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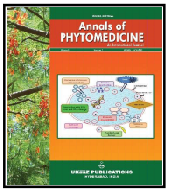

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Isolation and characterization of endophytic bacteria from ginger (*Zingiber officinale* Rosc.)

Dilfuza Jabborova◆*, Kannepalli Annapurna*, Mohina Fayzullaeva, Khurshid Sulaymonov, Dilbar Kadirova, Zafarjon Jabbarov*** and R. Z. Sayyed******

Laboratory of Medicinal Plants Genetics and Biotechnology, Institute of Genetics and Plant Experimental Biology, Uzbekistan Academy of Sciences, Tashkent, Uzbekistan

**Division of Microbiology, ICAR-Indian Agricultural Research Institute, Pusa, New Delhi-110012, India*

***Termez State University, Termez, Uzbekistan*

****Department of Soil Science and Agriculture, Faculty of Biology, National University of Uzbekistan, Tashkent, Uzbekistan*

*****Department of Microbiology, PSGVP Mandal's, Arts, Science and Commerce College, Shahada-425409, Maharashtra, India*

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Abstract

The present investigation was aimed to isolate and characterize endophytic bacteria from Ginger (*Zingiber officinale* Rosc.) plant with salt tolerance, antifungal activity, and plant growth-promoting traits. A total of 15 endophytic bacteria were isolated from ginger by standard microbiological culture methods. Nine endophytic bacterial isolates (GS1, GS2, GS3, GS4, GS6, GS7, GS8, GS9, and GS11) showed salt tolerance up to 10% NaCl concentration. Six isolates showed salt tolerance up to 7-8% NaCl concentrations. Studies on plant growth-promoting activity suggested that four endophytic bacterial isolates (GS2, GS5, GS8, and GS10) were positive for IAA production, siderophore production, and phosphate solubilization activity. All the endophytic bacterial isolates were screened for the production of enzymes. The result of the study revealed that maximum isolates have positive protease activity and this was followed by lipase and cellulase activities. While studying the antifungal activity of the bacterial isolates, it was determined that eight isolates showed antifungal activities against fungal strains *F. globosum* 905 and *F. graminearium* 611. Four endophytic bacterial isolates (GS4, GS6, GS8, and GS9) had antifungal activity against several fungal strains. The results indicated that endophytic bacteria were isolated from ginger with salt-tolerant and plant growth-promoting activities that were reported, that could be used as inoculants to establish a sustainable ginger production system.

1. Introduction

Ginger (*Zingiber officinale* Rosc.) is a spice and medicinal plant belonging to the *Zingiberaceae* family. Ginger has long been used in folk medicine in India and China. Especially, the wet and dry root of ginger is widely used in medicine and food industry in India. The wet root of *Z. officinale* is also used as a vegetable. It is used to make gingerbread, sweets, cakes, snacks, and soft drinks in many countries. It has been used in folk medicine for colds, sore throats, asthma, joint pain and stimulates appetite (Grzanna *et al.,* 2015). Ginger is also rich in beneficial nutrients for example phosphorus, potassium, and calcium, which play important roles in human physiological processes. These substances play an important role in boosting human immunity and maintaining health (Zadeh and Kor, 2014). The dry rhizome of ginger is medicinal contains biologically active compounds. The rhizome contains carbohydrates, fats, proteins, vitamins, minerals, amino acids, monoterpenoids (camphene, sineiol, borneol, citral curcumin, and linalool), gingerol, and sesquiterpenoids (Sharma, 2017). Microorganisms such as rhizobacteria and endophytic bacteria play an important role in increasing the growth, development, and productivity of plants (Egamberdieva *et al.,* 2013; Egamberdieva *et al.,* 2015, Egamberdieva *et al.,* 2016, Egamberieva *et al.,* 2017; Egamberdieva *et al.,* 2018; Jabborova *et al.,* 2018; Li *et al.,* 2018; Sayyed *et al.,* 2015).

Endophytic bacteria directly improve plant growth such as producing phytohormones IAA, gibberellins, cytokinins (Jabborova *et al.,* 2015), solubilizing phosphate (Sharma *et al.,* 2013), increase nutrients (Liu *et al.,* 2016; Liu *et al.,* 2017), increase stress tolerance in plants (Sagar *et al.,* 2020), biological control of plant pathogens (Sayyed and Chincholkar, 2009; Sayyed *et al.,* 2010; Shaikh *et al.,* 2014; Reshma *et al.,* 2018;) and nitrogen fixation (Li *et al.,* 2018). Phosphate solubilizing and phytohormone producing bacteria improve plant growth and yield of crops (Sharma *et al*., 2013; Jabborova *et al.,* 2015). The endophytic bacteria possess the capacity to solubilize and assimilate phosphates in plants (Kuklinsky-Sobral *et al.,* 2004). The objective of this study was to isolate and characterize the endophytic bacteria from Ginger (*Z. officinale*) medicinal plant with salt tolerance, antifungal activity, and plant growth-promoting traits.

