ENDOPHYTES OF TEA PLANTS FROM DARJEELING, WEST BENGAL

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Abstract:

A total of 14 bacterial and 6 fungal endophytes were isolated from Tea plant i.e. *Camellia sinensis* grown in famous Tea garden Happy valley, Darjeeling District, West Bengal, India. Endophytes are a group of microorganisms that grow within the tissue of higher plants and colonize them without causing any noticeable injury to the host. Both bacteria and fungi are considered as endophytes. Endophytes represent a potential hub of novel bioactive compounds such as antibiotics, anticancer, and other biological control agents. The bacterial population showed a high level of growth hormone production namely auxin and gibberellins to the level ranging from 160 to 300 µg/ml and 172 to 383 µg/ml respectively. None of the strains were found to solubilize phosphorus and fix nitrogen. The bacterial population also showed antimicrobial activity against human pathogenic strains such *as Escherichia coli, Vibrio cholera, Klebsiella sp. Pseudomonas aeruginosa, Acinetobacter baumannii* and *Burkholderia sepsia*. Most of the fungal genera showed amylolytic and proteolytic activity. Thus, the study suggests that these microbes have huge potential to synthesis of numerous novel compounds that can be explored in pharmaceutical, agricultural and other industries.

Keywords: Endophytes, Growth Hormones, Auxin, Gibberlins, antimicrobials, Tea plant

Introduction:

Endophytes are microorganisms that ubiquitously colonize the internal tissues of plants without causing any negative effects, and some endophytes are able to control plant pathogens and promote the growth of plants (Santoyo *et al.,* 2016; Kandel *et al.,* 2017, Wei *et al.,* 2018). They are considered as endosymbiont (Mukhopadhyay and Chakraborty, 2019). They enter in the plant body either through root or aerial parts (Kobayashi and Palumbo, 2000). Initially they remain localized and then spread in different tissues. During endophytes

colonization the microorganisms resides in almost every internal part of plant ranging from tissues of the underground roots to stem, leaf, flower, fruit and seed (Strobel and Daisy 2003).

Endophytic bacteria promote host plant's growth through direct mechanisms by producing plant hormones like IAA, gibberellins or indirectly by inhibiting plant pathogens (Kumar *et al.*,. 2016). Natural products from endophytic bacteria have been observed to inhibit or kill a wide variety of harmful disease-causing agents including bacteria, fungi, viruses, and protozoans that affect humans and animals (Lodewyckx, 2002).

Camellia sinensis commonly known as tea is a herbaceous plant of Family Theaceae cultivated in South Asia. It plays a very important role in Indian Economy as Tea is the most widely consumed as beverage in the world. Its polyphenolic compounds have been found for medinical properties (Devi and Wahab, 2012). Among the three varieties of tea found in India Assam Tea, Darjeeling Tea and Nilgiri Tea, Darjeeling tea is the most superior variety and having great demand throughout the World. Many Plant growth promoting (PGP) endophytic acitinobacteria are reported from *Camellia sinensis* showing PGP traits like, phosphate solubilization, indole-3-acetic acid (IAA), ammonia, siderophore and chitinase production (Borah and Thakur, 2020). Some endophytes showed direct growth promoting activity in tea plants by enhancing the vegetative parameters such as dry/fresh weight of root and shoot of tea plants in nursery conditions (Borah *et al.*, 2019).

Until a viable alternative can be accessible, the emergence of resistance to antimicrobials requires the constant development of new antibiotics. The actinomycetes isolated from plant parts are reported to produce antmicrobial substances and inhibited many human pathogens (Beiranvand *et al.*, 2017). Not only human pathogens these organisms are also helpful in controlling plant pathogens (Mohamd *et al.*, 2018). Various plant pathogens attack plant tissues which can be outcompeted and inhibited by the residing endophytic fungi with aid of extracellular lytic enzymes like chitinase, protease, cellulase etc. production (Choi *et al.*, 2005) which breaks down the plant pathogen cell wall constituting chitin, modified cellulose, starch, as storage material (de Bashan *et al.*, 2005).

Our current study focuses on the isolation of endophytes from important beverage plant - *Camellia sinensis* (Tea) and exploring their potential for plant growth promoters, extracellular enzymes and antimicrobial compounds.

