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ORIGINAL ARTICLE

New fungal pathogens and endophytes associated with *Salsola*

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Abstract

Species of the genus *Salsola* belong to the family Chenopodiaceae and are associated with large saline areas in eastern Iran. The aim of the study was to isolate and characterize the endophytic and phytopathogenic fungal communities from non-mycotrophic *Salsola* species. Sampling was done from different parts of *Salsola* plants in the Birjand region in 2017 and 2018. Isolation and identification of fungal isolates were done using biological characteristics and ITS region sequences. The pathogenicity of the representative isolates was investigated by cultivating disinfected *Salsola incanescens* seeds under greenhouse conditions and inoculating seedlings with a fungal spore suspension from 7 day old fungal colonies on PDA media. Based on morphological and molecular data, 27 isolates from 11 fungal species were isolated and identified from *Salsola* tissues. *Alternaria alternata, A. chlamydospora, Aspergillus terreus, Macrophomina phaseolina, Fusarium longipes, Ulocladium atrum,* and *Talaromyes pinophilus* caused root or stem rotting and yellowing leaf of *S. incanescens* under greenhouse conditions. *Aspergillus niger* induced *S. incanescens* crown swelling without any pathogenicity. *Clonostachys rosea, F. redolens* and *F. proliferatum* grew as endophytic fungi on *S. incanescens* roots. This is the first report of phytopathogenic *M. phaseolina, F. longipes, T. pinophilus*, endophytic *F. redolens* and *A. niger* as a swelling agent on *S. incanescens*.

Keywords: endophytes, fungal diversity, ITS-rDNA, sequences analysis

Introduction

Salsola species which belong to the family Chenopodiaceae are annual C4, drought and salinity tolerant plants (Rezvani Moghaddam and Koocheki 2003; Weber 2017). Forty species of *Salsola* have been identified in Iran which *Salsola turkestanica*, *S. crassa*, *S. kali*, *S. arbusculiformis*, *S. incanescens*, *S. tomentosa*, *S. dendroides*, and *S. orientalis* are the most commonly distributed (Assadi 1984; Ghahreman and Attar 2018).

Salsola is used for its high water use efficiency, drought and salinity tolerance, resistance to pests, diseases, and grazing, as an important crop forage in arid and salty regions (Seemann 1979). *Salsola* species as non-mycotrophic plants do not have symbiotic or endophytic relationships with fungi, but several mycorrhizal, endophytic and phytopathogenic fungi were isolated from these plants (Allen *et al.* 1989; Kolomiets *et al.* 2010).

Pugh and Williams (1968) introduced *Alternaria tenuis* species as the causal agent of *S. kali* root rot in England. *Cephalosporium* and *Fusarium* species were isolated from *S. kali* roots in the Lincolnshire region during 1964–1965 (Pugh and Williams 1968). *Alternaria alternata* and *A. tenuis* species in southern and eastern United Kingdom are *Salsola* species pathogens of which *A. alternata* has dominant populations (Pugh and Williams 1968). *Ulocladium atrum*, *U. chartarum*,

and *U. septosporum* species are *Salsola* endophytes in Iraq (Muhsin and Zwain 1989; Imran 2011). *Colletotrichum gloeosporioides* caused anthracnose disease of *S. tragus* in Greece (Berner *et al.* 2006). *Phoma medicaginis* species is a *Salsola* root endophyte in Mexico (Rivera-Orduña *et al.* 2010). Aletaha *et al.* (2018) isolated endophytic *Macrophomina phaseolina*, *Fusarium oxysporum* and *F. brachygibbosum* from *Salsola* roots in the Yazd desert and the Javar plain of Kerman, Iran.

Some species of *Salsola* are distributed in rangelands of Southern Khorasan province in eastern Iran and used as livestock and camel forage (Towhidi *et al.* 2011). On the other hand, *Salsola* is one of the dominant weeds in fields of eastern Iran and can be affected by the positive effects of endophytic fungi or the negative impacts of fungal pathogens. The purpose of this study was to investigate the microbiota of these plants and identify the phytopathogenic or endophytic fungi associated with them.

