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Original Article

ENDOPHYTES FROM THE AQUATIC PLANT *NELUMBO NUCIFERA*: DIVERSITY PROFILE AND ACTIVITY CHARACTERIZATION

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ABSTRACT

Objective: Endophytes represent a niche habitat for the study of novel bio-and chemo diversity. *Nelumbo nucifera* is an aquatic plant that has not been characterized for endophyte diversity. This study was undertaken with the objective of isolating endophytes from submerged and aerial part of *N. nucifera*, study the diversity profile of the isolated endophytes and their antimicrobial, antioxidant, and siderophore production capacity.

Methods: Endophytes were isolated from aerial and submerged parts of *N. nucifera* on different media (Starch Casein Nitrate, Glucose Yeast Extract, Nutrient and Potato Dextrose agar). These were further characterized for morphology (colony characteristics, Gram reaction), physiological characteristics (carbon, nitrogen utilization) and activity (antimicrobial, antioxidant, siderophore production). After dereplication, twelve isolates were studied further.

Results: All endophyte isolates were Gram-positive bacteria, and one was a fungus. Isolate L-300 showed the highest antioxidant capacity (238 AAE g FW^{-1}) and L-201 least (10 AAE g FW^{-1}. Antimicrobial activity was exhibited against bacteria and fungal targets, with 50% endophytes active against both bacteria and fungi. Isolates L-003 and L-207 exhibited activity against Gram-negative clinical isolates as also fungi. Siderophore production was shown by 58% isolates with L-208 showing maximum activity.

Conclusion: This is the first report on profiling of endophytes from *N. nucifera.* Results show that aquatic plants harbor diverse microbial population. Many promising isolates (such as L-003, L-211, L-214 and L-300) have been characterized in this study and results obtained of antioxidant, antimicrobial and siderophore production capacity demonstrate further utility in polypharmacological studies for identifying compounds of pharmaceutical and other industrial interest.

Keywords: *Nelumbo nucifera,* Endophyte, Microbial diversity, Antimicrobial, Antioxidant, Siderophore.

INTRODUCTION

Increasing incidence of drug resistance has led to a need for discovery of new antimicrobials and chemotherapeutic agents [1]. Unexplored habitats have been suggested as potential sources of new bioactive compounds. Amongst such habitats, endophytes occupy unique environment niches and constitute a group of less investigated microorganisms. Studies indicate that organisms which are constantly subjected to some metabolic or environmental interactions are likely to produce more diverse secondary metabolites that can be harnessed [2]. For unique natural antimicrobial compounds, the unique biological niche has to be selected. The plant growth promoting the activity of endophytes and their biocontrol properties assist the host plant in its survival, protection and growth. The plant growth promoting the activity of endophytes and their biocontrol properties assist the host plant in its survival, protection and growth. Endophytes, in return, are provided with a protected site for their establishment with direct accessibility to the nutrients [3]. Production of secondary metabolites by endophytes is influenced by their niche, the stresses they are subjected to and their interaction with the host.

A search in PubMed for endophyte diversity shows around 2000 results of which approximately 300 focus on products from endophytes, characterized for various activities such as antitumor, antioxidant and antimicrobial. About 70 % of the studies are on endophytic fungi and 30% studies on endophytic bacteria.

The opportunity to find new endophytes is high in uncultivated environmental diversity. Unique environmental conditions are selecting factors for different microbial assemblages that may lead to novel products [4]. *N. nucifera* is an aquatic plant with a rich ethnobotanical history [5] and has not been documented for endophyte diversity. Various parts of this plant have been shown to have astringent, cardiotonic, hypotensive, and vasodilator functions and used in the treatment of many diseases including cancer, depression, diarrhea and insomnia [5]. It has been documented to produce a number of important secondary metabolites, like alkaloids, flavonoids, steroids, triterpenoids, glycosides and polyphenols [6]. This study was undertaken with the following objectives: a) To isolate endophytes from submerged and aerial part of *N. nucifera*. (b) To study the diversity profile of the isolated endophytes and (c) To study the antimicrobial, antioxidant, and siderophore production capacity of the isolated endophytes.

MATERIALS AND METHODS

Sample collection

The plant was collected from village Karhera, district Ghaziabad, India (28 °41'07.5"N 77 °23'39.2"E). The plant was authenticated based on taxonomic characters by Professor Arun K. Pandey, University of Delhi, India. A voucher specimen (14168) was deposited at the herbarium of Department of Botany, University of Delhi, India. The water sample in which the plant was growing was analyzed for physicochemical properties.

