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Detection and Quantification of Plant Growth Hormones by High Power Liquid Chromatography from Purple Non Sulphur Bacterial isolates

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The Phototrophic Purple Non Sulfur Bacteria (PPNSB) are the best studied and most diverse group of the phototrophic bacteria. The pigments, metabolites, and nutrients made by PPNSB could give some positive effects on plant growth. The study on detection of growth hormones production from Purple non sulphur bacteria conducted during 2012-2013 in the department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad. The results revealed that totally two hundred isolates of NKPRPPNSB isolates subjected for rapid screening of Indole acetic acid (IAA) and cytokinine production, among them, 20 isolates found efficient for IAA and cytokinine production(. Further, efficient isolates qualitatively tested in HPLC by determining retention time and peak area to calculated the amount of IAA and Cytokinine production. The isolates NKPRPPNSB(H10) was shown IAA production 18 µÀg/ ml with 6.02 min retention time and NKPRPPNSB(S6) isolates produced 43.50 µÀg/ litre of cytokinine with retention time of 3.11 min.

Keywords: Pigments, Indole acetic acid, Cytokinine, Retention time.

The Phototrophic Purple Non Sulfur Bacteria (PPNSB) are the best studied and most diverse group of the phototrophic bacteria. All species grow best as anaerobic photoorganotrophs and have the capacity to grow also as facultatively microaerophilic to aerobic chemoorganotrophs (Imhoff *et al.,* 1995). PPNSB in the nitrogen nutrition of low land rice is often recognized (Kobayashi and Haque, 1971; Maudinas *et al.*, 1981) and auxin production (Rajasekhar and Ramana, 1999) by PPNSB have been reported. PPNSB were reported to have beneficial effects on growth and grain yield of rice under laboratory conditions as well as under pot and lysimeter conditions (Elbadry and Elbanna, 1999 and Harada *et al.,* 2003). The pigments, metabolites, and nutrients made by PPNSB could give some positive effects on plant growth (Sasikala and Ramana 1995). In addition to N_2 fixation, they possess the ability to solubilize the phosphorus and production of growth hormones *viz.*, indole-3-acetic acid (IAA), Cytokinin and 5-aminolevulinic acid (ALA) have been reported (Koh and Song, 2007). The property of promoting plant growth helped in recognizing them as plant growth promoting rhizobacteria (Sasikala and Ramana, 1995). This an attempt made to study the growth hormone production from PPNSB isolates which isolated from North Karnataka paddy rhizosphere soil.

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MATERIALS AND METHODS

The experiment conducted during 2012-2013 in the department of Agricultural Microbiology, University of Agricultural sciences, Dharwad. Totally 200 isolates of PPNSB isolated from rice rhizosphere soil of north Karnataka, the isolates coded as North Karnataka Paddy

Rhizosphere PPNSB (NKPRPPNSB). All the isolates subjected for morphological, physiological and molecular characterized after that plant growth promotion functionality studied among the functionality test plant growth hormone production is one of the important character. All the isolates subjected rapid screening of IAA by spectrophotometer method, procedure followed as per Gorden and Weber (1951) for rapid screening culture grown in both aerobic and microaerophillic condition. Based on the rapid screening by spectrophotometer method efficient isolates were selected for High Performance Liquid Chromatography (HPLC) analysis of IAA. All the selected isolates of NKPRPPNSB grown in 30ml capacity screw cap tubes under microaerophillic condition in light at 28±2°C for 7 days. After seven days of incubation, 25 ml of the sample was withdrawn and the culture was centrifuged at 12000 rpm for 30 min and the cell free culture filtrate was extracted twice with equal amount of ethyl acetate (Tien *et al.*, 1979). The ethyl acetate fractions of the cell free culture broth of NKPRPPNSB isolates were used for Indole-3-acetic acid quantification by HPLC. The ethyl acetate was evaporated, the sediment was redissolved in 2 ml of absolute methanol, membrane filtered and 10 µl volume was injected into the HPLC. Analysis was performed using a 10 mm particle size reverse phase column (C18) with a solvent gradient of 100 per cent methanol and one per cent acetic acid in water at a flow rate of 0.4 and 0.6 ml min-1 respectively and the operating pressure of 1600 PSI. The quantification of Indole-3-acetic acid compounds in the sample was done by comparison of retention time with peak height of chemical grade IAA (Sigma grade) (Tien *et al.*, 1979 and Sunayana *et al.*, 2005).

