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 isolate and characterize the culturable bacterial endophytes of *H. spinosa* and evaluate their antimicrobial properties.
Place and Duration of Study: The experiments were performed in the Department of Botany, Serampore College, Serampore as well as in the Microbiology Laboratory, Department of Botany, University of Calcutta, Kolkata during 2011 to 2012.
Methodology: Bacterial endophytes were isolated from healthy plant tissues following surface sterilization and plating on nutrient agar, glycerol asparagine agar and tryptic 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 pneumoniae* 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. The bacterial isolates belonged to the genera *Bacillus, Paenibacillus, Pseudomonas, Ralstonia, Staphylococcus, Micrococcus* and *Acidomonas*. Antibiotic sensitivity profile, however, have indicated that the isolates were mostly resistant to amoxycillin and bacitracin, while they were highly susceptible to tetracycline followed by neomycin and streptomycin. Interestingly, the bacterial endophytes of *H. spinosa* give a definite stamp on their antimicrobial activity against *E. coli* and *K. pneumoniae* followed by *S. aureus*. Two isolates, *Paenibacillus* HGS 202 and *Acidomonas* HGR 302 obtained from stem and root segments respectively showed antimicrobial activity against *B. subtilis, B. cereus, E. coli, K. pneumoniae* and *S. aureus*.
Conclusion: This study identified 11 bacterial endophytes harbored by the leaves, stem and root of *H. spinosa* which demonstrated antibacterial activity against Gram-positive as well as Gram-negative bacterial strains. Moreover these endophytic bacterial isolates could be exploited as sources of antibacterial substances.

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 helping with a wide variety of human health issues. *Hygrophila spinosa* T. Anders, belonging to the family Acanthaceae, is a promising medicinal herb mentioned in ancient ayurvedic literature as 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-O-glucoside, betulin, 25-oxo-hentriacontanyl acetate, methyl 8-*n*-hexyltetracosanoate, oleic acid, linoleic acid, etc. have exhibited antitumor, 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 benefit the host plant growth by fixation of atmospheric nitrogen, production of growth promoting substances, imparting effective disease management, plant protection and stress tolerance [4]. In addition recent studies have established that secondary metabolites elaborated by these microbial endophytes could serve as prospective resources of antimicrobial substances, antioxidants, cytotoxic compounds, growth hormones and hydrolytic enzymes of biotechnological applications [5, 6].

In view of the increasing prevalence of antibiotic-resistant human and plant pathogens, there is an escalating demand for newer antimicrobials from natural sources. Bacterial and fungal endophytes residing inside the healthy plant tissues are believed to carry out a resistance mechanism to overcome pathogenic attack and have emerged as a promising source of newer antimicrobial compounds. Several antimicrobial metabolites belonging to structural classes like alkaloids, peptides, benzopyranones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, xanthones, and others have been obtained from endophytes. 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 from endophytic fungi [3, 7, 10, 11]. However, little information is available on the occurrence as well as on the potential significance of bacterial endophytes from medicinal plants. Although, medicinal properties of *H. spinosa* have been studied in details by many researchers [1, 2], reports on the endophytic population of this medicinal herb is lacking. Biodiversity of both culturable and unculturable endophytic microbial communities of *H. spinosa*, therefore, needs to be determined. However, culturable endophytic bacterial isolates deserve special attention for further development of microbial-based biotechnological products and formulations. In the present study, we focused on the isolation, characterization and antimicrobial evaluation of bacterial endophytes from *H. spinosa*.

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

2. MATERIAL 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 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 and healthy 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]. 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. 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 were 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 follows: $H' = -\Sigma\ Pi \times \ln Pi$, where, Pi is the proportion of individuals that species "i" contributes to the total [7, 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: *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 Petri plate 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

3.1 Diversity of bacterial endophytes

Segments of surface sterilized leaf, stem and root of *Hygrophila spinosa* (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 phenotypically distinguishable 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, six were derived from leaf, while stem and root segments yielded three and two isolates respectively (Table 1). The colonization frequency was lower 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* harbor more diverse types of endophytic bacteria than in its stem (0.48) and root (0.41).

