metabolites of endophytic fungi were active against pathogens and founded an important noticeable source of biocontrol agents (Kongue Tatong et al. 2014; Lo Piccolo et al. 2015). *Acremonium* sp. isolated from Garcinia tree had maximum zone of inhibition against *Salmonella typhi*, *S. aureus* and *Klebsiella* (Ruma et al. 2013). *Acremonium* sp. that exist in *Taxus baccata* could produce leucinostatin, a peptide antifungal–anticancer agent (Strobel et al. 1997). We isolated *A. sclerotigenum* from *R. tinctorum* (isolate 6). This isolate had the highest gelatinase and pectinase activity among five studied isolates. These enzymes are capable of hydrolyzing sugarcane bagasse usable in industry (de Almeida et al. 2011; Bischoff et al. 2009). Endophytes required enzymes for degradation of host cell wall and

penetration (Schulz et al. 2002). *P. formosus* (isolate 11) also showed enzymatic and antimicrobial activity. It is previously reported that *P. formosus* isolated from the cucumber, *Boswellia sacra* and *Eugenia jambolana* produced bioactive compounds such as carbohydrates, alkaloids, phenols, amino acids, hormones and extracellular enzymes (Khan et al. 2016; Yadav et al. 2014; Khan et al. 2012). There is no report on *A. iranica* endophytes, and two salt-tolerant fungi, *P. formosus* and *M. ibericus,* are reported for the first time. The latter was already isolated from roots and stems of three halophyte plants in the Ebro Delta in Spain salt marshes in 2002 (Collado et al. 2002). Although *M. pyramidus* was already isolated from desert soil (Barron et al. 1961) and animal (Woudenberg et al. 2017), we identified that as a salt-tolerant isolate from plant, *S. tomentosa*.

Conclusion

The study on salt tolerance and enzymatic activity of fungal community in halophyte plants resulted in isolation of two species, *A. terreus* and *A. sclerotigenum* as halo-tolerant fungi with strong enzymatic and antibacterial activities. In addition, two species of *M. ibericus* and *M. pyramidus* were detected as salt-tolerant isolates in *A. iranica and S. tomentosa* for the first time. This study also reveals that *Ascomycota* phylum includes strains, which may be involved in plant salt tolerance.

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