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Native endophytic bacterial isolates from *Camellia sinensis* var assamica plantation in Tapesia (Assam) reveal distinct plant growth promoting potential

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Abstract

The present study was focused on screening of bacterial endophytes from *Camelia sinensis* for plant growth promoting activity along with phenotypic, biochemical characterization and identification. Plant tissue samples (leaves, stems and roots) were collected from the Tapesia tea garden which was thereafter surface sterilized followed by serial dilution for isolating the endophytic bacteria. The bacterial isolates were characterized biochemically and identified by VITEK –MS system. and thereafter screened for different plant growth promoting parameters *viz*. ammonia production, indole acetic acid production, siderophore production, L-asparaginase production. A total of twelve bacterial isolates were identified and their growth promoting features were studied. *Bacillus cereus* isolates ADBU 1 and ADBU 3 showed asparagine and ammonia production, while ADBU 2, ADBU 12 were found to be siderophore and ammonia producing. Similarly, ADBU 4 and ADBU 5 were only ammonia producers whereas ADBU 7 and ADBU 9 were siderophore, ammonia and asparaginase producing isolates. ADBU 6 was identified as *Lysinibacillus fusiformis* exhibited IAA and ammonia production property. ADBU 8 was *Acinetobacter johnsonii* which was found to be siderophore and ammonia producer. Hence, based on the present findings it may be concluded that the bacterial isolates associated with the tea plantation possess multifarous growth promoting properties which may be developed as bioformulation and/or biofertilizers.

Endophytes are a class of microorganisms that proliferate within plants and are known to play an important role in disease resistance, secondary metabolites synthesis, plant growth regulations, and help in withstanding environmental stress[18]. Endophytic bacteria have ability to promote plant growth by producing phyto-hormones including indole

acetic acid, gibberellins or by production of compounds which can inhibit the growth of other disease-causing microorganisms including bacteria, fungi, viruses, and protozoans. Endophytic bacteria cannot be limited to a particular class or family and belongs to the broad range of taxa which includes α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Firmicutes, Actinobacteria. Initially, endophytes studies were focused on rice, wheat, cotton and other staple crops, but gradually the attention has shifted to plants with therapeutic properties.

Camellia sinensis belonging to the family Theaceae is widely cultivated and economically important beverage of South East Asian countries which are known to be one of the richest source of polyphenols (*e.g.*, flavonoids and catechins), anthocyanin, theanine contributing to the antimicrobial, anticarcinogenic, anti-inflammatory, anti-arteriosclerotic properties of the plant. There has been great interest to investigate the endophytic association with tea as they might play an important role in production of the tea related plant compounds, endowing the tea plant to be disease resistant as well as promoting their growth. Bhattacharyya *et al*.[2], studied growth promotion properties and induction of anti-oxidative defense mechanism by tea rhizobacteria. Borah *et al*.,[3] isolated a total of 129 endophytic bacteria and studied the plant growth promoting traits[3]. Yan *et al.,*[19] showed endophytic bacteria from two closest cultivars Zijuan and Yunkang-10 of tea with various growth promoting properties[19]. Shan *et al.*,[17] also isolated a total of 46 actinobacteria from leaf, stem, and root samples of fifteen tea cultivars collected in Fujian province in China with varied growth promoting property[17]. Dutta *et al.,* assessed the growth promoting properties of culturable tea rhizobacteria[7]. Nath *et al*.,[11] reported a total of eighteen tea growth promoting endophytic bacterial isolates from tea roots.

Assam tea is well known all over the world for the flavor and is endowed with immense antioxidant potential. The clones available in Assam belong to *Camellia sinensis* var. assamica which is wide spread in Assam with 713 tea estates in total Assam (the Sentinel, 1st February, 2021). Tea harbors endophytic microbes which may directly affect the growth of tea plant as well as synthesis of different antioxidant phyto-compounds present in tea. This can affect tea quality and in turn influence the tea market all over the world as Assam tea is a dominant segment of world tea plantation. The present study was conducted at one of the plantation the Tapesia tea plantation of Assam. Tapesia tea plant plantation is embedded in the campus of Assam Don Bosco University, approximately 30 km from Guwahati, sharing its boundaries with Amchang Wildlife Sanctuary. It is believed locally that the name "Tapesia" is derived from an endophytic fungus which predominantly associated with this tea estate. Based on such reports and its unique location, the present research was formulated to identify the endophytic bacteria associated with tea plantation with potential growth promoting properties.

