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Multifunctional attributes of endophytic *Pseudomonas* strains isolated from the leaves of medicinal plants

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Abstract

Endophytic bacteria are responsible for improved plant growth due to its role in nitrogen fixation, indole acetic acid (IAA) production, phosphate solubilization etc and in plant protection through various mechanisms and production of bioactive compounds. The purpose of this study was to determine the plant growth promoting potential of endophytic bacteria isolated from medicinal plants namely, Adulsa, Amla, Bael, Kadamb, Mango, Neem, Tulsi. Endophytic bacteria isolated from the medicinal plants, comprised of 68% Gram positive and 29% Gram negative bacteria. Seventeen distinctly unique Gram-negative endophytes were selected for further analysis. The selected endophytes were tentatively identified as *Pseudomonas* sp. The multifarious endophytes were capable of nitrogen fixation, phosphate solubilisation, indole acetic acid (IAA) production, production of antimicrobial compounds and aromatic compound degradation. Some of the endophytic strains were found to harbor plasmids that may play a role in aromatic compound degradation. This study emphasizes the potential of endophytic *Pseudomonas* species in enhancing plant growth and plant protection.

Keywords

aromatic degradation, endophytic bacteria, IAA, nitrogen fixation, phytotoxicity

Introduction

Endophytic microorganisms inhabit plant tissues at least a period of its vital cycle. Apparently, they do not cause any damage to the host, which distinguishes them from the pathogenic microorganisms (1, 2). Plants harboring endophytes have an advantage since the endophytes promote growth as well as provide protection from phytotoxicity due to soil contaminants (3), and from phytopathogens. Endophytic bacteria promote plant growth in various ways, which includes secretion of plant growth regulators such as indole acetic acid (4-6), phosphate solubilizing activity (7), nitrogen fixation (1, 8), production of siderophores (9) and also supply essential vitamins to plants (10). The production of auxins and auxin like compounds increases seed production, germination, shoot growth and tillering. Moreover, a number of other beneficial effects on plant growth have been attributed to endophytes, which includes osmotic adjustment, stomatal regulation, modification of root morphology, enhanced uptake of minerals, alteration of nitrogen accumulation and metabolism (11), thereby contributing to plant stress resistance (12, 13). Endophytes are also reported to produce number of enzymes such as chitinases, β - glucanases, ACC deaminases etc that are re-

quired for their establishment in the plant tissue and may be beneficial to the host plant (14-17).

Endophytes confer profoundly enhanced fitness to the host plants by preventing disease development through endophyte-mediated *de novo* synthesis of novel compounds. The role of endophytes in plant-pathogen and plant-insect interactions is receiving increasing attention because of their potential use in pest control (2). Endophytic microorganisms have the capacity to control the pathogens, insects and nematodes (2, 18-22). This capacity is due to production of secondary metabolites such as antibiotics. Endophytic bacteria can thus prove to be efficient biocontrol agents. A number of endophytes have been discovered having the ability of synthesizing products that are extremely biologically active and selective against certain microbes harmful to the host plant (23). Thus, the endophytes are potential candidates that can be implicated in plant protection. Diseases of fungal, bacterial, viral origin and in some instances even damage caused by insects and nematodes can also be reduced by application of endophytic microorganisms (24). It is believed that certain endophytic bacteria trigger a phenomenon known as induced systemic resistance (ISR) leading to immunization of the plant towards the phytopathogens (25). Some protective compounds isolated from endophytes are taxol, munumbicins, oocydin A, cryptocin, ambuic acid, jesterone and volatile antimicrobials such as naphthalene (26-32). Reports are on an endophyte, *Streptomyces* NRRL 30562 having potential comparable to *Streptomyces griseoviridis*, a non-endophyte, included in the formulation for 'Mycostop', a commercial agricultural product of Butts International Inc., Fairfield, Connecticut, USA (33). This product is used in the control of plant diseases caused by *Alternaria*, *Fusarium* and *Phomopsis.*

Plant-bacteria combinations can increase contaminant degradations in the rhizosphere. These bacteria thus protect the plant from phytotoxic effects of the contaminants. Remnants of pesticides are phytotoxic to the plants. In order to minimize the phytotoxic effects, the plants may be selecting these plasmid bearing endophytic bacteria, which have the ability to mineralize aromatic compounds. Thus these bacteria have a role to play in bioremediation of contaminated soils. Endophytic strains with potential to enhance the phytoremediation of compounds like toluene, xylene as well as herbicides have been studied in poplar trees (34, 35). By promoting the establishment of the endophytes in the crop plants, plant growth improves as nutrients are supplied in the form of nitrogen, phosphates, production of plant growth promoting hormones, siderophore production, changes in root and stomatal behaviour etc.

