Macroalgae-Derived Fungal Endophytes Promoted The Growth of Mexican Lime Seedlings Under Heat Stress in Greenhouse Condition

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Abstract

Fungal endophyte associated with algae represents a rich source of bioactive metabolites and biostimulants, which can be used practically in agriculture as biofertilizers. Here, we aimed to study the associated symbiotic fungi collected from intertidal areas of the southern coastlines of the Persian Gulf and Oman Sea. The extracted endophytic fungi were identified based on morphological, physiological and molecular (based on ITS1-5.8S-ITS4rRNA regions) analyses. 566 fungal isolates were obtained from 190 seaweed segments. Results showed that the genera *Aspergillus* and *Penicillium* were the most frequent isolated fungi. The highest frequency of fungal isolates (48.29%) was observed in seaweeds collected from Bushehr province. In vitro, most of the fungal isolates (85%) could grow properly on a PDA medium incorporated with one molar NaCl concentration. Fungal isolates showing the highest resistance to NaCl in vitro assays and with highest frequency were used as biofertilizer agents to study their effects on the morphological characteristics of Mexican lime seedlings. The inoculation results showed that the fungal endophytes could increase the fitness of Mexican lime seedlings under heat stress by improving morphological attributes.

Keywords: Macroalgae, Endophytic Fungi, Biofertilizer, *Aspergillus*, *Penicillium*

Introduction

The marine ecosystem is a unique source of natural biological products from marine creatures with outstanding natural, biochemical, and biosynthesis characteristics (Subramani and Aalbersberg, 2013; Suriya et al., 2016; Jimenez, 2018). Endophytic microorganisms are a diverse group of organisms that live inside the host plant tissue and form colonies in the intercellu-

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lar spaces or between the xylem vessels of plants without any apparent symptoms (Rodriguez et al., 2009). Although, recently, a few studies have shown that infection by endophytes can increase the host plant's tolerance to abiotic and biotic stresses by inducing the activity of many defense-related genes of the host plants (Mejía et al., 2014; Miliute et al., 2015). Many questions remain unanswered regarding the biological attributes of endophytes (Suryanarayanan, 2013).

Algae in marine ecosystems adapt to frequent and sporadic environmental changes such as low oxygen content, high salinity, excessively high light, and nutrient limitation, which may be associated with endophytes to produce specific bioactive secondary metabolites to participate in the defense mechanisms of the hosts (Zhang et al., 2016).

Fungal endophytes constitute an inexplicably diverse group of polyphyletic fungi ubiquitous in plants and maintain an indiscernible dynamic relationship with their hosts for at least a part of their life cycle (Kusari and Spiteller, 2012). The endophytic fungi activate gene-silencing mechanisms, or vice versa, and start specific biosynthetic pathways. Hence, unique functional/bioactive metabolites will produce due to the reciprocal and advantageous relationship between endophytes and their hosts (Zhang et al., 2016). Symbiotic fungi have already been isolated from various marine habitats, including marine plants (algae and mangrove plants), aquatic invertebrates (sponges, holothurians, corals, and ascidians), and vertebrates (mainly fish). Among these organisms, algae are one of the most prevalent sources of marine-derived fungi for chemical studies (Rateb and Ebel, 2011).

Davati et al. (2013) studied the bioactive compounds extracted from endophytic fungi of macroalgae (Rhodophyta, Chlorophyta, and Phaeophyta) and reported different functions for these products. Anticancer, antibiotic, antiviral, antioxidative, and kinase inhibitory activities have been isolated from seaweed endophytic fungi (Strobel and Daisy, 2003; Kjer et al., 2010). Nevertheless, there is not much information regarding endophytic fungi in marine seaweeds, especially those are grown in tropical and subtropical regions (Suryanarayanan et al., 2010; Narayanan et al., 2013; Flewelling et al., 2015). Since no documented information is available regarding symbiotic fungi with seaweed species of the Persian Gulf and Oman Sea in the south of Iran, in the current study, we aimed to investigate the diversity of fungal endophytes of seaweeds of the Persian Gulf and Oman Sea and their role as stress-modifiers in Mexican lime seedlings under heat stress in greenhouse conditions.

Material and methods

Sampling and isolation of endophytic fungi

From April 2018 to March 2019, A total of 190 seaweed samples (37 species) belonging to 12 brown algae (62 samples), 15 red (79 pieces), and 10 green algae species

(49 pieces) were collected from 18 intertidal locations of three southern provinces of Iran including; Hormozgan, Bushehr and Sistan and Baluchistan (Table 1). Seaweeds were identified based on morphological keys (Sohrabipour and Rabiei, 2007; 2008; 2010).

The seaweed species in different sites were replicated. For example, we collected *Ulva* or *Gracilaria* from different sides and different seasons.

The physicochemical properties of seawater including EC, pH, and salinity were measured using the multimeter (HACH, HQ 40d USA) and refractometer (ATAGO, S/Mill-E Japan) devices (Table 2).

Fresh and healthy algal species were harvested and transferred to the biotechnology laboratory of Hormozgan University in sterile polyethylene bags. The surface of the samples was sterilized to isolate the exophytes from the collected seaweeds, as follows: washing the seaweeds with running tap water; soaking the illustrations in 70% ethanol for 5 S and, finally soaking in sterile distilled water for 10 S (Zhang et al., 2009). Sterilized tissues were cut into 0.5 cm^2 segments, then three segments of each seaweed sample were placed onto Petri plates containing Potato Dextrose Agar (PDA). Accordingly, 1674 sterilized segments of 190 seaweed specimens were used to isolate their endophytic fungi. The Petri plates were sealed and incubated in a light chamber at 26 ± 2 °C and a light period (12L:12D) for four weeks (Suryanarayanan, 1992). Most fungi grew within three days after incubation. The colonization frequency (CF) was calculated based on Yuan et al. (2010) as follows:

CF= total number of colonized endophytic fungi/ total number of incubated segments.

