Research paper

Bacterial endophytes of the medicinal herb *Hygrophila spinosa* T. Anders and their antimicrobial activity

Arundhati Pal[1] * and A. K. Paul[2]

[1]Department of Botany, Serampore College, 9, William Carey Road, Serampore, Hooghly, West Bengal 712 201, India

[2]Microbiology Laboratory, Department of Botany, University of Calcutta, 35, Ballygunge Circular Road, Kolkata 700 019, India

ABSTRACT

Aims: The ethnobotanical herb *Hygrophila spinosa* T. Anders (Acanthaceae) is native to India and used in traditional ayurvedic medicines for its pharmacologically important phytochemicals. This study aims to determine the endophytic bacterial diversity of *H. spinosa* and also to evaluate their antimicrobial properties.

Methodology: Bacterial endophytes were isolated from healthy plant tissues following surface sterilization and plating on nutrient agar, glycerol asparagines agar and trptic soy agar. They were characterized physio - biochemically following standard microbiological and biochemical methods. The endophytes were screened for production of antimicrobial compounds following cross-streak assay against test strains *Bacillus subtilis, B. cereus, Escherichia coli, Pseudomonas cepacia, Klebsiella pneumonia* and *Staphylococcus aureus* on nutrient agar plates.

Results: Eleven phenotypically distinguishable bacterial endophytes were isolated from surface sterilized leaf, stem and root tissues and Shannon Weaver diversity index clearly revealed more diverse (0.83) types of endophytes in leaves than in stem (0.48) and root (0.41) tissues. Physio-biochemical features of the isolates clearly indicated distinct variation in their sugar fermentation profiles along with NaCl tolerance. The endophytes produced important enzymes like catalase, amylase, gelatinase, nitrate reductase and lipase as well as plant growth promoting indole acetic acid. The bacterial isolates belonged to the genera *Bacillus, Penibacillus, Pseudomonas* and *Ralstonia*. Antibiotic sensitivity profile, however, have indicated that the isolates were mostly resistant to amoxycillin, while they were highly susceptible to neomycin and tetracycline. Interestingly, the bacterial endophytes of *H. spinosa* give a definite stamp on their antimicrobial activity against *Escherichia coli* and *Klebsiella pneumonia* followed by *Staphylococcus aureus*. Two isolates (*Bacillus* HGS 202 and *Pseudmonas* HGR 302) obtained from stem and root segments showed antimicrobial activity against *Bacillus subtilis, B. cereus, E. coli, K. pneumonia* and *S. aureus*.

Conclusion: This suggests that the bacterial endophytes of *H. spinosa* could be a potential source of extracellular enzymes and antibacterial substances for biotechnological application.

Keywords: Hygrophila spinosa, Endophytic bacteria, Antibacterial activity, Antibiotic sensitivity, Enzyme profile, NaCl tolerance

Tel.: +91 9830118047
E-mail: arundhatipalcu@gmail.com

1. INTRODUCTION

Medicinal plants provide valuable therapeutic agents in traditional medicines which are used on a global level for resolving wide variety of human health hazards. *Hygrophila spinosa* T. Anders, belonging to the family Acanthaceae, is a promising medicinal herb mentioned in ancient ayurvedic literature having great economic potential. The plant is indigenous to the Indian subcontinent and is reported to contain phytosterols, fatty acids, polyphenols, proanthocyanins, alkaloids, flavonoids, terpenoids, vitamins, and glycosides as major chemical constituents. In traditional medicine, *H. spinosa* is used mainly for the treatment of hyperdipsia, vesical calculi, flatulence, diarrhea, dysentery, leukorrhea, gonorrhea, asthma, blood diseases, gastric problems, cancer, rheumatism, etc. Many essential phytochemicals isolated from the whole plant including lupeol, stigmasterol, apigenin-7-O-glucuronide, apigenin-7-oglucoside, betulin, 25-oxo-hentriacontanyl acetate, methyl 8-*n*-hexyltetracosanoate, oleic acid, linoleic acid, etc. have exhibited antitumour, antibacterial, antidiabetic, antiinflamatory, antipyretic, antioxidant and hepatoprotective activity [1, 2].

