

Diversity of endophytic bacteria isolated from *Peganum harmala* distributed in arid regions in Uzbekistan

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In recent years, drought has become one of the most widespread global problems as a result of drastic climate change all over the world. In order to increase the efficiency of cultivation of agricultural crops under the conditions of drought, research was conducted aimed at determining the diversity of endophytic microorganisms of xerophytic and halophytic plants grown under the influence of this stress factor and evaluating their characteristics and potential is of great importance. This article describes the results of the research aimed at isolating, identifying and evaluating some properties of endophytic bacteria from *Peganum harmala* L., which is widespread in arid regions of Uzbekistan. These data are being published for the first time in terms of area and vegetation. The results of studying *P. harmala* endophytic bacteria diversity, species composition, resistance to pathogenic fungi confirm that this plant is a potential source of promising endophytic bacteria. In the experiments, 24 promising isotypes of endophytic bacteria were isolated from *P. harmala*, which were the research objects. Five strains were selected by evaluating the colonization potential of the isolated isolates under drought conditions and resistance of the selected strains to pathogenic fungi was evaluated. These promising strains were identified using molecular genetic methods.

Keywords: *Peganum harmala*; drought; bacteria; endophyte; plant growth-promoting properties.

Introduction

Endophytes are a group of microorganisms that live in the internal tissues of plants and have physiological mechanisms that positively affect the activity of the host plant. Their presence or the mechanisms controlled by these microorganisms may increase the plant's resistance to stressors (Kandel et al., 2017; Kondrasheva et al., 2022). Endophytic bacteria are bacteria that live inside the vegetative organs of plants, and they contribute to the growth and development of the host plant organism to a certain extent based on certain mechanisms (Shurigin et al., 2022). In the process, experts have noted that bacteria fix nitrogen, break down insoluble phosphates, produce phytohormones, produce siderophores, fight against phytopathogens, and use other mechanisms (Mamarasulov et al., 2022).

Endophytic bacteria isolated from different plants have been reported by several studies. In a number of studies conducted in domestic and foreign countries, endophytic bacteria were isolated and characterized from plants such as *Bolboschoenus planiculmis* (Hwang et al., 2022), *R. soongorica* (Li et al., 2021), *Tetragonia tetragonioides* (Egamberdieva et al., 2022), *Salicornia europaea* (Chebotar et al., 2022), *Haloxylon aphyllum* (Shurigin et al., 2022), *Helianthus annuus* (Forchetti et al., 2007), *Halocnemum strobilaceum* (Alikulov et al., 2022), *Seidlitzia rosmarinus* (Shurigin et al., 2020), *Halostachys belangeriana* (Alikulov et al., 2022), *Kochia prostrata*, *Potentilla eversmanniana* (Akramov et al., 2023). However, the analysis of scientific sources showed that the endophytic bacteria of *P. harmala*, which is common in arid regions, have not been thoroughly studied.

Peganum harmala L. is a perennial herb belonging to the Nitrariaceae family, which is naturally distributed in the Southern parts of Eurasia. There is also information that this species is acclimatized in some areas of South Africa and North America (Kartal et al., 2003) (Fig. 1).



Fig. 1. *Peganum harmala* L.

Peganum harmala has a multi-branched stem that can grow up to 100 cm tall. The leaves are divided into pieces. Flowers are white-yellow in color (Rechinger, 1982). *Peganum harmala* is widely used as an effective medicinal plant in several Asian countries. Carboline alkaloids extracted from various parts of the plant are included in many anti-inflammatory drugs (Hemmateenejad et al., 2006). On the other hand, *P. harmala* is also a poisonous plant. Its overdose has been reported to cause headache, dizziness, nausea, convulsions, hallucinations, paralysis, euphoria, indigestion, bronchodilation, hypothermia, and bradycardia (Moshiri et al., 2013).

Both *P. harmala* and its microorganisms are adapted to the drought conditions of Uzbekistan. Symbiotic relationships between plants and microorganisms are common in nature. However, endophytic bacteria associated with *P. harmala* have not yet been explored. Based on the above

analytical data, we aimed to isolate and identify endophyte bacteria from *P. harmala* distributed in arid regions of Uzbekistan.

