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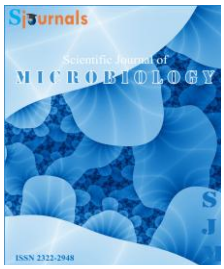

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Original article

Effect of endophytic bacterial isolates towards plant growth promoting activity

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ABSTRACT

Several microorganisms have been isolated from various plant sources which serve to improve the plant growth. Such plant associated endophytes protect their host against environmental stress, increase the tolerance against pathogens and induce growth by nitrogen fixation, through phosphate solubilization and also by producing growth hormone like substances. The present study was to elucidate the role of selected nine Endophytic Bacterial Isolates in plant growth promotion. These selected endophytic bacterial isolates were screened for their plant growth promoting activities like phosphate solubilization, nitrogen fixation, ammonia production, and protease, cellulase activity. Out of nine endophytic bacterial isolates, five of them showed positive results for all biochemical activities. The step by step isolation of endophytic bacteria with the features of auxin activity and growth promoting activity can makes a better formulation for maximum yield of crop plants.

1. Introduction

Endophytes are beneficial microbes that grow within the plants without showing any visible symptoms (Bandara et al., 2006). Being ubiquitous in plant tissues, they can be isolated from leaves, stem, roots, seeds, fruits and flowers(Barzanti et al., 2007).The direct effect of endophytes in promoting plant growth are thought to include phytohormone production (Zahir et al., 2003), asymbiotic nitrogen fixation (Şahin et al., 2004), solubilization of inorganic phosphate and mineralization of organic phosphate/ other nutrients(Ma et al., 2009). Endophytes have deleterious indirect effects on plant pathogens through siderophores production (Dobbelaere et al., 2002) and through induced systemic resistance (Pavlo et al., 2012).Certain nitrogen fixing endophytic bacteria are involved in various processes like increasing the nutrient availability in soil by improving the soil characteristics, activating the plant defense system by producing antibiotics and providing plant growth promoting substances (Reinhold-Hurek et al., 1993).

Phosphorus is one of the essential nutrients prevails in soil in two forms as organic and inorganic phosphates at levels of 400–1,200 mkg−1 of soil (Hayat et al., 2010). Endophytic bacteria can convert these insoluble phosphate compound into the available form to the host plants (Wakelin et al., 2004). Plants generally takes the soluble phosphate at lower level of 1ppm or even less but majority is absorbed in the form of HPO_4^{2-} or $H_2PO_4^-$ (Rodríguez and Fraga , 1999). Endophytic bacteria have the ability to solubilizing such inorganic phosphate (Rodríguez et al., 2007) by producing organic acids (Chen et al., 2006) and mineralizing these organic phosphorous compounds by secreting the enzymes like phosphatases or organic acid production (Adesemoye and Egamberdieva, 2013). Endophytic bacteria secreting enzymes can interfere with pathogenic survival. They are involved in hydrolyzing various polymeric compounds such as cellulose, protein, chitin and DNA and are effective in suppressing the fungal phytopathogens (Kamensky et al., 2003) and indirectly involved in disease resistance (Kilic-Ekici and Yuen, 2003). The study of endophytes and their role in plant growth promotion is important for the biotechnological application of these bacteria. Therefore, in the present study, a total of 9 selected endophytic bacteria isolated from leaf portion of various host plants were screened for plant growth promoting properties like nitrogen fixation, ammonia production, auxin production, phosphate solubilization and enzymatic activity like cellulase and protease.

2. Materials and methods

The nine selected endophytic bacterial isolates (EBIs) used for screening of nitrogen fixation, ammonia production, phosphate solubilization, protease and cellulose activity were listed in table 1.

Table 1
List of Endophytic Bacterial Isolates (EBIs) used in the present study.

S.no	EBI Id.no.	Name of the endophytes	Host plant	GB*
1.	6.PB.10	*Bacillus pumilus*	*Ficusbenjamina* L. (Benjamin's Plate)	HQ683833
2.	6.N17.1	*Bacillus subtilis*	*Sansevieriatrifasciata*Prain. (Snake Plant)	HQ694360
3.	9.P7.1	*Bacillus fusiformis*	*Cymbopogoncitratus*Spreng. (Lemongrass)	HQ694198
4.	6.NL.13	*Bacillus cereus*	*Nepheliumlappaceum* L. (Rambutan)	HQ670573
5.	9.P3.2	*Bacillus pumilus*	*Ananascomosus* L. (Pineapple)	HQ694116
6.	9.AC.9	*Pantoeaagglomerans*	*Artocarpuschampeden*Lour. (Chempedak)	HQ670625
7.	1.IC.3	*Bacillus megaterium*	*Ixoracoccinea* L. (Jungle Flame)	HQ634276
8.	11.P1.7	*Acinetobacter*sp.	*Polyalthialongifolia*Sonn. (Indian mast tree)	HQ683912
9.	5.HB.11	*Bacillus cereus*	*Heveabrasiliensis*Willd. (Rubber Tree)	HQ670437

*GB denotes GenBank accession number of 16S rDNA sequence of respective strain.