The grown fungi of each seaweed segment were periodically isolated and transferred to the fresh PDA plates. Identification of the isolated fungi was made based on morphological, physiological, and molecular methods. Microscopic characteristics of the isolates (hypha type, spore shape, and colony color) were also investigated to determine the morphological features of fungal isolates.

Molecular identification and phylogenetic analysis

DNA extraction

The fungal endophytes were identified molecularly using ITS primers (ITS1-5.8S-ITS4 region of rRNA). Genomic DNA was extracted according to the method proposed by Soltani and Moghaddam (2015). Colonies of each isolated fungi were grown at 28 °C and shaking rate of 90 rpm in the 15 ml Erlenmeyer flasks containing 2 ml Potato Dextrose Broth (PDB; Merck, Germany). After 15 days, the genomic DNA of each isolate was extracted using the CTAB method.

PCR amplification

The extracted DNA was subjected to Polymerase Chain Reaction (PCR) to amplify the ITS1-5.8S-ITS4rRNA region using the following primers, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′)

Table 1. List of seaweed species and their classification

No	Scientific name	Classification
1	*Stoechospermum marginatum*	Phaeophyta
2	*Padina australis*	Phaeophyta
3	*Padina crassa*	Phaeophyta
4	*Padina gymnospora*	Phaeophyta
5	*Rosenvingea orientalis*	Phaeophyta
6	*Sargassum boveanum*	Phaeophyta
7	*Sargassum bov.* Var. *aterrimum*	Phaeophyta
8	*Sargassum angustifolium*	Phaeophyta
9	*Dictyota* sp.	Phaeophyta
10	*Sirophysalis trinodis*	Phaeophyta
11	*Grateloupia lithophila*	Phaeophyta
12	*Iyengaria stellata*	Phaeophyta
13	*Galaxaura rugosta*	Rhodophyta
14	*Acanthophora spicifera*	Rhodophyta
15	*Hypnea* sp.	Rhodophyta
16	*Digenenia simplex*	Rhodophyta
17	*Gracilaria folliifera*	Rhodophyta
18	*Gracillaria corticata*	Rhodophyta
19	*Gracilaria* sp.	Rhodophyta
20	*Gracilaria salicornia*	Rhodophyta
21	*Gracilariopsis persica*	Rhodophyta
22	*Palisada perforata*	Rhodophyta
23	*Champia glublifera*	Rhodophyta
24	*Champia parvulla*	Rhodophyta
25	*Jania robens*	Rhodophyta
26	*Laurencia snideria*	Rhodophyta
27	*Grateloupia flicina*	Rhodophyta
28	*Halimeda tuna*	Chlorophyta
29	*Caulerpa sertularioides*	Chlorophyta
30	*Caulerpa peltata*	Chlorophyta
31	*Caulerpa taxifolia*	Chlorophyta
32	*Cladophoropsis* sp.	Chlorophyta
33	*Ulva ohnii*	Chlorophyta
34	*Ulva* sp.	Chlorophyta
35	*Chondrus crispus*	Chlorophyta
36	*Chaetomorpha* sp.	Chlorophyta
37	*Enteromorpha* sp.	Chlorophyta

Table 2. List of provinces, locations and their GPS coordinates as well as chemical properties of seawater in different sampling sites

Province	Location	Longitude (E)	Latitude (N)	EC (ms/cm)	pH	Salinity (PPT)
Hormozgan	Bandar Abbas	E56.27	N27.18	56.9	7.49	39.3
Hormozgan	Bandar-e Lengeh	E54.30	N26.18	56.6	7.97	38.4
Hormozgan	Bandar-e Kong	E54.54	N29.33	57.9	7.99	39.6
Hormozgan	Qeshm Island	E56.16	N26.57	56.6	7.94	38.9
Hormozgan	Hormoz Island	E56.27	N27.03	56.5	7.52	39.0
Hormozgan	Sirik	E57.10	N26.51	54.5	7.44	38.2
Sistan and Baluchistan	Chabahar	E60.39	N25.17	52.8	8.1	37.6
Sistan and Baluchistan	Gavader	E61.31	N25.10	54.5	7.12	36.9
Sistan and Baluchistan	Ramin	E60.25	N25.12	56.3	7.87	38.6
Sistan and Baluchistan	Great see	E60.39	N25.16	55.4	8.13	38.2
Sistan and Baluchistan	Kachu	E60.51	N25.15	56.4	8.2	38.1
Sistan and Baluchistan	Beris	E61.11	N25.08	55.5	7.98	38.3
Sistan and Baluchistan	Konarak	E60.23	N25.21	56.4	7.88	38.9
Bushehr	Bushehr	E50.83	N28.95	62.7	8.27	**42.0***
Bushehr	Shif Island	E50.52	N29.04	49.8	7.74	33.9
Bushehr	Nuclear Power plant	E50.53	N28.49	60.5	8.06	**41.0**
Bushehr	Sabz Abad park	E50.51	N28.44	60.2	8.06	**41.0**
Bushehr	Airport seaside	E50.50	N28.56	49.9	7.80	33.9

*Samples with ≥40 PPT salinity, were detected by refractometer device

(White et al., 1991). Each 25 µl reaction mixture included 2 µl of DNA, 12.5 µl of Taq DNA Polymerase (amplicon), 2 µl of primers, and 8.5 µl ddH_2O. Techno TC-572 thermocycler (Eppendorf, Hamburg, Germany) was used for PCR amplification which was programmed for 94 °C for 5 min, 35 cycles of 94 °C for 45 S, 53 °C for 30 S, 72 °C for 1 min, and 72 °C for 5 min. PCR products were subjected to electrophoresis on 1% agarose gel stained with blue stain.