Materials and Methods

Sampling was carried out during the period between 2017 and 2018 from *Salsola* parts including leaf, stem, crown and root, especially from symptomatic tissues, in different areas of the Birjand plain, South Khorasan province (32°52'N 59°12'E). The fungi were isolated from plant tissues by surface disinfection and cultured on general and selective media (Pugh and Williams 1968). Single-spore purification was performed on 2% water agar culture media (Pugh and Williams 1968; Choi *et al.* 1999).

Potato carrot agar (PCA), potato dextrose agar (PDA), Czapek agar, Czapek-yeast agar, Czapek-yeast 20% sucrose agar, malt extract agar and creatine agar media were used for fungal identification (Samson and Pitt 1986; Leslie and Summerell 2006; Mukherjee *et al.* 2013; Woudenberg *et al.* 2013). Macroscopic and biological characteristics were studied including the colony color from the front and back of culture plates, the growth rate of the colony, shape and size of chlamydospores, or conidia and phialides.

A pathogenicity test was performed by inoculation of *S. incanescens* seedlings as the dominant species in Birjand under greenhouse conditions. The selection of this species for the pathogenicity test was due to its widespread distribution in eastern Iran and the isolation of most isolates of this study was from this plant. The seeds of *S. incanescens* were collected from Amirabad village (32°52'50"N 59°07'31"E) and incubated after surface sterilization with 0.5% sodium hypochlorite in Petri plates under moist and sterile conditions at 25°C for 24 days (Zaman *et al.* 2010). The germinated seeds were sowed under greenhouse conditions (28 ± 2°C and 18 : 6 day/night) in sterile soil. Due to the high salinity requirements of this plant, the soil of the sampled region was sterilized and used for planting.

The pathogenicity of the isolates was investigated by inoculation of the pots' soil using spore suspension obtained from the 7 day old fungal colony on PDA media (Pugh and Williams 1968). The fungal spores' suspension which was obtained from PDA was added to the pots' soil several times, from the seed planting stage to 120-day-old plants (Park 1955). The inoculated plants were removed from the pots and the fungi were re-isolated after sterilization and culture on PDA medium.

The root colonization was studied by staining using the Kobae and Ohtomo method (2015) with slight modifications. The roots from the inoculated plants in the greenhouse were floated at 10% KOH for 24 h. After KOH removal and washing with water, 5% HCl solution was added for 1 to 2 min. HCl was removed and the tissues were stained for 24 h in a 2 $g \cdot l^{-1}$ trypan blue staining solution. Root staining was performed with a binocular microscope (Kobae and Ohtomo 2015).

The fungal isolates were identified based on morphological characteristics and sequencing of a part of the rDNA region. The product of polymerase chain reaction (PCR) of isolates using the internal transcribed spacer (ITS1/ITS4) (Bellemain *et al.* 2010) primers was purified and sequenced by Bioneer Co., South Korea. The UPGMA (unweighted pair group method with arithmetic mean) tree was constructed with Mega7 (Kumar *et al.* 2016) based on 1,000 Bootstrap replicates for each species.

Results

Twenty-seven isolates of 11 fungal species were isolated and identified on selective and general media (Table 1, Fig. 1).

Four isolates of *A. alternata* were isolated from *Salsola* root in Giuk (R3 and R7), Mezg (SH3) and Ark (D2) villages. The sequence of 508 bp of ITS region of D2 isolate (MK530695) had 100% similarity to the sequences of *A. alternata* (SS4 isolate) from NCBI GenBank (Accession number: MK226305). Crown rotting and blackening of *S. incanescens* stems were pathogenicity symptoms of these isolates under greenhouse conditions (Fig. 2A–B). Staining of the roots revealed the distribution of the mycelia and spores on the roots of *S. incanescens*.

