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Isolation and Characterization of Nitrogen Fixing Bacteria *Beijerinckia* from Tea Rhizosphere of South Assam, India

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Diverse nitrogen fixing microorganisms (aerobes, facultative anaerobes, heterotrophs, phototrophs) grow in the rhizosphere of tea and contribute to the soil nitrogen pools. The recent isolation and study of free-living nitrogen fixing bacteria represent an exciting period in the field of biological nitrogen fixation. The present investigation was carried out in tea gardens of south Assam in the month of August to December, 2010. Seventeen strains of nitrogen fixing bacteria of the genus *Beijerinckia* was isolated and identified based on their morphological and biochemical characteristics.

Key Words: Morphological and biochemical characteristics, Nitrogen fixing bacteria, Rhizosphere.

Tea (*Camellia sinensis*) (L.) O. Kuntze), a woody perennial tree crop, belongs to the family Theaceace. The genus Camellia includes 82 species[1], but only three of them are being used in commercial tea preparation. The Assam type *Camellia assamica* which is indigenous to South East Asia, Assam, Indochina, and China is characterized by tall trees with large leaves and is less resistant to cold. Microbes which pass independent life and fix atmospheric nitrogen are known as free living diazotrophs. There are two groups of such microbes: bacteria & cynobacteria (blue green algae).Further bacteria are divided into (i). Aerobic bacteria such as *Azomonas, Azotobacter, and Beijerinckia.* (ii). Facultative anaerobic bacteria such as *Bacillus, Enterobacter, Kleibsiella, etc.* (iii). Anaerobes such as *Clostridium, Desulfovibrio, etc.* and photosynthetic bacteria such as *Rhodomicrobium,* etc. Among Cynobacteria both heterocystous and non heterocytous forms, fix atmospheric nitrogen for example- *Nostoc, Anabaena* etc. The *Azotobacters* are the most intensive heterotrophic groups. They are aerobic bacteria possessing highest respiratory rates. Biological Nitrogen Fixation (BNF) occurs when atmospheric nitrogen gets converted to ammonia by a pair of bacterial enzyme called nitrogenase. Nitrogenase catalyses the reduction of N_2 to NH_3 in an ATP- & reductant dependent reaction.

$$N_2 + 8H^+ + 8e^- + 16\,ATP \rightarrow 2NH_3 + H_2 + 16\,ADP + 16\,Pi.$$

Nitrogenase is composed of two -oxygen -labile metalloprotein dinitrogenase and dinitrogenase reductase. Nitrogenase is a 240-KDa, alpha2 - beta2 tetramer of the nif D and nif K gene products. Dinitrogenase reductase is a 60-KDa alpha2 dimer of nif H gene products that contains a single 4Fe- 4s centre coordinated between the

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two subunits and various techniques such as PCR cloning, gel electrophoresis & fluorescently labeled gel electrophoresis have been used to analyze the composition of nif H gene pools in various environments [2].

In this present paper the nitrogen fixing bacteria of the genus *Beijerinckia* was isolated from tea rhizosphere of different tea estates of Southern Assam, India from August to December 2010, in nitrogen free *Beijerinckia* medium, their morphological, biochemical characteristics were taken into consideration and on the basis of the characteristics they were identified as *Beijerinckia*.

MATERIAL AND METHODS

Isolation of *Beijerinckia*

Rhizosphere soils were used for the isolation of *Beijerinckia* by following serial dilution and plating technique on *Beijerinckia* agar medium [3]. Plates were incubated for five days at 30^0 C. Small water droplet like colonies which appeared on the medium were purified by repeated streaking on *Beijerinckia* agar medium. The purified isolates were transferred to the slants of the same medium and stored for further studies. *Beijerinckia* isolates were examined for their colony morphology on *Beijerinckia* medium as well as for cell shape and Gram reaction. Utilization of different carbon sources was tested by replacing glucose in *Beijerinckia* medium with different carbon sources like starch, sucrose, dextrose, mannitol and citrate.

Morphological characterization

All the isolates were examined for the colony morphology, cell shape, gram reaction, as per the procedures described by Bartholomew and Mittewer (1950) [4, 5].

Biochemical characterization

The biochemical characterization of the isolates was done as per the procedures outlined by Cappuccino and Sherman (2004) [6].The tests conducted are as follows:

Starch hydrolysis

The test cultures were streaked on the starch agar plates and incubated at 30^0 C for 24 hours. After incubation, the plates were flooded with iodine solution. Formation of clear zone around the colony was taken as positive for the test.