Materials and methods

The collection of plant samples was carried out in the spring of 2023 based on segments isolated from the roots, leaves and stems of *P. harmala*. These plants are common in the arid lands of the southwestern regions of Uzbekistan, including Kamobchul and Yettitam (Fig. 2). First, samples were taken from plants growing at a distance of not less than 10 meters from each other. Next, the roots, leaves and stem were cleaned in sterile water to remove soil particles.

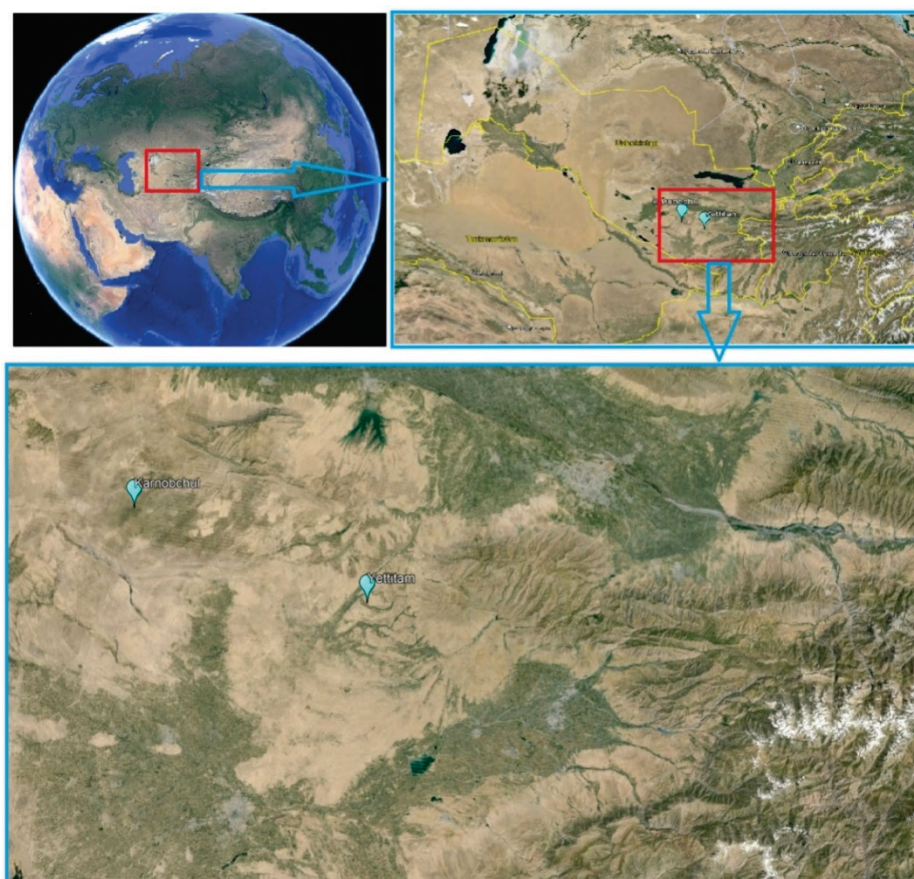


Fig. 2. Map with locations and coordinates of sample collection of arid regions in Uzbekistan

A total of 15 g stem, leaves and roots were sterilized in beakers filled with 99.9% ethanol for 2 minutes and 10% sodium hypochlorite for 1 minute. Afterward, they were placed in sterile water cups for 2 minutes (Coombs & Franco., 2003). Pieces of stem and roots were cut lengthwise into thin slices. For serial dilutions, 5 g of each sample was taken and transferred to test tubes containing 9 mL of sterile water (101–105). After each dilution, 100 mL of the suspension was taken and inoculated onto Luria-Bertani (LB) nutrient medium at 30 °C (Kuklinsky-Sobral et al., 2004). After four days, the colonies that changed in color and shape were transferred to petri dishes with LB for purification.

When selecting promising isolates of endophytic bacteria among isolated bacterial isolates, their colonization potential at different temperatures was taken into account. The isolates were grown in LB medium at different temperatures (24, 28, 32, 36, 40 °C) for 7 days. Isolates colonized at 40 °C were selected as promising isolates.