Rapid screening of Cytokinin production from the NKPRPPNSB isolates was done by Thin layer chromatography (TLC) as per Tien *et al.* (1979) based on the presence or absence of spots as compared with standard Zeatin (Sigma Aldrich company). Based on rapid screening efficient isolates were selected for High Performance Liquid Chromatography (HPLC) analysis of cytokinin (Zeatin) production. Rapid screening positive isolates were grown in BP,s medium for 7 days microaerophillically in light at 28±2°C. The cultures were centrifuged at 12000 rpm for 30 min and the cell free culture filtrate was extracted twice with equal amount of n-butanol (Tien *et al.*, 1979). The n-butanol fractions were pooled and allowed to

Table 1. Summary of the rapid screening test for the production of growth hormone from

IAA(µg/ml)	Aerobic	Number of isolates	Microaerophillic	Number of isolates
Light	Range		Range	
1	1-5	58	1-6	53
2	5-10	62	6-12	78
3	10-16	80	12-18	69
	Total	**200**	**Total**	**200**
Dark	Range		Range	
1	1-5	38	1-6	38
2	5-10	120	6-12	137
3	10-16	42	12-18	25
	Total	**200**	**Total**	**200**
	Light	Dark	Light	Dark
Ref.strain1	17.50	15.50	14.50	13.50
Ref.strain 2	9.70	18.00	7.50	15.50
Cytokinine	Positive	24		
	Negative	176		
	Total	**200**		
Ref.strain 1	Positive			
Ref.strain 2	Positive			

evaporate under N_2 gas at 40°C in a low volume concentrator (Model-Turbovap, UK) in order to prevent oxidation. After evaporation, the cytokinin fraction was dissolved in 2 ml of HPLC grade methanol and filter sterilized using 2 µm bacterial filters. In this study two reference strains used for comparison reference strain 1 *Rhodobacter capsulatus* from Hyderbad and reference strain 2 *Azospirillum* ACD 15 collected from department of Agricultural microbiology.

RESULTS AND DISCUSSION

The isolates belongs to PPNSB group confirmed by morphological, physiological and molecular characters data not shown. The rapid screening test result showed that IAA production ranged from 1.50 to 15.00 and 1.20 to 14.5 µg/ml in dark and light respectively under aerobic condition while under microaerophilic condition IAA production was less and ranged from 1.0 to 12.5 and 0.90 to 15.50 µg/ml in light and dark respectively (table 1). Out of two hundred isolates, twenty isolates were found to produce maximum IAA irrespective of the conditions under which they were grown. Among twenty isolates highest amount of IAA production was found in NKPRPPNSB(S6) (15 µg/ml) followed by this NKPRPPNSB (H10) and NKPRPPNSB (D7) (14.00, 12.5, 11.00, 10.00 and 6.5 µg/ml respectively) irrespective of the growth condition. The IAA production did not differ significantly with incubation in light and dark conditions while significant differences were found with aerobic and microaerophilic condition. The reason might be aerobic condition is effectively influences the IAA production

IAA production was qualitatively estimated by HPLC from twenty selected isolates based on rapid screening test. The peaks of IAA

Table 2. Qualitative estimation of IAA and Cytokinin by HPLC from selected native NKPRPPNSB isolates