It has been rationalized that plants having an ethnobotanical history and exploited for human use in traditional medicine may harbor an endophytic population which may produce a plethora of microbial metabolites related closely to the plant biochemistry [3]. Endophytes, by definition, are microorganisms colonizing living internal tissues of plant either symbiotically or in mutualistic relationship. They occur ubiquitously in all plant species on earth and indirectly benefit plant growth by producing abundant secondary metabolites which prevent the growth or activity of plant pathogens. It is believed that interactions of endophytes with the host plant significantly address their ecological relevance as plant growth promoting agents and endurance of defense mechanism [4]. Recent researches have proven that microbial endophytes are a new and potential source of novel natural products possessing antimicrobial, antifungal, antiviral compounds, antioxidants, cytotoxic activities, etc. for exploitation in modern medicine, agriculture and industry [5, 6].

It is believed that screening for antimicrobial compounds from endophytes is a promising way to overcome the increasing threat of drug resistant strains of human and plant pathogen. Antimicrobial metabolites isolated from endophytes belong to diverse structural classes, including: alkaloids, peptides, steroids, terpenoids, phenols, quinones, and flavonoids. The occurrence of endophytic bacteria in agricultural or medicinal plants has been reported quite extensively [7-9]. A comparison of different endophytic hosts shows that nearly 35% of the endophytes possessing antimicrobial activity have been isolated from medicinal plants followed by 29% from agricultural crops [6]. The diversity and ecological distribution of fungal endophytes associated with different medicinal plants native to China, Malaysia, Australia and India have been investigated with special emphasis on their antimicrobial efficacy. A mass of bioactive natural products isolated from endophytes have been reported in recent years and majority of them have been derived endophytic fungi [3, 7, 10, 11]. However, little information is available on the occurrence as well as potential significance of bacterial endophytes from medicinal plants. Although, medicinal properties of *H. spinosa* have been studied in details by many researchers, reports on the endophytic population of this medicinal herb is lacking. Our present study focuses attention towards isolation, characterization and antimicrobial evaluation of bacterial endophytes from *H. spinosa*.

2. MATERIALS AND METHODS

2.1 Collection of plant samples

Healthy plants of *Hygrophila spinosa* T. Anders (Acanthaceae) were collected from Medicinal Plant Garden of Serampore College, Hooghly, West Bengal and Department

Tel.: +91 9830118047
E-mail: arundhatipalcu@gmail.com

of Botany, University of Calcutta, Kolkata in sterile zip lock polythene bags. The collected plants were brought immediately to the laboratory and stored at 4°C until used for the isolation of bacterial endophytes.

2.2 Isolation and characterization of endophytes

Fresh leaf, stem and root segments were cut from the collected plants, washed thoroughly under running tap water. Surface sterilization was performed in sterile glass bottles by consecutive immersion in 70% ethanol (2 – 3 min), 0.5 % sodium hypochlorite (5 -10 min) and again in 70% ethanol for 30 sec [7](Sun *et al*., 2008). This was followed by repeated washing of plant samples in sterile distilled water for at least three times. Samples were blot dried on sterile towels and cut aseptically into small sections before plating 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, stem and root sections. Pure cultures of bacterial endophytes were developed by dilution-streaking on the same media and maintained on slopes of nutrient agar for further study. Bacterial strains were characterized and identified following micromorphological and physio-biochemical analysis following standard protocols [12, 13].

2.3 Diversity of endophytes

Based on the total number of samples plated and the number of samples yielding isolates, colonization frequency and isolation rate was calculated. Colonization frequency was calculated as the total number of plant samples infected by bacteria divided by the total number of samples incubated. Isolation rate was determined as the number of bacterial isolates obtained from plant samples divided by the total number of samples incubated. The Shannon Weaver biodiversity index H' was calculated as: $H' = -\Sigma\ Pi$ X ln Pi, where, Pi is the proportion of individuals that species "*i*" contributes to the total [14].

2.4 Antibiotic susceptibility spectrum

Antibiotic sensitivity test was performed following the Kirby Bauer disc diffusion assay method [15] using antibiotic impregnated discs (6 mm diameter) 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), Neomycin (30 µg/disc), Streptomycin (30 µg/disc) and Tetracycline (30 µg/disc).

