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Screening salt-resistant fungal endophytes to expand the cultivation of medicinal plants in saline areas

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Abstract

Endophytes, especially fungal types, are often useful for host plants, and by improving the production of specific metabolites of the host plant, they protect them against stresses, especially salt and drought stress. It is assumed that the plants that have adapted to harsh conditions and lived in these climatic conditions for many years are necessarily equipped with evolved salinity-resistant endophytes. Therefore, their isolation and then transfer to host plants could provide the possible cultivation of medicinal plants in saline soil and drought conditions. For this purpose, Yazd province was considered a good area for a pilot study. In this study, different parts (root, stem, and branches) of 112 species were selected, including ancient trees and perennials. Plant samples were transferred to the laboratory, and after sterilization, were cultured in potato dextrose agar (PDA). Out of 32 studied plant species, more than 70 endophytes were purified and cloned. Then, the isolated fungi were cultured in media with different salinity levels of 1, 2, and 3 M of sodium chloride. Results showed that among the investigated fungi, around 50 strains grew in the medium containing sodium chloride salt 1M. In the continuation of the screening process of the selected samples, 6 strains of the isolated fungal endophytes were able to grow in the medium containing NaCl in the concentration of 3 M. The ability of three selected endophyte isolates to be hosted on a basil plant (*Ocimum basilicum*) showed that all of the isolates have the ability to penetrate and spread in basil roots without any pathogenic symptoms. A study of the ITS genomic regions of ribosomal DNA of selected endophytes showed highly sexual and diverse species in the isolated endophytes. More research is needed to recommend the best endophytes in the basil plant culture in saline soil.

1. Introduction

Salinity is one of the most important sources of stress for plants (Angon *et al*., 2022). UNESCO's assessment indicates that more than 60% of the world's agricultural lands and 30% of the water used in this sector are affected by salinity (Devkota *et al.*, 2022). A high percentage of Iran's land, about 15% of the country's surface and 55% of agricultural land, is estimated to be affected by salinity (Behling *et al*., 2022). Salinity stress affects the growth of plants in two ways, directly by increasing the toxicity of ions and indirectly by increasing the osmotic stress

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(Isayenk *et al.*, 2019). In Yazd province, with an average rainfall of less than 60 mm per year and soils with different salinity levels, old trees whose life span is more than 4500 years can be found, such as the "Kohen Abarkoh cedar", or more than 2000 years in the case of the "old cypress of Mangabad Mehriz", as well as the "sycamore walnut" and "baneh trees", which are between 500 and 1000 years old and older (Shirvani *et al*., 2013). Based on this, it is possible that these plants get help from endophytes to increase resistance to stresses and adapt to harsh climates (Byregowda *et al*., 2022). This issue is especially true for the halophytic bushes and shrubs that grow in this region; they are able to retain high concentrations of osmolytes within themselves (Yasseen *et al*., 2022). NaCl is a major salinity agent in saline soil (Flowers *et al*., 2015). During the past 40 years, endophytic fungi have been biologically studied, and it was found that they continue to live inside healthy plants (Caruso *et al*., 2022). The cooperation of endophytic fungi with plants increases the plant's growth, resistance against pathogenic factors, and stability to environmental stresses, such as drought, temperature, and heavy metals (Sharma *et al*., 2021). Endophytes are among the microorganisms that grow inside the tissues of higher plants without causing external symptoms inside or outside the cells and are full of bioactive compounds (Sharma *et al*., 2021). Almost all plant species are host to at least one or more endophytes (Xia *et al*., 2022). Isolated endophytes from plants grown in warm climate soils and salty coasts have shown a high potential to increase the yield in warm environments using salty water (Gupta et al., 2021). The aim of the present study was to isolate salt tolerance endophytic fungi from long-lived plants and salt tolerance perennials and investigate the possibility of hosting the isolated fungal endophytes in the basil plant.

2. Materials and methods

The present study was carried out looking for salt tolerance endophytic fungi around the Yazd province in southern Iran. Plant samples were collected from stems, leaves, and roots of 112 long-lived trees and desert-healthy plants that were free from any signs of contamination. Three samples from each of the three parts, for a total of 9 samples, were collected from each plant (Table 3).