The present work highlights the various characteristics of endophytic bacteria isolated from medicinal plants.

Materials and Methods

Isolation of the endophytic bacteria

Young leaves of medicinal plants (Adulsa, Amla, Bael, Kadamb, Mango, Neem, Tulsi) were selected for isolation of endophytic bacteria using procedure as described earlier (36-38). Leaf sample (1 gm) was cut into small pieces. These were washed with distilled water and surface sterilized with 1% (v/v) Savlon for 10 min followed by treatment with 0.1% (w/v) $HgCl_2$ for 5-10 min. Leaf pieces were then rinsed 4-5 times with distilled water. The entire procedure of sterilization was done aseptically. The sterile leaves were crushed using the sterile, pre-cooled mortar and pestle. Sterile phosphate buffered saline was added (2 ml) and the extract collected in sterile container. The extract (0.1 ml) was spread plated on nutrient agar medium and the plates were incubated at 28°C ± 2°C, for 24 to 48 hr. Surface sterilized, uncrushed leaf pieces were placed on nutrient agar plate and also in nutrient broth tubes to check sterilization efficiency. After the incubation period, bacterial colonies obtained were purified, characterized and selected for further studies.

Identification of endophytic bacterial isolates

The isolated bacterial endophytes isolated on nutrient agar, were grouped based on their Gram character. The colonies showing Gram-negative character were selected for the present study. These cultures were streaked on cetrimide agar media and the plates were incubated for 24 hr at room temperature. The isolates growing on cetrimide agar were characterized morphologically and biochemically for identification as per the Bergey's Manual of Systematic Bacteriology (39).

Nitrogen fixation and phosphate solubilization (40, 41)

Isolated colonies of endophytic bacterial strains were streaked on the nitrogen free Ashby's agar medium (Himedia, India) to check their nitrogen fixing ability. To check phosphate solubilizing ability of these bacteria, the cultures were streaked on Pikovaskaya's agar medium (Himedia, India) which contains calcium phosphate as phosphorus source. Plates were incubated at 28°C ± 2°C for one week. Diameter of bacterial colony and zone of clearance were noted after the incubation period. Phosphate solubilizing efficiency was calculated using the formula: Diameter of clearance X 100/ diameter of colony (42).

Indole acetic acid production

Cultures were grown in nutrient broth supplemented with 10 mM tryptophan for 24 hr at 28°C ± 2°C. The concentration of IAA was quantitated by the standard method (43). Culture supernatant was used mixed with Salkowski reagent (2:1) and the developed color was measured at 530 nm on Shimadzu UV-Vis-Spectrophotometer. Concentration of IAA in culture supernatant was calculated from standard graph of IAA.

Antimicrobial activity

Endophytic bacterial strains were tested for antimicrobial activity against bacteria and fungi, by the modified method (44). Overnight grown culture broths of the endophytic strains were centrifuged at 10000 rpm for 10 min and the supernatant thus obtained was used as antimicrobial extract. In each well of nutrient agar plate, preplated with the test organisms, namely, *Bacillus* sp., *Streptococcus* sp., *E. coli, Pseudomonas* sp., *Fusarium* sp. and *Aspergillus* sp.,

100 µl of supernatant was added and the plates were incubated at room temperature. The test cultures were obtained from microbial culture collection of Department of Microbiology, Gogate Jogalekar College, Ratnagiri, Maharashtra (India). After the incubation period, plates were checked for zones of inhibition.

Aromatic compound utilization ability (45)

Aromatic compound degradation ability of the isolates was checked for 8 different aromatic compounds. Stock solutions were prepared by dissolving the aromatic compounds in distilled water. The bacterial strains were pregrown in glucose minimal medium and inoculated in minimal medium supplemented with 0.05 and 0.1% (w/v) of aromatic compound. To check the utilization ability, growth of the each isolate was observed after 7 days of incubation at 28°C ± 2°C. The isolates showing growth in the presence of the aromatic compounds were subcultured in the same medium and the cultures showing growth after 3 subcultures was taken as positive for aromatic compound utilization. Appropriate controls were maintained.

Plasmid isolation and curing

The bacterial strains were grown in nutrient broth for 24 hr followed by centrifugation using Remi C24 cooling centrifuge. The pelleted cells were used for plasmid extraction by alkaline lysis method (46). The plasmid positive cultures were further used for curing (36). Overnight grown cultures were pelleted and suspended in nutrient broth supplemented with varying concentrations of ethidium bromide (10 – 100 µg/ml). After overnight incubation at 28°C ± 2°C, the highest concentration of ethidium bromide showing turbidity was diluted and plated on nutrient agar plate. The colonies obtained were checked for presence of the plasmid and also for aromatic degradation ability as described above.