2.5 Production of antimicrobial substances

Bacterial endophytes were primarily screened for production of antimicrobial substances following cross-streak assay method using six test organisms like *Bacillus subtilis, B. cereus, Escherichia coli, Pseudomonas cepacia, Klebsiella pneumonia* and *Staphylococcus aureus* [16]. Nutrient agar plates were inoculated with bacterial endophytes as a single streak at the centre of the Petriplate and incubated for 5 days at 30ºC. Overnight grown cultures of the test organisms were streaked at right angle to the producer 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.

Tel.: +91 9830118047
E-mail: arundhatipalcu@gmail.com

3. RESULTS AND DISCUSSION

3.1 Diversity of bacterial endophytes

Segments of surface sterilized leaf, stem and root of *Hygrophila spinosa* T. Anders (Acanthaceae) 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 11 bacterial endophytes were isolated in pure form from 118 segments (39 leaf, 39 stem and 40 root) of *H. spinosa.* Out of these 11 isolates, 6 were derived from leaf, while stem and root segments yielded 3 and 2 isolates respectively (Table 1). Colonization frequencies was recorded low in leaf samples (17.9%) as compared to the stem (20.5%) and root (22.5%), while the isolation rate was poor in root (0.05) but increased gradually in stem (0.07) and leaf (0.15) samples.

The Shannon Weaver diversity index showed that leaves (0.83) of *H. spinosa* harbour diverse types of endophytic bacteria than in its stem (0.48) and root (0.41). Similar observations were also recorded in case of bacterial endophyte diversity from leaves and stem segments of the medicinal herb *Paederia foetida* [17]. In contrast, the colonization of endophytic fungi in Chinese medicinal plants *Eucommia ulmoides, Berberis poiretii,* and *Rhus potanini* showed high degree of host and tissue specificity [7].

Table 1. Diversity of endophytic bacterial isolates in leaf, stem and root tissues of *Hygrophila spinosa* T. Anders

Sl. No.	Parameters	Plant tissue			Total
		Leaf	Stem	Root	
1	Number of samples	39	39	40	118
2	Number of sample yielding isolates	07	08	09	24
3	Number of isolates	06	03	02	11
4	Colonization Frequency, % [a]	17.9	20.5	22.5	20.3
5	Isolation Rate [b]	0.15	0.07	0.05	0.09
6	Shannon Weaver Diversity Index [c]	0.83	0.48	0.41	0.68

[a] Colonization frequency was calculated as the total number of plant samples infected by bacteria divided by the total number of samples incubated.

[b] Isolation rate was calculated as the number of bacterial isolates obtained from plant samples divided by the total number of samples incubated.

[c] Shannon Weaver diversity index H' was calculated as: $H' = -\Sigma\ Pi\ X\ ln\ Pi$, where, Pi is the proportion of individuals that species "i" contributes to the total

3.2 Characterization of isolates

The bacterial endophytes of *H. spinosa* were characterized based on micromorphological (Table 2) and physio-biochemical characters (Table 3). Out of 11 isolates 7 were Gram +ve (3 cocci and 4 rod) and 4 were Gram –ve (all rod). Filamentous forms were not detected in any of the plant samples. Six isolates out of 11 showed motility and only 3 produced yellowish to green diffusible pigments during growth on tryptic soy agar plates. Only three Gram-positive isolates showed endospore formation.

Tel.: +91 9830118047
E-mail: arundhatipalcu@gmail.com

Table 2. Micromorphological characteristics of bacteria isolated from leaf, stem and root tissues of *Hygrophila spinosa* T. Anders

Tissue	Isolate no.	Cell morphology	Gram nature	Motility	Size, µm	Endospore	Diffusible pigments
Leaf	HGL 101	Cocci, single	+ve	-	0.5 dia	-	-
	HGL 102	Cocci, in chain	+ve	-	0.4 dia	-	Yellow
	HGL 103	Short rod	-ve	+	0.4 X 0.3	-	Green
	HGL 104	Rod, single	+ve	+	1.1 X 0.3	+	-
	HGL 105	Short rod	+ve	-	0.5 X 0.4	-	-
	HGL 106	Short rod	-ve	+	0.5 X 0.3	-	-
Stem	HGS 201	Rod, in chain	+ve	+	1.1 X 0.5	+	-
	HGS 202	Rod, single	+ve	+	0.8 X 0.4	-	-
	HGS 203	Cocci, single	+ve	-	0.5 dia	-	Yellow
Root	HGR 301	Short rod	-ve	+	0.5 X 0.4	+	-
	HGR 302	Short rod	-ve	-	0.5 X 0.4	-	-

"+" indicate positive response, "-" indicate negative response
Colony morphology was detected in Tryptic soy agar medium after 5 days of growth in 32ºC.