2.1. Collection of plant samples

Leaf, stem, and root samples of 112 plant species, including 18 old plant species and 96 salt-resistant desert plant species of the Amaranthaceae, Brassicaceae, Chenopodiaceae, Cupressaceae, Ephedraceae, Platanaceae, Polygonaceae, Tamaricaceae,and Zygophyllaceae from different regions of Yazd province, were collected within a distance of 1850 km, including Ardakan-Chah Afzal Desert, Abarkoh-Taghestan and Dasht Abarkoh to Marost, Herat and Marost, Mehriz, Bafaq, Taft and Mibd - Niuk and their subordinate areas (Fig. 1).

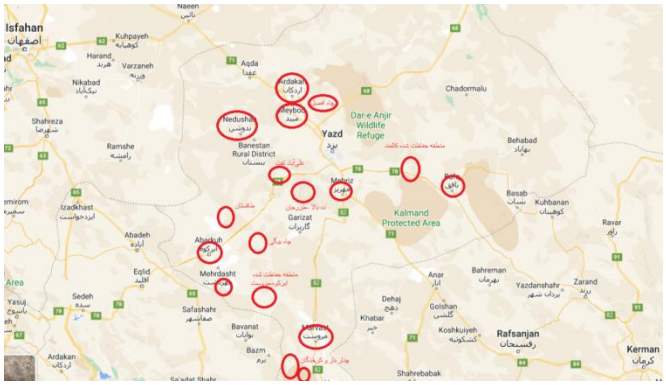

Figure 1. Plant sample collection areas for endophyte isolation in Yazd province.

2.2. Isolation of fungal endophytes

The healthy and disease-free sections of roots, stems, and leaves were packed in zipped plastic bags and transferred to the laboratory using ice boxes. The samples were first washed with running water. Then, the surface was sterilized using the following method: the samples were treated with 70% ethanol alcohol for 5 minutes under a laminar hood airflow cabinet, and then transferred and kept in a container containing 2% sodium hypochlorite for 15 min. Finally, the samples were kept in ethanol (96%) for 2 min and then washed three times using sterile distilled water (15). Samples were divided into 1cm

explants, cultured on potato dextrose agar (PDA), and incubated at 25±2°C for 4 weeks. Samples that displayed a contaminated appearance in the early days (within the first week) were considered saprophytic contaminated and removed. Fungal samples observed one week to ten days after cultivation were considered endophytes (Salazar *et al*., 2020).

To evaluate the isolated endophytes against salinity, the fungi were cultured in a PDA medium containing NaCl, at a concentration of 1M (Jalili *et al*., 2020). Fungi that were able to grow under a salinity of 1M NaCl were then cultured in a medium containing 2M NaCl, 3 M NaCl, and 4 M NaCl. The radius growth of fungi was measured and compared daily with the control (Aujla & Paulitz, 2017). The test continued for one week at a temperature of 25±°2C (Jalili *et al*., 2020).

2.3. Molecular identification of isolated fungi

For molecular identification of selected fungi, DNA extraction was done via the Murray and Thompson method (Murray & Thompson, 1980). Then, the extracted DNA was kept at -20 C (Rastogi et al., 2019) until the quantitative and qualitative assessments were conducted using the Glassell method (Glasel., 1995). The ITS1 (reverse) and ITS4 (forward) primers (Table 1) were used for isolated fungi identification (White et al., 1990).

ITS_1 and ITS_4 primers, which are designed based on the conserved sequences of ITS regions, produce a band of 550-750 bp during PCR with endophyte isolates. PCR program and device settings based on primer length are indicated in Table 2.

Table 1- Sequences of ITS primers used

Sequence	Nucleotide sequence
ITS 1	5' -TCCGTAGGTGAACCTGCGG -3'
ITS 4	5'-TCCTCCGCTTATTGATATGC-3'

All the PCR products of the selected fungi with specialized primers were sent to the Codon Genetic group–Armaghan Medical Lab for sequencing. The PCR products were first sequenced, then the data were edited with Chromas then the data were edited with Chromas software and finally compared with the sequences available in the NCBI gene bank (Aremu & Babalola, 2015).

