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Characterization and evaluation of plant growth promoting potentials of Actinobacteria from tea ecosystem

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Abstract The nutrient-binding abilities of six Actinobacteria strains native to tea ecosystems (designated AAS2, AAS7, APSA1, APSA4, APSA5, and CAS4) were investigated. Our findings demonstrated that all of the Actinobacterial strains examined exhibited strong solubilization abilities for both K and P. They also take advantage of the naturally occurring potassium and phosphorus to further their plant growth. Through nursery experiments can be established organic carrier materials for formulating the isolates and used as biofertilizers in the tea ecosystem as compared to other strains and untreated controls, the values of biometric growth parameters were significantly higher to provide the efficacy of the bio-formulation.

Keywords: Actinobacteria, Nutrient solubilization, Plant growth promotion, Bio-formulation, Carrier materials

Introduction

In agriculture, crop productivity is greatly influenced by the constant use of high doses of inorganic fertilizers Chemical fertilizers and pesticides harm soil health by creating nutrient imbalances in cultivable lands (Zhang *et al.,* 2018). Thus, a necessity to switch to eco-friendly farming practices arises. Several attempts have been made by scientists to devise a solution for this issue in various aspects; however, the use of microbes with multifunctional potentials as an alternative to inorganic fertilizers had proven to be the best choice to date (Khoshru *et al.,* 2020a, c). Soil microbes generally maintain a symbiotic relationship with plants by releasing several bioactive compounds, enzymes, antimicrobial substances, growth-promoting substances, and siderophores thereby upholding plant growth. In addition, microbial inoculants improve soil health by solubilizing inaccessible soil nutrients such as P, K, etc. and thereby enhancing nutrient mobility to plants. They also act against pathogens and pests

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and protect crops from biotic threats (Janardhan *et al.,* 2014; Kaur *et al.,* 2016; Liu *et al.,* 2018; Khoshru *et al.,* 2020b).

Actinobacteria is one such plant growth-promoting rhizobacteria with numerous plant growth-promoting characteristics. Actinobacteria possess both bacterial and fungal properties in them. This phylum Actinobacteria, being the largest, has six classes, while the class Actinobacteria is further grouped under bacteria with 20 orders (Nouioui *et al.,* 2018). They have demonstrated excellent plant growth-promoting activity by secreting a wide array of essential bioactive compounds in the soil which are paramount for crop productivity (Wahyudi *et al.,* 2019). They play key roles in N fixation, siderophore production, K solubilization, P solubilization, IAA production, etc. (Prakash and Cummings, 1988; Lee *et al.,* 2012; Anwar *et al.,* 2016; Myo *et al.,* 2019). They also manage plants to escape from pathogens. These bacteria release lignocellulosic enzymes which easily decompose dead and organic matter from the soil which results in efficient nutrient recycling in soil (Das *et al.,* 2007). They are known to well acclimatize extreme soils both alkaline and acidic (Phoebe *et al.,* 2001).

Bio-inoculants made out of soil Actinobacteria have demonstrated enhanced crop production under many abiotic stress conditions (Cheng *et al.,* 2018). Among all the Actinobacteria, *Streptomyces* is the most commonly found in all types of soil (Panneerselvam *et al.,* 2021) nevertheless, the diversity of soil microbes is endless.

Tea is one of the popular beverages in India and China. Tea is made from commercially cultivated tea plants (*Camellia sinensis* (L.) O. Kuntze). It is a cultivated crop in tropical to subtropical climates with even rainfall. Tea cultivation requires deep, acidic (pH 4.5–5.5), well-drained, fairly loamy, and lime-free soils (Zhang *et al.,* 2018). Generally, tea soils are noticeably acidic, rich in macronutrients, and classified as latosols (Natesan *et al.,* 1985). Nevertheless, the soil P supply in tea cultivation is usually poor owing to low intrinsic content and sturdy fixation by plentiful Al and Fe oxides found in the soil (Shen *et al.,* 2011). In addition, tea cultivation demands a relatively higher quantity of K for its better yield and quality.