Enzyme profile of endophytic bacteria showed that while all endophytes produced catalase, 6 and 7 isolates out of 11 produced amylase and gelatinase respectively. Few isolates showed production of lipase, nitrate reductase and indole. Strikingly, the isolates showed a wide variation in NaCl tolerance, which ranges from 2.5 – 10% of NaCl. 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 (Table 4). While dextrose is the best carbohydrate utilized by all the bacterial endophytes, lactose was fermented by only two isolates.

Table 3. Biochemical characterization of bacterial endophytes from leaf, stem and root tissues of *Hygrophila spinosa* T. Anders

Plant tissue	Isolate no.	Enzyme profile					Indole	NaCl, %
		Catalase	Amylase	Gelatinase	Lipase	NO_3 Reductase		
Leaf	HGL 101	+	+	+	+	-	-	10.0
	HGL 102	+	-	+	-	-	-	10.0
	HGL 103	+	-	+	+	-	+	3.5
	HGL 104	+	-	+	-	+	-	4.0
	HGL 105	+	-	-	+	+	+	4.0
	HGL 106	+	-	-	-	-	-	4.5
Stem	HGS 201	+	+	-	+	-	-	4.0
	HGS 202	+	+	+	-	-	-	4.0
	HGS 203	+	+	+	-	+	-	10.0
Root	HGR 301	+	+	+	+	-	+	3.0
	HGR 302	+	+	-	+	+	-	2.5

"+" indicate positive response, "-" indicate negative response
NaCl tolerance was tested in nutrient broth supplemented with sterile stock solution of NaCl.

Tel.: +91 9830118047
E-mail: arundhatipalcu@gmail.com

Based on microscopic and biochemical analysis the isolates were tentatively identified as species of *Bacillus, Penibacillus, Pseudomonas* and *Ralstonia*. Occurrence of similar endophytic bacterial genera have been reported from medicinal plants like *Gynura procumbens, Azadirachta indica, Boerhaavia diffusa, Phyllanthus emblica, Paederia foetida* etc. [17-20]. However, several authors have reported the presence of endophytic actinobacteria inside medicinal plants belonging to the genera *Streptomyces, Pseudonocardia, Promicromonospora, etc*. [21, 22].

Table 4. Fermentation of sugars by bacterial endophytes isolated from leaf, stem and root tissues of *Hygrophila spinosa* T. Anders

Plant tissue	Isolate no.	Fermentation of sugars				
		Dextrose	Fructose	Lactose	Maltose	Sucrose
Leaf	HGL 101	+	+	-	+	+
	HGL 102	+	+	-	-	-
	HGL 103	+	-	-	-	-
	HGL 104	+	+	-	-	+
	HGL 105	+	+	-	+	+
	HGL 106	-	-	+	-	-
Stem	HGS 201	+	-	-	-	-
	HGS 202	+	+	-	-	+
	HGS 203	+	+	+	+	+
Root	HGR 301	+	+	-	+	-
	HGR 302	+	-	-	-	-

"+" indicate positive response, "-" indicate negative response
Fermentation of sugars was screened in phenol red agar medium supplemented with 1% sugar.

3.3 Antibiotic sensitivity profile

Antibiotic sensitivity pattern of the endophytic bacterial isolates was studied against six different antibiotics like amoxycillin, bacitracin, chloramphenicol, neomycin, streptomycin and tetracyclin. Results as shown in Table 5 depicts that bacterial endophytes from leaf, stem and root tissues of *H. spinosa* were mostly resistant to amoxycillin, while they were highly susceptible to neomycin and tetracyclin. One leaf isolate, HGL 101 showed resistance to five different antibiotics while susceptibility against tetracycline only. The occurrence of similar antibiotic resistance character in bacterial endophytes from *Andrographis paniculata* and *Paederia foetida* leaves demonstrated that antibiotic resistance genes might have transferred horizontally amongst endophytes and plant hosts [17, 23].