DNA sequencing

All PCR products were sequenced directly by Macrogen Sequencing Service (Seoul, South Korea). The sequences of amplified ITS1-5.8S-ITS4rRNA regions were deposited in the GenBank database (NCBI, http://www.ncbi.nlm.nih.gov). The edited sequences were blasted against the NCBI GenBank nucleotide database (http://ncbi.nlm.nih.gov/blast/Blast.cgi) to search for closest relatives. Phylogenetic tree was reconstructed using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). The fungal isolates were archived as living vouchers at 4 °C.

Fig. 1. Fungal inoculum preparation using autoclaved water in 1×10^6 CFU mi^{-1} colony formation

Potential of endophytic fungi to grow under salt conditions

The potential of fungal endophytes to grow under salt conditions were investigated using the method proposed by Vatsyayan and Ghosh (2013). In brief, fungal isolates were grown in PDA medium supplemented with various concentrations of sodium chloride (1, 2 and 3 molar) and after 7 days their growth potential was scored from 1 to 8 (from weak to very severe), which was based on their ability to form a colony.

The effect of isolated fungi as modulator of heat shock factor

Three fungal species [*Aspergillus niger* (F1), *Penicillium chrysogenum* (F2), *Aspergillus flavus* (F3)] and their combination (F1 with F2) were used as modulator of heat shock factors and biofertilizers to inoculate eight months old Mexican lime seedlings under heat stress (45±2 ºC for seven days) (Chhabra et al., 2009). The seedlings were planted and grown in sterile soil. Fungal inoculum preparation using autoclaved water in 1×10^6 CFU mi^{-1} colony formation (Fig. 1). The study was carried out in completely randomized design with 4 treatments, and three replications. The endophytes were inoculated three times (over three weeks).

After 120 days, morphological characteristics of the seedlings, including the root length, root width, leaf fresh weight, leaf dry weight, root fresh weight, root dry weight, stem fresh weight, stem dry weight, stem

length, chlorophyll content (using SPAD-502; Konica Minolta, Osaka, Japan), trunk width, and numbers of leaves and branches were measured. The data were analyzed using a one-way analysis of variance (SAS Institute, 1988), and in the cases of significant differences, mean grouping was performed by the least significant difference (LSD) test ($P< 0.05$).

Results

Result of fungal isolation

From 1674 tissue segments belonging to 190 seaweed samples (79 red, 62 brown, and 49 green), in total, 566 colonies were recovered from red (285), brown (185), and green (96) seaweed species. The seaweeds collected from Bushehr province had the highest colonization rate (48.29%) (Table 3). In addition, the red seaweed species of Bushehr had the highest rate of colonization among three seaweed species both in summer (53.08%) and winter (50.61%) seasons. Likewise, red seaweed species, the brown seaweed species collected from Bushehr province, with 85 isolates, had a higher colonization rate than Hormozgan and Sistan and Baluchistan. Regarding green seaweed species, Hormozgan provinces with 43 isolates had a higher colonization rate than Bushehr and Sistan, and Baluchistan. Red seaweed species with 285 colonies had the highest isolation rate, followed by brown (185 colonies) and green (96 colonies) algae (Table 3).

Phylogenetic analyses

The phylogeny constructed using the sequence data of ITS1-5.8S-ITS4rRNA molecular region grouped all isolates in three distinct clusters (Fig. 2). Results of molecular identification, phylogenetic analysis, and GenBank accession numbers of all 20 sequences (Identified in the present study) and other supplementary information are shown in Table 4. Additional details regarding the endophytic fungi have been depicted in Table 5.

As descript in Table 5, all fungi isolation belonged to the Ascomycota phylum and had a transverse wall. Morphological and microscopy analysis showed that colonies' color varied from black to white (black, dark and light brown, gold, purple, dark and light green, yellow and white). Some fungi isolation like *Aspergillus niger* was mono-color (black), and some isolation like *A. carlsbadensis* was di color (white/purple). The spores' shapes were varied: Circle (in *Aspergillus*, *Penicillium* and *Paecilomyces* sp.), Spindle (in *Curvularia spicifera*) and Oval (in *Cladosporium macrocarpum*).

Spore array was in three forms, clustered (in *Aspergillus* and *Paecilomyces* sp. genus) (Fig. 3a), filamentous (in *Penicillium* genus) (Fig 3b), and sporadic (in *Curvularia spicifera* and *Cladosporium macrocarpum* genus) (Fig. 3c). All isolates have transverse walls in their septate hypha (Fig. 3d).

The fungi isolated by the F10 code (*Aspergillus carlsbadensis*) was extracted only from *G. folliifera* seaweed species collected from Bushehr province in the spring sea-

Table 3. Numbers of fungal colonies isolated from various seaweed samples collected from three province

Province	Season	No. of seaweed spices	Color	No. of segments	No. isolates generated	Colonization%
Hormozgan	Spring	18	5 red	45	14	31.11
			7 brown	63	9	14.28
			6 green	54	12	22.22
	Summer	21	8 red	72	12	16.66
			7 brown	63	16	25.39
			6 green	54	7	12.96
	Winter	37	15 red	135	46	34.07
			15 brown	135	31	22.96
			7 green	63	24	38.09
	Total	76	**76**	**684**	**171**	**25**
Sistan and Baluchistan	Spring	2	1 red	9	2	22.22
			1 green	9	1	11.11
	Summer	45	24 red	216	101	46.75
			6 brown	54	21	38.88
			15 green	135	13	9.62
	Winter	14	3 red	27	11	40.74
			7 brown	63	23	36.50
			4 green	36	10	27.77
	Total	61	**61**	**549**	**182**	**33.15**
Bushehr	Spring	11	5 red	45	15	33.33
			5 brown	45	23	51.11
			1 green	9	0	0
	Summer	17	9 red	81	43	53.08
			7 brown	63	25	39.68
			1 green	9	3	33.33
	Winter	25	9 red	81	41	50.61
			8 brown	54	37	68.51
			8 green	54	26	48.14
	Total	53	**53**	**441**	**213**	**48.29**
	Total+	190	**190**	**1674**	**566**	**33.81**

son (Fig. 4). It was individual isolation that did not found in any other seaweed species.