Corresponding author: Dr. Dilfuza Jabborova
Senior Scientific Researcher, Laboratory of Medicinal Plants Genetics and Biotechnology, Institute of Genetics and Plant Experimental Biology, Uzbekistan Academy of Sciences, Tashkent, Uzbekistan
E-mail: dilfuzajabborova@yahoo.com
Tel.: +91-8595092177; +998-909107761

2. Materials and Methods

2.1 Determination of soil properties

Soil samples were collected from Termez district, Surkhandaryo province (Figure 1). To determine the soil properties before experimenting, soil samples were collected from 0-10, 0-20, and 0-30 cm depth of soil. The total nitrogen content was determined by the Kjeldahl method. The phosphorus content by Machigin, the potassium content by Machigin-Protasov, and organic matter by Tyurin methods were analyzed (Table 1).

Figure 1: Map showing the location of the sampling sites. Surkhandaryo Region, Uzbekistan.

Table 1: Soil properties of field experiments

Soil properties	Total nitrogen (N), %	Phosphorus (P_2O_5), %	Potassium (K_2O), %	Organic matter, %
0-10 cm	0.094	0.180	0.73	1.69
10-20 cm	0.097	0.175	0.63	1.71
20-30 cm	0.088	0.175	0.54	1.59

2.2 Isolation of endophytic bacteria

For the isolation of endophytic bacteria, the ginger (*Z. officinale*) was collected from the Surkhandaryo Region, Uzbekistan (Figure 2). The bacterial endophytes were isolated from root, stem, and leaves of the healthy ginger plants. The plant samples were washed under running tap water for 10-15 min. to remove adhering soil particles, air-dried and roots, stem and leaves were separated. The separated plant root, stem, and leaves were weighed up to one gram on a weighing balance. The weighed samples were soaked in distilled water and drained. The samples were then surface-sterilized by dipping in 70% ethanol for 1 min, stem and leave with 4% sodium hypochlorite for 5 minutes and roots with 2% sodium hypochlorite for 10 min and then treated with 70% ethanol for 30 sec, followed by rinsing five times in sterilized distilled water. The surface-sterilized samples were blot dried using sterile filter paper. The samples were crushed. The root, stem, and leaves samples serial dilutions were prepared, up to 10^{-5} dilutions. One hundred microliters from each dilution of the respective sample were then poured in their respective Petri-plates so labeled from 10^{-1} to 10^{-5} containing tryptone soy agar medium, KB agar medium, and nutrient agar medium separately. The plating was done in triplicate for each dilution. The plates were incubated at 28 °C for 2-4 days for isolation of bacterial endophytes. The colonies of bacterial endophytes were picked and streaked on the 3 selective media for the selection of clone.

Figure 2: Ginger (*Zingiber officinale*) used in the present study for the isolation of endophytes.

2.3 Salt tolerance

NaCl was added into nutrient agar medium at various concentrations in the range of 1 to 10% and the test bacterial isolates were streaked.

2.4 Phosphate solubilization

Phosphate solubilization ability of bacterial endophytes was detected by spot inoculating pure bacterial endophytes on the Pikovskaya medium (Pikovskaya, 1948) and incubated at 28 °C for three to seven days along with the control plates. The uninoculated plates served as control. All the inoculations were done in triplicate. After seven days of incubation at 28 °C, the formation of clearing zones were evaluated.

2.5 Indole acetic acid (IAA) production

Bacterial isolates were grown for 48 h on their respective media at 28 °C. Fully grown isolates were centrifuged at 3000 rpm for 30 min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution). The development of pink color indicates IAA production (Brick *et al.,* 1991).

2.6 Siderophore production

The bacterial isolates were spotted on the Chrome azurol S agar media as described (Patel *et al.,* 2018). Isolates were incubated at 28 °C for 5-7 days. The development of yellow-orange hallow zone around the bacterial spot has been considered as a positive indication for siderophore production.

2.7 Lipase production

Production of lipase was carried out in a medium containing (gL^{-1}), (Ghodsalavi *et al.,* 2013), peptone; 10, calcium chloride; 0.1, NaCl; 5, agar; 15, distilled water 100 ml; 10 ml sterile Tween 20. All of the isolates were streaked on this medium and incubated at 27 °C for 48 h. Depositions around the bacterial colonies indicated the activity of the lipase enzyme.

