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Isolation And Characterization of Rhizospheric and Endophytic Microorganisms from Bacopa Moneri (Bramhi) For Indole Acetic Acid and Gibberellic Acid Production

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ABSTRACT

The plant *Bacopa monnieri* (Brahmi) is a time-honoured Indian herb, which has very big monetary cost due to memory revitalizing/enhancing capacity. The estimated annual market demand of *Bacopa monnieri* (Brahmi) was estimated at 1000 tons for the year 2000 and reported by Board of National Medicinal Plants. In recent years, *Bacopa monnieri* has received much attention worldwide due to its wide spectrum of pharmacologic activities including against Alzheimer disease, The study focused on isolating, screening, and characterizing plant growth-promoting bacteria (PGPB) obtained from *Bacopa monnieri* (Brahmi). The objective was to identify bacterial strains capable of producing essential plant growth regulators, including indole acetic acid (IAA), gibberellic acid (GA).

Keywords: *IAA (Indole acetic acid), GA (Gibberellic acid) for Bacopa monnieri (Brahmi). Rhizospheric and Endophytic microorganisms.*

1. INTRODUCTION

Bacopa monnieri, commonly known as Brahmi, is a botanical widely used in Ayurvedic medicine. It is primarily indicated for improving memory, treating insomnia, managing epilepsy, and acting as an anxiolytic. (Yadav & Reddy 2013). Plants are constantly involved in interactions with a wide range of bacteria. This plant associated bacteria colonize the rhizosphere (rhizobacteria), the phyllo sphere (epiphytes) and the inside of plant tissues (endophytes). Endophytes are sheltered from environmental stresses and microbial competition by the host plant and they seem to be ubiquitous in plant tissues, having been isolated from flowers, fruits, leaves, stems, roots and seeds of various plant species (Kumar *et.al*., 2017). Indole acetic acid (IAA) is one of the most physiologically active auxins. Indole acetic acid (IAA) production is a significant trait of rhizosphere bacteria that stimulates and facilitates plant growth (Khianngam *et al*., 2023). Gibberellins are biologically active, endogenous hormones in higher plants that play a crucial role in various developmental processes, including stem elongation, germination, seed dormancy, fruit senescence, and sex expression (Gupta *et al*., 2013). This study focuses on the isolation, characterization, and identification of IAA-producing & GA bacteria from rhizospheric soil

2. MATERIALS AND METHODS

Collection of soil & root sample: Soil & root sample were be collected from three different plants of *Bacopa monnieri*

- Sample preparation, Enrichment of Rhizospheric and Endophytic microorganisms of *Bacopa monnieri* : (Aneja 2003)

Sample Preparation: From Rhizospheric soil samples Plant debris was removed. Roots were surface-sterilized using ethanol and sodium hypochlorite solutions. Enrichment Culture: For Rhizospheric IAA and GA producing bacteria, 1 g of each soil sample was added to a sterile Erlenmeyer flask containing nutrient broth as a enrichment medium. and incubated aerobically

at a 30°C) for 48 hours. For Rhizospheric phosphate solubilizing bacteria, 1 g of each soil sample was added to a sterile Erlenmeyer flask containing Pikovasky's broth as an enrichment medium. and incubated aerobically at a 30°C) for 48 hours. The same process was applied for enrichment of Endophytic microorganisms.

- **Study of Cultural, Morphological & Biochemical characteristics of the isolates:** The colony characterization of well isolated colonies was recorded as their size, shape, margin, colour, elevation, consistency. The suspension of well isolated colony was prepared and was used to study its gram nature by gram staining and motility by hanging drop technique. The biochemical tests performed were Endospore staining, starch hydrolysis, Voges-Proskauer (VP), Oxidase test, and sugar fermentation test. Identification of isolates obtained in pure cultures were characterized by morphology, colony characteristics, and various biochemical tests

recommended in the Bergey's Manual of Determinative Bacteriology.

- Isolation of Rhizospheric and Endophytic microorganisms of *Bacopa monnieri*

(Aneja 2003):

1)Isolation and Subculturing: For IAA and GA producers, after enrichment, aliquots of the culture were streaked onto Nutrient agar plates and incubated at room temperature for 48 h. to obtain well isolated individual microbial colonies. Pure cultures were obtained by subculturintt5g. For Isolation of Phosphate solubilizing microorganisms after enrichment, aliquots of the culture were streaked onto Pikovasky's agar plates and incubated at room temperature for 48 h. to obtain well isolated individual microbial colonies. After incubation the colonies showing zone of hydrolysis around them were selected, picked up and restreaked on same medium to obtain the pure cultures. Each isolate was purified by repeated sub -culturing method. Colonies Will be picked and maintained on nutrient agar slant and stored at 4°C.

Refrigerator for further use.

- **Gibberellin Production Screening of Rhizospheric Isolates: (Desai 2017)** The isolates (Rhizobacteria) were subjected to a screening process to assess their capacity for gibberellin production. Specifically, 100 mL of nutrient medium was dispensed into 250 ml conical flasks and inoculated with Rhizospheric isolates. The culture flasks were incubated at 35°C for 48 h. After 48 hours, the bacterial culture was centrifuged at 10,000 rpm for 15-20 minutes. The pH value of the culture supernatants was adjusted to 2.5 using 3.75 N HCl. The culture supernatants were

then extracted using the liquid-liquid (ethyl acetate and NaHCO3) extraction method.