The study's objectives were to find a suitable carrier material for actinobacterial strains to multiply in large numbers, characterize the actinobacteria, and investigate any possible consequences for the development of nursery tea plants.

Materials and methods

Soil sampling and isolation of Actinobacteria

The Anamallais and the Nilgiris, two agroecological regions of south India, provided the soil samples (0–9 inches), which were collected aseptically. The soil samples were dried in the air and sieved before being used to isolate actinobacteria using the SCNA medium (Starch 10.0g, Casein 0.3g, KNO3 2.0g, NaCl2 2.0g, K2HPO4 2.0g, MgSO4.7 H2O 0.01g, Agar 20g, and Distilled Water 1000 ml). In order to prevent bacterial contamination, nalidixic acid was added to the media, while nystatin was added to prevent fungal contamination. The pH was lowered to 7.2 in the medium. Soil samples were serially diluted (from 10-4 to 10-7). The inoculums were then gently mixed by rotating 1 ml of each dilution while it was being plated onto the starch-casein agar (SCA) plates. To see aerial mycelium, mature aerial mycelium, and slow-growing isolates, 30 days of incubation at 37 ℃ were required for mature aerial mycelium to be visible on the plates. On starch-casein nitrate agar plates, the isolates were repeatedly subcultured until single colonies appeared.

Cultural and morphological characterization of the chosen strains

Actinobacteria were streaked on sterile SCA plates and cultivated at 28 ℃ for 7 days in order to study their cultural characteristics. Gram staining was used to examine the morphological characteristics. From each isolate, one colony was selected, streaked onto an uncontaminated slide that had been coated with SCNA, and then cultured for 48 hours at 37 ℃. Then, a couple of drops of methylene blue dye was added and the area was allowed to sit for one minute. Their morphology was examined under a microscope after the slide had been covered with a cover slip. Diffusible pigments, aerial hyphae, hyphal colour, and colony form were observed (Jayanthi et al., 2016). Under a high power and oil immersion microscope, the spore chain morphology of actinobacteria was examined.

Actinobacterial molecular identification

Each of the six actinobacteria antagonists was grown in a culture for one night before genomic DNA was extracted employing the QIAGEN DNA extraction kit (QIAGEN, Valencia, CA). After putting it in 100 l of elution buffer (10 mM/L Tris-HCl, pH 8.5), the amount was found by measuring the optical density at 260 nm. The PCR reaction mixture was made up of 100 ng of template DNA, 20 mol of 16S rRNA primers made from 200 M dNTPs, 1.5 mM of MgCl2, 1 U of Taq DNA polymerase, and 2 L of 10x Taq polymerase buffer. To carry out the amplification, 35 cycles of denatured state at 94 ℃ for 45 seconds, annealing at 56 ℃ for 45 seconds, extension at 72 ℃ for one minute, and the last extension at 72 ℃ for 5 minutes were utilized in a

thermocycler. 16S rRNA amplicons have been examined as PCR results on a gel consisting of 1% agarose at 100 V.

PCR product purification, sequencing, and sequence analysis

The 16S rRNA amplicons were purified using the Qiagen QIA Quick Gel Extraction Kit from Valencia, California. The recovered components were then connected to the pGEM®-T Easy vector (Promega Corporation, Madison, USA) in accordance with the manufacturer's instructions. With the addition of ampicillin (50 g/mL), X-Gal (20 g/mL), and IPTG (isopropyl—D-thiogalactopyranoside; 0.1 mM/L), the ligated products were then transformed into the E. coli strain DH5 and grown on Luria Bertani agar medium (Sambrook et al.,1989). The presence of insert DNA encoding 16S rRNA was verified in recombinants using automated DNA sequencing (Model 3100, Applied Biosystems, USA) and PCR amplification. The sequences were deposited in GenBank. Searches on the internet for similarities in sequences and phylogenetic analysis were carried out using the Basic Local Alignment Search Tool (BLAST) tool (http://www.ncbi.nlm.nih.gov/blast).