Results and Discussion

Isolation and Identification of endophytic bacteria

Medicinal plants namely, Adulsa, Amla, Bael, Kadamb, Mango, Neem, Tulsi harbored Gram-negative endophytes along with other diverse groups of bacteria. Among the 83 endophytic bacteria isolated from leaves of the medicinal plants, 58.43% were Gram positive rods, 10% Gram positive cocci, 19.10% Gram negative rods, 10% Gram negative cocci and 2.25% yeast (Unpublished data). The dominance of Gram positive bacterial species has been observed by others workers as well (47, 48). Seventeen of the Gram negative short rods that grew on cetrimide agar media were selected for the present study. Based on their morphological and biochemical characterization, these strains were tentatively identified as *Pseudomonas* species and designated as Adulsa 1, Adulsa 3, Amla 1, Amla 2, Amla 5, Bael 1, Bael 4, Bael 7, Bael 9, Kadamb 1, Kadamb 4, Mango 1, Mango 4, Tulsi 3, Tulsi 1, Neem1, Neem 2, according to the host plant from which they were isolated. However, the identity confirmation using molecular tools needs to be carried out. Gram negative bacteria in general and *Pseudomonas* sp. in particular have been reported to have favorable characteristics for its applications in agriculture. *Pseudomonas* is one of the 5 taxa of microbial community showing promising levels of colonization, the others being *Cellulomonas, Clavibacter, Curthobacterium* and *Microbacterium* (49). *Pseudomonas stutzeri,* endophytic in *Echinacea* plants is an auxin producer (6). Similarly, isolation and identification of endophytic *Pseudomonas* species have been reported by number of workers (6, 49-56).

Nitrogen fixation and phosphate solubilization

All the 17 leaf endophytic isolates used in the present study showed ability to fix atmospheric nitrogen when grown on Ashby's nitrogen free medium. Thus, the strains can grow in nitrogen free medium by fixing atmospheric nitrogen. Thus, they have a role to play in plant nutrition by making nitrogen available to the host plant and this property is beneficial to the host plant when the bacteria are applied as biofertilisers. Endophytic bacteria isolated from leaves having nitrogen fixing bacteria have been reported (57, 58). A renewed interest in endophytic diatrophism, such as *Acetobacter*, *Azoarcus,* and *Herbaspirillum* in gramineous plants has arisen because of their occurrence within plant tissues and involvement significant nitrogen fixation (8, 59). Colonization of nitrogen fixing endophytes in the stem and root tissues but their absence in leaf tissues has been reported (60). Studies are on the nitrogen fixing ability of endophytes isolated from stem and root tissue only (1, 61). Endophytic distributions and penetration of young leaf tissues in case of *Herbaspirillum* sp. strain B501 in aerial parts of wild rice was *via* apoplastic spaces. Motility and pectinase and cellulase production by the endophyte might be involved in its spread throughout shoot tissues (61). Once endophytic diazotrophs such as *Azoarcus* sp. infect plants, they spread systemically and reach aerial tissues of the plant (59). This explains the presence of diazotrophic endophytes in the leaf tissues.

The endophytic bacterial isolates demonstrated good phosphate solubilizing efficiency seen from the results depicted in Table 1. Sixty % of the isolates tested in the present study could solubilize phosphate. Isolates Adulsa 3, Amla 1, Amla 5, Kadamb 4, Tulsi 1, Tulsi 3 showed very good phosphate solubilizing activity, with Tulsi 1 showing highest efficiency. Ten cultures showed very negligible or no activity. It was reported that phosphate solubilizing activity of endophytic *Pseudomonas* sp. isolated from various plant species (62). The activity was measured by the standard method to determine soluble phosphate and the solubilization efficiency was correlated to production of gluconic acid. In a combination technique, synchrotron X-ray spectro-microscopy, inorder to prove the direct role of endophytes in phosphorus uptake in poplar plants (63). As in the present study, nitrogen fixing ability as well as phosphate solubilisation and other plant growth promoting activities were observed and reported in endophytic bacterial strains of indigenous rice varieties (64) and endophytic isolates of Chinese fir seedlings (65). The authors noted and increased concentration of inorganic phosphates and potassium in soil as well as growth promoting effects.

