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Research Article

DIVERSITY AND ANTIMICROBIAL SPECTRUM OF ENDOPHYTIC BACTERIA ISOLATED FROM *PAEDERIA FOETIDA* L.

ARUNDHATI PAL*[1], APARNA CHATTOPADHYAY[2] AND A. K. PAUL[2]

[1]Department of Botany, Serampore College, 9, William Carey Road, Serampore, Hooghly, West Bengal 712201, [2]Microbiology Laboratory, Department of Botany, University of Calcutta, Ballygunge Circular Road, Kolkata 700019, India. Email: arundhatipalcu@gmail.com

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ABSTRACT

The pharmacologically important perennial shrub *Paederia foetida* L. (Rubiaceae), native to temperate and tropical Asia has been investigated for its endophytic bacterial diversity as well as evaluation of antimicrobial activity. A total of 20 phenotypically distinguishable bacterial endophytes were isolated from surface sterilized stem and leaf tissues. Shannon Weaver diversity index has clearly revealed more diverse (1.06) types of endophytes in leaves of *P. foetida* than in stem (0.68) and the bacterial isolates belonged to *Bacillus, Paenibacillus, Pseudomonas, Ralstonia, Micrococcus* and *Alcaligenes*. Physio-biochemical features of these isolates clearly indicated distinct variations in their enzymatic and sugar fermentation profiles along with NaCl tolerance. Antibiotic sensitivity profile, however, have shown that the isolates were mostly resistant to amoxicillin but were sensitive to streptomycin and tetracycline. Interestingly, the antimicrobial activity of the bacterial endophytes of *P. foetida* gives a definite stamp on the inhibitory effect of majority of the isolates against *Escherichia coli, Staphylococcus aureus* and *Klebsiella pneumonia*. This suggests that the bacterial endophytes of *P. foetida* could be a potential source for antimicrobial substances for biotechnological application.

Keywords: *Paederia foetida* L., Bacterial endophytes, Antimicrobial activity, Antibiotic sensitivity, Enzymatic profile, NaCl tolerance.

INTRODUCTION

Microbial endophytes are organisms which colonize living internal tissues of plants and live in a symbiotic or mutualistic relationship with the host. Endophytes are ubiquitous in all plant species on earth and contribute to their host plants by producing abundant metabolites that are harnessed for plant defense and endurance. Studies on interaction and functions of endophytes inside their hosts are significant in addressing the ecological relevance of endophytes [1]. Since they occupy unique biological niches, endophytes are viewed as an exceptional source of novel bioactive products for exploitation in medicine, agriculture and industry. To date, several antibacterial, antifungal, antiviral compounds, antioxidants, cytotoxic substances, etc. have been isolated from endophytes and it is believed that screening of newer endophytes for their functional roles is a promising way to overcome the increasing threat of drug resistant strains of human and plant pathogen [2, 3]. It has been rationalized that plants having an ethnobotanical history are likely candidates since the medical uses to which the plant may have been selected relates more to its population of endophytes than to the plant biochemistry itself [4].

Bacterial endophytes colonize inner tissues of plants in high numbers and are not usually present inside plant cells or surrounded by a membrane compartment [5]. They have been reported to produce plant growth promoting hormones (auxin, cytokinin and gibberellin); possess aminocyclopropane-1-carboxylate (ACC) deaminase activity (suppress stress-related ethylene synthesis); fix biological nitrogen or mobilize phosphorus [6]. Moreover, they confer plant protection against pathogens by inducing host defense mechanisms, producing pathogen-antagonistic substances or through competition for colonization sites and nutrients. The occurrence of endophytic bacteria in agricultural or medicinal plants have been reported quite extensively [7-9]. Archaea do not appear to be associated with plants. However, culture-independent methods for community analysis and functional genomics will provide a better understanding of community dynamics, signaling, and functions in endophyte–plant associations.

Paederia foetida L. (Rubiaceae) commonly known as skunkvine, is a perennial climbing shrub native to temperate and tropical countries of Asia. Therapeutically the plant is attributed in folk medicine as an antibacterial, anti-inflammatory and anti-diarrhoeal agent. The aerial parts of the plant rich in iridoid glucosides, methyl mercaptan, alkaloids, etc. are mainly used for gastrointestinal troubles including dyspepsia, flatulence, gastritis and enteritis. However, information on the diversity of endophytes in *P. foetida* is missing. With an elaborate pharmacological record, *P. foetida* was chosen for investigating the diversity of its bacterial endophytes and evaluate their antimicrobial activity.