Tel.: +91 9830118047
E-mail: arundhatipalcu@gmail.com

Table 5. Screening of bacterial endophytes from *H. spinosa* for their antibiotic susceptibility following disc-diffusion assay

Plant tissue	Isolate no.	Diameter of inhibition zone, mm					
		Antibiotics					
		Amoxycillin	Bacitracin	Chloramphenicol	Neomycin	Streptomycin	Tetracyclin
Leaf	HGL 101	08 (R)	NI (R)	9.5 (R)	12 (R)	11 (R)	40 (S)
	HGL 102	14 (I)	12 (I)	22 (S)	20 (S)	32 (S)	10 (R)
	HGL 103	22 (S)	14 (S)	NI (R)	22 (S)	32 (S)	NI (R)
	HGL 104	23 (S)	NI (R)	26 (S)	18 (S)	18 (I)	19 (S)
	HGL 105	14 (I)	12 (I)	18 (S)	24 (S)	30 (S)	26 (S)
	HGL 106	11 (R)	12 (I)	18 (S)	28 (S)	36 (S)	44 (S)
Stem	HGS 201	25 (S)	13 (S)	14 (I)	20 (S)	27 (S)	24 (S)
	HGS 202	20 (S)	16 (S)	17 (I)	16 (I)	32 (S)	20 (S)
	HGS 203	09 (R)	NI (R)	9.5 (R)	21 (S)	NI (R)	19 (S)
Root	HGR 301	7.5 (R)	NI (R)	26 (S)	14 (I)	NI (R)	20 (S)
	HGR 302	11 (R)	08 (R)	21 (S)	16 (I)	25 (S)	22 (S)

NI=No inhibition, R=Resistant, I=Intermediate, S=Sensitive

Antibiotic susceptibility was tested on nutrient agar plates using antibiotic impregnated discs (6 mm) from HIMEDIA, India

Tel.: +91 9830118047
E-mail: arundhatipalcu@gmail.com

3.4 Evaluation of antimicrobial activity

Antimicrobial activity of all eleven bacterial endophytes were assessed against six bacterial test organisms, *Bacillus subtilis, B. cereus, Escherichia coli, Pseudomonas cepacia, Klebsiella pneumoniae* and *Staplycoccus aureus* following cross-streak method on nutrient agar plates. 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 6). Out of 11 endophytes screened, majority showed antibacterial activity against *Escherichia coli* and *Klebsiella pneumoniae* followed by *Staphylococcus aureus*. Two isolates (*Bacillus* HGS 202 and *Pseudmonas* HGR 302) obtained from stem and root tissues showed antimicrobial activity against five test organisms. However, isolates HGL 102 and HGL 103 did not possess antimicrobial activity. Although numerous reports on the antimicrobial evaluation of endophytic fungi from medicinal plants have been presented [24-26], antimicrobial activity of endophytic bacteria are rare [17, 18, 23]. Li *et al*. [27], however, have 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 non-ribosomal peptide synthetases (NRPS) sequences.

Table 6. Evaluation of antimicrobial activity of bacterial endophytes of *Hygrophila spinosa* following cross-streak method

Plant tissue	Isolate no.	Length of inhibition zone, mm					
		Test organisms					
		Bacillus subtilis	***Bacillus cereus***	***Pseudomonas cepacia***	***Escherichia coli***	***Klebsiella pneumoniae***	***Staphlococcus aureus***
Leaf	HGL 101	-	-	-	20	10	-
	HGL 102	-	-	-	-	-	-
	HGL 103	-	-	-	-	-	-
	HGL 104	-	-	-	20	10	-
	HGL 105	-	-	5	-	-	5
	HGL 106	-	-	-	-	5	-
Stem	HGS 201	-	-	-	20	20	-
	HGS 202	1	1	3	6	-	3
	HGS 203	-	-	-	20	8.5	8
Root	HGR 301	-	-	-	20	5	-
	HGR 302	4	2	-	20	5	3

"-" means no inhibition

Tel.: +91 9830118047
E-mail: arundhatipalcu@gmail.com

4. CONCLUSION

There was high diversity of endophytic bacterial isolates associated with leaves, stem and root of the medicinal plant, *Hygrophila spinosa* 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,* and *Klebsiella pneumonia*. Thus bacterial endophytes of traditional medicinal plants appear to be promising sources of bioactive compounds which can be further exploited for biotechnological applications.

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Tel.: +91 9830118047
E-mail: arundhatipalcu@gmail.com

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Tel.: +91 9830118047
E-mail: arundhatipalcu@gmail.com