Table 2- Thermocycler settings used in the present study

Reaction stage	Temperature (Celsius)	duration (seconds)
Primary denaturation	95	300
Secondary annealing	95	60
Annealing	58	45
Extension	72	40
Final extension	72	420

2.4. Sweet basil (*Ocimum basilicum*) hosting feasibility test for selected endophytes

At this stage, healthy and strong sweet basil *(Ocimum basilicum)* seedlings with 4-6 leaves were selected. A suspension containing selected fungi spores in a concentration of 5x710 spores/ml was prepared, and the roots of the seedling were kept in the suspension for 3 h. Treated seedlings were transferred to pots (10 pots with 10 cm diameter and height) containing sterile perlite and were fed with Hoagland and Arnone solution (1950) for 3 weeks to evaluate for endophyte colonization. The staining technique and microscopic observation were used to investigate endophyte penetration and growth in the basil (Vierheilig *et al*., 1998). Also, a molecular investigation was done using ITS_1 and ITS_4 primers (Table 1) to prove the presence of the inoculated endophyte.

3. Results and discussion

The isolated endophytes of 112 long-lived trees and desert plants were purified in the new medium (Fig. A-1). To evaluate the resistance of the isolated fungi to salinity, the pure cultures were subcultured in a medium containing a concentration of 1, 2, 3, and 4 M NaCl salt, respectively, (Fig. 1-B).

Table 3- Selected species from old trees, halophytes, the number of isolated endophytes, their stability to different environmental salt levels, and the possibility of settling in host basil (Ocimum basilicum) roots.

Row	The scientific name of the primary host species of the endophyte	The fragment where the endophyte is isolated			frequency	Salt concentration in PDA				Colonization
		Root	Stem	Leaf		1M	2M	3M	4M	
1	*C .sempervirens*	-	-	1*	1	1	-	-	-	
2	*Tribulus longipetalum*	1	1	1	3	3	1	1	-	
3	*Anabasis setifera*	1	1	1	3	2	1	1	1	1
4	*Fortynia bungei*	-	1	1	2	2	1	1	-	
5	*Seidlitzia rosemarinus*	-	1	1	2	2	1	1	-	
6	*Salaola tomentosa*	-	1	1	2	1	–	–	-	
7	*Atriplex canesens*	-	1	1	2	2	1	–	-	
8	*Jugland regia*	-	1	1	2	2	1	1	1	1
9	*Platanus orientalis*	-	1	1	2	2	1	1	1	1
10	*C. sempervirens*	-	1	1	2	1	1	–	-	
11	*Tamarix aphylla*	-	1	1	2	2	2	2	-	
12	*Ephedra strobilasa*	-	1	1	2	2	1	–	-	
13	*Suaeda aegyptiaca*	-	1	1	2	2	1	1	-	
14	*V.phlomoides*	-	1	1	2	2	2	1	-	
15	*Anabasis setifera*	1	1	1	3	3	2	-	-	
16	*C. sempervirens*	-	1	1	2	1	1	–	-	
17	*Platanus orientalis*	-	1	1	2	3	2	1	-	
18	*Platanus orientalis*	-	1	1	2	2	2	-	-	
19	*Cupressus sempervirens*	-	1	1	2	2	1	-	-	
20	*Juglans regia*	-	1	1	2	1	1	-	-	
21	*Salix alba*	-	1	1	2	1	–	–	-	
22	*Amygdalus scoparia*	-	1	1	2	1	–	–	-	
23	*Pistacia atlantica*	-	1	1	2	1	1	–	-	
24	*Platanus orientalis*	-	1	1	2	1	1	1	-	
25	*Salix alba*	-	1	1	2	1	2	–	-	
26	*Pistacia atlantica*	-	1	1	2	1	1	1	-	
27	*Juglans regia*	-	1	1	2	1	1	–	-	
28	*Pistacia atlantica*	-	1	1	2	1	1	–	-	
29	*Salix alba*	-	1	1	2	1	1	–	-	
30	*Platanus orientalis*	-	1	1	2	1	1	–	-	
31	*Juglans regia*	-	1	1	2	1	1	–	-	
32	*C. sempervirens*	-	1	1	2	1	1	–	-	
33	*Juglans regia*	-	1	1	2	1	–	–	-	
34	*Juglans regia*	-	1	1	2	1	1	1	-	
The total number of endophytes		3	33	34	70	50	34	12	3	3