Salinity test

Although, most of fungal endophytes (85%) could grow properly on PDA medium incorporated with one molar NaCl concentration and got the number 8 and 7 ranking scores in salinity test, in 2 molar NaCl, only 60% of the isolates were able to grow decently. In 3 molar NaCl, only 35% of the isolates were grown (Table 6, Fig. 5 and Fig. 6).

Result of fungal endophytes inoculation as biofertilizer

After 120 days post-inoculation, Mexican lime seedlings were analyzed. As shown in Figure 8, fungal endophytes could improve the morphological characteristics of the Mexican lime seedlings. Selected fungal endophytes (F1, F2, F3, and F1F2),

Table 4. Name, identification code and accession number of the isolated endophytic fungi along with other additional information regarding their seaweed host, sampling site and sampling time

No	Fungi isolates	Code	Accession No.	Seaweed host	location	Season
1	*Aspergillus niger*	F1	MT420720	*Cladophoropsis* sp.	BU	Spring
2	*Penicillium chrysogenum*	F2	MT420723	*S. bov,* var. *attrimum*	BU	Spring
3	*A. flavus*	F3	MT420731	*Chaetomorpha* sp.	B. S	Summer
4	*P. chrysogenum*	F4	MT476156	*Gracilaria* sp.	RA	Summer
5	*Penicillium* sp.	F5	MT476164	*Champia parvulla*	BU	Spring
6	*A. terrus*	F7	MT476158	*Champia parvulla*	BU	Spring
7	*A. puniceus*	F8	MT476159	*Champia parvulla*	BU	Spring
8	*A. terrus*	F9	MT420850	*Hypnea* sp.	B. S	Summer
9	*A. carlsbadensis*	F10	MT476165	*G. follifera*	BU	Spring
10	*P. chrysogenum*	F11	MT476166	*S. angustifolium*	B. L	Summer
11	*A. terrus*	F14	MT420880	*Gracilaria* sp.	BE	Summer
12	*A. niger*	F15	MT420884	*Cladophoropsis* sp.	Q. I	Spring
13	*Curvularia spicifera*	F16	MT420887	*Iyengaria stellata*	B. L	Winter
14	*A. egyptiacus*	F17	MT420890	*Gracillaria* sp.	BU. P	Winter
15	*A. chevalieri*	F18	MT420889	*Gracilaria* sp.	Q. I	Winter
16	*P. chrysogenum*	F20	MT420891	*Gracilaria* sp.	B. A	Winter
17	*Cladosporium macrocarpum*	F24	MT420892	*S. boveanum*	B. A	Spring
18	*Paecilomyces* sp.	F25	MT476163	*Iyengaria stellata*	B. L	winter
19	*P. chrysogenum*	F29	MT420908	*G.persica*	B. A	Winter
20	*P. chrysogenum*	F33	MT476157	*Grateloupia lithophila*	B. A	Winter

BU, B. S, RA, B. L, BE, BU. P, Q. I, B. A denote Bushehr, Bandar-e Sirik, Ramin in Sistan and Baluchistan province, Bandar-e Lengeh, Beris in Sistan and Baluchistan province, Bushehr power plant, Qeshm Island and Bandar Abbas, respectively.

Table 5. Endophyte isolate code and Supplementary information of the endophytic fungi associated with some Iranian Macroalgae

Code	Taxa	Fugal order	phylum	Fungal class	Family
F1	*Aspergillus niger*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F2	*Penicillium chrysogenum*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F3	*A. flavus*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F4	*P.chrysogenum*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F5	*Penicillum* sp.	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F7	*A. terrus*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F8	*A. puniceus*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F9	*A. terrus*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F10	*A. carlsbadensis*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F11	*P. chrysogenum*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F14	*A. terrus*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F15	*A. niger*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F16	*Curvularia spicifera*	Pleosporales	Ascomycota	Dothideomycetes	Pleosporaceae
F17	*A. egyptiacus*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F18	*A. chevalieri*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F20	*P. chrysogenum*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F24	*Cladosporium macrocarpum*	Capnodiales	Ascomycota	Dothideomycetes	Cladosporiaceae
F25	*Paecilomyces* sp.	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F29	*P. chrysogenum*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae
F33	*P. chrysogenum*	Eurotiales	Ascomycota	Eurotiomycetes	Trichocomaceae

were able to increase the root length, root width, leaf fresh weight, leaf dry weight, root fresh weight, root dry weight, stem fresh weight, stem dry weight, stem length, trunk width, SPADE number, leaf number and branches number in the inoculated Mexican lime seedlings (Fig. 7). The combination of F1F2 isolates resulted in higher trunk width, leaf number, leaf fresh/dry weight, root fresh/dry weight, stem fresh weight, and root length compared to when they were used alone (Fig. 7). The highest chlorophyll SPAD and root width were obtained by F1 inoculation. The highest stem length was obtained by F3 inoculation (Fig. 7).

Significant differences were found in all morphological characteristics measured by colonizing the Mexican lime seedling with endophytic fungi (Table 7 and Fig. 8).