Collection and preparation of samples :

The shoots, leaves and roots were collected from Tapesia tea plantation located

at latitude 26.128 N and longitude 91.901 E from five different locations. Healthy and disease-free young plant parts were collected in poly bags and stored at 4°C for further use.

Surface sterilization and isolation :

Shoots, roots and leaves were washed in running tap water and graded by size and surface appearance in order to exclude samples that showed symptoms of disease or superficial damage. The plant parts were washed with 70% ethanol for 30 seconds and 2% sodium hypochlorite for 5 minutes and washed five times with sterilized water. The sterilized stems and leaves were aseptically cut into 1-2 mm sections. The samples of shoots, leaves and roots was cut into small pieces and macerated separately in phosphate buffer of pH 7.2 with a sterile pestle and mortar. Tissue extract was then prepared for tenfold dilution in sterile saline. Serial dilutions (10^{-5}, 10^{-6}, and 10^{-7}) was prepared from this extract. For inoculations 0.1ml of the aliquot was used on nutrient agar medium. To confirm that the disinfection process was successful, the plant organs pieces were pressed onto tryptic soya agar medium plates and aliquots of the sterile distilled water used in the final rinse were also plated onto the same medium.

Phenotypic and Biochemical characterization :

The pure culture of bacterial isolates was streaked on the tryptic soya agar plates to observe the colony characteristics such as size, color, form, surface, texture and elevation. Different colonies were subjected to Gram's staining in order to identify the type of bacteria. Further, catalase test, oxidase test, nitrate reduction test, urease test, c*itrate utilization assay,* triple sugar iron test were carried out.

Identification of bacterial isolate

The bacterial isolates were also identified by the automated system VITEK MS system (Nazareth Hospital, Shillong). VITEK- MS is an automated mass spectrometry microbial identification system that uses Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) technology.

Screening for plant growth promoting properties :

Indole acetic acid production :

The various bacterial isolate was grown in 5ml of nutrient broth medium in test tube for 24 hrs. After incubation period, bacterial culture was harvested and centrifuged at 10000g for 15min at 4°C. Two drops of orthophosphoric acid was added to 2ml of cell free supernatant and the development of colour was observed. The presence of a pink colour indicate positive reaction for indole acetic acid and yellow colour indicate negative reaction[4].

Siderophore production :

Ability of the bacteria to produce siderophore was determined qualitatively using the chrome azurol S (CAS) based agar medium (Schwyn and Neilands, 1987). Bacteria was spot inoculated on the test medium and allowed to grow for 48-72 hours at 30°C. The colonies surrounded by orange to yellow halo was identified as siderophore producers.

L-Asparaginase production :

Modified M-9 agar media supple-

mented with the pH indicator, phenol red 0.027 g (0.009%) used for screening purpose. Endophytes was inoculated into the media and after incubation for 48 hours. At a temperature of 37°C, the pink zone developed around the colony was appeared[15].

Ammonia production :

Bacterial isolates were grown in 10ml peptone water medium for 72 hours at 30°C. After 0.6 OD growths 0.5ml of Nessler's reagent was added. Development of brown to yellow coloration indicated ammonia production[5].

Phenotypic, biochemical characteristics and identification of isolates by VITEK- MS