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

Parameters	Plant tissue			Total
	Leaf	Stem	Root	
Number of samples	39	39	40	118
Number of sample yielding isolates	07	08	09	24
Number of isolates	06	03	02	11
Colonization Frequency, % [a]	17.9	20.5	22.5	20.3
Isolation Rate [b]	0.15	0.07	0.05	0.09
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 / = -Σ Pi X ln Pi, where, Pi is the proportion of individuals that species "i" contributes to the total [7, 14].

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

Tissue	Isolate no.	Cell morphology	Gram nature	Motility	Size, µm	Endospore	Diffusible pigments
Leaf	HGL 101	cocci, in cluster	positive	non-motile	0.5 Ø	absent	none
	HGL 102	cocci, single	positive	non-motile	0.4 Ø	absent	yellow
	HGL 103	short rod	negative	motile	0.4 x 0.3	absent	green
	HGL 104	rod, single	positive	motile	1.1 x 0.3	present	none
	HGL 105	short rod	positive	non-motile	0.5 x 0.4	present	none
	HGL 106	short rod	negative	motile	0.5 x 0.3	absent	none
Stem	HGS 201	rod, in chain	positive	motile	1.1 x 0.5	present	none
	HGS 202	rod, single	positive	motile	0.8 x 0.4	present	none
	HGS 203	cocci, single	positive	non-motile	0.5 Ø	absent	yellow
Root	HGR 301	short rod	negative	motile	0.5 x 0.4	absent	none
	HGR 302	short rod	negative	non-motile	0.5 x 0.4	absent	none

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

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

3.2 Characterization and identification 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, seven were Gram-positive (three cocci and four rod) and four were Gram-negative (all rod). Filamentous forms were not detected in any of the plant samples. Six isolates out of 11 showed motility and only three produced yellowish to green diffusible pigments during growth on tryptic soy agar plates. All Gram-positive rods showed endospore formation.

Enzymatic profile of endophytic bacterial isolates showed that all of them produced catalase, while about 55 and 64% of the isolates produced amylase and gelatinase respectively (Table 3). Lipolytic (55%) and nitrate reductase (36%) activities were not uncommon amongst the endophytic isolates. Production of indole by the enzyme tryptophanase was evident only in isolates HGL 103, HGL 105 and HGR 301. The isolates showed wide degree of tolerance to NaCl (2.5 – 10%) in the growth medium. 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 was the best carbohydrate utilized by all most all the bacterial endophytes, lactose was fermented by only two isolates. The endophytic isolates were moderate in fermenting fructose, sucrose and maltose.

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

Plant tissue	Isolate no.	Enzyme profile					Indole production	NaCl tolerance, %
		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

"+" presence; "-" absence

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

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.

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

Based on microscopic and biochemical analysis, the bacterial isolates were tentatively identified as species of *Bacillus* (HGL 104, HGS 201), *Paenibacillus* (HGL 105, HGS 202), *Pseudomonas* (HGL 103, HGR 301), *Ralstonia* (HGL 106), *Staphylococcus* (HGL 101), *Micrococcus* (HGL 102, HGS 203) and *Acidomonas* (HGR 302).

3.3 Antibiotic sensitivity profile

Antibiotic sensitivity pattern of the endophytic bacterial isolates was determined by disc-diffusion method against six different antibiotics (amoxycillin, bacitracin, chloramphenicol, neomycin, streptomycin and tetracycline). Results as shown in Table 5 depict that bacterial endophytes from leaf, stem and root tissues of *H. spinosa* were mostly resistant to amoxycillin and bacitracin, while they were mostly sensitive to tetracycline followed by neomycin and streptomycin. One leaf endophyte, *Staphylococcus* HGL 101 was highly resistant to five antibiotics and was followed by *Micrococcus* HGS 203 showing resistance to four of the six tested antibiotics. On the contrary, the isolates from leaf and stem (*Paenibacillus* HGL 105, *Bacillus* HGS 201 and *Paenibacillus* HGS 202) showed sensitive to intermediate response towards all the tested antibiotics.