The pathogenic fungi *Fusarium oxysporum*, *Rhizoctonia solani* and *Alternaria alternata*, used in antifungal studies were collected from the Department of Microbiology and Biotechnology of the National University Uzbekistan. The bacterial endophyte isolations were checked *in vitro* for the presence of antagonistic activity against the fungi mentioned earlier by using the plate method. Within 5–7 days, the fungi were grown on Czapek Dox Agar medium at 28 °C. Agar discs containing grown fungi

cultures were cut into small squares (7–8 mm on each side) and placed in the center of the petri dishes (9 cm in diameter). Bacteria were grown in a LB medium and then passed to test plates in the same medium as the fungi. The plates were incubated at 28 °C for seven days until the fungi covered the control plates without bacteria. Antifungal activity was measured as the width of the growth inhibition zone between fungi and test bacteria.

The genomic DNA used for molecular identification was extracted as per the method given by Dashti et al. (2009). The 16S rRNA gene of the extracted DNA was amplified using PCR with the following primers: 27F 5'-GAGTTTGATCCTGGCTCAG-3' (Sigma-Aldrich, St. Louis, Missouri, USA) and 1492R 5'-GAAAGGAGGTGATCCAGCC-3' (Sigma-Aldrich, St. Louis, Missouri, USA). The PCR program was used as follows: a primary heating step for 30 s at 94 °C, followed by 30 cycles of denaturation for 15 s at 94 °C, annealing for 30 s at 55 °C and extension for 1.5 min at 68 °C, then followed by the final step for 20 min at 68 °C. The PCR products were checked by electrophoresis using GelRed (Dashti et al., 2009). The ABI PRISM BigDye 3.1 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) was used for the sequencing. The obtained sequences were compared with the sequences of the closest relatives from GeneBank at the National Center for Biotechnology Information (NCBI).

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. This analysis involved 16 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1632 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

Results

The study of *P. harmala* distributed in the arid regions of Uzbekistan as a source of endophytic bacteria and the assessment of their diversity was carried out by us for the first time (Fig. 3).

The activity and growth of microorganisms, including bacteria, is mainly determined by the temperature of the environment. Minimum, optimum and maximum growth temperatures are usually indicated for each microorganism. The temperature dependence of the growth rate of microorganisms, including bacteria, is individual for each microbial culture. In our research, a total of 570 isolates that grew well on the surface of nutrient media were isolated from roots, stems and leaves of *P. harmala* (Table 1). The isolates were grown at different temperatures to select

isolates capable of growing at higher temperatures. The analysis of growth results showed that 74% (141 isolates) of bacterial isolates isolated from plant roots were able to grow at 24 °C, while this indicator was equal to 66.8% and 69.5% in leaves and stems. It was noted that 450 (79%) of the studied isolates grew at 28 °C, 156 of the grown isolates were isolated from roots, 146 from leaves and 148 from roots. During the experiments, the optimal growth temperature for bacterial isolates isolated from *P. harmala* was found to be 32 °C. At 32 °C, 93% (177 isolates) of bacterial isolates isolated from plant roots were able to grow, while in leaves and stems, this indicator was equal to 86.8% and 88.0%. It was noted that the growth potential of bacterial isolates at 36 °C was reduced compared to the values at 32 °C. Of the total isolates studied, 430 (75.4%) were found to grow in this variant. It was observed that the growth potential of bacterial isolates was drastically reduced at 40 °C. Of the total isolates studied, 4.2% (24) showed the ability to grow under this variant of extreme temperature. Of the isolates resistant to high temperature, 11 are isolated from roots, 5 from leaves, and 8 from stems. We continued with these 24 bacterial isolates for our future studies (Table 1).

One of the mechanisms by which endophytic bacteria stimulate the growth of the host plant and increase its resistance to abiotic and biotic stress factors is the fight against phytopathogens or biological control of its activity. This mechanism plays an important role in the fight of endophytic microorganisms against pathogens considered dangerous to plants in agricultural crops. Therefore, in the first stage of our research, among the selected endophytic bacterial isolates, screening was carried out to select active isolates that are antagonistic to pathogenic fungi (Fig. 4).