S. No.	Isolate	IAA		Cytokinin(Zeatin)	
		µg/ ml	Retention time (min)	µg/ litre	Retention time (min)
1	NKPRPPNSB(H10)	18.00	6.02	43.50	3.11
2	NKPRPPNSB(S6)	15.50	6.10	43.50	3.01
3	NKPRPPNSB(D7)	17..50	6.10	46.50	3.10
4	NKPRPPNSB(R16)	13.20	6.08	41.00	3.10
5	NKPRPPNSB(H2)	12.30	6.07	40.00	3.11
6	NKPRPPNSB(B6)	13.50	6.06	34.00	3.12
7	NKPRPPNB(B10)	11.50	6.05	38.00	3.11
8	NKPRPPNSB(K10)	13.00	6.10	39.50	3.12
9	NKPRPPNSB(S2)	14.20	6.10	40.50	3.09
10	NKPRPPNSB(D15)	12.40	6.10	38.50	3.08
11	NKPRPPNSB(S8)	14.10	6.08	40.80	3.02
12	NKPRPPNSB(G1)	14.00	6.09	42.50	3.04
13	NKPRPPNSB(H8)	14.50	6.15	41.50	3.11
14	NKPRPPNSB(B3)	13.50	6.15	43.50	3.12
15	NKPRPPNSB(R10)	13.78	6.15	42.50	3.13
16	NKPRPPNSB(H16)	13.50	6.10	33.50	3.12
17	NKPRPPNSB(D12)	13.10	6.10	31.50	3.15
18	NKPRPPNSB(H18)	12.50	6.10	32.30	3.16
19	NKPRPPNSB(R19)	11.50	6.15	25.60	3.18
20	NKPRPPNSB(H22)	12.50	6.16	21.00	3.11
21	Ref. strain 1	14.50	6.12	39.50	3.11
22	Ref. strain 2	18.50	6.15	40.00	3.115

Note: Ref. strain 1- *Rhodobacter capsulatus* KU002,
Ref. strain 2- *Azospirillum* ACD 15; IAA- Indole acetic acid

production are presented in fig.1 (table.2) . The results revealed that all twenty isolates were able to produce higher IAA as compared to spectrophotometric method which ranged from 11.50 to18.50 µg/ml (table 2).

Similarly two hundred isolates were tested rapidly for the production of cytokinin (Zeatin) by thin layer chromatography (TLC). Twenty four isolates were positive for cytokinin production (Table 1.). Later twenty isolates were subjected for HPLC analysis to quantify the amount of cytokinin production (fig.2). The amount of cytokinin produced ranged from 20.00 to 45.5 µg per liter (table.2). The maximum amount of cytokinin production was noticed in NKPRPPNSB(S6) followed by NKPRPPNSB (H10), NKPRPPNSB (D7) NKPRPPNSB (R16) NKPRPPNSB (H2), NKPRPPNSB (B6) (46.50, 40.00, 34.00, 36.00. 34.00 and 30.00 µg per liter respectively. Ref. strain 2 produced 45.50 µg per liter which was on par with other isolates producing maximum amount of cytokinin while the other ref. strain 1 produced lower amount of cytokinin (Zeatin) (20 µg per litre.).

IAA production results in diverse physiological effects in plants. It stimulates the division, extension and differentiation of plant cells, enhances root formation by promoting the conversion of parenchyma into xylem and phloem and regulates the leaf fall and fruit ripening. Many epiphytic and soil microorganisms are able to synthesize and secrete auxin, primarily IAA due to which they influence the growth of the plants. Microorganisms like *Azospirillum*, *Rhizobium* and *Pseudomonas*, may exert beneficial effects on plants. The classical phytohormone auxin (indole-3 acetic acid) was produced and secreted by different strains of purple nonsulphur bacteria (Sasikala and Ramana, 1995 and Rajashekar *et al*. 1998). The production of IAA by phototrophic bacteria was reported by many workers (Sasikala and Ramana, 1995; Rajasekhar *et al*., 1999). Mujahid *et al.* (2011) and Sunayana *et al.* (2005) documented production of IAA by PPNSB in presence or absence of light under both aerobic and anaerobic condition. The other plant growth regulators like zeatin, trans-zeatin and trans-zeatin riboside and related cytokinins are known to influences the seed germination and seedling growth in plants (Tien *et al*., 1979). The cytokinin production from PPNSB group of organism was first reported by Serdyuk *et al.* (1997). They confirmed cytkinin production by HPLC. The plant growth promotional activity