MATERIALS AND METHODS

Collection of plant samples

Healthy plants of *Paederia foetida* L. (Rubiaceae) were collected from Medicinal Plant Garden of Serampore College, Hooghly and Medicinal Plant Garden of Ramkrishna Mission Ashram, Narendrapur, West Bengal, India in sterile zip lock polythene bags and brought immediately to the laboratory for isolation of bacterial endophytes.

Isolation and characterization of endophytes

Fresh leaves and stem segments were cut from the collected plants, washed thoroughly under running tap water and transferred to sterile glass bottles for surface sterilization. The samples were sterilized by consecutive immersion in 70% ethanol (2 – 3 min), 0.5 % sodium hypochlorite (5 -10 min) and again with 70% ethanol for 30 sec [8]. After washing for several times in sterile distilled water, the samples were cut into sections and plated aseptically on previously prepared nutrient agar, glycerol asparagine agar and tryptic soy agar plates for isolation of bacteria including actinomycetes. The plates were incubated at 30ºC for 2 – 4 days and observed for growth of bacterial colonies surrounding the leaf and stem sections. Pure cultures of bacterial endophytes were developed by dilution-streaking on the same media and characterized and identified following micromorphological and physio-biochemical analysis according to standard protocols [10, 11].

Data analysis

Colonization rate was calculated as the total number of plant segments infected by bacteria divided by the total number of segments incubated. Isolation rate was determined as the number of bacterial isolates obtained from plant samples divided by the total number of samples incubated. The Shanon Weaver diversity index H' was calculated as: $H' = -\Sigma\ Pi \times \ln Pi$, where, Pi is the proportion of individuals that species "i" contributes to the total [12].

Antibiotic sensitivity assay

Antibiotic sensitivity test was performed following the Kirby Bauer disc diffusion assay method [13] using antibiotic impregnated discs (6

mm dia) from Himedia (India). Based on the diameter of inhibition zone recorded to nearest mm, the organisms were categorized as resistant, intermediate and sensitive following DIFCO Manual 10th edition (1984). Antibiotics used include: Amoxycillin (30 µg/disc), Bacitracin (10 U/disc), Chloramphenicol (30 µg/disc), Erythromycin (15 µg/disc), Neomycin (30 µg/disc), Streptomycin (30 µg/disc) and Tetracycline (30 µg/disc).

Antimicrobial evaluation

Bacterial endophytes were primarily screened for production of antimicrobial substances following cross-steak method using six test organisms like *Bacillus cereus, B. subtilis, Escherichia coli, Pseudomonas cepacia, Klebsiella pneumonia* and *Staphylococcus aureus* [14]. Nutrient agar plates were inoculated with bacterial endophytes as a single streak at the centre of the Petri plate and incubated for 5 days at 30ºC. Overnight grown cultures of the test organisms were streaked at right angle to the producer bacterial endophyte and observed for its growth /inhibition after 24 – 48 h of incubation at 30ºC. The length of inhibition zone was measured to nearest mm.

RESULTS AND DISCUSSION

Colonization and isolation rate of endophytes

Segments of surface sterilized leaf and stem of *Paederia foetida* L. incubated on nutrient agar, glycerol asparagine agar and tryptic soy agar plates showed growth of morphologically distinguishable bacterial colonies surrounding the segments after 48-96 h. Avoiding the repetitive strains a total of 20 bacterial endophytes were isolated in pure form from 129 segments (70 leaf and 59 stem) of *P. foetida,* of which 12 and 8 isolates were obtained from leaf and stem segments respectively (Table 1).

Table 1: Diversity of endophytic bacteria in stem and leaf tissues of *Paederia foetida* L.