Isolation of endophytes

The plant was washed under running tap water for 15 min and separated into aerial and submerged parts. These parts of the plants were cut into pieces and weighed; submerged parts [root (2.6g) and stolon (10.5g)] and aerial stem (10.1g) were then surface sterilized with 70% (v/v) ethanol for 1 min followed by 0.1% $HgCl_2$ (w/v) for 5 min, again washed with 70% (v/v) ethanol for 30 sec and then with sterile distilled water 5-6 times [7 & 8]. Subsequently, the sample (2-3 pieces of each part) was placed on nutrient agar as a control. Remaining parts were crushed with 1/4 strength of Ringer's solution in 1:1 ratio using sterile mortar and pestle. Then 200 µl of

these crushed samples were plated onto-Starch Casein Agar (SCA) to isolate actinomycetes [in gL^{-1}: starch 10.0, casein 0.3, KNO_3 2.0, NaCl 2.0, K_2HPO_4 2.0, $MgSO_4.7H_2O$ 0.05, $CaCO_3$ 0.02, $FeSO_4.7H_2O$ 0.01, Agar 15, antibiotics: cycloheximide (50 µg/ml) and rifampicin (20 µg/ml)], Glucose Yeast Extract (GYE) [Yeast extract 10.0, Glucose 10.0, Agar 15.0, antibiotics: rifampicin 20µg/ml), Nutrient Agar (NA) for bacteria (nutrient broth 20.0, Agar 15.0,] and Potato Dextrose Agar (PDA) for fungi [Potato dextrose broth 24.0, Agar 15.0,] The plates were then incubated at 37 °C for up to two weeks. The colony counts were estimated, and single clones of the isolates were studied further.

Characterization of endophytes

The endophytes were characterized for morphology (colony characteristics, Gram reaction), physiological characteristics (carbon, nitrogen utilization) and activity (antimicrobial, antioxidant, siderophore production). Obtained bacterial colonies were further selected and cultured on NA/GYE. The bacterial and fungal colonies were enumerated, and pure cultures of 12 distinct colonies were made for further studies.

Carbon and nitrogen source utilization profile

Carbon utilization by the isolates was tested using sucrose, fructose, raffinose, lactose, maltose, D-glucose (positive control) and no carbon source (negative control), as per Pridham & Gottlieb [9]. Nitrogen utilization was tested based on the growth of isolates on media containing ammonium chloride, ammonium sulfate, urea, proline, alanine, potassium nitrate (positive control) and no nitrogen source (negative control). In both cases, the growth of the isolates on plates was scored as positive and no growth as negative.

Activity characterization

Total antioxidant capacity

Total antioxidant capacity was measured according to Prieto *et al.* [10]. The isolates were grown in 5 ml nutrient broth overnight, centrifuged at 5000 rpm for 10 min, supernatant collected and 0.1 ml of the supernatant assayed with 1 ml reagent solution (0.6M H_2SO_4, 28 mM sodium phosphate, 4 mM ammonium molybdate). Using ascorbic acid as standard, total antioxidant capacity was expressed as Ascorbic Acid Equivalents with equation ($y = 0.097x + 0.005$) derived from the calibration curve.

Antimicrobial activity

Agar-well diffusion method was used for testing the antibacterial activity of the isolates [11]. The twelve isolates were tested against Gram-negative bacteria (*Pseudomonas fluorescens* [MTCC2421], *Escherichia coli* [MTCC1679]) and Gram-positive bacteria (*Staphylococcus epidermidis* [MTCC435], *Bacillus subtilis* [MTCC121]). Clinical isolates (*Klebsiella pneumonia* from sputum, *E. coli* from urine and pus and *Pseudomonas aeruginosa* from blood) were also used as target organisms. Antifungal activity was tested by using modified cross-streak method [12] against *Penicillium chrysogenum* [MTCC161], *Rhizopus oryzae* [MTCC 246] and *Saccharomyces cerevisiae* [MTCC1874]. Antimicrobial activity was quantified by measuring the diameters of the inhibition zones formed around the target organism [12].

Siderophore production

The assessment of siderophore production was done by Chrome Azurol Sulfonate (CAS) assay [13]. Using cork borer, wells were made in CAS agar plates, cultures seeded, incubated at 37°C for two days and quantified by measuring the diameter of orange zones. *E. coli* was used as positive control and *Streptomyces rimosus* as a negative control. For each experiment, readings were taken from three replicates, and each experiment repeated thrice.