* Number of isolated endophytes

3.1. Screening the isolated endophytes based on the salinity tolerance

The results showed that among the isolated fungi, 3 fungi were from the roots, 33 from the stem, and 34 fungi were from the leaves of the studied plants. In total, 48.57% of endophytes were isolated from leaves, 47.1% from stems, and 4.3% from roots. Results of the fungi screening based on the PDA medium salinity level showed that from the 70 studied fungi, around 50 (~71%) were able to grow up in PDA medium containing salinity at a concentration of 1M (Table 3). Among these, 31 fungi (62%) were able to grow in media containing 2 M sodium chloride salt. In the next step, the selected fungi were cultured in a

media containing 3M NaCl, and only 3 accessions (19%) were able to colonize the cultured media and grow. The selected fungi were then cultured in a medium containing NaCl in a concentration of 4M, no colonization was observed (Fig. C-1). Contamination at the early stages of isolation was the main reason for the low success in isolating the endophyte from the root samples.

3.2. Molecular identification of selected fungi

Molecular identification of the three endophytic fungi (from the 70 isolates) capable of growing in media containing 3M was done. In this regard, the extracted DNA was evaluated quantitatively and qualitatively (Lahlali *et al*., 2022) and their sequences were obtained (Table 4). Results of obtained sequences identified *Cladosporium limoniforme* as one of the isolates that separated from the *Anabasis setifera* obtained from the border between Yazd and Nadoshan, and it was registered in NCBI under accession number 473942.1. *Cladosporium sinuosum* was the second endophytic fungi with high tolerance against salinity recognized; it was isolated from the old walnut tree of "Deh-Bala" located in "Deh-Bala" village of Shirkoh in Yazd province. This species was registered in NCBI with the identification number 473975.1. The endophytic fungus *Neocamarosporium goegapense* was isolated from the *Platanus orientalis* in Korkhengan (II) located near the city of Marvast, in the Korkhengan protected area, Yazd province. This species was registered in NCBI with the identification number 473972.1.

3.3. Sweet basil (*Ocimum basilicum*) plant colonization potential of endophyte fungi

Roots of sweet basil (*Ocimum basilicum*) seedlings were used to test the possibility of colonization of selected fungi (Fig. D-1, E-1, and F-1). Any symptoms of disease, withering, or wilting were considered pathogenic signs. No symptoms of disease in the treated plants for all of the used fungi indicate the selected isolates are not a pathogen, at least for sweet basil (Fig. 1, G-1). Staining results of the healthy root tissues after 3 weeks showed the presence of fungi around the root tip (Fig. 1, L- 1). This means that all of the selected salt-resistant endophytes are capable of colonizing the sweet basil root without visible symptoms. Thus, sweet basil, as an important essential oil-bearing plant, is an appropriate host for salt-resistant isolates.

Table 4- Identified endophyte species and their host plants.

Row	The scientific name of endophyte	GenBank Accession Number No. / GenBank Accession number No. Rev.	Endophyte family	Host species profile		Identity (%)	
				The scientific name	Family	ITS1	ITS4
1 1	*Cladosporium limoniforme*	473942.1 542461.1	*Cladosporiaceae*	*Anabasis setifera*	*Amaranthaceae*	100	100
2 2	*Cladosporium sinuosum*	473975.1 542475.1	*Cladosporiaceae*	*Juglans regia*	*Juglandaceae*	100	100
3 3	*Neocamarosporium goegapense*	473972.1 542469.1	*Pleosporaceae*	*Platanus orientalis*	*Platanaceae*	100	100