Parameter	Plant parts		
	Stem	Leaf	Total
Number of samples	59	70	129
Number of sample yielding isolates	18	20	38
Number of isolates	8	12	20
Colonization frequency, %	30.5	28.6	59.1
Isolation rate	0.13	0.17	0.3
Shannon Weaver diversity index	0.68	1.06	1.34

Colonization frequency was recorded low in leaf samples (28.6%) as compared to the stem (30.5%), while the isolation rate was 0.17 and 0.13 for leaf and stem respectively. The Shannon Weaver diversity index showed that leaves (1.06) of *P. foetida* harbour diverse types of endophytic bacteria than its stem (0.68). In contrast, Chinese medicinal plants *Eucommia ulmoides, Berberis poiretii,* and *Rhus potanini* showed high colonization frequency (47 – 63%) and isolation rates (0.7 – 0.9) of endophytic fungi which showed certain degree of host and tissue specificity [8].

Characterization of the isolates

The bacterial endophytes of *P. foetida* were characterized based on micromorphological (Table 2) and physio-biochemical features (Table 3). Out of 20 isolates 7 were Gram +ve (3 cocci and 4 rod) and 13 were Gram –ve rods. Filamentous forms were not detected in either leaf or stem samples. Ten out of 20 isolates were motile and few (25%) produced yellow to orange diffusible pigments during growth on tryptic soy agar. Enzyme profile of endophytic bacteria showed that while all endophytes produced catalase, 90% of them produced amylase. Few isolates showed production of lipase (60%), gelatinase (45%), nitrate reductase (40%) and indole (40%). The endophytes were also screened for their ability to utilize and ferment dextrose, fructose, maltose, sucrose and lactose in phenol red agar medium supplemented with 1% sugar. Glucose and fructose were best utilized by most of the isolates, while only 3 showed fermentation of lactose and maltose. Strikingly, the isolates showed a wide variation in NaCl tolerance, which ranges from 2.5 – 12% of NaCl.

Based on micromorphological and biochemical analysis, the isolates were tentatively identified as species of *Bacillus, Paenibacillus, Pseudomonas, Ralstonia, Micrococcus* and *Alcalignes*. Occurrence of similar endophytic bacterial genera has been reported from medicinal plants like *Gynura procumbens, Azadirachta indica, Boerhaavia diffusa* and *Phyllanthus emblica* [15, 16]. However, several authors have reported the presence of endophytic actinobacteria inside medicinal plants belonging to the genera *Streptomyces, Pseudonocardia* and *Promicromonospora* [17, 18].

Antibiotic sensitivity profile

Antibiotic susceptibility pattern of endophytic bacteria was studied against seven different antibiotics. Figure 1 showed that bacterial endophytes from leaf and stem of *P. foetida* were mostly resistant to amoxicillin, while they were highly susceptible to streptomycin and tetracycline. Out of 20 isolates, 10 and 9 bacteria showed intermediate response to bacitracin and neomycin respectively. One leaf isolate, PAL 103 showed resistance to five different antibiotics while intermediate response was exhibited against bacitracin and streptomycin. The occurrence of similar antibiotic resistance character in bacterial endophytes from *Andrographis paniculata* leaves demonstrated that antibiotic resistance genes might have transferred horizontally amongst environmental isolates [19].

Table 2: Micromorphological characteristics of bacteria isolated from stem and leaf tissues of *Paederia foetida* L.

Part	Isolate no.	Cell morphology	Gram nature	Motility	Size, µm	Endospore	Diffusible pigments
Stem	PAS 101	Cocci	G+ve	-	0.5 (dia)	-	-
	PAS 107	Rod	G+ve	-	0.8 X 0.3	+	-
	PAS 203	Rod	G-ve	-	0.8 x 0.4	-	-
	PAS 207	Rod	G-ve	-	0.7 X 0.4	-	-
	PAS 302	Rod	G+ve	+	0.8 x 0.5	+	Yellow
	PAS 303	Rod	G-ve	+	1.4 x 0.6	-	-
	PAS 309	Rod	G-ve	+	1.3 x 0.4	-	Orange
	PAS 311	Rod	G-ve	-	0.9 x 0.5	-	Yellow
Leaf	PAL 102	Rod	G-ve	+	0.9 x 0.5	-	-
	PAL 103	Rod	G+ve	-	0.9 x 0.6	+	-
	PAL 105	Rod	G-ve	-	0.6 x 0.3	-	Yellow
	PAL 106	Cocci	G+ve	+	0.4 (dia)	-	-
	PAL 110	Rod	G-ve	+	0.9 x 0.4	-	-
	PAL 111	Rod	G-ve	+	0.6 x 0.4	-	-
	PAL 201	Rod	G-ve	-	1.5 x 0.3	-	-
	PAL 203	Rod	G+ve	+	1.7 x 0.5	-	-
	PAL 206	Rod	G-ve	+	1.1 x 0.4	-	-
	PAL 207	Cocci	G+ve	+	0.5 (dia)	-	-
	PAL 208	Rod	G-ve	-	0.7 x 0.3	-	-
	PAL 209	Rod	G-ve	-	1.5 x 0.4	-	Orange