Screening of K solubilizing actinobacteria

The streaking method was used to study how actinobacterial isolates dissolved potassium in Alexandrov medium. 2016 (Princy *et al*.). Muriate of potash is used in the Alexandrov medium as a potassium source. Actinobacteria cultures that were 48 hours old were streaked in a loop onto the mentioned plates. For four to five days, plates were incubated at 37 degrees. Based on their capacity for k solubilization and halo zone development, actinobacterial strains investigated the detection of k solubilization.

Screening of P solubilizing actinobacteria

Strains were streaked over Pikovskaya's agar medium, containing (per liter): 0.5g yeast extract, 10g dextrose, and 5g Ca3(PO4), to identify the actinobacteria that solubilize phosphate. 2’ 0.5g (NH4)15 agar, 2SO4, 0.2 g of KCl, 0.1 g of MgSO4.7H2O, 0.0001 g of MnSO4.7H2O, and 0.0001 g of FeSO4.7H2O. PSA (Phosphorus Solubilizing Actinobacteria) strains were those that produced a clear zone surrounding the colony after 7 days at 30 ℃.

Estimation of phosphatase activity

The purified PSA strains were infused into Pikovskaya's broth, which contained p-glycerophosphate instead of TCP, and cultured for 72 hours in an

environmental shaker. A sample of 10 ml of the medium was taken out and centrifuged for 10 minutes at 10,000 rpm. The pellets were re-suspended in 5 ml of sterilised distilled water after the supernatant was discarded. These cells acted as a supply of enzymes. One millilitre of the sample was placed in a 50-millilitre Erlenmeyer flask. Toluene, modified universal buffer, one millilitre of p-nitrophenyl phosphate solution, and 0.25 millilitres of toluene were then added. After that, the flask was sealed with a stopper and put in an incubator set at 37 ℃. The stopper was taken off after an hour, and 1 ml of 0.5 M CaCl2 and 4 ml of 0.5 M NaOH were added. Another brief swirling motion was used to filter the flask mixture through Whatman No. 2 filter paper. At 410 nm, the mixture's intensity of yellow colour was measured. A calibrated graph with a standard comprising 0, 10, 20, 30, 40, and 50 mg of p-nitrophenol was used to determine the filtrate's p-nitrophenol content. According to Eivazi and Tabatabai (1977), the phosphatase activity was measured as moles of PNP released per ml of filtrate per hour.

Assessment of actinobacterial strains' capacity to produce IAA

According to the procedure outlined by Tien *et al*. (1979), the in-vitro measurements of the indole acetic acid formation by actinobacteria strains were made.

Standardization of carrier materials

Two kinds of carrier materials were tested for the mass multiplication of actinobacteria such as Coir pith and Vermicompost. Carrier material was autoclaved at 121℃ for 15 minutes. Seven days old cultures of six proven strains of actinobacteria were individually immobilized with vermicompost (VC) and composted coir pith (CP) mixture and kept under incubation for 60 days (Jayanthi *et al.,* 2022). The moisture was maintained at 40% and kept away from sunlight and direct heat. The bio formulations were stored at room temperature. Using the dilution plate technique, the shelf life of the bio formulations was measured at 15-day intervals for a maximum of two months and represented as colony-forming units/g.

Nursery treatment

To test the effectiveness of the bio-formulations, a nursery experiment was carried out using the high-yielding cultivar UPASI-9 of the Tea Research Institute (TRI). *Streptomyces chartreusis, Streptomyces flavogriseus, Streptomyces crystallinus, Streptomyces xanthocidicus, Streptomyces albus*, and

Streptomyces sp. were used in the nursery experiment to study the effects of different actinobacteria formulations on the growth of vegetatively propagated (VP) nursery plants using the well-known clone UPASI-9. Eight treatments, three replications, and fifty nursery plants were used in the nursery experiment. Nursery soluble mixes (NSM) (30 g/L water) were given out in accordance with the schedule specified in the suggested practise. 5 g of actinobacteria formulations were given to each plant. Nursery-soluble mixes were given once a month, followed by actinobacteria at the previously mentioned concentrations every ten days. Following were the treatments:T1 – *Streptomyces chartreusis,* T2 – *Streptomyces crystallinus,*T3 – *Streptomyces flavogriseus,* T4 – *Streptomyces albus,*T5 – *Streptomyces* sp., T6 – *Streptomyces xanthocidicus,* T7 –Recommended practice and T8 –Untreated Control (water)