A total of twelve isolates were obtained from different tissue parts of *Camellia sinensis* from the tea planatation, referred as ADBU 1- ADBU 12 and their colony characteristic, features were recorded. All the isolates showed creamish white colony. ADBU 1, ADBU 2, ADBU 3, ADBU 4, ADBU 5, ADBU 7, ADBU 9, ADBU 12 are circular; ADBU 6, ADBU 8, ADBU 10, ADBU 11 which are irregular in contour. Gram's staining of the isolate showed that ADBU-1, ADBU-2, ADBU-3, ADBU-4, ADBU-5, ADBU-6, ADBU-7, ADBU-9 and ADBU-12 were gram positive whereas ADBU-8, ADBU-10, ADBU-11 were gram negative. All the isolates except ADBU 8 were rod shaped; ADBU 8 was cocci shaped. Further biochemical analysis revealed that all the twelve organisms were catalase producing. Out of the twelve, ADBU 1, ADBU 2, ADBU 3, ADBU 4, ADBU 5 ADBU 6, ADBU 7, ADBU 9, ADBU12 were oxidase producers. All the isolates except ADBU 8 showed nitrate production and ADBU 6, ADBU 10, ADBU 11 were demonstrating urease production. The isolates ADBU 1, ADBU 8, ADBU 10 and ADBU 11 showed red slant/red butt, with no production of gas and H_2S indicating absence of carbohydrate fermentation results, whereas ADBU 2, ADBU 3, ADBU 4 ADBU 5, ADBU 6, ADBU 7, ADBU 9, ADBU 12 showed red slant/ yellow butt with no production of gas and H_2S which mean it indicates that the organism is a dextrose fermenter but is unable to ferment lactose and/ or sucrose. Further, isolates were identified as ADBU 1, ADBU 2, ADBU 3, ADBU 4, ADBU 5, ADBU 7, ADBU 9, ADBU-12 were identified as *Bacillus cereus*, ADBU 6 as *Lysinibacillus fusiformis*, ADBU 10, ADBU 11 as *Pseudomonas fluorescens* and ADBU 8 as *Acinetobacter johnsonii*.

Screening for plant growth promoting properties :

Indole Acetic Acid production is one of prominent plant growth promoting compound secreted by endophytic bacteria**.** The isolate ADBU 6 which is identified *Lysinibacillus fusiformis* showed the presence of pink colour which indicated the production of indole acetic acid whereas rest of the isolates were not positive as it remained yellow in colour. Ammonia production is another plant growth promoting trait which is involved in cell signaling role between plant and bacterial interactions and further help in uptake of nitrogen in form of ammonia released by endophytic microbes. In ammonia production test, all the isolates were positive as on addition