Gelatin liquefaction

To the pre-sterilized nutrient gelatin deep tubes, the test cultures were inoculated and tubes were incubated at 30^0 C for 24 hours. Following this, the tubes were kept in a refrigerator at 4^0 C for 30 minutes. The tubes with cultures that remained liquefied were taken as positive and those that solidified on refrigeration were taken as negative for the test.

Urease test

The overnight cultures were inoculated to the test tubes containing sterilized urea broth and incubated for 24 to 48 hours at 30^0 C. The development of pink colour was taken as positive.

Catalase test

Nutrient agar slants were inoculated with overnight growth of test organisms and were incubated at 30^0 C for 24 hours. After incubation, the tubes were flooded with one ml of 3 per cent hydrogen peroxide and observed for gas bubbles. The occurrence of gas bubbles was scored positive for catalase.

Methyl red test

Test tubes containing MR-VP broth were sterilized and inoculated with the test cultures. The tubes were incubated at 30^0 C for 48 hours. After incubation, five drops of methyl red indicator was added to each tube and gently shaken. The production of red colour was taken as positive for the test and production of yellow colour was taken as negative for the test.

Voges – Proskauer test

To the pre-sterilized tubes containing MR-VP broth the test cultures were inoculated. The tubes were incubated for 48 hours at 30^0 C. After incubation, ten drops of Barrit's reagent – A was added and gently shaken followed by addition of ten drops of Barrit's Reagent-B. The development of rose colour in the broth was taken as positive for the test.

Acid production

To the yeast extract mannitol (YEM) agar plates, overnight culture of the test isolates were streaked and incubated at 30^oC.After overnight of incubation ,in the next day any observation in colour change from white to yellow is taken as positive for the test that means acid are produced by the microbes.

Oxidase test

To the trypticase soy agar plates,

Table 1. Morphological characteristics of the isolates and their tentative identification

			Utilization of Carbon sources					
Isolate	Colony morphology and shape	Gram reaction	sucrose	dextrose	mannitol	starch	citrate	Probable genus
BEIJ 1	Small transparent raised water droplet like, rod shaped.	Gr-ve	++	+	_	_	_	*Beijerinckia*
BEIJ 2	Water droplet like with white center, rod.	Gr-ve	++	+	_	_	_	*Beijerinckia*
BEIJ 3	Water droplet. like with raised center, rod	Gr-ve	++	+	+	+	_	*Beijerinckia*
BEIJ 4	Small, circular water droplet like	Gr-ve	+	++	+	_	_	*Beijerinckia*
BEIJ 5	Medium, circular water droplet like, rod.	Gr-ve	+++	+++	+	_	_	*Beijerinckia*
BEIJ 6	Water droplet like with raised center, rod.	Gr-ve	++	+	_	_	+	*Beijerinckia*
BEIJ 7	Small, circular, water droplet like, rod.	Gr-ve	+	+	_	_	_	*Beijerinckia*
BEIJ 8	Small, circular, raised water droplet like, rod	Gr-ve	++	+	_	_	_	*Beijerinckia*
BEIJ 9	Water droplet like with white center, rod.	Gr-ve	+	+	_	_	_	*Beijerinckia*
BEIJ10	Small, circular water droplet like, rod.	Gr-ve	+	+	_	_	_	*Beijerinckia*
BEIJ11	Small, circular water droplet like, rod.	Gr-ve	+	++	+	+	_	*Beijerinckia*
BEIJ12	Small, raised, water droplet like, rod.	Gr-ve	++	+	+	+	_	*Beijerinckia*
BEIJ13	Medium, white transparent, water droplet like, rod.	Gr-ve	+	+	+	_	_	*Beijerinckia*
BEIJ14	Medium, white transparent, water droplet like, rod.	Gr-ve	++	+++	+	_	_	*Beijerinckia*

Table 1. Continues

BEIJ15	Small, circular, water droplet like, rod.	Gr-ve	++	++	+	_	+	*Beijerinckia*
BEIJ16	Medium, round, water droplet like, rod.	Gr-ve	+	+	+	_	_	*Beijerinckia*
BEIJ17	White, round water droplet like, rod.	Gr-ve	+	+	+	_	_	*Beijerinckia*