2.8 Production of protease

The qualitative assay for protease production was performed on sterile skim milk agar containing (gL^{-1}) pancreatic digest of casein; 5.0, yeast extract; 2.5, glucose; 1.0, agar; 15.0, distilled water 1000 ml, skim milk (inducer), 7%. Isolates were spot inoculated grown at 30 °C for 48 h. After incubation plates were observed for the appearance of the zone of clearance around the colony indicating the enzymatic degradation of protease (Malleswari and Bagyanarayana, 2013).

2.9 Production of cellulase

Screening and production of the cellulose-degrading ability of bacterial isolates were performed by separately streaking each isolate on the cellulose congo red agar media containing (gL^{-1}) KH_2PO_4; 0.5, $MgSO_4$; 0.25, cellulose; 2, agar; 15, congo red; 0.2, gelatin; 2; distilled water 100 ml. pH was set to 6.8 with the help of 0.1 N HCl. Plates were incubated at 30 °C for 48 h. After incubation clearance of halos around and beneath the colony was taken as an indication of the enzymatic degradation of cellulose (Gupta *et al.,* 2012).

2.10 Antifungal activity

The bacterial isolates were tested for *in vitro* inhibitory effects against common phytopathogenic fungi namely: *Fusarium sporotrichiodes* 404, *F. globosum* 905, *F. oxysporum* 328, *F. culmorum* 903, *F. graminearium* 611, *F. solani* 528, and *F. proliferatum* 516. Fungal cultures were grown on the ISP_2 agar plate at 28 °C for 5 days until the fungi had grown over control plates without bacteria. Antifungal activity was recorded as the width of the zone of growth inhibition between the fungus and the test bacterial isolates. All bacterial isolates were used in triplicate for the determination of antifungal activity.

2.11 Statistical analysis

Stat View software packages were used to perform Fisher's PLSD test following an ANOVA (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1 Salt tolerance test of bacterial isolates

Fifteen bacterial isolates obtained from ginger showed varied tolerance to salt levels (Table 2). Nine endophytic bacterial isolates (GS1, GS2, GS3, GS4, GS6, GS7, GS8, GS9, and GS11) tolerated 10% of NaCl. Four isolates (GS5, GS10, GS12, GS13, GS14, and GS15) tolerated up to 7% of NaCl and were inhibited at 9% and 10% NaCl concentration.

Table 2: Salt tolerance of bacterial endophytes from Ginger (*Z. officinale*)

Bacterial isolates	1% NaCl	2% NaCl	3% NaCl	4% NaCl	5% NaCl	6% NaCl	7% NaCl	8% NaCl	9% NaCl	10% NaCl
GS1	+	+	+	+	+	+	+	+	+	+
GS2	+	+	+	+	+	+	+	+	+	+
GS3	+	+	+	+	+	+	+	+	+	+
GS4	+	+	+	+	+	+	+	+	+	+
GS5	+	+	+	+	+	+	+	-	-	-
GS6	+	+	+	+	+	+	+	+	+	+
GS7	+	+	+	+	+	+	+	+	+	+
GS8	+	+	+	+	+	+	+	+	+	+
GS9	+	+	+	+	+	+	+	+	+	+
GS10	+	+	+	+	+	+	+	-	-	-
GS11	+	+	+	+	+	+	+	+	+	+
GS12	+	+	+	+	+	+	+	-	-	-
GS13	+	+	+	+	+	+	+	+	-	-
GS14	+	+	+	+	+	+	+	-	-	-
GS15	+	+	+	+	+	+	+	+	-	-

3.2 Plant growth-promoting traits

A total of 15 bacterial isolates were isolated from the medicinal plant *Z. officinale* were screened for multiple plant growth-promoting traits such as phosphate solubilization, IAA production, siderophore production, and enzymes activities (Table 3). Most bacterial isolates exhibited one or more plant growth-promoting activities. Four bacterial isolates (GS2, GS5, GS8, and GS10) were able to solubilize phosphate and produce IAA, siderophore, lipase, protease, and cellulase.