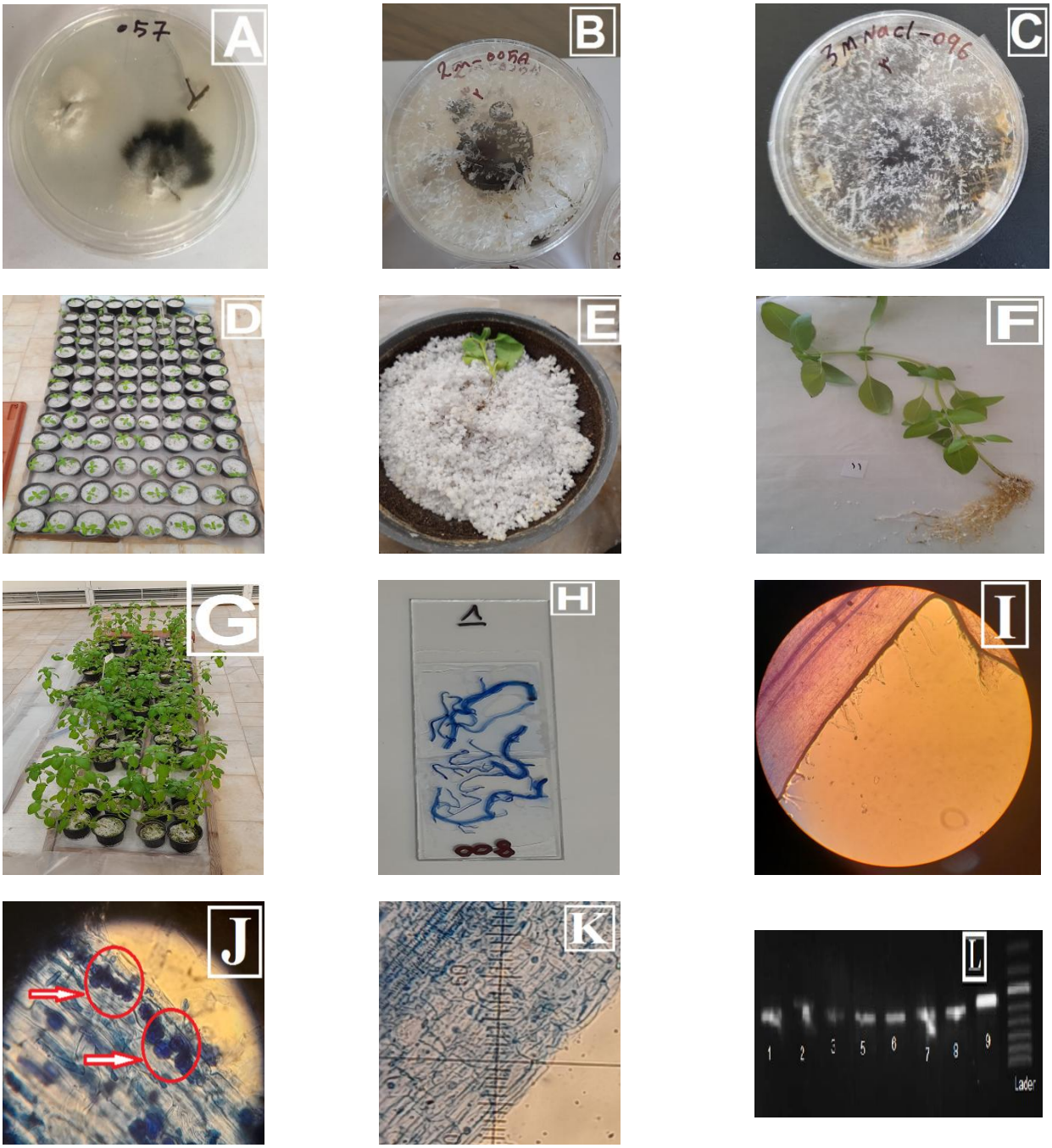

Figure 1- The results from different stages of the research. A: Purification and preservation of the endophyte isolated from the culture. B: Endophytes resistant to the environment containing 2 M sodium chloride concentration of Potato Dextrose Agar Medium with 2 Molar NaCl. C: Three endophytes with the ability to grow in the environment of 3 M sodium chloride salt and above, D: seedlings adapting after treatment with Fungal spores with a concentration of 5×10^7 spores per milliliter, E: appearance of disease symptoms in the treated graft, and F: graft transformed into a healthy plant and free of disease symptoms, G: treatments with no disease symptoms or discoloring of the roots. To observe the extent of endophyte fungus penetration, H: stained root on a slide for microscopic observation, I: stained root surface: J: endophytes penetrating into the root tissue, K: Control root, and L: PCR product of fungal DNA sample.