Results

Identification of actinobacteria

A total of 30 actinomycetes strains were discovered in South India's various tea-growing agroclimatic zones. Five strains—AAS2, AAS5, AAS6, AAS7, APSA4, and CAS4—isolated from the Anamallais and one isolate from the Nilgiris, respectively, were shortlisted for more research. Figure 1 illustrates microscopic observations of the six strains that distinguish the spore chain morphologies, including flexible, spiral, straight hyphae, and spores with zigzag fragmenting hyphae (Figure 1).

Species identification

The six isolates AAS2, AAS7, APSA1, APSA4, APSA5, and CAS4 each have partial 16S rRNA gene sequences that have been identified and deposited in the GenBank with the accession numbers listed in Table 1. Analysis of the sequences using BLAST showed that these strains have an identical sequence at least 99% to *Streptomyces,* further confirming this.

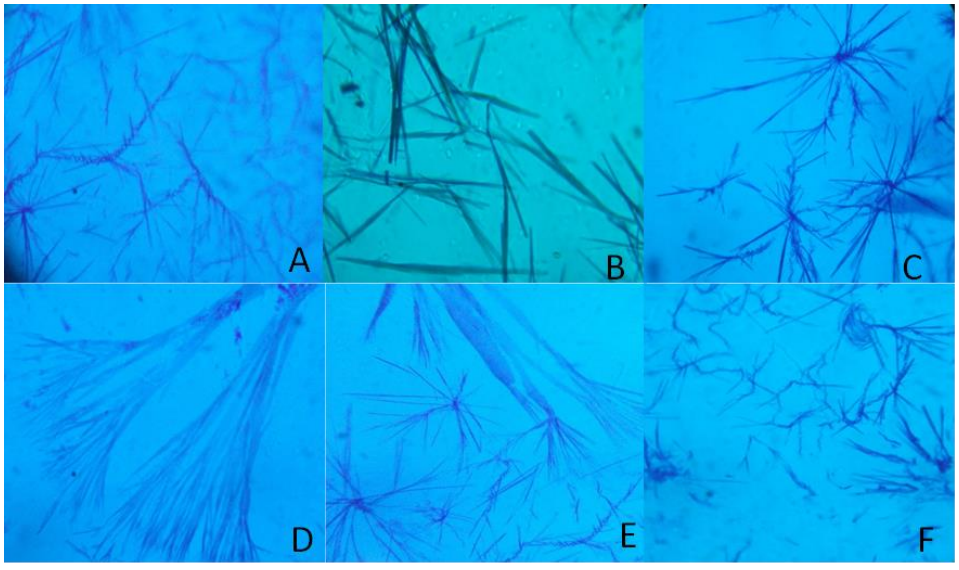

Figure 1. Microscopic appearance of spore chain morphology of actinobacteria strains A – AAS2, B – AAS7. C – APSA1, D – APSA4, E – APSA5 AND F – AAS7

Table 1. Molecular identification of Actinobacteria

Isolates	***Species***	***NCBI accession No.***
AAS2	*Streptomyces chartreusis*	*KP00444*
AAS7	*Streptomyces flavogriseus*	*KM 06711*
APSA1	*Streptomyces crystallinus*	*KM067119*
APSA4	*Streptomyces xanthocidicus*	*KM067120*
APSA 5	*Streptomyces sp*	*IMI No. 504703*
CAS4	*Streptomyces albus*	*KM067121*

Potassium solubilization of the isolates on Alexandrov medium

All the six selected strains (AAS2, AAS7, APSA1, APSA4, APSA5, and CAS4) demonstrated halo zone formation on the Alexandrov medium (Figure 2)**.** The solubilization zone ranged from 16 mm to 20 mm. The isolate AAS7 demonstrated a maximum zone of solubilization of 20 mm followed by the other strains (Table 2). All the strains were able to solubilize potassium.