Table 3: Overview of plant beneficial properties of the fifteen isolated bacterial isolates

Bacterial isolates	Plant growth-promoting traits					
	P solubilization	IAA production	Siderophore production	Lipase	Protease	Cellulase
GS1	+	-	-	+	+	-
GS2	+	+	+	+	+	+
GS3	+	+	+	+	+	-
GS4	+	+	-	-	-	+
GS5	+	+	+	+	+	+
GS6	+	+	-	-	-	-
GS7	+	-	-	+	-	-
GS8	+	+	+	+	+	+
GS9	-	+	-	+	+	+
GS10	+	+	+	+	+	+
GS11	-	+	-	+	+	+
GS12	-	-	+	-	-	+
GS13	+	+	+	-	+	+
GS14	+	-	+	+	+	-
GS15	-	+	-	+	+	+

The results of the plant promoting growth test revealed that three isolates (GS9, GS11, and GS15) produce IAA, lipase, protease, and cellulase enzymes. Among all isolates, about 26% of them did not produce IAA (GS1, GS7, GS12, and GS14), lipase (GS4, GS6, GS12,

and GS13), and protease (GS4, GS6, GS7, and GS12) and did not solubilize phosphate (GS9, GS11, GS12, and GS15). About 47% of the isolates (GS1, GS4, GS6, GS7, GS9, GS11, and GS15) did not produce siderophore. About 33% of bacterial isolates (GS1, GS3, GS6, GS7, and GS14) did not produce cellulase.

3.3 Antifungal activity of endophytic bacterial isolates

The endophytic bacterial isolates showed antifungal activity against selected fungal strains (Table 4). Thirteen isolates did not show antagonistic activity against fungal strain *F. sporotrichiodes* 404. Eight bacterial isolates had antifungal activity against fungal *F. globosum* 905 (GS1, GS3, GS4, GS6, GS7, GS8, GS9, and GS15) and *F. graminearium* 611 (GS1, GS2, GS3, GS4, GS8, GS11, GS12, and GS13) strains. Three endophytic bacterial isolates exhibited antifungal property against fungal strains *F. oxysporum* 328 (GS4, GS11, and GS15) and *F. culmorum* 903 (GS2, GS6, and GS15). A total of 5 bacterial isolates exhibited antifungal property against fungal strains *F. solani* 528 (GS5, GS6, GS9, GS12, and GS13) and *F. proliferatum* 516 (GS5, GS6, GS7, GS8, and GS9).

Table 4: Antagonistic activity of bacterial endophytes from Ginger (*Z. officinale*) against some pathogenic fungi

Bacterial isolates	Antifungal activity (fungal growth inhibition)						
	F. sporotrichiodes 404	*F. globosum 905*	*F. oxysporum 328*	*F. culmorum 903*	*F. graminearium 611*	*F. solani 528*	*F. proliferatum 516*
GS1	-	+	-	-	+	-	-
GS2	-	-	-	+	+	-	-
GS3	-	+	-	-	+	-	-
GS4	-	+	+	-	+	-	-
GS5	-	-	-		-	+	+
GS6	+	+	-	+	-	+	+
GS7	-	+	-	-	-	-	+
GS8	-	+	-	-	+	-	+
GS9	-	+	-	-	-	+	+
GS10	+	-	-	-	-	-	-
GS11	-	-	+	-	+	-	-
GS12	-	-	-	-	+	+	-
GS13	-	-	-	-	+	+	-
GS14	-	-	-	-	-	-	-
GS15	-	+	+	+	-	-	-

growth inhibition zone "+",
"–" - no inhibition

4. Discussion

The endophytic bacterial isolates were isolated from root, stem, and leaves of ginger. Endophytic bacteria previously reported in various medicinal plants such as *Momordica charentia* (Singh *et al.,* 2013), *Cassia tora* (Kumar *et al.,* 2015), *Glycyrrhiza uralensis* (Li *et al.,* 2018), *Talinum triangular*e (Ali and Rante, 2018) and *Thymes vulgaris* (Mohamad *et al.,* 2020).

Endophytic bacteria are particularly tolerant of environmental stresses such as high salinity, heat, and cold. Our results showed that the nine endophytic bacterial isolates (GS1, GS2, GS3, GS4, GS6, GS7, GS8, GS9, and GS11) tolerated 10% of NaCl. Several other investigations have also reported that endophytic bacteria efficiently tolerated the high salt concentration (Kumar *et al.,* 2015; Mohamad *et al.,* 2020). Rashid *et al.* (2012) reported that *Pseudomonas* sp. tolerated up to 4% NaCl and *Bacillus* sp. 2% NaCl. The endophytic bacterial strains of *Curcuma longa* L showed tolerance to the increasing salt concentration. *B. thuringiensis* (ECL2) and *B. pumilus* (ECL4) tolerated higher salt level (8% NaCl) whereas *B. cereus* ECL1 and *Bacillus* sp. ECL3 tolerated 7% of NaCl. *Pseudomonas putida* (ECL5) and *Clavibacter michiganensis* (ECL6) tolerated 6% of NaCl concentration (Kumar *et al.,* 2016).