Table 1. Phosphate solubilizing efficiency of Gram negative endophytic bacterial isolates

Culture name	Phosphate solubilizing efficiency (E)
Adulsa 1	112
Adulsa 3	230
Amla 1	180
Amla 2	127
Amla 5	225
Bael 1	98
Bael 4	83
Bael 7	155
Bael 9	118
Kadamb 1	85
Kadamb 4	198
Mango 1	167
Mango 4	95
Tulsi 1	245
Tulsi 4	214
Neem 1	148
Neem 4	100

Indole acetic acid production

Out of the 17 endophytic *Pseudomonas* strains tested, 10 strains showed IAA producing capacity (Fig. 1). Isolate

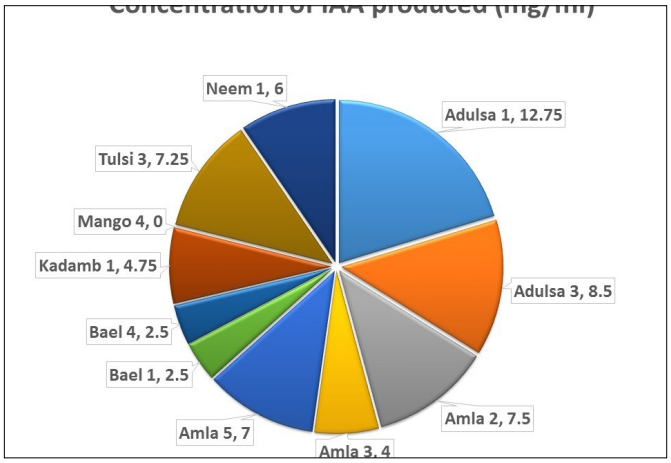

Fig. 1. Production of Indole Acetic Acid by endophytic *Pseudomonas* strains.

Adulsa 1 produced highest amount of IAA *i.e.* 12.75 µg/ml, followed by Adulsa 3, Amla 2, Tulsi 3, Neem 1 and Amla 5. Indole production was not detected in Bael and Mango isolates. The results indicate that these endophytic *Pseudomonas* strains have an important role to play in plant growth. Endophytic microorganisms play an important role in the plant growth by producing growth hormones, which are produced as secondary metabolites by the microorganisms. Production of plant hormones by endophytic bacteria has been reported in number of plants (5, 66). Indole acetic acid (IAA) is the main auxin in plants, controlling many important physiological processes including cell enlargement and division, tissue differentiation, and responses to light and gravity (67). Bacterial IAA producers contribute to the input of IAA into the plant auxin pool. Excessive production of IAA and cytokinins by *Pseudomonas savastanoi* results in knot or tumor development in the olive tree (6).

Antimicrobial activity of endophytic bacterial strains

The antagonistic activity of the endophytic *Pseudomonas* strains was checked and except for antifungal activity shown by Bael 4 and Kadamb 1 against *Fusarium* sp., no antagonism was seen against the other test cultures used. Fungi causes majority of infectious plant diseases. The plant diseases caused by fungi include all white and true rusts, smuts, needle casts, leaf curls, mildew, sooty molds, wood rots, wilts, galls etc. *Fusarium* species are responsible for the wilts, crown rot, stem rot etc in number of crops. Biological control of the fungal pathogens can be successfully done using endophytic bacteria. It was reported that the use of endophytic bacteria against *Fusarium* sp (19, 68). Antifungal and antibacterial activities of endophytic microorganisms has also been reported by number of workers from the identification of taxol producing endophytic fungi till date (69-74). Treatment of crops with endophytes prior to plantation reduces the incidence of infection by pathogens.

Aromatic compound utilization ability

The response of endophytic *Pseudomonas* strains to aromatic compounds is given in Table 2. All the strains, except Kadamb 4, Neem 1, Neem 4, Mango 1, Tulsi 1, grew in the presence of phenol. Similarly, in the case of resorcinol, the above cultures could not grow and utilize it along with Amla 2. Growth was observed after 4 days in phenol and resorcinol, showing that the cultures took time to get adapted to the aromatic compounds. Kadamb 4, Neem 1, Neem 4, Mango 1, Tulsi 1 and Mango 4 did not grow in the presence of tannic acid, while all other cultures showed good growth. Tryptophan, 1-naphthol and 2-nitrophenol supported growth of most of the strains. While 4-nitrophenol and sodium benzoate were toxic to most of the cultures at 0.1% concentration. Four cultures, namely, Adulsa 3, Amla 2, Kadamb 1, Tulsi 2 grew in the presence of 4-nitrophenol and only Amla 2, Mango 4 and Bael 9 could grow in benzoate medium. Six days of incubation at 28°C ± 2°C was required for growth in 4-nitrophenol and tannic acid. Whereas, growth was observed after 2 days of incubation in sodium benzoate and tryptophan. Among the endophytic strains used in the present study, Kadamb 4, Neem 1, Neem 4, Mango 1, Tulsi 1, could not utilize any of the aromatic compounds. The plants readily take up organic xenobiotic water-soluble volatile compounds present in the soil. These compounds enter the xylem and some of them are metabolized and transformed into more toxic volatile pollutants (75). Plants release these volatile pollutants and their metabolites into the environment by evaporation via the leaves, resulting in air pollution. Using engineered endophytic bacteria, studies on water-soluble volatile organic pollutants such as toluene demonstrated that endophytic bacterium possessing the aromatic degradation pathway protects its host plant against the phyto-