Two isolates (R4M and R2M) belonging to *A. chlamydospora* species were isolated from the root and crown of *Salsola* in the village of Amirabad. The

Table 1. Fungal isolates from *Salsola* species: identification and pathogenicity on *Salsola incanescens*

Isolate	Species	Origin	Date	Source	Symptom on *Salsola incanescens*	GenBank accession no.
R3	*Alternaria alternata*	Giuk	30 January 2018	root	crown rot	
R7	*A. alternata*	Giuk	30 January 2018	root	crown rot	
SH3	*A. alternata*	Mezg	February 2018	root	crown rot	
D2	*A. alternata*	Ark	26 November 2017	root	crown rot	MK530695
R2M	*A. chlamydospora*	Amirabad	25 October 2017	root	black stem, yellow leaves, leaf drop	MG589492
R4M	*A. chlamydospora*	Amirabad	25 October 2017	root	black stem, yellow leaves, leaf drop	
R1D	*Macrophomina phaseolina*	Amirabad	25 October 2017	root	black stem, crown rot, yellow leaves, leaf drop	
M1	*Fusarium longipes*	Amirabad	16 December 2017	stem	crown rot	
Z8	*F. longipes*	Amirabad	16 December 2017	root	crown rot	
M6	*F. longipes*	Hajiabad	16 December 2017	root	crown rot	
M2	*F. longipes*	Hajiabad	16 December 2017	root	crown rot	
M3	*F. longipes*	Shamsabad	18 December 2017	root	crown rot	
H4	*Ulocladium multiformis*	Mud	8 January 2018	root	black stem, crown rot	MK530696
A4	*Aspergillus niger*	Chahardeh	3 February 2018	root	endophyte	
A2	*A. terreus*	Aliabad	9 January 2018	root	yellowing, leaf drop	
ZS5	*A. terreus*	Aliabad	9 January 2018	stem	yellowing, leaf drop	
B1N	*A. terreus*	Chahardeh	February 2018	stem	yellowing, leaf drop	
B5	*Clonostachys rosea*	Ark	February 2018	root	endophyte	MK530694
O2	*C. rosea*	Behdan	February 2018	root	endophyte	
ZR1	*C. rosea*	Razg	December 2017	root	endophyte	
RB	*Penicillium pinophilus*	Behdan	February 2018	root	yellow leaves	
RB5	*P. pinophilus*	Behdan	February 2018	root	yellow leaves	MK530699
B10	*P. pinophilus*	Behdan	January 2018	root	yellow leaves	
BM	*P. pinophilus*	Bojd	January 2018	root	yellow leaves	
R12M	*P. pinophilus*	Bojd	January 2018	root	yellow leaves	
R9	*F. proliferatum*	Amirabad	December 2017	root	endophyte	MK530698
O4	*F. redolens*	Behdan	February 2018	root	endophyte	MK530697

morphological characteristics and sequence of 372 bp of ITS region of R2M isolate (Accession number: MG589492) had 100% similarity to the sequence of *A. chlamydospora* HTCR38 species of NCBI GenBank (Accession number: Mk809953). These two isolates caused rot and darkening of the crown, wilting as well as a reduction of the number of *S. incanescens* seedling leaves (Fig. 2C–D).

Based on morphological characteristics and sequence similarity (96% of 700 bp of ITS region) with NCBI GenBank (Accession number: KU578313), R1D isolate from *Salsola* roots from the Amirabad region was grouped to *M. phaseolina* 274 species. Inoculation of this isolate on *S. incanescens* led to root rot, leaf yellowing and wilting (Fig. 2E–F).

M1 and Z8 isolates from stems and roots of *Salsola* in Amirabad, M2 and M6 in Hajiabad and M3 in the village of Shamsabad from the root of this plant were identified as *F. longipes* species with infected roots and stems of *S. incanescens* (Fig. 2G–H). The characteristics of isolates were consistent with the characteristics described for *F. longipes* species in the references.