2.1. Screening of EBIs for nitrogen fixation

Nitrogen fixing ability of the isolates was assessed by streaking the individual culture on Jensen's medium which contains (per liter): 20 g Sucrose, 1 g Dipotassium phosphate, 0.5 g $MgSO_4$, 0.5 g NaCl, 0.1 g $FeSO_4$, 0.005 g

Na_2MoO_4, 2 g $CaCO_3$ and 15 g agar. The petriplates were incubated at 28°C for 5 days. Growth on medium confirms the presence of nitrogen fixing bacteria (Gayathri et al., 2010).

2.2. Screening of EBIs for Ammonia production

Individual isolates were inoculated in test tubes containing 10 ml of sterile peptone broth. The tubes were then incubated at 37°C for 48 hrs in shaking incubator at 120rpm. After incubation 0.5 ml Nessler's reagent was added to each tube and observed for the development of faint yellow to dark brown colour (Gayathri et al., 2010).

2.3. Screening of EBIs for phosphate solubilizing activity

To detect the phosphate solubilizing bacteria, strains were streaked onto Pikovskaya's agar medium, which contains (per liter): 0.5 g yeast extract, 10 g dextrose, 5 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)2SO_4$, 0.2 g KCl, 0.1 g $MgSO_4.7H_2O$, 0.0001 g $MnSO_4.H_2O$, 0.0001 g $FeSO_4.7H_2O$ and 15 g agar. After 3 to 5 days of incubation at 28°C, strains that induced clear zone around the colonies were considered as positive (Pandey et al., 2008).

2.4. Analysis of EBIs for protease activity

The protease activity was determined using skim milk agar medium, which contains (per liter): 5 g pancreatic digestof casein, 2.5 g yeast extract, 1 g glucose, 7% skim milk solution and 15 g of agar. Bacterial cells were spot inoculatedand after 2 days incubation at 28°C, proteolytic activity was identified by clear zone around the cells (Naik et al., 2008).

2.5. Analysis of EBIs for cellulase activity

To determine the cellulase activity, endophytic bacterial culture were inoculated on Carboxy Methyl Cellulose (CMC) agar medium. Inoculated plates were incubated at 35°C in an inverted position. After 2–5 days, the cellulase activity was observed (Wenzel et al., 2002).

3. Results and discussion

The present study deals with endophytic bacterial isolates in evaluating their plant growth promoting activities.All nine isolates viz., 6.PB.10, 6.N17.1, 9.P7.1, 6.N1.13, 9.P3.2, 9.AC.9, 1.IC.3, 11.P1.7 and 5.HB.11 were studied for assessing the presence of other plant growth promoting abilities such as phosphate solubilisation, nitrogen fixation, ammonia production, protease activity and cellulase activity. Out of nine endophytic isolates screened for plant growth promoting activity, maximum of 5 isolates (56%) showed positive results for phosphate solubilization (Plate 1), nitrogen fixation (Plate 2) and ammonia producing activity (Plate 3). Of the isolates studied for enzymatic activity, 8 isolates (89%) showed protease activity(Plate 4) and cellulase activity (Plate 5). Plant growth promoting activities of 9 endophytic bacterial isolates were mentioned in Table 2.

Table 2
Plant growth promoting activities of selected 9 endophytic bacterial isolates.

s.no	Strains	Phosphate solubilization	Nitrogen fixation	Ammonia production	Protease activity	Cellulase activity
1.	6.PB.10	-	-	-	+	+
2.	6.N17.1	-	+	+	+	+
3.	9.P7.1	+	+	+	+	+
4.	6.N1.13	+	-	-	+	-
5.	9.P3.2	-	-	-	-	+
6.	9.AC.9	+	+	+	+	+
7.	1.IC.3	+	+	+	+	+
8.	11.P1.7	+	+	+	+	+
9.	5.HB.11	+	+	+	+	+

+ = positive activity and - = negative activity.