Several endophytic bacteria interact positively, *via.,* various mechanisms with their host plant. They produce plant growth, phosphate solubilization, IAA production, siderophore production, and activities of enzymes. In the present study, it was observed that several bacterial isolates isolated from medicinal plant *Z. officinale* were able to produce IAA, siderophore, enzymes, and solubilize phosphate (Table 2). Similar investigations indicated that endophytic bacteria exhibited plant beneficial traits (Singh *et al.,* 2013; Ali and Rante, 2018; Mohamad *et al.,* 2020). In this study production of IAA, siderophore, and solubilization phosphate was observed in four bacterial isolates namely GS2, GS5, GS8, and GS10, similar to the previous report by Mohamad *et al.* (2020). Mohamad *et al*. (2020) reported *Bacillus* sp. and *Pseudomonas* sp. with plant growth promotion and their activity was associated with the production of IAA and siderophore. Earlier research reported endophytic bacteria *B. pumilus*a and *P. protegens* produce siderophore (Etminani and Harighi, 2018).

Phosphorus is an important macronutrient necessary for plant growth and development. Phosphate solubilizing bacteria are capable of solubilizing the insoluble phosphate; enhance soil quality and plant growth and development of different plants (Sharma *et al*., 2013 and 2016; Jabborova *et al.,* 2015). Kumar *et al.* (2016) reported solubilization in *Bacillus cereus* ECL1, *Bacillus* sp. ECL3, *Bacillus pumilis* ECL4, and *Pseudomonas putida* ECL5. In this research, all seven endophytic bacterial isolates (GS2, GS5, Gs8, GS9, GS10 GS11, and GS15) were able to produce different enzymes such as lipase, protease and cellulase enzymes (Table 2). These results are in general agreement with (Mohamad *et al.,* 2020) who reported that endophytic bacteria associated with the medicinal plant, *Thymes vulgaris* capital were able to produce enzymes. Li *et al.,* (2018) reported endophytic bacteria with the same capacity of producing plant growth-promoting traits.

Many endophytic bacteria exhibited antifungal properties that inhibit the growth of fungal pathogens. This study has demonstrated the antifungal activity of bacterial isolates (GS1, GS3, GS4, GS6, GS7, GS8, GS9, and GS15) against *F. globosum* 905 and *F. graminearium* 611 (GS1, GS2, GS3, GS4, GS8, GS11, GS12, and GS13) strains in Table 3. Our results showed that three endophytic bacterial isolates (GS4, GS11, and GS15) inhibited the growth of fungal strain *F. oxysporum* 328. Endophytic bacteria isolated from different medicinal plants have been reported to inhibit the growth of fungi (Kumar *et al.,* 2016; Mohamad *et al.,* 2020). Endophytic bacteria isolated from medicinal plant *Glycyrrhiza uralensis* have been reported to exhibit antifungal activity. Many of the previous studies have shown that endophytic bacteria control fungal pathogens, including *Bacillus* sp. ECL3 (Kumar *et al.,* 2016), *Bacillus*

atrophaeus (Mohamad *et al.,* 2018) and *Bacillus* sp. and *Pseudomonas* sp. (Mohamad *et al.,* 2020).

5. Conclusion

Nine endophytic bacterial isolates (GS1, GS2, GS3, GS4, GS6, GS7, GS8, GS9, and GS11) tolerated high salt (10% of NaCl) concentration. Endophytic bacterial isolates have different abilities related to plant growth promotion such as solubilization of phosphate, IAA, siderophores, protease, lipase, and cellulase. Only four bacterial isolates (GS2, GS5, GS8, and GS10) were able to solubilize phosphate and produce IAA, siderophore, lipase, protease, and cellulase. All the bacterial isolates variously showed antifungal activity against selected fungal strains *F. sporotrichiodes* 404*, F. globosum* 905, *F. graminearium* 611, *F. oxysporum* 328, *F. culmorum, F. solani, F. proliferatum* 516. Four endophytic bacterial isolates (GS4, GS6, GS8, and GS9) had antifungal activity against several fungal strains. This study provides future encouragement for the plant growth-promoting endophytic bacterial isolates (GS2, GS5, GS8, and GS10) for the improvement of eco-friendly biofertilizers to increase the ginger yield and enhance plant tolerance to salt stress.

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Conflict of interest

The authors declare that there are no conflicts of interest in the course of conducting the research. All the authors had final decision regarding the manuscript and decision to submit the findings for publication.

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