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

Plant tissue	Isolate	Diameter of inhibition zone, mm					
		Antibiotics					
		Amoxycillin	Bacitracin	Chloramphenicol	Neomycin	Streptomycin	Tetracycline
Leaf	*Staphylococcus* HGL 101	08 (R)	0 (R)	9.5 (R)	12 (R)	11 (R)	40 (S)
	Micrococcus HGL 102	14 (I)	12 (I)	22 (S)	20 (S)	32 (S)	10 (R)
	Pseudomonas HGL 103	22 (S)	14 (S)	0 (R)	22 (S)	32 (S)	0 (R)
	Bacillus HGL 104	23 (S)	0 (R)	26 (S)	18 (S)	18 (I)	19 (S)
	Paenibacillus HGL 105	14 (I)	12 (I)	18 (S)	24 (S)	30 (S)	26 (S)
	Ralstonia HGL 106	11 (R)	12 (I)	18 (S)	28 (S)	36 (S)	44 (S)
Stem	*Bacillus* HGS 201	25 (S)	13 (S)	14 (I)	20 (S)	27 (S)	24 (S)
	Paenibacillus HGS 202	20 (S)	16 (S)	17 (I)	16 (I)	32 (S)	20 (S)
	Micrococcus HGS 203	09 (R)	0 (R)	9.5 (R)	21 (S)	0 (R)	19 (S)
Root	*Pseudomonas* HGR 301	7.5 (R)	0 (R)	26 (S)	14 (I)	0 (R)	20 (S)
	Acidomonas HGR 302	11 (R)	08 (R)	21 (S)	16 (I)	25 (S)	22 (S)

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, *B. subtilis, B. cereus, E. coli, P. cepacia, K. pneumoniae* and *S. 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 *E. coli* and *K. pneumoniae* followed by *S. aureus*. Isolates *Paenibacillus* HGS 202 and *Acidomonas* HGR 302 obtained from stem and root tissues respectively showed comparatively broad spectrum of antibacterial activity inhibiting both Gram-positive and Gram-negative test organisms.

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

Plant tissue	Isolate	Length of inhibition zone, mm					
		Test organisms					
		Bacillus subtilis	***Bacillus cereus***	***Pseudomonas cepacia***	***Escherichia coli***	***Klebsiella pneumoniae***	***Staphylococcus aureus***
Leaf	*Staphylococcus* HGL 101	-	-	-	20	10	-
	Micrococcus HGL 102	-	-	-	-	-	-
	Pseudomonas HGL 103	-	-	-	-	-	-
	Bacillus HGL 104	-	-	-	20	10	-
	Paenibacillus HGL 105	-	-	5	-	-	5
	Ralstonia HGL 106	-	-	-	-	5	-
Stem	*Bacillus* HGS 201	-	-	-	20	20	-
	Paenibacillus HGS 202	1	1	3	6	-	3
	Micrococcus HGS 203	-	-	-	20	8.5	8
Root	*Pseudomonas* HGR 301	-	-	-	20	5	-
	Acidomonas HGR 302	4	2	-	20	5	3

"-" means no inhibition zone produced

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

DISCUSSION

Studies on the diversity of culturable microbial endophytes in medicinal and vegetative crop plants are essential to understand their potentials and importance in different fields of biotechnology. This study is the first attempt to isolate microbial endophytes from the traditional medicinal herb *H. spinosa*. We have screened only the medicinally important plant organs like root, stem and leaf of *H. spinosa*, although endophytes could also occur in flower, fruit and seeds. The leaves of *H. spinosa* were found to harbor more diverse types of bacterial endophytes than stem or root segments (Table 1). Such a higher species richness in leaves may be attributed to their anatomical and micro-environmental peculiarities, as specific conditions in essential nutrients drive the survival of tissue specific endophytic taxa. Similar prevalence of endophytes in leaf tissues have been observed in *Paederia foetida* [17], *Kigelia pinnata* [18] and *Quercus ilex* [19].