The results are illustrated in Figure 1 and Table 1

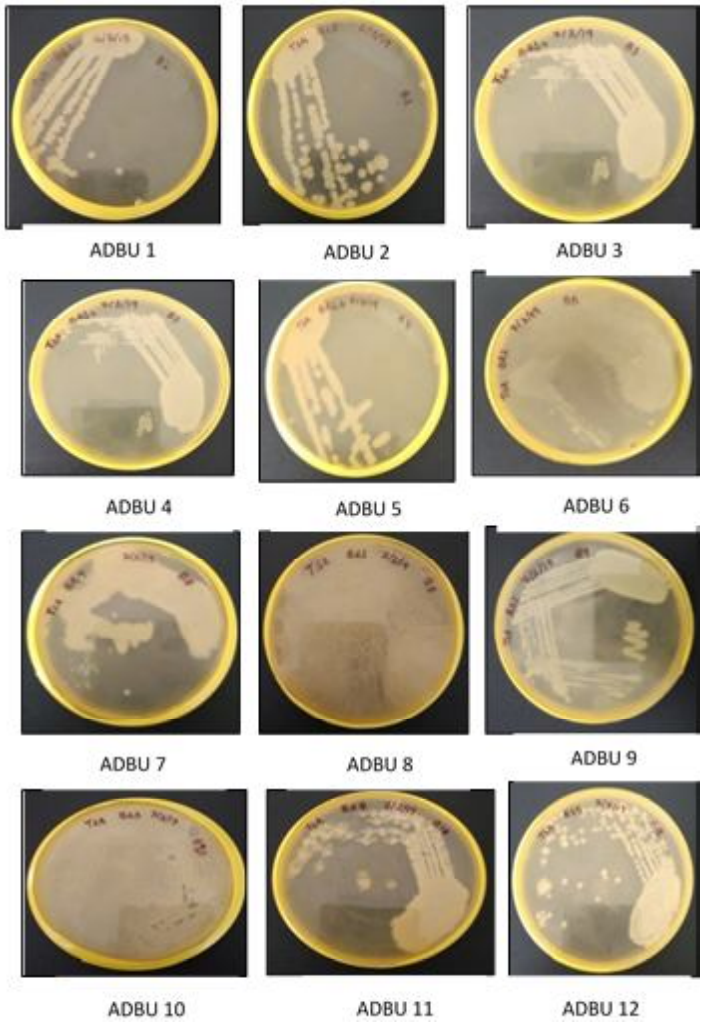


Figure 1 Colony characteristics of the isolates

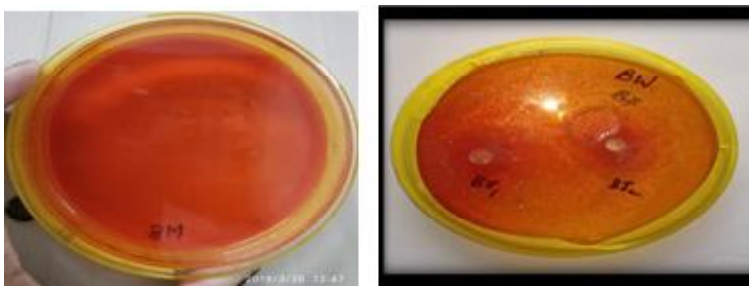
of Nessler's reagent, yellow colour appeared, indicating all the organism produce ammonia exhibiting that they are involved strongly in nitrogen supply to the plants.

Out of the twelve isolates, ADBU 2, ADBU 7, ADBU 8, ADBU 9, ADBU 10, ADBU 11, ADBU 12 were positive for siderophore production indicated by appearance of orange to yellow halo whereas isolates ADBU1, ADBU 3, ADBU 4, ADBU 5, ADBU 6 were negative as there was no appearance of the halo Qualitative analysis of asparaginase production showed that ADBU

Table-1. Phenotypic, biochemical features and identity of the isolates

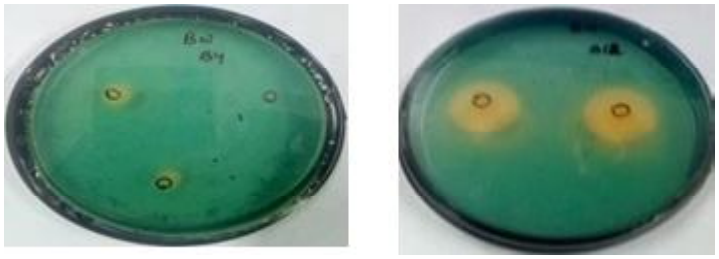
Isolate ID	Colony features	Gram staining	ViteK Identity	Catalasetest	Oxidase test	Nitrate reduction test	Urease Test	Citrate utilization	Triple Sugar Iron test
ADBU-1	Creamish white, Circular, Flat, Undulate	Rod+ve	*Bacillus cereus*	+ve	+ve	+ve	-ve	+ve	Red slant/butt, No Gas, No H_2S
ADBU-2	Creamish white, Circular, Flat, Undulate	Rod +ve	*Bacillus cereus*	+ve	+ve	+ve	-ve	+ve	Red slant/ Yellow butt, No Gas, No H_2S
ADBU-3	Creamish white, Circular, Flat, Undulate	Rod +ve	*Bacillus cereus*	+ve	+ve	+ve	-ve	+ve	Red slant/ Yellow butt, No Gas, No H_2S
ADBU-4	Creamish white, Circular, Flat, Undulate	Rod +ve	*Bacillus cereus*	+ve	+ve	+ve	-ve	+ve	Red slant/ Yellow butt, No Gas, No H_2S
ADBU-5	Creamish white, Circular, Flat, Undulate	Rod +ve	*Bacillus cereus*	+ve	+ve	+ve	-ve	+ve	Red slant/ Yellow butt, No Gas, No H_2S
ADBU-6	Creamish white, Irregular, Flat, Lobate	Rod+ve	*Lysinibacillus fusiformis*	+ve	+ve	-ve	+ve	+ve	Red slant/ Yellow butt, No Gas, No H_2S
ADBU-7	Creamish white, Circular, Flat Undulate	Rod +ve	*Bacillus cereus*	+ve	+ve	+ve	-ve	+ve	Red slant/ Yellow butt, No Gas, No H_2S
ADBU-8	Creamish white, Irregular, Raised, Entire	Cocci -ve	*Acinetobacter johnsonii*	+ve	-ve	-ve	-ve	-ve	Red slant/Red butt, No Gas, No H_2S