Discussion

Numerous research studies have shown that asymptomatic, systemic fungi that colonize the healthy leaves, stems, roots, reproductive organs of the host significantly affect the physiology, ecology, and reproductive biology (Bonnet et al., 2000; Clay

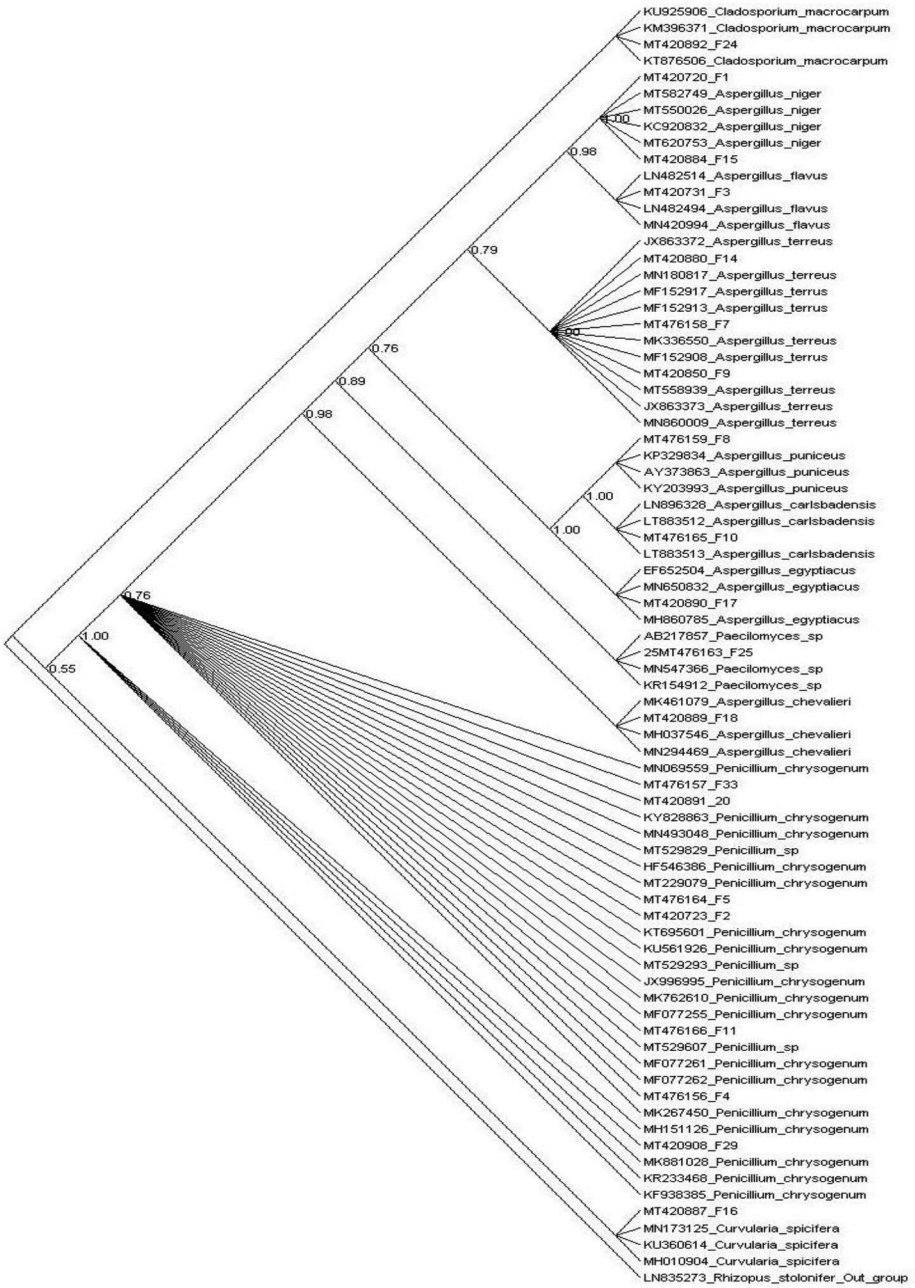

Fig. 2. The 50% majority rule consensus tree inferred from Bayesian analysis under the GTR + G + I model. The accession numbers of strains and their reference codes are shown

and Schardl, 2002; Clay et al., 2005; Malinowski and Belesky, 2006; Knop et al., 2007; Alfaro and Bayman, 2011) of the host plants. It showed that applying microbial products as inoculants can improve crop production under stress conditions or enhance disease resistance. Endophytic fungi can confer protection to hosts against insect pests and abiotic stresses (Thrower and Lewis, 1973; Clay and Schardl, 2002). The simulations of plant growth executed by plant growth promoters could be attributed to tolerance to biotic and abiotic stresses and improved plant nutrition (Machungo et al., 2009).

DNA analyzing method is a rapid approach

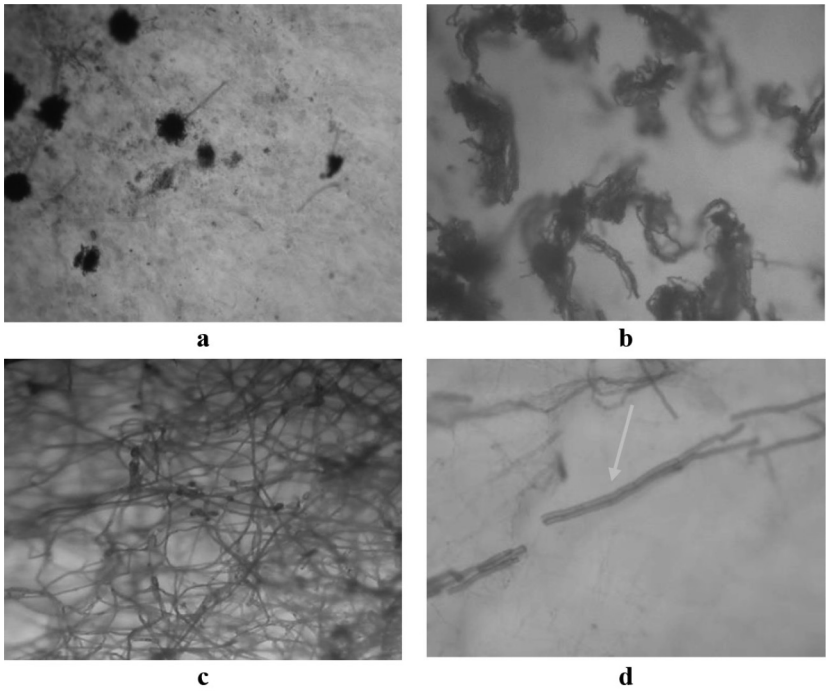

Fig. 3. Microscopic results for fungal isolates spore array; **a**: clustered form in *Aspergillus* genus, **b:** filamentous form in *Penicillium* genus and **c**: sporadic form in *Curvularia spicifera* genus, **d**. septate hypha in fungal isolates (a the isolates have transverse wall in their septate hypha).