In recent years, the application of non-chemical methods in the management of plant stresses has received attention (El-Saadony *et al*., 2022). For example, induced resistance activates the natural defense mechanism of the plant (Singh et al., 2021). Verma1 et al. found that endophytic fungi may establish a mutualistic association with diverse plant species (2020). They concluded their varied association with the host plant under dynamic environments boosts the endogenic tolerance mechanism of the host plant against various stresses via overall modulations of local and systemic mechanisms accompanied by higher antioxidant secretion. Under biotic stress plants mitigate and elevate phytohormone levels to be used as biocontrol agents and as biofertilizers against various pathogens, promoting crop improvement and soil improvement, respectively (Verma1 et al., 2022). Plants show different defensive responses against the attack of pathogenic microorganisms that threaten their life (Aremu & Babalola, 2015). Broad-spectrum endophytic fungi follow direct or indirect mechanisms to benefit the plants. The direct beneficial mechanisms involve phytohormone production, nitrogen fixation, phosphate solubilization, siderophore production, and anti-microbial metabolite production (El Enshasy et al., 2020). The indirect beneficial mechanisms involve abiotic and biotic resistance (modifying the metabolism process), biocontrol, bioremediation, and phytoremediation (Dubey et al., 2020; Singh et al., 2021).

One of these responses is acquired systemic resistance, which can be induced by non-pathogenic gene products of pathogens or non-living agents (Byregowda *et al*., 2022). Endophytic fungi can greatly stimulate plant growth and increase its resistance to environmental stresses, including salinity, drought, and plant diseases (Sharma *et al*., 2021) by causing genetic, physiological, and ecological changes in its host plants (Nicoletti *et al*., 2021). Also, they provide the possibility of growing plants in saline and dry soils that have abiotic and biotic stressors (Byregowda *et al*., 2022).

Stress-adapted fungal endophytes can reduce the harmful effects of salinity by increasing other types of physiological and biochemical reactions in the plant, such as the rate of transpiration, the activity of antioxidant enzymes, and the concentration of osmotic protective molecules, i.e., proline and soluble sugars (Kamran et al., 2022). It has been reported that both endophytic bacteria and fungi were able to improve the plant's lifestyle via the direct production of secondary metabolites (Xia et al., 2022). Nowadays, the utilization of microorganism-plant symbiosis is strongly considered in planning sustainable agricultural systems. In addition to the presence of a sufficient number of active microbial strains in the rhizosphere, an increase in the level of plant root contamination by microbial symbiosis is necessary to achieve the maximum efficiency of the useful power of symbiotic systems (Byregowda *et al*., 2022).

Examining the results obtained in this research shows the importance of a symbiotic relationship between the fungus and the basil (*Ocimum basilicum*) plant, with the identified fungi stimulating the growth of the plant, which in turn, increases its yield. A comparison of the dry weight of the shoot and root of plants inoculated with fungi compared to the control plants in optimal conditions in terms of soil salinity confirms the fungi's effect of stimulating plant growth. Compared with control plants, the results show that the dry weight of shoots and roots of contaminated plants increased by 37% and 97%, respectively.

Verma *et al.*'s study showed that the endophyte *Piriformospora indica* increased the biomass of tobacco (*Nicotiana tabacum*), corn plants (*Zea mays* L.), parsley (*Petroselinum crispum* L.), *Artemisia (annula* L.), and water hyssop (*Bacopa monnieri* L.) (1998). The results of their studies indicated that the biomass of the plant's aerial parts and roots inoculated with the fungus increased by two times compared to the uninoculated control plants (Verma et al., 1998). Soil microorganisms, including endophytic fungi, interact with plants by establishing cooperative and symbiotic relationships (Shrivastava et al.,

2021). Their activities include the production of numerous types of metabolites, the breakdown of various organic compounds, atmospheric nitrogen fixation, the production of growth-promoting substances, and increasing the availability of mineral nutrients for the plant to improve biomass (Xia et al., 2022). In addition to the ability to stimulate plant growth, the results of this research indicate the effective role of endophytes on sweet basil (*Ocimum basilicum*) by improving plant growth and performance under salt-stress conditions (unpublished data). Understanding the mechanism of plant resistance to environmental stresses (saltiness, drought, plant pathogens, etc.) with endophyte is a subject that has attracted the attention of researchers (Verma *et al*., 2022).

3.4. Identified endophytes

In this research, three species belonging to two genera were identified as salt-resistant endophytes with the ability to penetrate the root of sweet basil (*Ocimum basilicum*), the included *goegapense* species were from the *Neocamarosporium* genus, as well as *limoniforme* and *sinuosum* species from the *Cladosporium* genus.