Table 2. Screening of endophytic *Pseudomonas* strain for degradation of aromatic compounds (0.1%) and presence of plasmid

Culture	Aromatic compound used for growth (0.1 %)								Presence of plasmid
	Phenol	1-Naphthol	4-Nitro phenol	2-Nitro phenol	Resorcinol	Sodium benzoate	Tryptophan	Tannic acid	
Adulsa 1	+	+	+	-	+	-	-	++	+
Adulsa 3	+	-	-	+	+	-	-	+	+
Amla 1	+	-	-	+	+	D	+++	++	+
Amla 2	+	-	-	-	-	+++	+	+	+
Amla 5	+	+	+	+	++	-	D	+++	+
Bael 1	+	+	D	+	+	-	D	+++	+
Bael 4	+	+	D	+	+	-	D	+++	+
Bael 7	++	+	-	+	+	-	D	++	-
Bael 8	+	+	-	-	+	+	+++	+++	-
Kadamb 1	+	+	++	+	+	-	++	++	+
Mango 4	+	-	D	-	++	+	++	-	-
Tulsi 3	+	-	++	+	+	-	-	+	+

Key: +++, Very good growth; ++, good growth; +, satisfactory growth; -, no growth; D, doubtful

toxic effects of an environmental contaminant (76). These endophytes protect the plant from toxicity caused by xenobiotic compounds by degrading these compounds.

Plasmid isolation and curing

Presence of plasmid has been detected in nine of the 13 strains that showed utilization of aromatic compounds (Table 1). It was observed that Neem 1, Mango 4 did not show the presence of plasmid, which explains its inability to utilize most of the aromatic compounds. However, Bael 7 and Bael 9 also did not show presence of plasmid but could utilize some aromatic compounds. The plasmid bearing cultures were cured using ethidium bromide (concentration range between 10 - 100 µg/ml) and upon curing, the aromatic degradation capacity was lost. Enzymes for aromatic degradation are plasmid encoded (77). Strains growing in the presence of aromatic compounds indicate the presence of plasmids and thus presence of enzymes of degradation pathway for the aromatic compounds. Plasmid curing results confirm that the enzymes for aromatic degradation are plasmid encoded. Reports are on the studies on selection of aromatic compound degrading endophytic bacteria by plants (3). Their studies showed that plants recruit bacteria that contain genes for toxic compound degradation. These help in protecting the plant from phytotoxic effects of the contaminants such as petroleum hydrocarbons and nitro aromatics by its mineralization.

Natural transfer of degradative plasmids to number of endophytes *inplanta* increase further increase the efficiency aromatic compound degradation in plants harboring these endophytes (78). Horizontal gene transfer (HGT) among the endophytes of the medicinal plants taken up in the present study need to be researched. The endophytic bacteria having degradation capacity can be used in the bioremediation of soil and water pollutants.

Conclusion

This study focused on the Gram negative isolates of medicinal plants and highlighted the multifunctional agricultural potential of endophytic isolates as assessed by their ability to produce plant growth hormone indole 3-acetic acid, to fix atmospheric nitrogen, to solubilize phosphates, to produce antagonistic compounds and to degrade aromatic compounds. All endophytic strains used in the study were diazotrophic with 80% of the strains showing phosphate solubilizing activity and 58% were IAA producers. More than 70% of the tested endophytic strains could degrade various aromatic compounds and 50% of these degraders were plasmid bearers. The potential prospects of finding multiple applications make the endophytic microbiome effective candidates not only for agriculture but also for industry and medicine.

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Authors contributions

CB hypothesized the concept and drafted the research article, VB carried out sample collection, carried out experiments, referencing and helped in writing the manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare that there is no conflict of interest.

Ethical issues: None

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