The amount of gibberellic acid (GA3) in the ethyl acetate phase was measured using a UV spectrophotometer at 254 nm against a control flask. For GA3 estimation by the DNPH (2,4- Dinitrophenyl hydrazine) method, equal volumes of the cell-free extract and ethyl acetate were mixed in a test tube and shaken vigorously for 10 minutes. This process was repeated thrice. Ethyl acetate was allowed to evaporate at room temperature, and the remaining contents were dissolved in absolute alcohol.

This suspension was then mixed with 1 mL of DNPH and incubated at 100°C for 5 minutes, followed by cooling in a water bath. To this, 5 ml of 10% potassium hydroxide was added and allowed to stand until a red wine color developed. Then, 15 mL of sterile distilled water was added, and the content was finally diluted to 1:2 using sterile distilled water. The colour intensity was measured at 430 nm using a UV-VIS Spectrophotometer. For the standard curve, different aliquots of standard gibberellic acid (0.8 mg/ml) were prepared using absolute alcohol and estimated similarly. (Desai, 2017)

- **Production of IAA:** IAA production was carried out by inoculating the isolate in the nutrient broth with tryptophan(0.1 g/l) and was incubated at 300C for 72 hrs. The IAA produced was harvested by centrifuge at 3000rpm for 20 min. obtained supernatant was than detected for presence of IAA by Salkowski method. This was carried out by mixing 2 drops of orthophosphoric acid with 2 mL of Salkowski reagent (50 mL, 35% perchloric acid; 1mL of 0.5% FeCl3) the formation of pink color indicates test to be positive. The optical density was measured at 540 nm by using clear supernatant as blank.
- **Production of GA:** For production of gibberellic acid submerged fermentation was used where sterile nutrient Broth is used. Inoculated isolated single colony in separate 250 mL yeast nutrient broth and incubated for 7 days at 30^0 C at 120 rpm samples were removed aseptically and analysed for GA production at regular interval.
- **GA3 estimation:** For GA estimation 5mL cell free extract and 5mL ethyl acetate was taken in test tube and shake vigorously for 10 min and separate ethyl acetate layer and remaining ethyl acetate was evaporated from organic layer and This organic layer was dissolved in alcohol. 1 mL of DNPH was mixed with 2mL organic suspension and Incubated in water bath at 100 °C for 5 min and cooled and in cooled extract add 5 ml of 10% potassium Hydroxide and wait until red wine colour developed. Add 15mL of sterile distilled water the content was Diluted to 1:2 using sterile distilled water. Colour intensity was measured at 430nm in UV-VIS Spectrophotometer. For standard GA (0.8 mg/mL) was prepared in absolute alcohol and estimated (Desai 2017)

3. RESULTS AND DISCUSSION

- Result of cultural & morphological characteristics: In all isolates were obtained and designated as

Table No.1 Results of, Isolation of Rhizospheric and Endophytic microorganisms

Sr.no.	Isolate	Type	Source
1	IS1	Rhizospheric	Soil
2	IS2	Rhizospheric	Soil
3	IS3	Rhizospheric	Soil
4	IS4	Endophytic	Root
5	IS5	Endophytic	Root
6	IS6	Endophytic	Root

Table No.2 Result of morphological and cultural characteristics of isolates

Isolate	Size	Shape	Colour	Margin	Opacity	Elevati on	Consist ancy	Gram natur e	Motility
IS1	0.5 mm	circular	Creamy white	Irregular	opaque	Raised	Smooth	Gram positiv eRods	Motile
IS2	1-2 mm	circular	white	Rough	opaque	Convex	Mucoid	Gram positiv ecocci	Non motil e
IS3	1mm	circular	Cream	Regular	opaque	Flat	Moist	Gram positiv e	Motile
IS4	2-3 mm	irregular	Blue green	Regular	Opaque	Convex	Smoot h mucoi d	Gram negativ erod	Motile
IS5	1 mm	circular	White	Regular	Opaque	Flat	Mucoid	Gram negativ e	Motile
IS6	1mm	circular	Pinkish	Regular	Opaque	Convex	Mucoid	Gram negativ erod	Motile

From the study of cultural, morphological and biochemical characteristics of the isolates the Isolate IS2 was tentatively identified as *Bacillus subtililis*

From the study of cultural, morphological and biochemical characteristics of the isolates the Isolate IS3 was tentatively identified as *Micrococcus agilis*

From the study of cultural, morphological and biochemical characteristics of the isolates the Isolate IS4 was tentatively identified as *Pseudomanas aeruginosa* From the study of cultural, morphological and biochemical characteristics of the isolates the Isolate IS5 was tentatively identified as *Azotobacter spp*

From the study of cultural, morphological and biochemical characteristics of the isolates the Isolate IS6 was tentatively identified as *Serratia spp*

- **Biochemical characters:** Some biochemical characters were studied for all the isolates including Indole production test, carbohydrate utilization test, enzymatic properties & amino acid decarboxylation test.