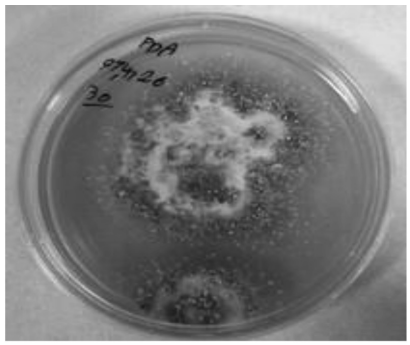

Fig. 4. *Aspergillus carlsbadensis* (F10), a fungal endophyte that was isolated just from *G. folliifera* species that collected from Bushehr Province in spring

to identifying fungi, especially in no sporulation endophytes (Lee et al., 2008; Zhang et al., 1996). The ITS1-5.8S-ITS4rRNA is a highly conserved region in fungi and can differentiate higher taxonomic levels. In contrast, ITS regions are highly variable and can be used to analyze lower taxonomic ones (Sugita and Nishikawa, 2003). Marine-derived endophytic fungi have been widely studied due to their bioactive metabolites (Bhadury et al., 2006; Newman et al., 2006). In the present study, the iden-

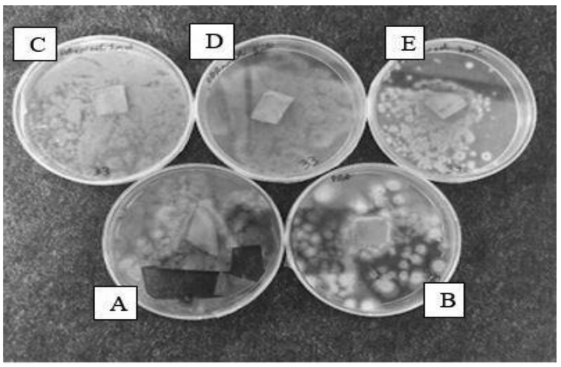

Fig. 5. Result of salinity test of the fungal endophyte (F33) on mediums incorporated with different NaCl concentrations (0, 1, 2, and 3 mol NaCl) after seven days; a, b, s, d, and e means: main stock, medium without NaCl, medium with 1 mol NaCl, medium with 2 mol NaCl and medium with 3 mol NaCl respectively

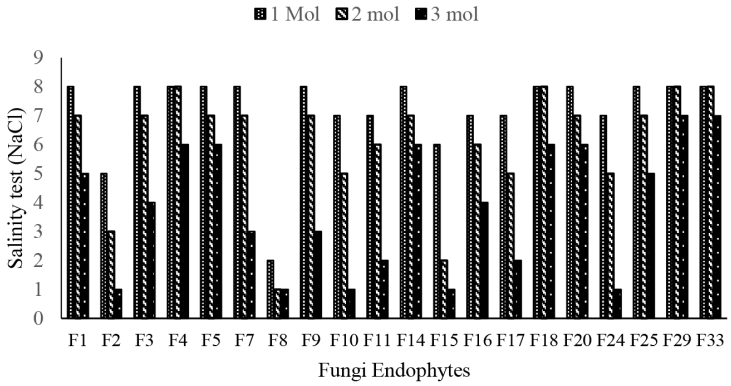

Fig. 6. Result of salinity test of the fungal endophytes on mediums incorporated with different NaCl concentrations (0, 1, 2 and 3 molar NaCl) after seven days

tification of fungi endophytes associated by different seaweed species (Rhodophyta, Chlorophyta, and Phaeophyta) collected from coastal regions of the Persian Gulf and Oman Sea was confirmed by PCR assay using universal primers (ITS1-5.8S-ITS4r-RNA sequence). Most isolates belonged to *Aspergillus* and *Penicillium* genera. The type of endophytes varied based on seaweed species, sampling site, and sampling season. All isolates belonged to the Ascomycota phylum (Table 5).

Phosphate is one of the essential compounds needed for plant growth, generally found in insoluble form and not utilized by plants. Plant growth-promoting fungi with phosphate solubilizing ability belong to *Aspergillus* and *Penicillium* genera (Noor-

Table 6. Physiological and morphological characteristics reports of endophytic fungi isolations

No	Code	1 mol	2 mol	3 mol	Colony color	Mono/Di color	Spore shape	Septate hypha
1	F1	8	7	5	Black	Mono	Circle/Clustered	+
2	F2	5	3	1	green	Mono	Circle/filamentous	+
3	F3	8	7	4	Green	Mono	Circle/Clustered	+
4	F4	8	8	6	Green	Mono	Circle/filamentous	+
5	F5	8	7	6	Green/creamy	Di	Circle/filamentous	+
6	F7	8	7	3	Brown/gold	Di	Circle/Clustered	+
7	F8	2	1	1	Brown	Mono	Circle/Clustered	+
8	F9	8	7	3	Brown	Mono	Circle/Clustered	+
9	F10	7	5	1	White/purple	Di	Circle/Clustered	+
10	F11	7	6	2	Black/white	Di	Circle/filamentous	+
11	F14	8	7	6	Yellow/green	Di	Circle/Clustered	+
12	F15	6	2	1	White	Mono	Circle/Clustered	+
13	F16	7	6	4	Dark gray	Mono	Spindle/sporadic	+
14	F17	7	5	2	Gray green	Di	Circle/Clustered	+
15	F18	8	8	6	Yellow	Mono	Circle/Clustered	+
16	F20	8	7	6	Purple	Mono	Circle/filamentous	+
17	F24	7	5	1	Dark green	Mono	Oval/ sporadic	+
18	F25	8	7	5	Dark green	Mono	Circle/Clustered	+
19	F29	8	8	7	Light brown	Mono	Circle/filamentous	+
20	F33	8	8	7	Dark green	Mono	Circle/filamentous	+