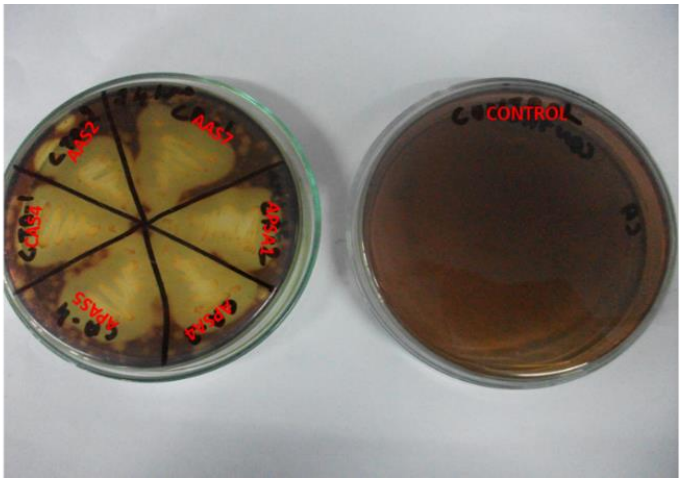

Figure 2. Potassium solubilization on Alexandrov medium

Table 2. K solubilizing ability of the isolated strains

Strains	Zone of clearance (mm)
AAS2	14.5
AAS7	20.0
APSA1	17.2
APSA4	18.0
APSA 5	16.5
CAS4	19.0
Control	-

Phosphorus solubilization of the isolates

All the six isolates streaked on the Pikovskaya's agar demonstrated clear halo zones around the colonies. Among six isolates, AAS7 produced a better zone of clearance (Figure 3). Phosphatase assay and soil available P estimation were determined for all six isolates. All the strains showed higher enzyme activity compared to the control (Table 3).

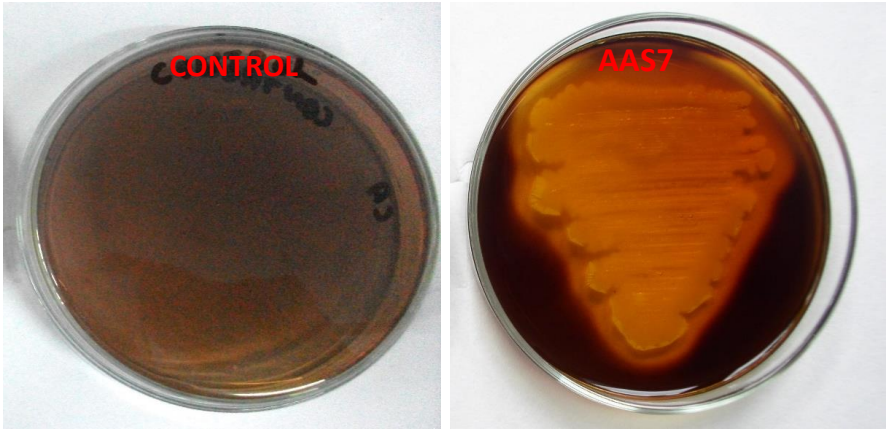

Figure 3. Phosphorous solubilization on Pikovskaya medium

Table 3. P solubilizing ability of the isolated strains

Strains	Zone of clearance (mm)	Soil available P (mg/kg)	Acid/alkaline Phosphatase	
			Acid	Alkaline
AAS2	10.5	26.0 ±0.4	1448	1541
AAS7	22.2	32.9 ±0.8	1444	858
APSA1	11.7	22.7 ±0.3	1400	176
APSA4	13.5	34.2 ±0.5	919	5068
APSA 5	14.5	28.3 ±0.6	1867	1640
CAS4	17.5	76.4 ±0.6	2785	931
Control	-	8.9 ±0.1	285	307

Production of Indole Acetic Acid (IAA) by the isolates

Actinobacterial strains that can solubilize potassium and phosphorus and create indole acetic acid (IAA) can also manufacture plant growth hormones in a lab setting. IAA production peaked in the APSA1 and APSA4 strains, then in other strains (Table 4).