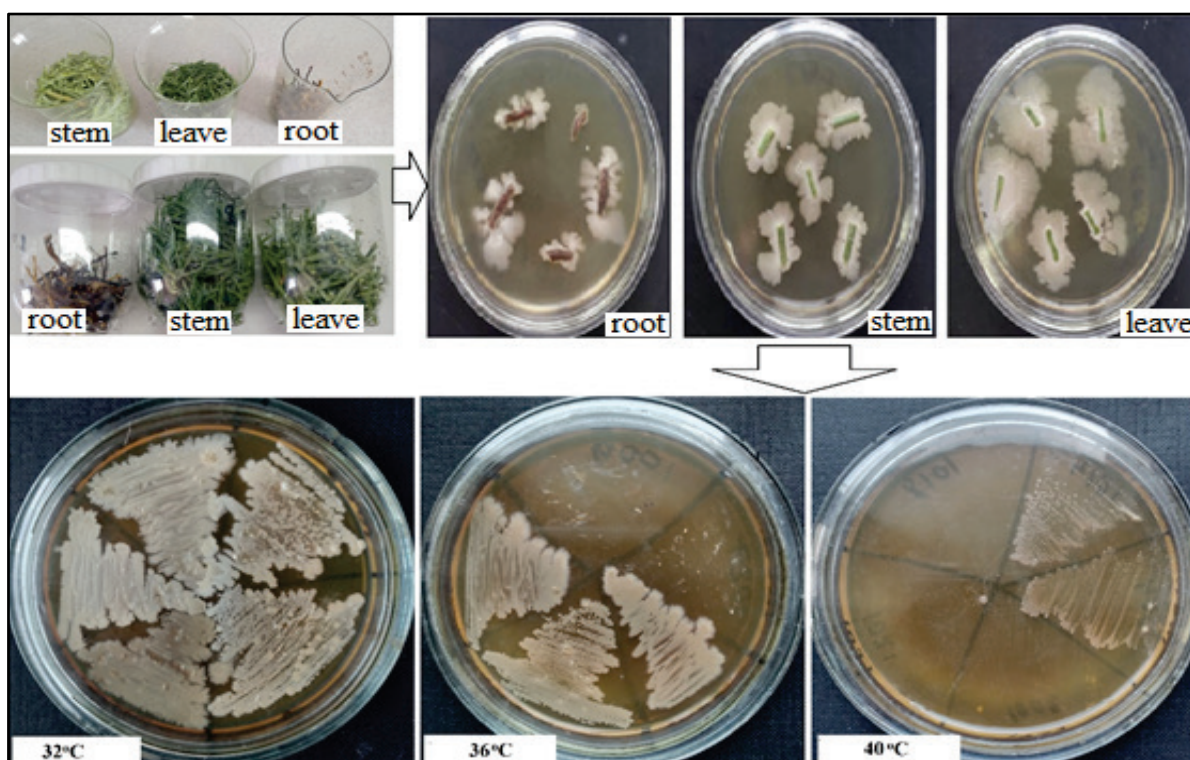


Fig. 3. Selection of promising isolates from endophytic bacterial isolates isolated from *P. harmala*

Table 1
Effect of temperature on the growth of endophytic bacteria isolated from *P. harmala*