H4 isolate from *Salsola* root in the village of Mud, based on morphological characteristics and sequence similarity of 519 bp of ITS region (Accession number: MK 530696) with NCBI GenBank sequences (*A. multiformis*, Accession number: EU30458) were identified as *U. multiformis* species. Inoculation of *S. incanescens* seedlings with H4 isolate resulted in blackening, complete crown rot and plant death (Fig. 2I).

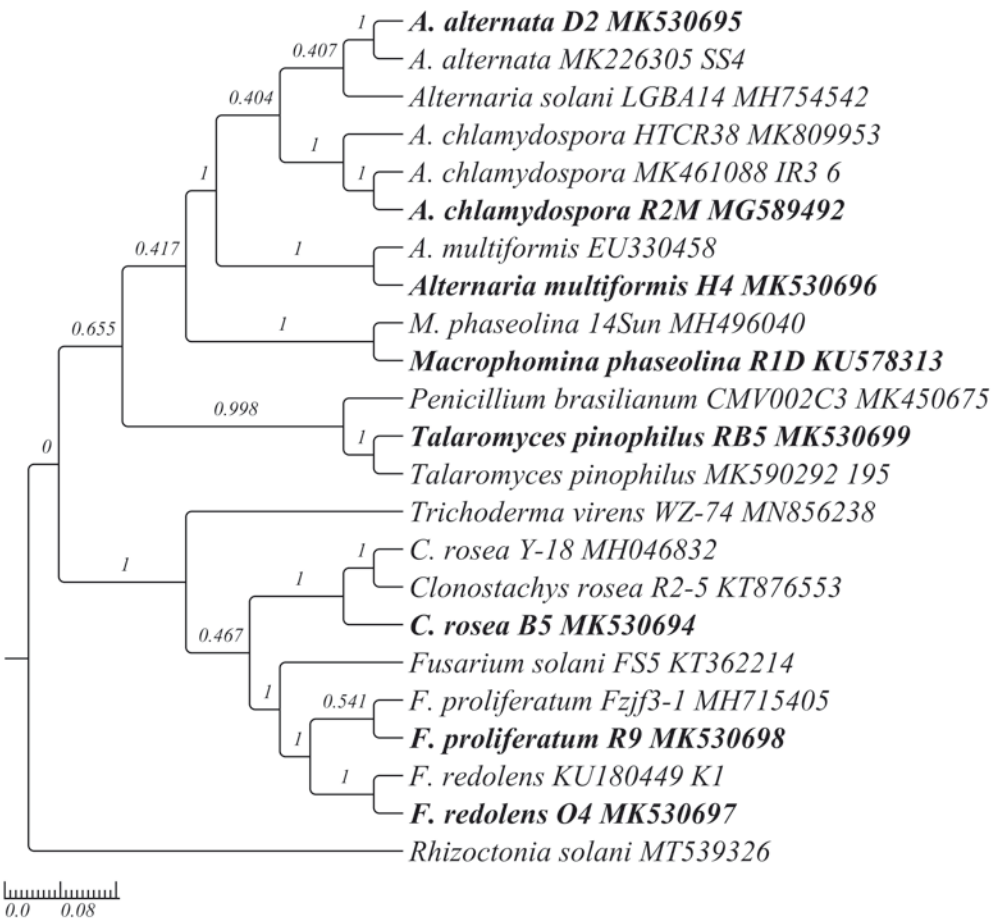

Fig. 1. A UPGMA phylogenetic tree was constructed with Mega7 from analysis of ITS sequences of fungi isolated from *Salsola* in eastern Iran based on 1,000 bootstrap replicates

A4 isolate (*A. niger*) from the root of *Salsola* in the village of Chahardeh did not cause disease or rotting on *S. incanescens* plants, but in the crown of inoculated seedlings, swelling was induced (Fig. 2J). This swelling did not have any negative impact on seedling growth. The root stain indicated the presence of fungal mycelial on crown and root tissues.