ADBU-9	Creamish white, Circular, Flat, Undulate	Rod +ve	*Bacillus cereus*	+ve	+ve	+ve	-ve	+ve	Red slant/ Yellow butt, No Gas, No H_2S
ADBU-10	Creamish white, Irregular, Raised, Undulate	Cocci -ve	*Pseudomonas fluorescens*	+ve	-ve	-ve	+ve	+ve	Red slant/Red butt ,No Gas, No H_2S
ADBU-11	Creamish white, Irregular, Raised, Undulate	Cocci-ve	*Pseudomonas fluorescens*	+ve	-ve	-ve	+ve	+ve	Red slant/Red butt ,No Gas, No H_2S
ADBU-12	Creamish white, Circular, Flat, Undulate	Rod -ve	*Bacillus cereus*	+ve	+ve	+ve	-ve	+ve	Red slant/ Yellow butt, No Gas, No H_2S



A *B*

Figure 2 Siderophore production of the of the various isolates from leaves, roots and shoots. Non appearance of the halo (A) indicates negative result, whereas positive result is indicated by appearance of orange to yellow halo (B).



A *B*

Figure 3. Asparaginase production of the of the various isolates from leaves, roots and shoots of *Camellia sinensis* Non appearance of the halo (A) indicates negative result, whereas positive result is indicated by appearance of orange to yellow halo (B).

1, ADBU 3, ADBU 7 and ADBU 9 identified as *Bacillus cereus* were found to be asparaginase producer. The results are illustrated in Table-2.

Endophytes are unique class of microbes which help in the growth of the plant by helping in making nutrients available for plants, alleviating the harsh environmental conditions on the plants. Endophytic microbiota are known to produce various compounds which have insect repelling, disease preventing effects which result in luxuriant growth pattern of the plants. In the present study, twelve bacterial endophytes were isolated belonging to the genus of *Bacillus spp, Lysinibacillus spp*, *Pseudomonas spp* and *Acinetobacter spp* with varied biochemical characteristics from Tapesia tea plantation. There are various reports of rhizospheric as well as endophytic bacteria being isolated and identified from tea plants. Isolation of rhizospheric bacteria from seven different tea estates of Darjeeling, West Bengal, belonging to different genus of *Bacillus, Staphylococcus, Ochrobactrum, Pseudomonas, Lysinibacillus, Micrococcus, Leifsonia, Exiguobacterium,* and *Arthrobacter* have been reported[2]. Borah *et al*.,[3] identified endophytic bacteria genera of *Bacillus, Brevibacterium, Paenibacillus and*

Table-2. Identification of various isolates and screening of plant promoting activities from different parts of *Camellia sinensis*

Sl No.	Codes	Bacterial species (Vitek identity)	Indole acetic acid (IAA) production	Siderophore Production	L-Aspari ginase Production	Ammonia production
1	ADBU 1	*Bacillus cereus*	-	-	+	+
2	ADBU 2	*Bacillus cereus*	-	+	-	+
3	ADBU 3	*Bacillus cereus*	-	-	+	+
4	ADBU 4	*Bacillus cereus*	-	-	-	+
5	ADBU 5	*Bacillus cereus*	-	-	-	+
6	ADBU 6	*Lysinibacillus fusiformis*	+	-	-	+
7	ADBU7	*Bacillus cereus*	-	+	+	+
8	ADBU 8	*Acinetobacter johnsonii*	-	+	-	+
9	ADBU 9	*Bacillus cereus*	-	+	+	+
10	ADBU 10	*Pseudomonas fluorescens*	-	+	-	+
11	ADBU 11	*Pseudomonas fluorescens*	-	+	-	+
12	ADBU 12	*Bacillus cereus*	-	+	-	+

Lysinibacillus associated with Assam tea plantation[3]. Yan *et al.,*[17] reported isolates from tea belonging to Proteobacteria, Firmicutes, and Bacteroidetes genera from Zijuan and from Yunkang mainly belonging to phylums Proteobacteria and Firmicutes. Dutta *et al.*,[7] reported rhizobacterial isolates obtained from six different tea estates of India.