Spatial distribution of endophytic genera also depends on seasonal variation, precipitation, soil parameters and location of plants, plant age and genotypes [4]. Here, we have tested only one genotype from cultivated soil of two different localities which does not reflect the true portrait of culturable endophyte diversity of *H. spinosa*. The phenotypically distinguishable bacterial endophytes harbored by leaves, stem and root tissues of *H. spinosa* were characterized in details (Tables 2 - 4) and tentatively identified as members of the bacterial genera *Bacillus*, *Paenibacillus, Pseudomonas, Ralstonia*, *Staphylococcus, Micrococcus* and *Acidomonas*. These isolates belong to a class of fast growing endophytes and were also reported to colonize several other host plants. Occurrences of similar endophytic bacterial genera have been reported from medicinal plants like *Gynura procumbens, Azadirachta indica, Boerhaavia diffusa, Phyllanthus emblica, P. foetida* etc. [17, 20-22]. In addition, several authors have reported the presence of endophytic actinobacteria inside medicinal plants belonging to the genera *Streptomyces, Pseudonocardia, Promicromonospora, etc*. [23, 24]. However, such filamentous forms have not been recorded during the present study.

Information pertaining to the production of enzymes by microbes of plant origin is few. Endophytic bacteria isolated from leaves of maize [25], leaves and stem of *Jacaranda decurrens* [26], roots of *Chlorophytum borivilianum* [27] and leaves of mangrove plants [28] have been reported to produce hydrolytic enzymes of diverse types. All the aerobic endophytic isolates of *H. spinosa* possessed catalase responsible for the decomposition of hydrogen peroxide to less reactive oxygen and water molecules. Production of hydrolytic enzymes, gelatinase, amylase and lipase (Table 3) also supports earlier observations on production of such enzymes by bacterial endophytes of maize, *Jacaranda, Chlorophytum, etc.* [25-28]. The presence of nitrate reductase and tryptophanase in some of the isolates suggests they play a key role in the nitrogen cycle, thereby having important agricultural, environmental and public health implications. The emergence of antibiotic resistance is not only limited to pathogenic microorganisms but also found amongst environmental isolates as a result of horizontal transfer of antibiotic resistance genes. Majority of the endophytes from *H. spinosa* showed resistance to amoxycillin and bacitracin (Table 5) similar to those encountered in bacterial endophytes of *P. foetida* [17], *Andrographis paniculata* [29] and mangrove plants [28].

In view of the ever increasing demand for novel antimicrobial substances, the endophytes have been identified as a potential source of antibiotics [6]. Several reports on the antimicrobial evaluation of endophytic fungi from medicinal plants have been presented [30-32]. Furthermore, antimicrobial activities of endophytic bacteria are not uncommon [17, 20, 29]. Li *et al*. [30] 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*. In the present study, nine bacterial endophytes out of 11 from *H. spinosa* showed antibacterial activity against *B. subtilis, B. cereus, E. coli, P. cepacia, K. pneumoniae* and *S. aureus* following cross-streak assay (Table 6) and two of them showed broad spectrum antimicrobial activity indicating possible biotechnological applications. However, isolation, purification and detection of active compound(s) are in progress for their further utilization.

4. CONCLUSION

Endophytic bacterial isolates was found to be associated with leaves, stem and root of the medicinal plant, *H. spinosa* and they differed significantly in their morphological, physiological and biochemical characters. The endophytes also produced several hydrolytic enzymes of commercial importance. Antimicrobial evaluation of these culturable endophytes of *H. spinosa* has shown that they possess antibacterial activity against various bacterial species. The endophytes of traditional medicinal plants appear to be a source of antimicrobial metabolites as well as enzymes for potential biotechnological applications in health, agriculture and industry.