A2 and ZS5 isolated from roots and crowns of *Salsola* in Aliabad and B1N from the crown of the plant in the village of Chahardeh, was identified as *A. terreus* species according to morphological characteristics. Inoculation of these three isolates caused yellowing of the leaves and the number of leaves of some inoculated *S. incanescens* seedlings was reduced (Fig. 2K).

The three isolates of B5, O2 and ZR1 isolated from the villages of Ark, Behdan and Razg, respectively, were identified as *Clonostachys rosea* species. Sequencing of 511 bp fragment (Accession number: MK 530694) confirmed morphological identification of this species. These three isolates were inoculated into plants but did not result in disease or growth rate changes.

The B10, RB and RB5 isolates from the village of Behdan, BM and R12M from the village of Bojd from Salsola root, were assigned to the *Penicillium* genus. Based on the morphological characteristics and similarity (99,8%) of RB5 ITS sequences (Accession number MK 530699), this group belonged to *Talaromyes pinophilus*. Inoculation of these five isolates into the soil caused yellowing of the leaves of the inoculated plants (Fig. 2L).

An isolate (R9) belonging to *F. proliferatum* species in Amirabad was isolated from the root tissue. The morphological characteristics and sequence of the ITS region at 506 bp (Accession number: MK 530698) were the criteria for identifying this species. Inoculation of *S. incanescens* with this isolate showed that it was endophytic or epiphytic and non-pathogenic.

One isolate of *F. redolens* (O4) was isolated from *Salsola* root in Behdan. According to morphological characteristics and sequence of 505 bp fragment of ITS region (Accession number: MK530697), this isolate was consistent with the sequence of the NCBI GenBank (*F. redolens* K1, Accession number: KU180449). Inoculation of this isolate into *S. incanescens* did not lead to pathogenicity. It seems that it exists in endophytic or epiphytic forms on *Salsola*.

Fig. 2. Pathogenicity assays on *Salsola incanescens* under greenhouse conditions: *Alternaria alternata* (A–B), *Alternaria chlamydospora* (C–D), *Macrophomina phaseolina* (E–F), *Fusarium longipes* (G–H), *Ulocladium multiformis* (I – the arrow points crown rot), *Aspergillus niger* (J – the arrow points crown swelling), *Aspergillus terreus* (K) and *Talaromyes pinophilus* (L)

Discussion and Conclusions

Salsola as a halophytic plant is compatible with salty soil and absorption of salty compounds increases the salinity of its tissues (Lieth and Mochtchenko 2003). The specific conditions of the *Salsola* tissue are not often suitable for fungal growth, and endophytic or a pathogenic fungal association with these plants is very limited (Pugh and Williams 1968). As a result, few fungal species have been isolated from this plant. The study results showed that some of the fungal isolates

of Birjand can act as endophytes or pathogens on the tissues of this plant. A total of 27 fungal isolates was isolated in this study, of which *Fusarium* species had the highest frequency (Table 1). The fungi of this study are often isolated from the root tissue, which is probably due to low moisture content. High salinity of the shoot causes the activity of the fungus on this part of the plant to be less.

A pathogenicity test was done on *S. incanescens* species seedlings. In order to prepare *S. incanescens* seedlings, seed germination, which was a very long process (24 days), was carried out in Petri plates. During the pathogenicity test, providing appropriate conditions for seed germination and seedling health is essential. It was attempted to use the same field soil after sterilization. However, due to high salinity, sometimes salinity increased on the pot surface and affected the plant growth and crown tissue. The seedlings were grown for more than 4 months in pots and inoculated approximately four times, allowing for sufficient time for pathogenicity or endophytic relationships between the fungus and plant to develop.

Eleven fungal species were isolated from *Salsola* tissues so that *A. alternata*, *A. chlamydospora*, *M. phaseolina*, *F. longipes*, *U. multiformis*, and *A. terreus* were pathogenic to *S. incanescens*, leading to yellowing, rotting or wilting. Some of these species have already been introduced as *S. incanescens* endophytes or pathogens, but pathogenicity and endophytic relationships of some isolates with *S. incanescens* were determined for the first time.