Table No.3 Results of study of biochemical characteristics of the Isolates

Sr.No.	Test	IS1	IS2	IS3	IS4	IS5	IS6
1	Indole	+	+	_	+	+	_
2	MR	+	+	+	_	+	_
3	VP	+	+	_	_	_	+
4	Oxidase	_	+	_	_	+	_
5	Catalase	+	+	+	+	+	+
6	Urease	+	+	_	+	+	_
7	Gelatinase	+	+	+	+	+	+
8	Citrate	+	+	_	_	+	_
9	Sucrose	+	+	_	+	+	+
10	Mannitol	+	+	_	+	+	+
11	Lactose	+	+	_	-	+	_
12	glucose	+	+	_	+	+	+
13	Starch hydrolysis	+	+	+	+	+	_

IS 1showed the positive test result for Indole, Methyl red (MR), Voges-Proskauer (VP), Catalase, urease, Gelatinase, Citrate, Sucrose, Mannitol, Lactose, Glucose, and Starch hydrolysis and negative test for oxidase.

IS2 showed the all biochemical test are positive.

IS3 showed the positive test for MR, Catalase, Gelatinase and starch hydrolysis and negative test for Indole, VP, Oxidase, Urease, Citrate, Sucrose, Mannitol, Lactose and Glucose.

IS4 showed the positive test result for Indole, Catalase, Urease, Gelatinase, Sucrose, Mannitol, Glucose and Starch Hydrolysis. And negative test for MR, VP, Oxidase, Citrate and Lactose.

IS5 showed the positive test result for Indole, Methyl Red(MR), Oxidase, Catalase, Urease, Gelatinase, Citrate, Sucrose, Manitole, Lactose, Glucose. And negative test for VP.

IS6 showed the positive test for VP, Catalase, Gelatinase, Sucrose, Manitol, Glucose. And negative test for Indole, MR, Oxidase, Urease, Citrate, Lactose

4. RESULT OF SCREENING OF THE ISOLATES FOR IAA PRODUCTION

In terms of IAA production, all four isolates (IS1, IS2, IS3, IS4,IS5 and IS6) exhibited positive results, indicating their capability to synthesize IAA. Indole-3-Acetic Acid is a key plant growth hormone known for its role in stimulating root development and overall plant growth. The positive IAA production by these isolates suggests their potential to enhance plant growth through the production of this important hormone

Table No.4 Result of Quantitative analysis of IAA produced by isolates

Tube No.	Std IAA µg/mL(100ug/mL)	D/W	Concentration of IAA (µg/mL)	Salkowski reagent (2mL)		OD at 530 rpm
1	0.1	9.9	10	2	Incubation 25 min in room temperature	0.12
2	0.2	9.8	20	2		0.23
3	0.3	9.7	30	2		0.34
4	0.4	9.6	40	2		0.36
5	0.5	9.5	50	2		0.36
6	0.6	9.4	60	2		0.46
7	0.7	9.3	70	2		0.49
8	0.8	9.2	80	2		0.72
9	0.9	9.1	90	2		0.74
10	1	10	100	2		0.89
IS1	1	10	-	2		0.38
IS2	1	10	-	2		0.24
IS3	1	10	-	2		0.25
IS4	1	10	-	2		0.24
IS5	1	10	-	2		0.26
IS6	1	10	-	2		0.27

In terms of IAA production, all six isolates (IS1, IS2, IS3,IS4,IS5 and IS6) exhibited positive results, indicating their capability to synthesize IAA. Indole-3-Acetic Acid is a key plant growth hormone known for its role in stimulating root development and overall plant growth. The positive IAA production by these isolates suggests their potential to enhance plant growth through the production of this important hormone.

Table No.5 Result of IAA production

Sr. No.	Isolate	Amount of IAA produced (µg/ mL)
1	IS1	34
2	IS2	22
3	IS3	23
4	IS4	22
5	IS5	24
6	IS6	26

- **Result of screening of the isolates for GA production:** In terms of GA production, all six isolates (IS1, IS2, IS3, IS4, IS5 and IS6) exhibited positive results, indicating their capability to synthesize GA

Table No.6 Result of IAA production

Sr. No.	Isolate	GA Production
1	IS1	+
2	IS2	+
3	IS3	+
4	IS4	+
5	IS5	+
6	IS6	+

5. DISCUSSION

This study successfully isolated and characterized several microbial isolates from the Rhizospheric and endophytic environments of *Bacopa monnieri* (Brahmi). The isolated bacteria, specifically *Bacillus subtilis* strains (IS1 and IS2), *Micrococcus agilis* (IS3), and *Pseudomonas aeruginosa* (IS4), *Azotobacter spp* (IS5), *Serratia spp*.(IS6) exhibited the valuable capability to produce plant growth-promoting hormones IAA and GA.

Maximum IAA production ability 34 µg/mL. isolate IS1 (S1) would be a promising isolate

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