Table 2. Biochemical Characteristics of the isolates

S. No.	Isolate	A	B	C	D	E	F	G	H	Probable genus
1.	BEIJ1	+	_	+	+	_	_	+	+	
2.	BEIJ2	+	_	+	+	_	_	_	+	
3.	BEIJ3	_	_	+	+	_	_	_	+	
4.	BEIJ4	+	_	_	+	_	_	+	+	
5.	BEIJ5	+	_	_	+	_	_	+	+	
6.	BEIJ6	+	_	_	+	_	_	+	+	
7.	BEIJ7	+	_	_	+	_	_	+	+	*Beijerinckia*
8.	BEIJ8	+	_	_	+	_	_	+	+	
9.	BEIJ9	_	_	_	+	_	_	+	+	
10.	BEIJ10	+	_	_	+	_	_	+	+	
11.	BEIJ11	+	_	_	+	_	_	+	+	
12.	BEIJ12	+	_	_	+	_	_	+	+	
13.	BEIJ13	+	_	_	+	_	_	+	+	
14.	BEIJ14	+	_	_	+	_	_	+	+	
15.	BEIJ15	_	_	_	+	_	_	+	+	
16.	BEIJ16	+	_	_	+	_	_	+	+	
17.	BEIJ17	+	_	_	+	_	_	_	+	

A. Starch hydrolysis, B. Gelatin liquefaction, C. Urease test, D. Catalase test, E .Methyl red test, F. Voges-Proskauer, G .Acid production, H .Oxidase test.

overnight cultures of the test isolates were spotted and plates were incubated for 24 hours at 30^0 C. After incubation, 2 to 3 drops of tetra methyl – phenylenediamine dihydrochloride was added to the surface of the growth of each test organism. The colour change on to maroon was taken as oxidase positive.

RESULTS AND DISCUSSION

The present investigation was carried out in the month of August to December, 2010 on isolation of diazotrophs from tea rhizosphere of different tea estates of Southern Assam, India. The isolates were identified on the basis of their morphological and biochemical characteristics. The results obtained on these aspects are presented hereunder.

All seventeen strains were isolated on the plates containing *Beijerinckia* agar medium. The colonies on the plates were examined as water droplet like colonies ranging from small to medium in size. All of them were Gram negative and rod shaped. They showed good growth on medium containing dextrose and sucrose, moderate growth on mannitol and poor growth on starch as sole

carbon source. All the isolates were catalase positive, most were oxidase positive, gelatin negative and most of them were Urease negative , MR –VP negative and a few were starch negative Based on their growth on N-free *Beijerinckia* medium and on the basis of their morphological, physiological and biochemical characteristics all the isolates were identified of the of the genus *Beijerinckia*. The diazotrophs as well as the plant growth promoting rhizobacteria contribute greatly to the turnover of organic matter and, while present in soil, are abundant on the surface of plant roots. Of the plant colonizing strains, some isolates positively affect plant health and nutrition[7, 8].The mechanistic bases of these effects remain unclear, but are known to include the production of plant growth hormones, the suppression of pathogens harmful to plant health, and the direct elicitation of plant defense responses [9].It has been argued that exploitation of the plant growth promoting bacteria and diazotrophs in agriculture requires an improved understanding of the determinants of ecological performances[10].

The morphological, biochemical and physiological tests identified different strains within the genus *Beijerinckia* .But the identification at species level could not be confirmed using the morphological, biochemical and physiological analysis. In the present study, seventeen *Beijerinckia* isolates were obtained from tea rhizosphere of different tea estates of south Assam in the month of August,2010.The isolates were purified and subjected to identification based on morphological ,physiological and biochemical characters. The isolates obtained on *Beijerinckia* medium were tentatively identified as *Beijerinckia* based on colony morphology and utilization of different carbon sources as suggested by Becking (1961).

CONCLUSION

An attempt was made to isolate and characterize rhizomicroorganisms from tea rhizosphere in tea growing regions of South Assam,India.Based on morphological ,physiological and biochemical characters all the seventeen isolates were identified as rhizomicroorganisms of the genus *Beijerinckia* .So far no molecular work has been done on the diazotrophs isolated from tea rhizosphere of South Assam .It has become the need of the hour to extend the molecular tools in conjunction with microbiological methods for analyzing the genetic diversity of free living diazotrophs from rhizosphere and non rhizospheric soil of tea and to study the host genes that are mainly responsible for correlation between the plant roots and bacteria and the best genes helping in nitrogen fixation for achieving a significant breakthrough in this approach.

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