3.1. Screening of EBIs for nitrogen fixation

The ability of the five EBIs, 1.IC.3, 9.AC.9, 9.P7.1, 5.HB.11 and 11.P1.7 that grown in Jensen's medium showed that they have the ability to fix nitrogen (Plate 1). Endophytes are responsible for the accumulation of nitrogen even in non-legumes by the process of Biological Nitrogen Fixation (BNF) (Oliveira et al., 2002). Nitrogen fixing activity has been reported for endophytic bacteria isolated from various plant sources such as rice and wheat (Ding et al., 2005; Feng et al., 2006; Sachdev et al., 2010). Similar results are reported by Forchetti et al. (2007), who isolated *B.pumilus* from sunflower roots. The results are also in accordance with the findings reported by Ding et al. (2005), who confirm the ability of endophytes such as *B.megaterium* and *B.cereus* in nitrogen fixing activity and were found to contain*nif H* gene.

Plate 1. Nitrogen fixation halo produced by 5 EBIs such as A) 1.IC.3 (*Bacillus megaterium*); B) 9.AC.9 (*Pantoeaagglomerans*); C) 9.P7.1 (*Bacillus fusiformis*); D) 5.HB.11 (*Bacillus cereus*); and E) 11.P1.7 (*Acinetobacter* sp.). Control F), does not show any halo.

3.2. Screening of EBIs for Ammonia production

In addition, the production of ammonia by EBIs (Plate 2) indirectly booststhe host plant growth as the dinitrogen gas in the atmosphere is converted into ammonia and nitrate for plant absorption (Saini et al., 2015). While function of ammonia by EBIs is not clear, it is known to act as a nitrogen source (Vishal Kumar et al., 2013). The results also revealed that EBIs that are positive in the nitrogen fixation test also produces ammonia. Furthermore, they also exhibited cellulase activity (Plate 5).

Plate 2. Results of EBIs analysis for ammonia production; A) control (uninoculated peptone broth); B) 5.HB.11 (*Bacillus cereus*); C) 9.P7.1 (*Bacillus fusiformis*); D) 9.AC.9 (*Pantoeaagglomerans*); E) 1.IC.3 (*Bacillus megaterium*) and F) 11.P1.7 (*Acinetobacter* sp.).

3.3. Screening of EBIs for phosphate solubilizing activity

Development of clear zone around the EBIs colonies such as 1.IC.3, 9.AC.9, 9.P7.1, 5.HB.11 and 11.P1.7 after growth on PVK medium indicates the isolate's ability to solubilize phosphate (Plate3). Endophytic bacteria can transform the insoluble form of phosphate into a soluble form and influence the plants to utilize phosphate (Richardson et al., 2001). The most common mechanism used by PSB in solubilizing the tri-calcium phosphate is through biosynthesis and release of a wide variety of organic acids thereby causing acidification of the medium (Goldstein, 2007).

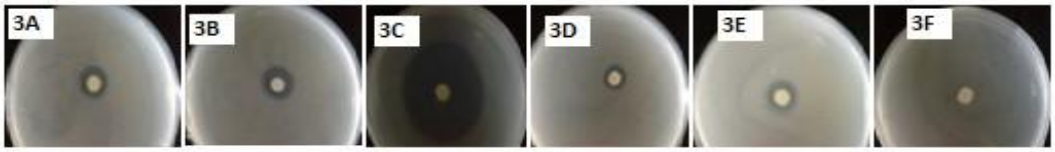

Plate 3. Phosphate solubilization halo produced by 5 EBIs such as A) 1.IC.3 (*Bacillus megaterium*); B) 9.AC.9 (*Pantoeaagglomerans*); C) 9.P7.1 (*Bacillus fusiformis*); D) 5.HB.11 (*Bacillus cereus*); and E) 11.P1.7 (*Acinetobacter* sp.). Control F), does not show any halo.

3.4. Analysis of EBIs for protease activity

Based on Plate 4, eight of the EBIs, 6.PB.10, 6.N17.1, 9.P7.1, 6.NL.13, 9.AC.9, 1.IC.3, 11.P1.7, and 5.HB.11 showed protease activity. This enzyme activity in endophytic bacteria has been reported by Szilagyi et al. (2014). This enzyme has hyperparasitic activity and has the ability to degrade structural fungal cell walls and are therefore indirectly involve in plant disease control (Kim and Chung, 2004; Podile and Kishore, 2006). EBIs with protease activity also known to be involved in IAA production and antagonistic activity against fungi and insects(Naik et al., 2008). In addition, Protease activity of *Serratiaplymutica is involved in suppressing the microbes such as Sclerotinia sclerotiorum* and *Botrytis cinerea* (Compant et al., 2005).