Sample collection area	Vegetative organ of a plant	Number of isolates isolated	Number of isolates grown (%)					The name of temperature-resistant isolates	
			Temperature, °C						
			24	28	32	36	40		
Kamobchul (Nurabad District, Samarkand Region, 39°36'46.2" N 65°29'00.9" E)	Root	100	83 (83)	85 (85)	94 (94)	87 (87)	7 (7)	MsHpR101-MsHpR107	
	Leaf	98	65 (66)	77 (79)	89 (91)	72 (74)	3 (3)	MsHpL101-MsHpL103	
	Stem	80	56 (70)	68 (85)	70 (88)	59 (74)	3 (4)	MsHpS101-MsHpS103	
	<i>Total by area</i>	<i>278</i>	<i>204 (73.4)</i>	<i>230 (82.7)</i>	<i>253 (91.0)</i>	<i>218 (78.0)</i>	<i>13 (4.7)</i>	-	
Yettitam (Chirakchi District, Kashkadarya Region, 39°21'40.9" N 66°14'02.1" E)	Root	90	58 (64)	71 (79)	83 (92)	65 (72)	4 (4)	MsHpR108-MsHpR111	
	Leaf	92	62 (67)	69 (75)	76 (83)	63 (69)	2 (2)	MsHpL104-MsHpL105	
	Stem	110	76 (69)	80 (73)	98 (89)	84 (76)	5 (5)	MsHpS104-MsHpS108	
	<i>Total by area</i>	<i>292</i>	<i>196 (67.0)</i>	<i>220 (75.0)</i>	<i>257 (88.0)</i>	<i>212 (72.6)</i>	<i>11 (3.8)</i>	-	

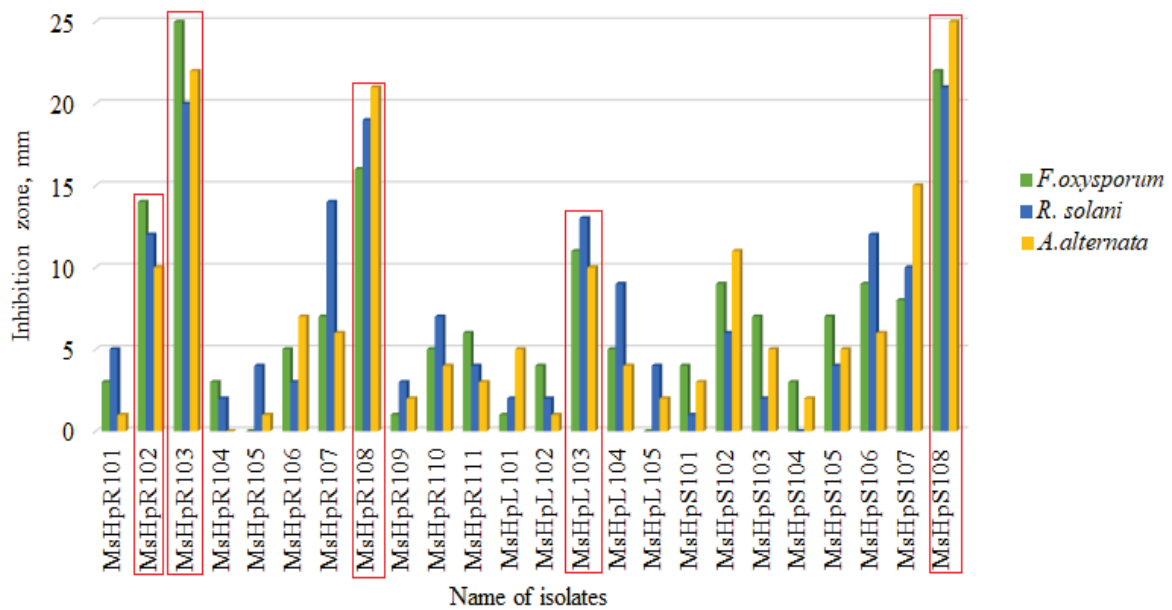


Fig. 4. Antifungal activity of isolates of drought-resistant endophytic bacteria isolated from *P. harmala*

It was noted that 20 of the 24 isolates selected in the experiments were somewhat resistant to phytopathogens *F. oxysporum*, *R. solani* and *A. alternata*. Among the remaining 4 isolates, isolate MsHpR104 did not show antifungal activity against *A. alternata*, isolates MsHpR105 and MsHpL105 against *F. oxysporum*, isolate MsHpS104 against *R. solani*. In order to select promising isolates among 20 isolates with certain resistance to phytopathogens, the width of inhibition zones between pathogenic fungal and bacterial isolates was comparatively analyzed. According to the results of the analysis, MsHpR102, MsHpR103, MsHpR108, MsHpL103 and MsHpS108 isolates were recorded as isolates with higher inhibition zones between pathogenic fungal and bacterial isolates than other isolates. In these isolates, it was found that the inhibition zones between pathogenic fungi and bacterial isolates were not less than 10 mm. In particular, the width of the inhibition zone between MsHpR102 isolate and *F. oxysporum* was 14 mm, while this index was 12 and 10 mm in relation to *R. solani* and *A. alternata*. The width of inhibition zone bet-