"+" indicate positive response, "-" indicate negative response

Colony morphology was detected in Tryptic soy agar medium after 5 days of growth in 32ºC.

Table 3: Physio biochemical characteristics of bacterial endophytes isolated from stem and leaf tissues of *Paederia foetida* L.

Plant Part	Isolate no.	Enzyme profile					Indole production	NaCl tolerance, %	Fermentation of sugars				
		Catalase	Amylase	Gelatinase	Lipase	Nitrate Reductase			Dextrose	Fructose	Lactose	Maltose	Sucrose
Stem	PAS 101	+	+	+	+	+	+	10	+	+	-	-	-
	PAS 107	+	+	+	+	+	+	3.5	+	-	+	-	-
	PAS 203	+	+	-	+	-	-	5	+	+	-	-	-
	PAS 207	+	+	+	-	+	-	4	+	-	-	-	-
	PAS 302	+	+	-	+	+	+	5	-	-	-	-	-
	PAS 303	+	+	-	-	-	-	10	+	+	-	-	-
	PAS 309	+	-	-	+	+	-	5	-	-	-	-	+
	PAS 311	+	+	+	+	+	+	2.5	+	+	-	-	-
Leaf	PAL 102	+	+	-	+	-	-	10	-	+	-	-	-
	PAL 103	+	+	+	-	-	-	12	+	-	-	-	-
	PAL 105	+	+	-	-	-	-	2.5	-	-	-	-	-
	PAL 106	+	+	+	+	-	-	3	+	+	-	+	-
	PAL 110	+	+	-	-	-	-	3	+	+	-	+	+
	PAL 111	+	+	+	+	-	-	10	-	+	-	-	+
	PAL 201	+	-	+	-	+	+	3.5	+	+	+	-	-
	PAL 203	+	+	-	-	-	+	10	+	-	+	-	-
	PAL 206	+	+	-	+	+	+	4	+	+	-	-	-
	PAL 207	+	+	-	+	-	+	11	+	+	-	+	+
	PAL 208	+	+	-	+	-	-	12	+	+	-	-	-
	PAL 209	+	+	+	-	-	-	12	-	-	-	-	+

"+" indicate positive response, "-" indicate negative response

Sodium chloride tolerance was tested in nutrient broth supplemented with sterile stock solution of NaCl.

Fermentation of sugars was screened in phenol red agar medium supplemented with 1% sugar.