Materials and Methods

Sample collection:

Samples were obtained from Tea plant (*Camellia sinensis*) collected from Happy Valley Tea Estate, Darjeeling , West Bengal, India. The roots, stems and leaves of the collected plants were taken aseptically to the laboratory and kept refrigerated until use.

Isolation of Endophytes:

Surface sterilization of the root, stem and leaves was done with tap water, Tween 20, Sodium hypochlorite, 70% alcohol and sterile distilled water.

Slurry was prepared in isotonic saline solution and then plated in Luria Bertani agar and Czapek Dox agar, incubated at 37ºC and 30°C for consecutive days. Isolated colonies are sub cultured in respective slant and morphology and Gram nature were determined by standard method.

Gibberellin production assay:

Gibberellic acid was estimated by (Borrow *et al.,* 1955). The isolates were grown in Nutrient broth for 4 days then were centrifuged at and the supernatant was used for extraction of Gibberellin. pH of the supernatant was adjusted to 2.8 by 1N HCL and to 1.5ml supernatant 0.2ml of Zinc acetate solution and 0.2ml of Potassium ferrrocyanide solution was added and centrifuged, 0.5ml of supernatant was then added to 0.5 ml of 30% HCL and the mixture was then incubated at 27°C. Absorbance was measured at 250 nm in UV-Vis spectrophotometer and compared to a standard curve.

Auxin production assay:

Auxin was estimated by (Gordon *et al.,* 1951). The isolates were made to grow in IAA production media for 10 days and then centrifuged and the supernatant was used for IAA production. 1 part of the supernatant was added to 2 parts of Salkowsky reagent and incubated for 30 minutes to observe red colour. Absorbance was measured at 530 nm and compared to a standard curve for quantification.

Phosphate solubilization:

The isolates were streaked onto plates containing the Pikovskaya's agar medium and were incubated for 7 days at 28°C.

Nitrogen fixation:

In order to screen the bacterial isolates for nitrogen fixation ability, they were made to grow on slants of glucose nitrogen free mineral media. Slants were prepared of the mentioned agar and isolates were streaked onto it and incubated at 28°C for 7 days.

Antimicrobial assay:

Isolated bacterial strains were cultured in Luria Bertani for 5 days then were centrifuged and the filtrate was used for the assay. The assay was done by agar well diffusion method on Luria Agar plates containing test pathogenic organisms namely, *Vibrio cholerae, Pseudomonas aeruginosa, Burkholderiacepacia, Acenatobacterbaumanii, Klebsiella sp.* and *Escherichia coli.*

Screening of fungal isolates for Extracellular Enzyme production:

a)Cellulase Activity - The fungal isolates were assessed for cellulase activity by streaking on CMC Agar Media(K_2HPO_4-1gm, $MgSO_4$-0.5gm, NaCl-0.5gm, $FeSO_4.7H_2O$-0.01gm,$MnSO_4$-

0.01gm,NH_4NO_3-0.03gm, CMC-10gm, Agar-20gm,dis.H_2O-1lit.) and incubated for 5 days. After fungal growth appearance, the plates were flooded with 0.1% Congo red solution for 15 mins with shaking and destained with 1M NaCl solution for 15 minutes. Appearance of clear zones around fungal colony indicated cellulase activity.

b)Amylase Activity - The fungal isolates were assessed for amylase activity by inoculating on Starch Agar Media (Beef Extract-3gm, Soluble Starch-10gm, Agar-20gm, dis.H_2O-1lit.) and incubated at 28°C for 24 - 48hrs . After incubation, the plates were flooded with 1%Gram's iodine solution and observed for the appearance of a clear zone of hydrolysis around the fungal growth.

Results and Discussion:

In the present study, 14 bacterial and 6 fungal isolates resulted from the root, stem, young leaf and mature leaf samples collected from tea garden of Happy Valley, Darjeeling, West Bengal which signifies a diverse amount of residing endophytes in Tea plant. Bacterial endophytes are either rod or coccus in shape and Gram positive in nature. 23 bacterial endophytes were reported from Rice plant were found mostly belong to Gram positive reaction (Mukhopadhyay and Chakraborty, 2019). The bacterial endophytic isolates were microscopically characterized as gram positive rods, which is consistent with those of *Curcuma longa* L.(Kumar *et al.*, 2016). Also, *Aspergillus sp*. are identified as endophytes in *Andrographis paniculata* (Elfita *et al.*, 2015).