RESULTS

Characteristics of water

Characteristics of the water body from which *N. nucifera* was obtained showed it was slightly acidic, and concentration of minerals like chloride, manganese, nitrate, fluoride, and sulfate was within limits as per IS: 10500-1993 standard (table 1). Pesticide and cadmium content was marginally higher.

Isolation of endophytes

Microorganism colony was not observed on control plates confirming that the cultures obtained were of endophytes. A maximum number of colonies were obtained on nutrient agar and starch casein agar. No isolates were obtained from GYE and PDA. Endophyte isolates obtained from various parts of *N. nucifera* on different media are shown in table 2**.** After dereplication, based on colony and microscopic characteristics, twelve isolates were used for further studies.

Table 1: Characteristics of water sample

Characteristics	Units	Value	IS: 10500-1993
Turbidity	NTU	5.95	5.0-10.0
pH	-	6	6.5–8.5
Total Dissolved Solids	mg/l	337	500-1000
Total Alkalinity	,,	250	200-600
Total Hardness	,,	154	300-600
Chloride	,,	50	250-1000
Sulfate	,,	51.82	200-400
Cadmium	,,	0.03	0.01
Manganese	,,	0.09	0.10-0.30
Nitrate	,,	22.4	45
Fluoride	,,	0.79	1.0-1.5
Pesticides	µg/l	0.004	0.001

Table 2: Enumeration of endophyte isolates obtained from aerial and submerged parts of *N. nucifera* on different media

Media	Plant Part	CFU(g FW^{-1})	Number of distinct colonies
SCA	Aerial stem	4.9×10^3	4
	Root	0	0
	Stolon	9.476×10^3	12
GYE	Aerial stem	0	0
	Root	0	0
	Stolon	0	0
NA	Aerial stem	4.1×10^3	6
	Root	3.8×10^4	7
	Stolon	9.5×10^4	3
PDA	Aerial stem	0	0
	Root	0	0
	Stolon	0	0

Table 3: Characterization of endophytes obtained from fresh *N. nucifera* plant material

Media	Isolate code	Plant part	Colony characteristics	Gram reaction
SCA	L-001	Stolon	Circular, white	Gram positive, cocco-bacilli
	L-002	Stolon	Circular, white	Gram positive, cocco-bacilli
	L-003	Aerial	Irregular, white	Gram positive, coccus
	L-004	Aerial	Irregular, white	Gram positive, coccus
	L-005	Aerial	Irregular, white	Gram positive, coccus
	L-006	Aerial	Circular, white	Gram positive, bacilli
	L-007	Stolon	Circular, white	Gram positive, cocco-bacilli
	L-008	Stolon	Irregular, white	Gram positive, bacilli
	L-010	Stolon	Irregular, white, opaque	Gram positive rods and coccus
	L-011	Stolon	Irregular, white	Unstained, coccus
	L-012	Stolon	Elongated, white	Gram positive, bacilli
	L-013	Stolon	Irregular, white	Gram positive, bacilli
	L-014	Stolon	Round, cream	Unstained, bacilli
	L-015	Stolon	Round, white, opaque	Gram positive, bacilli
	L-016	Stolon	Round, cream	Gram positive, bacilli
	L-017	Stolon	Irregular, white, opaque	Gram positive, bacilli
NB	L-201	Aerial	Round, peach	Unstained, bacilli
	L-202	Aerial	Round, white, opaque	Gram positive, coccus
	L-203	Aerial	Round, white, opaque	Gram positive, coccus
	L-204	Aerial	Round, white, opaque	Gram positive, coccus
	L-205	Aerial	Round, white, opaque	Gram positive, coccus
	L-206	Stolon	Round, white	Gram positive, bacilli
	L-207	Aerial	Round, shiny yellow	Gram positive, bacilli
	L-208	Stolon	Round, yellow	Gram positive, bacilli
	L-209	Root	Circular, white, shiny	Gram positive, bacilli
	L-210	Root	Circular, white, shiny	Gram positive, bacilli
	L-211	Root	Irregular, yellowish	Gram positive, cocco-bacilli
	L-212	Root	Irregular, white	Gram positive, bacilli
	L-213	Root	Circular, white	Gram positive, coccus
	L-214	Root	Irregular, orange	Gram positive, coccus
	L-215	Root	Irregular, white	Gram positive, bacilli
	L-300	Stolon	Grey fungus	-

Characterization of endophyte

Morphological characterization on the basis of colony color, shape and Gram reaction was done. All isolates were Gram positive bacteria, and one was fungi (table 3). Out of 32 isolates, 50% were bacilli, 31% coccus, 3% mixed, 3% fungi and 12% cocco-bacilli. As shown in table 3, colonies showing similar characteristics (such as L-001, L-002) were considered as separate clones since they were from different replicate plates. However, during dereplication, only one representative colony was chosen for further subculture and studies.