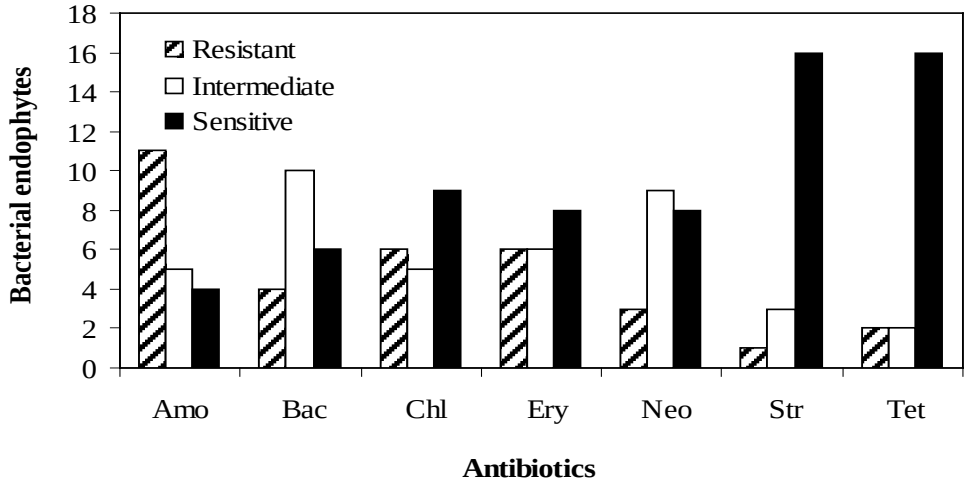

Fig. 1: Antibiotic sensitivity profile of bacterial endophytes from *Paederia foetida* L. (Antibiotics used are Amo = Amoxycillin; Bac = Bacitracin; Ery = Erythromycin; Chl = Chloramphenicol; Neo = Neomycin; Str = Streptomycin; Tet = Tetracycline)

Table 4: Evaluation of antimicrobial activity of bacterial endophytes of *Paederia foetida* L. following cross-streak method

Plant part	Isolate no.	Length of inhibition zone, mm					
		Test organisms					
		Bacillus cereus	***Bacillus subtilis***	***Pseudomonas cepacia***	***Escherichia coli***	***Klebsiella pneumoniae***	***Staphylococcus aureus***
Stem	PAS 101	-	-	-	-	3	-
	PAS 107	-	-	-	-	-	-
	PAS 201	3	-	-	-	3	-
	PAS 203	-	-	-	12	-	6
	PAS 302	-	-	8	7	-	-
	PAS 303	-	-	-	-	-	-
	PAS 309	-	-	-	10	-	5
	PAS 311	-	-	-	18	-	-
Leaf	PAL 102	-	-	13	-	4	3
	PAL 103	-	-	-	-	-	-
	PAL 105	-	5	3	8	6	5
	PAL 106	-	-	-	12	-	12
	PAL 110	10	6	12	3	8	-
	PAL 111	-	11	15	8	6	3
	PAL 201	-	-	-	15	-	12
	PAL 203	-	-	-	-	-	-
	PAL 206	-	-	-	14	-	14
	PAL 207	-	-	-	-	-	4
	PAL 208	4	5	-	-	10	5
	PAL 209	-	-	-	15	-	4

"-" means no inhibition

Evaluation of antimicrobial activity

Antimicrobial activity of the bacterial endophytes were assessed against six bacterial test organisms, *Bacillus cereus, B. subtilis, Escherichia coli, Pseudomonas cepacia, Klebsiella pneumoniae* and *Staphylococcus aureus* following cross-streak method. The isolate which inhibited growth of any of the test isolate(s) was considered having antibacterial activity and the length of inhibition zone was measured (Table 4). Out of 20 endophytes screened, majority showed antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* followed by *Klebsiella pneumoniae*. Three isolates (PAL 105, PAL 110 and PAL 111) obtained from leaf tissues showed antimicrobial activity against five test organisms. However, isolates PAS 107, PAS 303, PAL 103 and PAL 203 did not show any antimicrobial activity. Although numerous reports on the antimicrobial evaluation of endophytic fungi from medicinal plants have been presented [20-23], antimicrobial activity of endophytic bacteria are rare [17]. Li *et al*. [24] explored endophytic actinomycetes associated with pharmaceutical plants in rainforest of Yunnan, China and detected endophytic *Streptomyces* displaying antimicrobial activities against *S. aureus, E. coli and C. albicans*. Moreover, occurrence of antitumour and antimicrobial activities in these bacteria was confirmed through the presence of either polyketide synthases (PKS-I, PKS-II) or nonribosomal peptide synthetases (NRPS) sequences.

CONCLUSION

There was high diversity of endophytic bacterial isolates associated with leaves and stem of the medicinal plant, *Paederia foetida* and they differed significantly in their morphological, physiological and biochemical characters. Antimicrobial evaluation revealed that majority of the bacterial endophytes showed significant antibacterial activity against *Escherichia coli, Staphylococcus aureus* and *Klebsiella pneumonia*. Thus bacterial endophytes of traditional medicinal plants are promising sources of bioactive compounds which can be exploited for biotechnological application.

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