Table 4. Production of indole acetic acid by the actinobacterial strains

Name of the strain	IAA (ppm)
AAS2	1483.28
AAS7	2011.9
APSA1	2067.1
APSA4	2092.2
APSA 5	1002.2
CAS4	1928.2

Optimization of organic carrier materials for bio-formulation

Sampling was done at periodical intervals and the colonies were enumerated using the dilution plate technique. Results indicated that the colony count had gradually improved in vermicompost carrier materials as the incubation days increased. After the 45th day of incubation, the population tends to decline (Table 5). To conclude, vermicompost carrier materials were suitable and supported the growth of actinobacteria while the incubation period may be optimized as 45 days to get the maximum number of colonies.

Among the eight treatments, T3 *Streptomyces flavogriseus* (AAS7) and T4 *Streptomyces albus* (CAS4) demonstrated dramatically improved biometric parameters (Table 6). T3 has shown a drastic change in shoot length and leaf weight. T4 also had shown similar effects. The overall growth of tea plants had been well influenced by our bio-formulation T3 and T4.

Table 5. Population level of actinobacteria in Vermicompost and Coir pith

Strains	Vermicompost					Composted Coir pith				
	5th	15th	30th	45th	60th	5th	15th	30th	45th	60th
AAS2	9.3	8.4	7.3	3.1	0.3	5.5	4.2	1.8	1.8	0.1
AAS7	6.1	2.5	2.1	2.8	0.7	4.8	3.8	2.6	1.8	0.8
APSA1	8.0	6.2	2.5	2.0	1.0	8.2	5.2	3.4	1.9	0.8
APSA4	7.1	5.3	2.5	1.9	0.9	8.0	4.8	3.3	1.7	1.0
APSA 5	6.0	5.8	2.2	2.0	0.4	6.7	4.8	3.7	2.1	1.0
CAS4	8.8	6.1	4.8	2.2	2.0	7.8	5.8	4.1	2.0	1.5

Values indicate Colony forming unit (x10^{9}) per gram

Table 6. Evaluation of bio-formulations on the growth of nursery plant in tea (ten months)

Treatment	Leaf count (No.)	Collar Diameter (mm)	Shoot length (cm)	Root length (cm)	Shoot dry weight (g)	Root dry weight (g)	No. of lateral root
Streptomyces chartreusis	16.3	2.5	49.6	32.5	4.2	3.8	2.5
Streptomyces crystallinus	18.5	2.8	44.2	36.1	3.6	2.8	2.8
Streptomyces flavogriseus	23.2	3.1	55.1	34.3	6.2	6.0	3.1
Streptomyces albus	20.8	3.1	50.0	31.7	6.6	5.9	3.1
***Streptomyces* sp.**	20.0	3.3	56.1	34.5	6.8	8.0	3.3
Streptomyces xanthocidicus	18.8	2.5	42.8	33.3	4.1	3.9	2.5
Recommended practice	12.8	2.4	49.8	30.0	4.0	2.9	2.4
Control (Untreated)	13.8	2.4	33.0	27.1	3.8	4.4	2.4
CD @0.05%	9.2	0.6	4.4	7.8	0.4	0.7	0.6

Discussion

Tea (*Camellia sinensis* (L.) O. Kuntze.) cultivation in India is frugally vital and perpetual. Widespread usage of agrochemicals to encounter the universal demand for tea gave rise to an altered microbiome in tea soil and tea plants (Cernava *et al.,* 2019; Bishnu *et al.,* 2008). Intriguingly, the Indian tea rhizosphere has been explored only a little. Numerous culture-dependent studies have demonstrated that the tea soil is highly diverse with metabolically beneficial PGPR that has the prospective to be used as a biofertilizer (Chakraborty and Chakraborty, 2015; Datta *et al.,* 2015; Dutta and Thakur, 2017). Additionally, some of these soil-borne bacteria established the potential to be used as biocontrol agents (Chakraborty *et al.,* 2013).