ween MsHpR103 isolate and studied pathogens (*F. oxysporum*, *R. solani* and *A. alternata*) was found to be 25, 20 and 22 mm, respectively. In this regard, MsHpR108, MsHpL103, and MsHpS108 isolates had similar values to MsHpR102 and MsHpR103 isolates (Fig. 4). As a result of evaluation of antifungal activity of the selected isolates, isolates MsHpR102, MsHpR103, MsHpR108, MsHpL103 and MsHpS108 were selected as isolates that should be assigned a promising systematic position (Fig. 5).

Therefore, it is also necessary to identify MsHpR102, MsHpR103, MsHpR108, MsHpL103 and MsHpS108 isolates of endophyte bacteria isolated from *P. harmala* (Table 2). The 16S pRNA nucleotide sequences of 5 promising strains of endophytic bacteria isolated from *P. harmala* were deposited in the National Center for Biotechnology Information (NCBI) database: OR841349, OR841350, OR841351, OR841352, OR841353 listed with (www.ncbi.nlm.nih.gov).

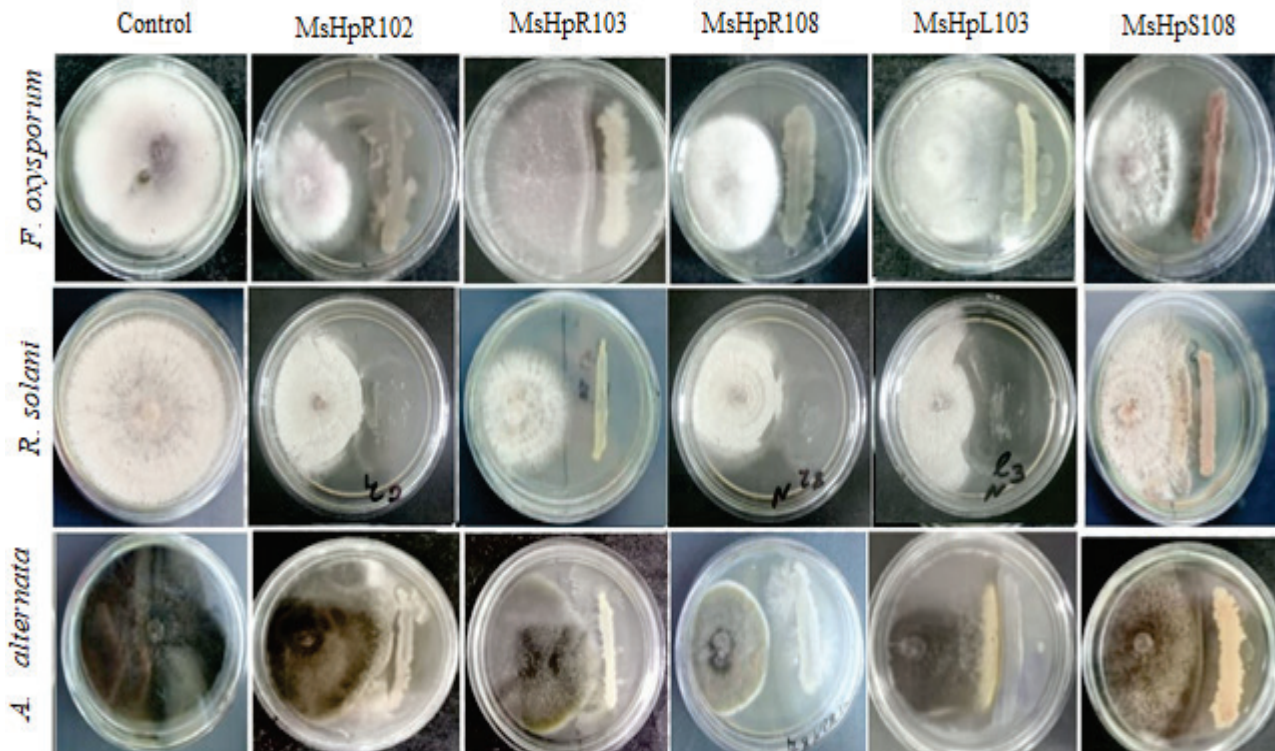
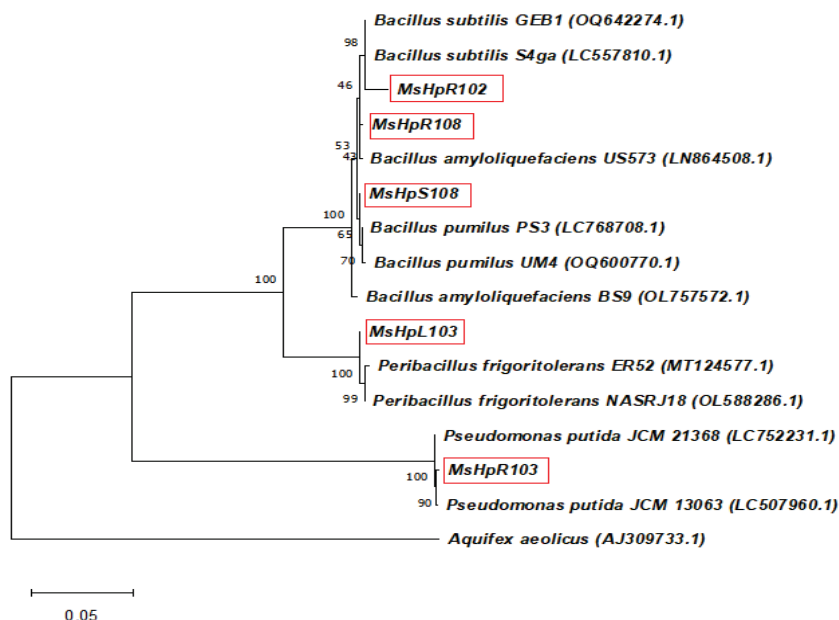