Alternaria alternata is the biocontrol of *S. kali* in Eurasia (Kolomiets *et al.* 2010) and the United Kingdom (Pugh and Williams 1968). Aletaha *et al.* (2018) isolated *A. chlamydospora* species in Gorgan from Iranian *Salsola* as an endophytic fungus. Muhsin and Zwain (1989) isolated *A. chlamydospora* from *S. baryosma* in Iraq. This study is the first report of pathogenicity of the two above mentioned species on *S. incanescens* in Iran. *Macrophomina phaseolina* species is the cause of stem rot in several plants, but it has not been introduced on *S. incanescens* as the host of this pathogen in Iran. Endophytic *Fusarium* have been isolated from *Salsola* in Iran (Aletaha *et al.* 2018) but *F. longipes* species has not been introduced as a *Salsola* pathogen and its pathogenicity on *S. incanescens* was reported in this study. Imran (2011) in Iraq reported *U. multiformis* as a *Salsola* pathogen.

Aletaha *et al.* (2018) isolated endophytic *A. terreus* from *S. crassa* and *S. incanescens* from Iran but the results of this research showed its pathogenicity on *S. incanescens*. *T. pinophilus* in Mexico (Rivera-Orduña *et al.* 2010) has been introduced as a *Salsola* pathogen, and an endophyte in Iran (Aletaha *et al.* 2018).

Fusarium proliferatum, *F. redolens* and *C. rosea* species had no negative impact on seedlings, and it seems that these species are endophytic or epiphytic on *Salsola*. *Fusarium redolens* species has not been isolated from this plant and has been introduced as a *S. incanescens* endophyte in this study for the first time.

Aspergillus niger induces swelling symptoms on the seedling crown which may be due to the plant's response to the fungal activity or plant hormone production. There is no report on *A. niger* pathogenicity of this species on *Salsola*.

After root staining, the mycelia of *A. chlamydospora* and *A. niger* were observed in root tissues. In some species, such as *Fusarium* and *C. rosea*, fungal hyphae were not observed on/in root tissues. It is possible that no endophytic relationship was found or it was not recognizable using this staining method.

Some of the isolates of this study were pathogens of *S. incanescens*, which can be used as *Salsola* biocontrol agents in eastern Iran. Kolomiets *et al.* (2010) inoculated many *Uromycese salsolae*, *C. gloeosporioides*, *Phomopsis oblonga*, *A. alternata*, *Epicoccum nigrum*, *Pythium* sp., and *F. fujikuroi* on *S. kali.* Of these the first three species had the highest efficiency on controlling this weed. The secondary metabolites of *C. rosea*, which were isolated in this research, may be effective on the growth and development of *Salsola* plants.

Several isolates develop as endophytes on *Salsola*, and the use of this endophytic relationship probably leads to more adaptation of the plant in rangelands. In recent years, on the one hand, *Salsola* in eastern Iran has been used as a source of feed for livestock and camels. *Salsola* as a non-mycotrophic plant has conditions that prevent a relationship with fungi. However, some mycorrhizal species can interact with the roots of mature and immature *S. kali* plants to form a symbiotic and mycorrhizal relationship. Johnson (2002) showed that the mycorrhizal relationship between *S. kali* and mycorrhizal fungi reduced plant growth and its negative response. Therefore, the isolates of this study may also reduce the growth of these plants under rangeland conditions.

The basis of this study was the identification of necrotrophic fungi that were able to grow and develop on the culture medium. Therefore, the biotrophic and/or mycorrhizal species were not investigated. Although it seems that the population of fungi on the roots of this plant is not highly diverse, this group of fungi should also be studied by adding the roots of the plants collected from the field to the sterilized soil and planting the plant in that soil.

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