Carbon and nitrogen source utilization profiling

Carbon and nitrogen utilization by the isolates were studied by comparing growth on glucose or potassium nitrate as positive controls. As seen from table 4, maltose was the carbon source preferably utilized by all the isolates, though to varying degrees. Sucrose was utilized by only 41% of isolates. L-211 and L-207 were able to utilize all carbon sources. Ammonium chloride was the preferred nitrogen source for 92% isolates and ammonium sulphate least preferred (33%). L-211 utilized all nitrogen sources.

Table 4: Utilization of the selected carbon and nitrogen sources

Isolate code	Carbon source*#					Nitrogen source*#				
	Fructose	Lactose	Maltose	Raffinose	Sucrose	Alanine	Ammonium chloride	Proline	Urea	Ammonium sulfate
L-003	+	-	(+)	+	+	-	+	-	-	+
L-007	(+)	+	+	+	-	-	+	-	+	+
L-014	+	-	+	(+)	+	-	+	-	-	-
L-201	+	+	+	-	-	-	+	++	-	-
L-206	+	++	+	-	-	(+)	+	++	-	-
L-207	+	+	+	+	+	-	+	-	+	-
L-208	+	+	++	-	-	(+)	+	+	+	-
L-209	-	-	+	(+)	+	+	+	+	-	-
L-211	+	+	+	+	+	+	+	+	+	++
L-213	+	+	+	+	-	(+)	+	-	+	++
L-214	(+)	+	(+)	-	-	+	+	-	+	-
L-300	+	+	+	-	-	-	-	-	-	-

***-**No growth, **(+)** Growth less than control, +Growth same as the control,**++**Growth more than control, #Glucose control carbon source, potassium nitrate control nitrogen source

Activity characterization

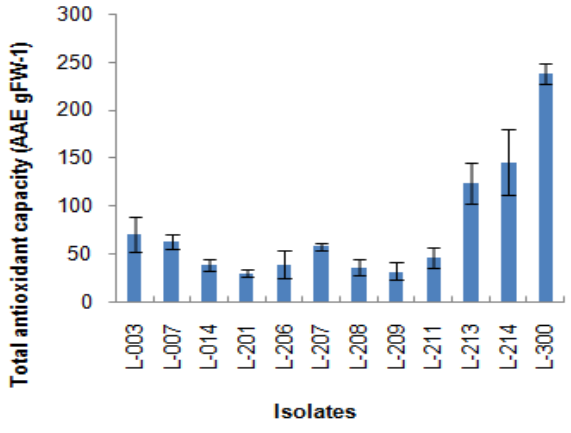
Total antioxidant capacity

Antioxidant capacity was tested using ascorbic acid as standard. It was observed that all the isolates showed the antioxidant capacity to varying degrees (fig. 1a). L-300 showed the highest antioxidant capacity followed by L-214. Least antioxidant capacity was exhibited by L-201 (10 AAE g FW^{-1}.

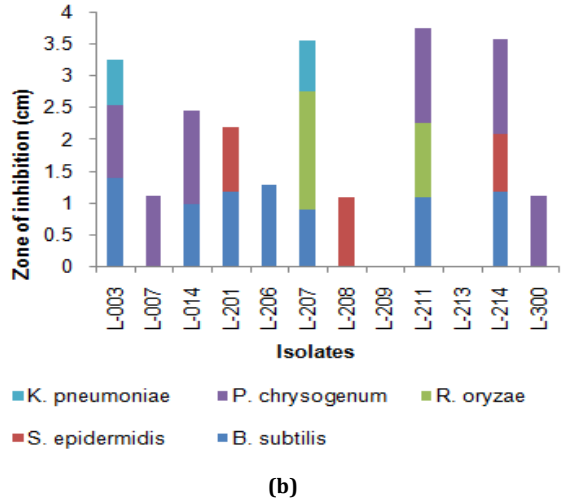
Antimicrobial activity

As seen from fig. 1b, when tested for antimicrobial activity, most of the isolates showed activity against Gram-positive bacteria (*S. epidermidis*