Fig. 5. Resistance of promising isolates of drought-tolerant endophytic bacteria isolated from *P. harmala* to pathogenic fungi

Table 2The effective endophytes isolated from *P. harmala* and their closest relatives from GenBank

Isolated strains deposited to GenBank			Closest match (16S ribosomal RNA genes) (GenBank)		
Strain	Length, bp	Accession number	Reference strains	Accession number	Percent identity
MshpR102	1425	OR841349	<i>Bacillus subtilis</i> GEB1	OQ642274.1	99,79
MshpR103	1451	OR841350	<i>Pseudomonas putida</i> JCM 21368	LC752231.1	99,86
MshpR108	1450	OR841351	<i>Bacillus amyloliquefaciens</i> US573	LN864508.1	99,79
MshpS108	1433	OR841352	<i>Bacillus pumilus</i> PS3	LC768708.1	99,86
MshpL103	1399	OR841353	<i>Peribacillus frigoritolerans</i> NASRJ18	OL588286.1	99,79

**Fig. 6.** Phylogenetic tree of promising strains of salinity-tolerant endophytic bacteria isolated from *P. harmala* based on 16S rRNA gene sequence

The results of molecular genetic analysis and the data obtained from the gene bank, *Bacillus amyloliquefaciens*, *B. subtilis*, *Pseudomonas putida*, *B. amyloliquefaciens*, *B. pumilus* and *Peribacillus frigoritolerans* of promising strains isolated from *P. harmala* (Fig. 6) are as follows. According to the evolutionary analysis of the phylogenetic tree constructed by the Maximum Likelihood method in the Mega 4 program, species of the genus *Bacillus* were formed in one cluster with 100% similarity. 3 strains of the genus *Bacillus* were found to be a separate branch, separated from the species *Peribacillus frigoritolerans*, which is located in a different genus. In the next branch, it can be seen that *Pseudomonas putida* species is located in a separate joint. Another group in the family tree contains *Aquifex aeolicus*, which is an outgroup for the identified species.