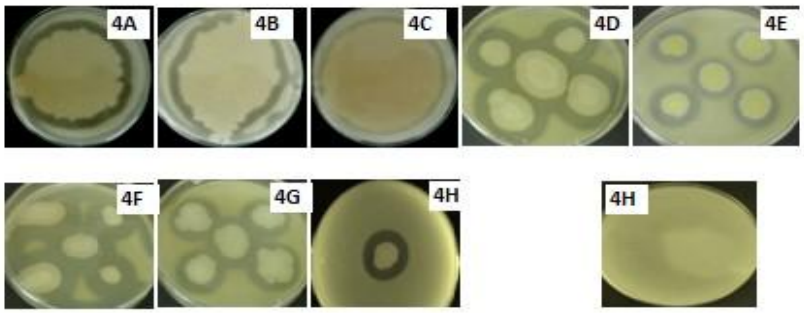

Plate 4. Proteolytic activity test results for 8 EBIs; A) 6.PB.10 (*Bacillus pumilus*); B) 6.N17.1 (*Bacillus subtilis*); C) 6.NL.13 (*Bacillus cereus*); D) 1.IC.3 (*Bacillus megaterium*); E) 9.AC.9 (*Pantoeaagglomerans*); F) 9.P7.1 (*Bacillus fusiformis*); G) 5.HB.11 (*Bacillus cereus*); H) 11.P1.7 (*Acinetobacter* sp.) and I) control.

3.5. Analysis of EBIs for cellulase activity

As shown in Plate 5, the isolated EBIs,6.PB.10, 6.N17.1, 9.P3.2, 9.P7.1, 9.AC.9, 1.IC.3, 11.P1.7 and 5.HB.11 also exhibited cellulase activity. The results are in agreement with the findings of Szilagyi-Zecchin et al. (2014) thatreported cellulase activity. This result correlates with the findings of Mostajeran et al. (2007) thatsuggested nitrogen fixing bacteria may also produce hydrolytic enzymes like cellulase to enter the plant roots as endophytes. Endophytic bacteria secrete lytic enzyme like cellulase are involved in hydrolyzing polymeric compound such as cellulose and is therefore indirectly involved in disease resistance mechanism of plants (Kilic-Ekici and Yuen, 2003).

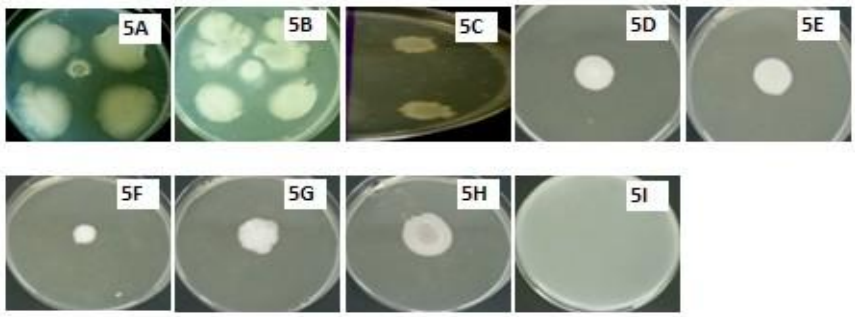

Plate 5. Cellulase activity test results for 8 EBIs; A) 6.PB.10 (*Bacillus pumilus*); B) 6.N17.1 (*Bacillus subtilis*); C) 9.P3.2 (*Bacillus pumilus*); D) 1.IC.3 (*Bacillus megaterium*); E) 9.AC.9 (*Pantoeaagglomerans*); F) 9.P7.1 (*Bacillus fusiformis*); G) 5.HB.11 (*Bacillus cereus*); (H) 11.P1.7 (*Acinetobacter* sp.) and I) control.

4. Conclusion

The study was able to observe phosphatesolubilization, nitrogen fixation, ammonia production, IAA production, protease and cellulase activities by the EBIs. This activities are highly effective for improving the growth and development of plants. Therefore, endophytic bacterial isolates may possess plant growth promoting activity and can be used as biofertilizers, phytostimulator and biopesticides.

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