The species *N. goegapense* was isolated from the old sycamore of Korkhengan-Marvast. This species has the ability to change resistance to salinity by making changes in the antioxidant system of the host plant (Hosseyni Moghadam *et al*., 2021). Hosseyni Moghadam *et al*. reported that *Neocamarosporium* goegapense, *Neocamarosporium chichastianum*, and *Periconia macrospinosa* endophytes isolated from a desert plant reduced drought stress in wheat and cucumber by enhancing antioxidant defense enzymes activity (2021). In another study, it has been shown that the endophytic bacteria *Bacillus safensis* and *Ochrobactrum pseudogregnonense* increased the growth of wheat under drought conditions by strengthening the antioxidant defense enzymes and their antioxidant activity (Shrivastava *et al*., 2021). Similar mechanisms of endophyte-mediated drought tolerance have been reported in maize (Mohammad Kamran *et al*., 2022), wheat (Jalili *et al*., 2020), Chinese cabbage (Lahlali *et al*., 2022), and other crops (Khan *et al*., 2016). Mohammad Kamran *et al*. (2022) reported that some endophytes help the host plant to remain safe from oxidative stress caused by drought. The genus *Kamarosporiums* was shown to be polyphyletic or have different subspecies, which led to the introduction of several new genera (Crous *et al*., 2014).

Neocamarasporium was first isolated in South Africa from the leaves of *Mesembryanthemum* sp and then introduced to the species *N. goegapense* (Crous *et al*., 2014). Most *Neocamarasporium* species have been found in association with halophytes (salt-resistant plants) in marine habitats or river mouths (Papizadeh *et al*., 2018). In this research, this type of endophytic fungus was isolated from an elder Platanus tree in Korkhengan, Marvast, with an estimated age of more than one thousand years (Shirvani *et al*., 2013).

Two *limoniforme* species of the genus *Cladosporium* were isolated from the *Anabasis setifera* in the Niuk-Mibod region, and the *sinuosum* species was isolated from the old walnut of Deh-Bala located in the Deh-Bala village of Shirkoh, Taft city.

C. limoniforme has also been previously isolated from grapes (Bensch *et al*., 2015), acuminate banana (Papizadeh, *et al*., 2017) very salty water (Kamran *et al*., 2022), and *Eucalyptus* sp. (Bensch *et al*.2015). *C. sinuosum* was previously isolated from *Fuchsia* excorticate (Khan *et al*., 2016), *unidentified moss* (Becchimanzi *et al*., 2021), saffron (*Crocus sativus*), *Eryngium maritimum*, and *Iris pseudacorus* (Salvatore *et al* 2021). The *Cladosporium* genus is a rich source of diverse and bioactive natural compounds (Salvatore *et al* 2021). The molecular tools for identification at the species level have made it possible to accurately investigate and express the existing diversity. In the last genus update, 218 species were classified under the *Cladosporium* genus (Bensch *et al*., 2018), and more have been

recently added to the list (Bensch *et al*., 2015. Iturrieta-González *et al*., 2021).

Due to the importance of secondary metabolites as mediators of biological interactions, their versatility has created significant research activities on the metabolome of these fungi (Salvatore *et al*., 2021). Species of this genus have been isolated from land and aquatic sources. More than 75% of the land species of this genus are endophytes (Salvatore *et al*., 2021).

4. Conclusion

Many biological activities of secondary metabolites produced by *Cladosporium* species, including antimicrobial (Iturrieta-González *et al*., 2021), antibacterial (Zimowska *et al*., 2021), phytotoxic (Lu *et al*., 2016), inhibition (Salvatore *et al* 2021), cytotoxic (Liu *et al*., 2016), acetylcholinesterase (Zhang *et al*., 2019), anti-toxic (Bensch *et al*., 2018), antioxidant (Amin *et al*., 2020), anti-fungal (El-Saadony *et al*., 2022), and antifat (Lu *et al*., 2016), have been reported so far. The ability of Cladosporium to destroy extensive disulfide crosslinks of keratin polypeptides and to dissolve keratin by secreting specialized enzymes can be used as plant biostimulants (El-Saadony *et al*., 2022).

The present study resulted in the isolation of Cladosporium, which needs additional research to recommend as a biostimulant agent in enhancing plant growth in saline conditions. Evaluation of this fungus on sweet basil provided important information about the characteristics of *Cladosporium* isolates, which will be important for its application as plant biostimulants in agriculture.

Conflict of interest

The authors declare that there is no conflict of interest.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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