Discussion

Specific communities of endophytic bacteria are found in the tissues of various plants. In addition to stimulating plant growth, there is information that they are important in protecting against various stress factors (Hassan, 2017). Endophytic bacteria have several advantages over rhizosphere bacteria. When they enter the tissues, they are in direct contact with the plant, and as a result, easy communication between cells is established, which has a direct beneficial effect on the host plant (Jayakumar et al., 2020). According to the results of a number of studies, the optimal temperature for growing endophytic bacteria isolated from plants is 28–37 °C. Chebotar et al. (2022) isolated 28 endophytic bacterial isolates from *Salicornia europaea* L., *Salsola australis* (R. Br.), *Bassia sedoides* (Pall.) and *Kochia prostrata* (L.) Schrad grown at seven different temperatures between 10–55 °C and observed that bacteria rapidly formed colonies at 30–37 °C (Chebotar et al., 2022). Enquahone et al. (2022) evaluated the growth potential of 167 isolates of endophytic bacteria isolated from *Sporobolus specatus* (Wahr) Kunth and *Cyperus laevigatus* L. plants growing in Ethiopia at different temperatures and concluded that the optimal growth temperature for these isolates is 28–32 °C. Also, as a result of this study, 6 bacterial isolates resistant to 50 °C temperature were isolated from among the studied isolates (Enquahone et al., 2022).

A number of important scientific results have been recorded by local and foreign researchers in determining the antagonistic properties of endophytic bacteria of plants. Endophytic bacteria isolated from *Ajuga turkestanica* by Mamarasulov et al. (2023) have antagonistic activity against pathogenic fungi *F. oxysporium*, *F. proliferatum*, pathogenic bacteria *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa* were screened, among which strains resistant to pathogens were selected (Mamarasulov et al., 2023). Analysis of endophytic bacteria isolated from *Hylocereus undatus*, *H. costaricensis* (Vietnam), *Lilium davidii* (China), a number of medicinal plants (Iran), *Tinospora cordifolia*, *Catharanthus roseus*, *Tectona hamiltoniana*, *Boscia variabilis* (Myanmar) and their pathogenicity shows them to have relatively antibacterial and antifungal properties (Beiranvand et al., 2017; Myo et al., 2020; Luu et al., 2021; Gao et al., 2022). In these studies, the high activity of some strains of endophytic bacteria is explained by the nature and effects of the secondary metabolites secreted by them.

The taxonomic composition of endophytic bacteria of xerophytic plants has been studied by a number of researchers. In particular, Alikulov et al. (2023) found that halophytic plants such as *H. aphyllum*, *H. strobilaceum* and *H. belangeriana* were dominated by *Bacillus*, *Pseudomonas* and *Priestia* species of endophytic bacteria (Alikulov et al., 2023). In another study aimed at determining the species composition of endophytic bacteria of *S. specatus* and *C. laevigatus* plants, it was reported that endophytic bacteria such as *Agrobacterium taibaishanense*, *Pseudomonas alcaliphila*, *Pseudomonas toyotomiensis*, *Halomonas johnsoniae* and *Halomonas alkaliantarctica* were found in these plants (Enquahone et al., 2022). In general, a detailed study of endophytic bacteria isolated from *P. harmala* distributed in arid regions in Uzbekistan, showed that these plants are new sources for isolation of promising bacterial strains.

Conclusion

Based on the results, *P. harmala* contains promising bacterial endophytes having resistance to severe drought and phytopathogens. Molecular analysis confirmed that these endophytic bacteria belong to genus *Bacillus*, *Pseudomonas*, and *Peribacillus*. Among the identified endophytes,

Bacillus subtilis MsHPr102, *Pseudomonas putida* MsHPr103, *Bacillus amyloliquefaciens* MsHPr108, *Bacillus pumilus* MsHPrL103 and *Peribacillus frigiditolerans* MsHPrS108 have high inhibition capacity against plant pathogenic fungi. These endophytic bacteria can be used as biological control of fungal pathogens in crops in arid regions.

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