Gibberellin is an important plant hormone that regulate various developmental processes, including stem elongation, germination, dormancy, flowering, flower development, and leaf and fruit senescence. All of the 14 isolates could produce appreciable amounts of Gibberellin ranging from 172-383µg/ml (Figure 1). The strains HVR1, HVLY2 and HVLM1 could produce maximum amount of gibberellic acid. Figure 1 shows the amount gibberellin production by the isolates. Ambawade and Pathade (2013) estimated Gibberellic acid production from banana plant endophyte to the levels of 0.240 mg/ml where as in rice plant it maximally produced at 250µg/ml (Mukhopadhyay and Chakraborty, 2019). Giberellic acid produced from Endophytic *Fusarium oxysporum* was reported to affect positively on morphological and physiological parameters in Tomato plant (Rohuma *et al.*, 2020).

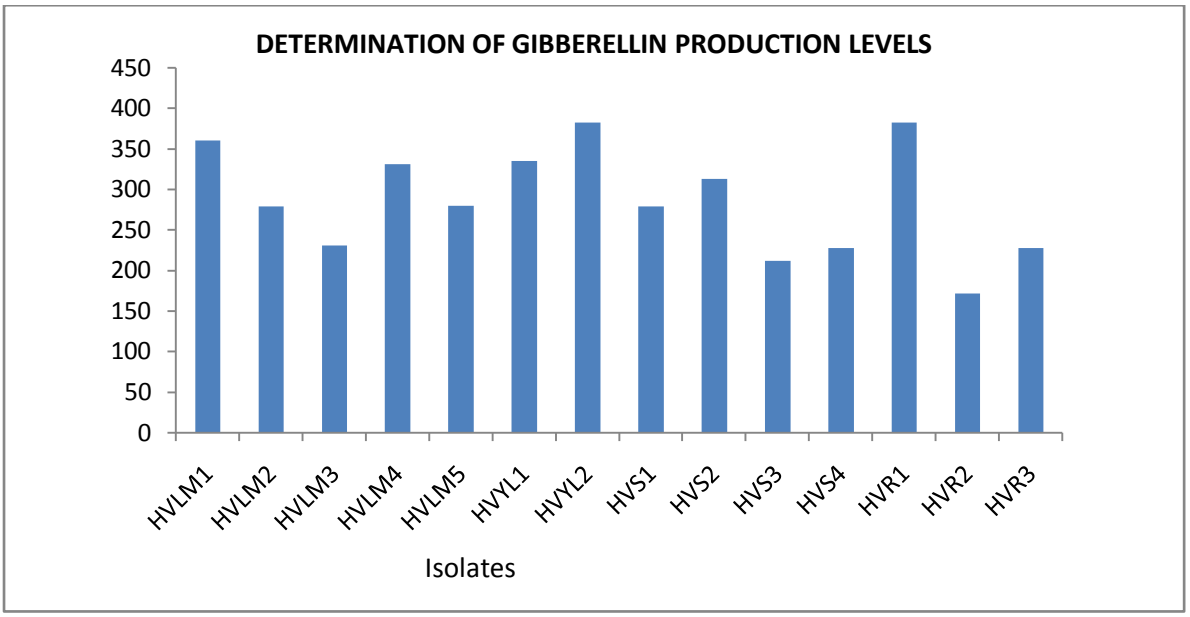

Figure 1: Production of Giberellin by Endophytc Bacerial Isolates

Table 1: Amount of Auxin produced by each isolate

Isolate	Auxin producing ability	Conc. of IAA produced (µg/ml)
HVLM1	+	300
HVLM2	+	200
HVLM3	+	300
HVLM4	+	200
HVLM5	+	300
HVLY1	+	300
HVLY2	+	160
HVS1	+	160
HVS2	+	160
HVS3	+	300
HVS4	+	200
HVR1	+	182
HVR2	+	200
HVR3	+	182

Under natural condition endophytes are reported to promote growth by IAA production (Khan *et al.*, 2020; Mukhopadhyay and Adhikari, 2020). All the isolates produced auxin at

appreciable levels of 160-300 µg/ml which was confirmed by production of red colouration of the supernatant (Table 1). The strains isolated from tea leaf could produce more auxin compared to other parts. Diverse microorganisms including bacteria (Arshad and Frankenberger, 1998; Khalid *et al.*, 2004), filamentous fungi (Kaldrof and Ludwig-Muller, 2000) and yeasts (El-Tarabily, 2004) are capable of producing physiologically active quantities of auxins and which have pronounced effects on plant growth and development.