These microbes have a variety of abilities that aid in the growth of plants, including the fixation of nitrogen and the solubilization of macronutrients like potassium and phosphorus with micronutrients like zinc. Additionally, they create plant growth hormones like IAA. Having these properties, these microbes are being widely used as bio-fertilizers and bio-inoculants for viable agriculture. Indeed, these bio-formulations or microbes-based formulations are highly reliable for developing disease resistance, and plant growth promotion through several mechanisms (Mendes *et al.,* 2011).

It was discovered that the strain that was isolated belongs to the genus Actinobacteria after the study was designed to isolate native strains of PGPR from the tea environment and characterise them morphologically and molecularly. Actinobacteria were discovered to be the most prevalent PGPR (Bal *et al*., 2013; Ali *et al*., 2016). These bacteria have been identified in a range of crop fields and have several potential uses for boosting plant growth. Almost all of the strains we identified had whitish-grey aerial masses, and only strain D1 displayed a yellow colour. Based on the descriptions of recognised genera found in Bergey's Manual of Determinative Bacteriology, the genera of those six pure cultures were identified (Rajesh Muthu *et al*., 2013). All of the strains we saw shared the same morphological characteristics, which was consistent with earlier studies on the structure of the polysporous, most prevalent type of actinobacteria, the genus Streptomyces. Singh and Rai (2012); Jain *et al*. (2011).

In our study, among all the isolates tested, *Streptomyces flavogriseus* (AAS7) showed more zone of clearance while *Streptomyces albus* (CAS4) demonstrated higher enzyme activity when compared to the control. These observations are in agreement with a previous finding that reported a high level of P solubilizing activity by *Streptomyces* (Nandimath *et al.,* 2017).

Moreover, earlier studies showed that the species *Streptomyces* was able to producing IAA, which is responsible for promoting plant growth by supporting plants with nutrient uptake mechanisms, absorbing water, increasing germination of seeds rates, and extending roots (El-Tarabily, 2008). In another instance, *Streptomyces* collected from the soil of wheat, maize and beans were assessed for their capacity to make IAA, and this demonstrated their potential (Abd-Alla *et al*., 2013). In this current study, all six strains selected exhibited IAA production in a good range. Similarly, all the strains tested in this study exhibited potassium solubilization ability which was in line with the previous reports demonstrating the K solubilizing potential of *Streptomyces sp*. (Boubekri *et al.,* 2021).

Furthermore, an appropriate inert carrier material aids the growth of living microbial cells, ensures their tranquil establishment in and around the field of the crop, and enhances the probability of improving plant growth (Arora *et al.,* 2016). Our observations are in line with previous studies on bio-fertilizers based on organic manure combined with beneficial microbes. The organic amendments used for bio-formulation should be rich in organic matter content, have enhanced water retaining ability, and have neutral pH to be successful (Radhakrishnan and Mahendran, 2007, 2010).

One of India's finest crops, tea provides major subsidies to the national economy and generates foreign cash. Unjudged application of chemical fertilisers, weed killers, insecticides, and fungicides as well as decreased use of organic resources deplete soil health. Predominantly owing to the demand of importers for organic tea, there is a necessity to enrich the organic production of tea crops. Thus, switching to bio-fertilizers that are organic carrier based would be the best alternative. Bio-formulations are inoculants that are cultured from beneficial microbes, which are then immobilized on organic carriers such as vermicompost, coir pith, farm yard manure, vermiwash, etc. These bio-fertilizers boost soil productivity and promote plant development by making nutrients easier to access. Although almost all soils include nitrogen-fixing and nutrient-solubilizing microorganisms, their number may not be sufficient to raise these biological changes to a significant level. Due to their many functions in the soil, organic fertilisers are among the main sources of plant nourishment in organic farming. Thus, the combined use of bio-fertilizers and organic manure bid unlimited chances to upturn crop productivity in a cost-effective manner. Our study is one such attempt to evaluate the efficacy of our bio-formulation made out of native PGPR isolated from tea soils on organic tea cultivation. This study would be a preliminary attempt to make use of these strains in bulk production of crop-specific or soil-specific bio-fertilizers.

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