Investigation on the plant growth promoting property showed that all the twelve isolates of endophytic bacteria were ammonia producers. Ammonia can be produced by mechanism of degradation of various amino acids and decarboxylation of amino acids in plants, however production of ammonia by endophytic microbial colonies is one of major source of nitrogen that vastly contribute to the growth of plant. Similar reports of ammonia producing tea endophytes have been reported by Borah *et al*.,[3] and Nath *et al*.,[11]. Another important compound, produced by endophytes using tryptophan dependent or tryptophan independent pathways is the plant growth promoting hormone indole acetic acid commonly known as auxin. In the present study, one of the isolate *Lysinibacillus fusiformis* was found to be IAA producer from the tea samples. Reports show that IAA producing *Lysinibacillus fusiformis* were isolated from local plants of Thailand[8], wheat and soyabean crops in China[18]. Similar studies related to tea were carried out by Yan *et al*.,[19]; Shan *et al*.,[17]; Borah *et al*.,[3] Dutta *et al*.[7], Shan *et al*.,[17] Yan *et al*.,[19] & Yu *et al.*,[20] recorded IAA producing culturable endophytes from tea. In the present study, seven of the endophytes were siderophore producing endophytes belonging to the genus of *Bacillus*, *Pseudomonas* and *Actinobacter*. Siderophores are iron chelating compounds helping in uptake of iron which in turn helps in growth of the plants. Additionally, the production of siderophores

has been reported to be one of the mechanisms to compete with pathogens (O'Sullivan & O'Gara, 1992), and protect the plants from diseases[18]. These are low weight molecules (between 500 and 1500 dalton) with great affinity and selectivity to bind and complex Fe^{3+}. They are produced by microorganisms, as well as by some gramineous plant, as part of a strategy to obtain iron from the environment because of the low bioavailibility of iron. Siderophore production from bacterial strains is considered one of the direct mechanisms of plant growth promotion. Various groups of siderophore producing *Acinetobacter spp* has been reported for siderophore production from wheat plants (Maindad *et al.,*)[10], rhizosphere of *Pennisetum glaucum*[15,16]. Shan *et al*.,[17], Yan *et al*., 2018 and Dutta *et al*., 2015 also reported various groups of bacteria producing siderophore isolated from tea[7,17,19]. Another interesting, amino acid degrading enzyme L-Asparaginase (E. C 3.5.1.1) responsible for conversion of L-Asparaginase to aspartic acid and ammonia has been reported from *Bacillus subtilis* strain hswx88, has been isolated from Taptapani hotspring of Odisha, India[14], from *Bacillus licheniformis* isolated from the Red Sea, Saudi Arabia has also been reported[1], from soil samples collected from various places near Aurangabad which were found to be asparaginase producer[13]. Joshi *et al.,*[9] reported isolation of endophytic bacteria with asparaginase producing activity from medicinal plants with anticancer properties. Nongkhlaw and Joshi[12] reported endophytic bacteria associated with ethnomedicinal plants of North-east India exhibiting significant asparaginase producing capacity. Asparaginase enzyme is gaining attention as it possesses therapeutic mechanism for tackling cancer. Several types of tumour cells require L-asparagine, an essential amino acid and in the presence of L-asparaginase, they are deprived of an essential growth factor. It is found that effective depletion of L-asparagine results in cytotoxicity for leukemic cells. In the present study four isolates belonging to the genus *Bacillus* which exhibited asparaginase producing property. Reports related to such asparaginase producing endophyte isolated from tea are scanty and these endophytes might be explored for their therapeutic benefits.

The study revealed potential gateway for developing plant growth prompting bioformulation and biofertilizer which may aid in *in situ* nutritional management in local plantations of Assam tea and thereby reduce dependence on chemical fertilizers. The collateral benefits to such microbial formulations such as disease and pest resistance can be derived, However the identity and the diversity of these isolates needs to be ascertained at molecular level by 16s rRNA sequencing for further confirmation. This study can be the basis for further studies on plant growth promoting like asparaginase and sideraphore in Assam tea for the sustainability of the industry.

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