Table 2: Antimicrobial activity of selected endophytic bacterial isolates against selected test organisms

Endophytic bacterial Isolates	Test organisms	Sensitivity	Diameter of inhibition zone (cm)
HVLM1	*Pseudomonas aeruginosa*	Resistant	-
	Klebsiella sp.	Resistant	-
	Acenetobacter baumanii	Resistant	-
	Vibrio cholerae	Sensitive	1.4
	Escherichia coli	Resistant	-
	Burkholdaria sepsia	Sensitive	1.3
HVLY1	*Pseudomonas aeruginosa*	Resistant	-
	Kleipbsiella sp.	Resistant	-
	Acenetobacter baumanii	Resistant	-
	Vibrio cholerae	Resistant	-
	Escherichia coli	Resistant	-
	Burkholdaria sepsia	Resistant	-
HVS1	*Pseudomonas aeruginosa*	Sensitive	0.6
	Kleipbsiella sp.	Resistant	-
	Acenetobacter baumanii	Resistant	-
	Vibrio cholerae	Sensitive	1
	Escherichia coli	Resistant	-
	Burkholdaria sepsia	Resistant	-
HVR1	*Pseudomonas aeruginosa*	Sensitive	0.8
	Kleipbsiella sp.	Resistant	-
	Acenetobacter baumanii	Resistant	-
	Vibrio cholerae	Resistant	-
	Escherichia coli	Resistant	-
	Burkholdaria sepsia	Sensitive	0.3

Unlike other endophytic organisms (Goldstein *et al.*, 1995; Tonooka *et al.*, 2008) these strains were not efficient in solubilizing phosphate or fixing nitrogen.

Antimicrobial potential of the endophytic bacteria isolate were evaluated against six pathogenic bacteria (*E.coli, Burkholderia sepsia, Acinetobacter baumannii, Kleibseilla sp., Vibrio cholera, Pseudomonas aeruginosa*) (Table 2).

In primary screening 3 bacterial isolates (HVLM 1, HVR 1, HVS1) were found to show antimicrobial activity against 3 pathogenic bacteria out of the 6 and appeared to have a broad spectrum of antimicrobial activity in vitro. Several endophytic isoaltes are reported to produce antimicrobial substances against different pathogens (Hussain and Mustakim, 2017; Morris, 2003; Mukhopadhyay and Adhikari, 2020).

Apart from various primary and secondary metabolites, antioxidants, anticancer agents (Gunatilaka *et al.*, 2006), the endophytic fungi also serve as potent sources of industrially important enzymes with invaluable roles in biotechnology. They extracellularly produce hydrolases like pectinase, lipase, proteinase, amylase, laccase, xylanase etc. to resist pathogen invasion and nutrient acquisition from the host (Sunitha *et al.*, 2013) which are available for mankind with industrial and biomedical potentialities (Strobel *et al.*, 2003).

Of the isolated 6 strains 4 of the isolates could produce protease indicated by the hydrolysis zone in the casein agar medium. Fungal amylases have been widely used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production (Burhan *et al.*, 2003). All the 6 fungal isolates could degrade starch by amylase production, which is shown by significant area of clear zone around fungal mycelial growth (Plate 1).

Plate 1: Fungal isolate showing amylolytic activity

Conclusion:

In conclusion, the endophytes of important beverage plant -*Camellia sinensis* are novel and diverse. They exhibit several Plant Growth Promoting traits that results in improvement of growth and development in tea plant and serve as a resource of medicinally important

compounds against different human pathogens and help the plants in pathogen resistance and plays an important role in nutrient cycling as well. Thus, the endophytes are promising sources of various bioactive compounds in agricultural, biotechnological and pharmaceutical fields.

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