ACKNOWLEDGEMENTS

Financial support from University Grants Commission, New Delhi (UGC-Minor Research Project PSW – 061 / 10-11 ERO) to A. Pal is duly acknowledged.

COMPETING INTERESTS

Authors have declared that no competing interest exists in performing this research and preparation or publication of the results.

AUTHORS' CONTRIBUTIONS

This research was carried out through joint collaboration of authors AP and AKP. The authors took equal responsibilities to design the study, writing protocols, literature survey, performing the experiments and preparation of manuscript.

REFERENCES

1. Misra TN, Singh RS, Pandey HS, Singh BK, Pandey RP. Constituents of *Asteracantha longifolia*. Fitoterapia. 2001;72(2):194–96.
2. Kshirsagar AD, Ingale KG, Vyawahare NS, Thorve VS. *Hygrophila spinosa*: A comprehensive review. Pharmacogn Rev. 2010;4(8): 167–171.
3. Strobel G, Daisy B, Castillo U, Harper J. Natural products from endophytic microorganisms. J Nat Prod. 2004;67(2): 257-268
4. Rosenblueth M, Martínez-Romero E. Bacterial endophytes and their Interactions with hosts. Mol Plant Microbe Interact. 2006;19(8): 827-37.
5. Tan RX, Zou WX. Endophytes: A rich source of functional metabolites. Nat. Prod. Rep. 2001,18(4); 448-59.
6. Yu H, Zhang L, Li L, Zheng CA, Guo L, Li W. et al. Recent developments and future prospects of antimicrobial metabolites produced by endophytes. Microbiol Res. 2010;165(6): 437-49.

7. Sun JQ, Guo LD, Zang W, Ping WX, Chi DF. Diversity and ecological distribution of endophytic fungi associated with medicinal plants. Sci China Ser C - Life Sci 2008;51(8): 751-59
8. Chelius MK, Triplett EW. The diversity of archaea and bacteria in association with the roots of *Zea mays* L. Microbiol Ecol. 2001;41(3): 252–63.
9. Van der Lelie D, Taghavi S, Monchy S, Schwender J, Miller L, Ferrieri R, *et al.* Poplar and its bacterial endophytes: coexistence and harmony. Crit Rev Plant Sci. 2009;28(5): 346–358
10. Radu S, Kqueen CY. Preliminary screening of endophytic fungi from medicinal plants in Malaysia for antimicrobial and antitumour activity. Malaysian J Med Sci. 2002;9(2): 23-33.
11. Raviraja NS, Maria GL, Sridhar KR. Antimicrobial evaluation of endophytic fungi inhabiting medicinal plants of the western ghats of India. Engg in Life Sci. 2006;6(5): 515-20.
12. Smibert RM, Krieg NR. Phenotypic characterization. In*:* Gerhardt P, Murray RGE, Wood WA, Krieg NR, editors. Methods for General and Molecular Bacteriology, Washington, D.C.: American Society for Microbiology; 1995.
13. Sneath PHA. Bergey's Manual of Systematic Bacteriology. 2nd ed. Baltimore, Williams and Wilkins; 2001.
14. Pielou EC. Ecological diversity. New York: John Wiley and Sons Inc.; 1975.
15. Bauer AW, Kirby WMM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 1996;45(4): 493–6.
16. Williston EH, Zia-Walrath P, Youmans GP. Plate methods for testing antibiotic activity of actinomycetes against virulent human type tubercle bacilli. J Bacteriol. 1947;54(5): 563 – 8.
17. Pal A, Chattopadhyay A, Paul AK. Diversity and antimicrobial spectrum of endophytic bacterial isolated from *Paederia foetida.* L. Int J Curr Pharm Res. 2012;4(3): 123-7.
18. Maheswari S, Rajagopal K. Biodiversity of endophytic fungi in *Kigelia pinnata* during two different seasons. Curr. Sci. 2013;104(4):515-8.
19. Fisher PJ, Petrini O, Petrini LE, Sutton BC. Fungal endophytes from the leaves and twigs of *Quercus ilex* L. from England, Majorca and Switzerland. New Phytol. 1994; 127(1):133–7.
20. Miller KI, Qing C, Sze DMY, Roufogalis BD, Neilan BA. Culturable endophytes of medicinal plants and the genetic basis for their bioactivity. Microbial. Ecol. 2012;64(2):431-449
21. Bhore SJ; Ravichantar N, Loh CY. Screening of endophytic bacteria isolated from leaves of Sambung Nyawa [*Gynura procumbens* (Lour.) Merr.] for cytokinin-like compounds. Bioinformation 2010;5(5): 191–7.
22. Chandrasekhara, Niranjanraj S; Deepak SA; Amruthesh KA; Shetty NP, Shetty HA. Endophytic bacteria from different plant origin enhance growth and induce downy mildew resistance in pearl millet. Asian J. Plant Pathol. 2007;1(1): 1-11.
23. Zhao K, Penttinen P, Guan T, Xiao J, Chen Q, Xu J, Lindstrom K, Zhang L, Zhang X, Strobel GA. The diversity and anti-microbial activity of endophytic actinomycetes isolated from medicinal plants in Panxi Plateau, China. Curr. Microbiol. 2011;62(1): 182-90.
24. Li J; Zhao GZ; Huang HY; Qin S; Zhu WY; Zhao LX; *et al*. Isolation and characterization of culturable endophytic actinobacteria associated with *Artemisia annua* L. Antonie van Leeuwenhoek 2012;101(3):515–27.
25. Stamford T, Stamford N, Coelho L, Araujo JM. Production and characterization of a thermostable glucoamylase from *Streptosporangium* sp. endophyte of maize leaves. Biores. Technol. 2002;83(2):105–9.