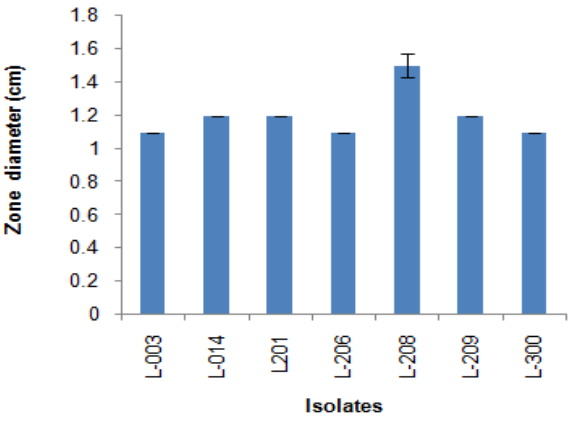
and *B. subtilis*) and fungi (*P. chrysogenum* and *R. oryzae*). Amongst these, 25% isolates were active against only Gram-positive bacteria, and 50% were active against both Gram positive bacteria and fungi. L-003 showed maximum antibacterial activity against *B. subtilis* and L-208 showed maximum activity against *S. epidermidis* while L-201 and L-214 showed antibacterial activity against both of these. L-003, L-007, L-014, L-211, L-214 and L-300 showed antifungal activity against *P. chrysogenum;* L-207 was antagonistic to *R. oryzae* while L-211 showed antifungal activity against both these fungi. None of the endophyte showed activity against the tested Gram negative bacteria.



(a)



(b)



(c)

Fig. 1: Bioactivity of endophyte isolates from *N. nucifera*. a. Total antioxidant capacity b. Antimicrobial activity c. Siderophore production (For each activity, readings were taken from three replicates and experiments repeated thrice)

Siderophore production

Siderophore production was exhibited by 58% of the endophyte (fig. 1c). L-003, L-014, L-201, L-206, L-208, L-209 and L-300 showed siderophore production. L-208 showed the maximum activity, as measured by the diameter of the orange halo zones.

DISCUSSION

The distribution and function of endophytic bacteria have been less studied in aquatic plants [14]. Our study focused on characterization of endophytes from various parts of *N. nucifera* for antimicrobial, antioxidant and siderophore activities. Different parts of the same plant may harbor different types of endophytes producing different compounds [14], as also seen from our results. In this study, 70% of the total endophytes obtained were from submerged parts of the plant suggesting that these parts harbor more population of endophytes. Different parts of the plant may interact with the aquatic environment in different ways. This could explain submerged parts harboring more endophytes. Variations amongst isolates from submerged and aerial part were also shown by analysis of antimicrobial, antioxidant and siderophore producing capacity. It was seen that 83% of the endophytes showing antioxidant activity were obtained from submerged parts and only 17% of the endophytes from aerial parts. L-300, showing maximum antioxidant capacity, was obtained from submerged part of the plant. L-003, which showed maximum antibacterial activity against *B. subtilis*, was obtained from an aerial part of the plant.

It was also interesting that 59% of the endophytes showed siderophore activity, out of which 42% were from submerged and 17% from aerial parts of the plant. As with antioxidant activity, L-208, which showed maximum siderophore activity, was obtained from submerged part. Our results suggest that more antioxidant and siderophore activities were exhibited by endophytes from submerged parts of the plant. The presence of heavy metal may stimulate bacterial siderophores that help the plant to reduce the toxicity of heavy metal by increasing the supply of iron to the plant [15]. However, siderophore production may be a general phenotype of the plant to maintain sufficient level of free iron ions to plant tissue as the quantity of metals present in the water in which the plant was growing, was within limits as per IS: 10500-1993. In conclusion, endophytes isolated from *N. nucifera* showed significant antioxidant, antimicrobial and siderophore production activities. Further studies will help us to purify and identify these compounds.

CONCLUSION

Screening for endophytes from the aquatic plant *N. nucifera* has revealed the presence of many distinct endophytes (bacteria and fungi). It was also seen that most of the endophytes were bacteria and only one fungus (L-300). This initial screening provides a platform for further studies focusing on promising isolates (such as L-003, L-211, L-214 and L-300) for identifying compounds of pharmaceutical and other industrial interest.

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CONFLICT OF INTERESTS

Declared None

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