For salinity test, 1, 2, 3, 4, 5, 6, 7 and 8 means; very weak, weak, relatively weak, relatively intermediated, intermediated, relatively severe, severe and very severe, respectively.

jahan et al., 2019). Numerous studies indicate that *Aspergillus* and *Penicillium* are two common endophytes in different seaweed species (Suryanarayanan et al., 2010; Narayanan et al., 2013; Venkatachalam et al., 2015; Flewelling et al., 2015). Among the different genera identified in the seaweed species, the *Aspergillus* and *Penicillium* genus had the highest number of colonies (55% and 30%, respectively). Out of 190 seaweed samples, 32 isolates (16.84%) belonged to *A. niger*. This is the first study that describes indigenous fungal endophytes isolated from Iranian seaweeds. It has been revealed that most endophytic fungi isolated from plants are members of the Ascomycota or their anamorphs (Rungjindamai et al., 2008).

Seven different species of *Aspergillus* were identified in the present study, including *A. niger*, *A. terras*, *A. flavus*, *A. puniceus*, *A. carlsbadensis*, *A. egyptiacus* and *A. chevalieri*. We also found differences in endophyte colonization between different sam-

Table 7. Mean (± SE) of the studied attributes in the fungi colonized Mexican lime seedlings compared to non-inoculated seedlings

Characterizes	N	F1	IRCC	F2	IRCC	F1F2	IRCC	F3	IRCC
SPAD	37.56±0.04^{***b}	59.73±0.04^{***a}	59.02	59.66±0.03^{***a}	58.83	59.13±0.01^{***a}	57.42	59.53±0.04^{***a}	58.49
Trunk width	2.91±0.00^{***d}	3.93±0.00^{***b}	35.05	3.6±0.01^{***bc}	23.71	4.3±0.01^{***a}	47.76	3.37±0.01^{***c}	15.80
Leaf no	22.00±0.1^{***d}	30.00±0.27^{***c}	36.36	40.66±0.21^{***b}	84.81	66.33±0.08^{***a}	201.5	40.00±0.1^{***b}	81.81
Branches	2.66±0.03^{**b}	3.33±0.03^{**b}	25.18	3.66±0.03^{**b}	37.59	3.66±0.03^{**b}	37.59	5.33±0.03^{**a}	100.37
Stem length	15.33±0.03^{***d}	30.33±0.08^{***b}	97.84	25.66±0.11^{***c}	67.38	34.66±0.13^{***a}	126.09	38.00±0.1^{***a}	147.87
Leaf FW	4.99±0.02^{***c}	9.94±0.02^{***b}	99.19	10.06±0.01^{***b}	101.60	13.87±0.01^{***a}	177.95	10.02±0.09^{***b}	100.80
Leaf DW	0.45±0.00^{***c}	2.11±0.00^{***b}	368.88	2.10±0.00^{***b}	366.66	3.29±0.00^{***a}	631.11	2.16±0.01^{***b}	380
Root FW	5.03±0.02^{***d}	12.28±0.02^{***c}	144.13	16.66±0.01^{***b}	218.54	17.8±0.00^{***a}	253.87	15.99±0.04^{***b}	217.89
Root DW	1.36±0.02^{***d}	2.47±0.00^{***c}	81.61	3.19±0.00^{***b}	134.55	4.31±0.00^{***a}	216.91	3.44±0.01^{***b}	152.94
Stem FW	3.11±0.00^{***d}	6.06±0.02^{***c}	94.85	7.01±0.00^{***ab}	125.40	8.71±0.05^{***a}	180.06	7.81±0.01^{***ab}	151.12
Stem DW	1.53±0.00^{*b}	2.37±0.00^{*ab}	54.90	2.63±0.01^{*a}	71.89	2.59±0.06^{*a}	69.23	2.83±0.02^{*a}	84.96
Root length	29.5±0.16^{***c}	32.16±0.05^{***c}	9.01	35.83±0.08^{***b}	21.45	44.73±0.1^{***a}	51.62	44.16±0.04^{***a}	49.69
Root width	27.00±0.1^{***b}	55.5±0.16^{***a}	105.55	55.33±0.15^{***a}	104.92	51.5±0.07^{***a}	90.74	52.16±0.06^{***a}	93.18

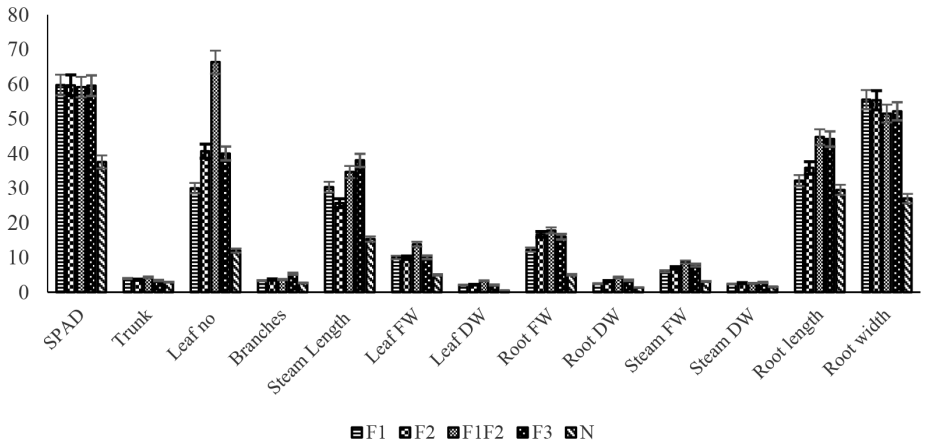

Fig. 7. Morphological analysis (Chlorophyll SPAD number, trunk width, leaf and branch number, stem length, leaf fresh weight, leaf dry weight, root fresh weight, root dry weight, stem fresh weight, stem dry weight, root length and root width) of Mexican lime seedling after 120 days. N, means; without inoculation. F2 is the F4 isolate in Table 3.