26. Carrim A, Barbosa E, Vieira J. Enzymatic activity of endophytic bacterial isolates of *Jacaranda decurrens* Cham. (Carobinha-do-campo). *Braz. arch. biol. technol.* 2006, 49(3):353-9.
27. Panchal H, Ingle SS. Isolation and characterization of endophytes from the root of medicinal plant *Chlorophytum borivilianum* (Safed musli). J. Adv. Dev. Res. 2011; 2(2):205-9.
28. Gayathri S, Saravanan D, Radhakrishnan M, Balagurunathan R, Kathiresan K. Bioprospecting potential of fast growing endophytic bacteria from leaves of mangrove and salt-marsh plant species. Indian J. Biotechnol. 2010;9(4):397-402.
29. Arunachalam C, Gayathri P. Studies on bioprospecting of endophytic bacteria from the medicinal plant of *Andrographis paniculata* for their antimicrobial activity and antibiotic susceptibility. Int J Curr Pharm Res. 2010;2(4): 63-8.
30. Li H, Qing C, Zhang Y, Zhao Z. Screening of endophytic fungi with antitumour and antifungal activities from Chinese medicinal plants. W J Microbiol Biotechnol. 2005;21(8-9): 1515- 9.
31. Verma VC, Gond VC; Kumar SK, Mishra A, Kharwar RN, Gange AC. Endophytic actinomycetes from *Azadirachta indica* A. Juss.: Isolation, diversity, and anti-microbial activity. Microb Ecol 2009;57(4):749–56.
32. Sumarah MW, Kesting JR, Sorensen D, Miller JD. Antifungal metabolites from fungal endophytes of *Pinus strobes*. Phytochemistry 2011;72(14-15), 1833-7.
33. Li J, Zhao GZ, Chen HH, Wang HB, Qin S, Zhu WY, *et al.* Antitumour and antimicrobial activities of endophytic streptomycetes from pharmaceutical plants in rainforest. Lett Appl Microbiol. 2008;47(6): 574–80.