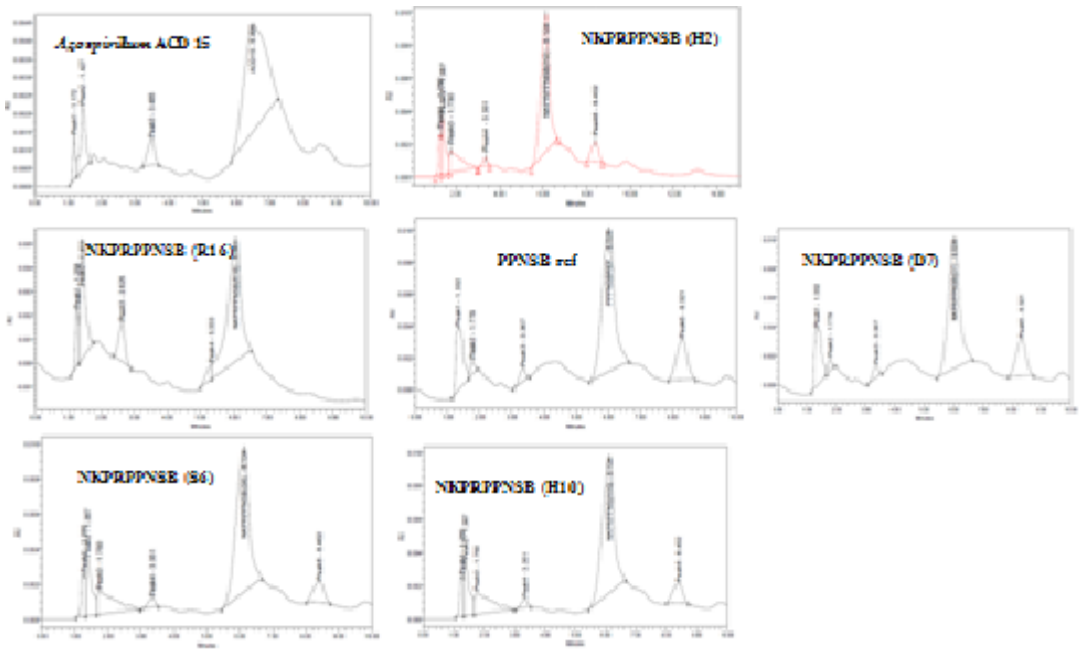

Fig. 1. HPLC chromatograms of IAA produced by selected NKPRPPNSB isolates

of cytokinin produced from this group of organism was observed on amaranth cotyledons, radish leaf cuts, tobacco pith tissue callus and strawberry, cherry and kiwifruit leaf transplants by Serdyuk (2000).

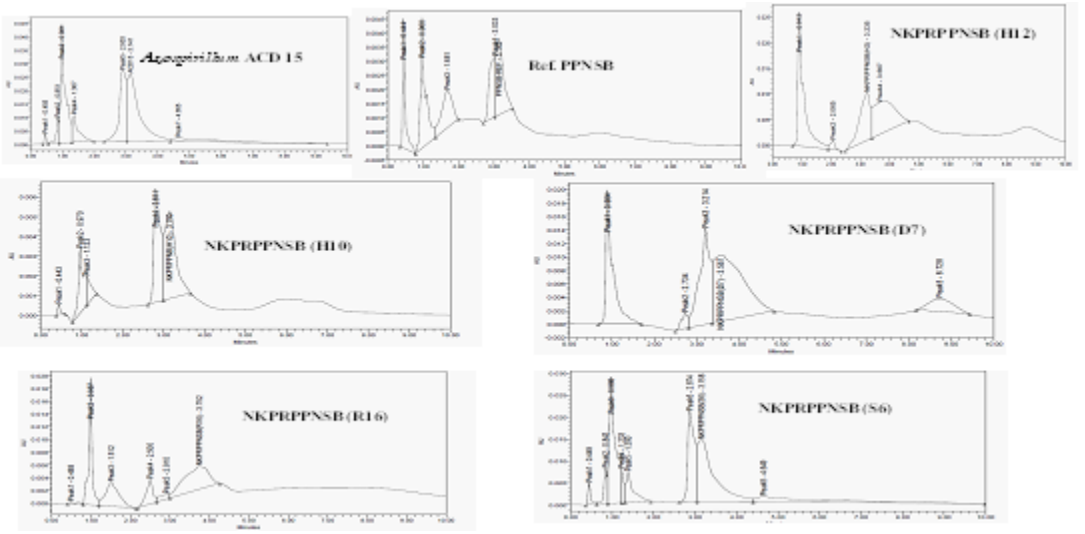

Fig. 2. HPLC chromatograms of cytokinine (Zeatin) produced by selected NKPRPPNSB isolates

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