Fig. 8. Mexican lime seedling inoculated with fungal endophytes (F1+F2) compared to uninoculated seedling (N), 120 days post inoculation and seven days heat stress (45±2 ℃)

pling sites. High differences in the number of colonization were found between the three provinces in which the sampling was made. The highest number of isolates were found in the seaweeds collected from Bushehr province (213 isolates), followed by Sistan and Baluchistan (182 isolates) and Hormozgan (171 isolates) provinces. Also, Bushehr had the highest colonization rate (48.29%), followed by Sistan and Bal-

uchistan (33.15%) and Hormozgan (25%). These results could be due to Bushehr's position in the Persian Gulf region.

In this study, it was found that some endophytic fungi were restricted only to one seaweed species. For instance, some fungal endophytes such as *A. carlsbadensis* was observed only in *G. folliifera* collected from Bushehr in the spring season. The ecological and environmental conditions such as seawater temperature or salinity rate may affect the colonization of host tissues by endophytes. In another example, the species *G. persica* collected from Qheshm Island in spring had no endophytes. When it was collected from Bandar Abbas in winter, it was associated with four fungal endophytes.

In the genus *Gracilaria*, the highest number of isolates was observed in summer (66 isolates) in Ramin (38 isolates) and Beris (13 isolates) regions. The association of these seaweed species with high salinity-resistant endophytes may be one of the reasons for their abundance in the sea. Interestingly, some seaweeds (*Gracilariopsis persica*) had no endophyte in warm seasons whereas, in cool winter, several fungal endophytes were isolated from them. *Gracilariopsis persica* is an endangered species that has limited growth in warm seasons. The association of endophytes with this species in cold seasons seems to be an adopted defense mechanism to save this species from extinction.

In the current study, the ability of endophytic fungi to produce colony on the PDA medium incorporated with different concentrations of NaCl was studied and found, in one mol NaCl; most of the isolates (85%) were able to colonize the medium and allocated the score of 7 and 8 in the test of resistance to salinity conditions. In 2 mol NaCl, 60% of the studied isolates could produce a colony. The species *Penicillium* chrysoganum (F4), *A. chevalieri* (F18), *P. chrysogenum* (F29), and *P. chrysogenum* (F33) were able to colonize in the PDA culture over the experiment fully. These fungal endophytes were isolated from *Gracilaria* sp., *Gracilariopsis persica*, and *Grateloupia lithophila,* indicating the resistance to salinity in endophytes can vary based on seaweed species (Koch et al., 2017). In addition, it seems that the genus *Penicillium* was more tolerable to salinity than the other studied genus (Yadav et al., 2018).

Identifying the relationship between seaweeds and their associated endophytes provides important opportunities for research. Some endophytes like *Aspergillus* and *Penicillium* genus have shown potential to produce pectinases, cellulases, xylanases, and proteases (Bezerra et al., 2012). Over 300 natural endophyte-derived products were identified from 32 endophytic fungi, with 22% of the investigated fungi being from the *Aspergillus* genus (Flewelling et al., 2015).

Aspergillus niger (MT420720), *P. chrysogenum* (MT476156), and *A. flavus* (MT420731) for F1, F2, and F3 isolates, respectively, were used for inoculation purposes. We selected these isolates due to their high frequency in the studied seaweed

species in the current study.

We showed that the fungal endophytes F1, F2, F3, and F1F2 could improve the root length, root width, leaf fresh weight, leaf dry weight, root fresh weight, root dry weight, stem fresh weight, stem dry weight, stem length, trunk width, SPAD number, the number of leaf and branches in inoculating Mexican lime seedlings. Therefore, the fungal community present in the internal parts of the roots and foliage of Mexican lime seedlings has the potential role of promoting plant growth. One of the problems of lemon trees in summer is susceptibility to the fungus *Nattrassia*. In the present study, the endophyte-inoculated seedlings did not have any exposure to *Nattrassia* and had a higher growth rate than the control. Ngamau et al. (2014) showed that endophytes, through P solubilization or siderophore production (iron chelation), can help plants grow better. For instance, Nadeem et al. (2010) studied the plant growth-promoting activity and stress resistance capability of the genus *Penicillium* and *Aspergillus*. They found these two endophytic fungi produced physiologically active gibberellins. A previous study showed that seaweeds with high endophyte colonization had more ability to metal (Fe, Cu, Zn) absorption (Baghazadeh et al., 2020). So, some endophytes have a high capacity to metal absorbance from soil to host plants. It needs more investigation to understand the skills of the other seaweeds fungi endophytes obtained in this study, especially for biofertilizer aims.

Overall, our findings indicate that the fungal endophyte diversity in the seaweeds can be affected highly by seaweed species, sampling sites, and sampling season. Although this is the first study on fungal endophytes of Iranian seaweed, more research is needed to identify the functional and ecological significance of these fungal endophytes. This study showed that some of the identified species have a high potential to increase plant tolerance to salinity conditions that can be used to produce bio-fertilizers in the future. Accordingly, to reach this critical goal, establishing microbial reservoirs of seaweed endophytes is highly recommended.

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